Genetic diversity, candidate genes and gene expression in relation to drought tolerance in Ghanaian cowpeas (*Vigna unguiculata*)

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By

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Declaration

I hereby declare that no part of this thesis has been previously submitted to this or any other University as part of the requirements for a higher degree. The content of this thesis is the result of my own work unless otherwise acknowledged in the text or by reference.

The work was conducted in the department of Biology, University of Leicester, during the period October 2003 to October 2006.

Signed.....

Emmanuel Plas Otwe, December, 2007

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Abstract

Cowpea (*Vigna unguiculata*) is grown mainly for its protein-rich grains, which is consumed in various forms in sub-Saharan Africa. Average grain yield in farmers' fields is generally low due to a number of biotic and abiotic stresses. A phenotype to gene approach aimed at identifying DNA polymorphisms linked to drought stress responses that could be adopted for drought tolerance breeding programmes in cowpea was used for this study. One hundred and six cowpea accessions from Ghana were evaluated for seedling drought tolerance on an individual plant basis under greenhouse conditions using the pot screening method. The results suggested that there were more drought tolerant accessions in the germplasm than the susceptible ones. The cluster results from the morphological analysis were informative but inadequate to effectively determine variability of germplasm. Three multi-locus PCR based molecular markers; SSR (simple sequence repeat), IRAP (inter-retrotransposon amplified polymorphism) and REMAP (retrotransposon – microsatellite amplified polymorphisms) were used for the diversity analysis. The results indicated the highly polymorphic nature of the DNA markers used as small groups could be used to identify each of the accessions and the diversity recorded by each marker showed good correlation coefficient values. The fragment candidate gene cloning and sequencing analysis indicated the enormous diversity amongst the accessions. Numerous single nucleotide polymorphisms (SNPs) were identified in both exonic and intronic regions as well as some deletions and insertions. The SNPs were largely nucleotide transitional in character and comparative analysis with other plant species indicated that some correlated with previously identified genes and polymorphisms related to drought stress. Some abiotic or drought inducible genes in cowpea from the differential display PCR (DD-PCR) analysis may have been identified, but needs Northern hybridization analysis for confirmation. The thesis gives a broad insight into genetic diversity, fragment candidate genes and gene expression in relation to drought tolerance in cowpeas from Ghana, for future progress towards gene discovery and exploitation for plant breeding.

Dedication

I wish to wholeheartedly dedicate this thesis, which signifies all the research work that I did here at the University of Leicester, biology department, to my lovely, caring and understanding wife Mrs Rebecca Plas Otwe, and to our wonderful children, Priscilla, Emmanuel (Jnr.), and Patrick.

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Abbreviations

ABA	Abscisic acid
AFLPs	Amplified fragment length polymorphisms
APS	Ammonium persulphate
BC	Back cross population
bp	base pairs
CAPS	Cleaved amplified polymorphic sequence
cDNA	Complementary DNA
cM	centiMorgans
cm	Centimeter
CTAB	Cetyltrimethyl-ammoniumbromide
COS	Conserved Orthologous sets
DAF	DNA amplification fingerprinting
dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytosinetriphosphate
dGTP	Deoxyguanosinetriphosphate
dNTPs	Deoxynucleotidetriphosphates
DNA	Deoxyribonucleic acid
dTTP	Deoxythymidinetriphosphate
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
F_1	First filial generation, produced by crossing two parental lines
FAO	Food and agriculture organization of the United Nations
g	Gram
H	Hour(s)
IRAP	Inter-retrotransposon amplified polymorphism
IITA	International institute for tropical agriculture
KI	Potassium iodide
LTRs	Long terminal repeats
M	Molar

MAS	Marker-assisted selection
min	Minute(s)
mm	Millimeters
mRNA	Messenger RNA
µl	Microliter
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PV	Phenotypic variance
QTL	Quantitative trait loci
RAPD	Random amplified polymorphic DNA
REMAP	Retrotransposon-microsatellite amplified polymorphism
RFLP	Restriction fragment length polymorphism
RILs	Recombinant inbred lines
RNA	Ribonucleic acid
RNAse	Ribonuclease
s	Second(s)
SCARs	Sequence characterized amplified regions
SDS	Sodium dodecyl sulfate
SNPs	Single nucleotide polymorphisms
SSRs	Simple sequence repeats or microsatellite
STRs	Short tandem repeats
STS	Sequence tagged site
TBE	Tris-Borate-EDTA buffer
TE	Tris-EDTA (TE) buffer
Tris	2-amino-2-(hydroxymethyl)-propane-1,3-diol
WUE	Water use efficiency
w/v	Weight/Volume
v/v	Volume/Volume
U	Unit
%	Percentage

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Chapter One

1.0. General introduction and literature review

1.1. Legumes and the changing face of agriculture

It is anticipated that the population of the earth will approach 10 billion by 2040 (Khush, 1999). The demographics of this rise are not encouraging. Based on this population projections, food production will need to increase by as much as 400% in Africa and Asia and 200% in Latin America (Khush, 1999). In sub-Saharan Africa, the population may reach 1.2 billion by 2025 and be combined with a demographic shift from about 30% of the population (in 1990) in urban areas to at least 50% (Winrock, 1992). Without an increase in food production, starvation and malnutrition especially among women and children of the developing countries will become more and more prevalent. Currently, according to Borlaug (1997), more than one billion people are living in hunger, mainly in Africa and Asia, which adds more credence to the need for increased food production.

Even as the earth's human population continues to grow, global warming has the potential to dramatically alter earth's climate and to have negative impacts on world food production. New ways to grow more and better food on a limited amount of land with limited water must be vigorously pursued. A tremendous variety of production systems and environmental constraints are found between different developing countries and even within individual countries. Four broad agro-ecological zones namely, humid and peri-humid, hill and mountain areas, irrigated and naturally flooded areas, dry lands and areas of uncertain rainfall, account for 90% of agricultural production in developing countries (FAO, 2000). Within each of the zones, a range of farming systems is found as well as a mixture of traditional and modern production systems. The increase in food

production needed to cover the increased population size projected cannot come from recruiting new land for agricultural purposes as most land suitable for agriculture is already in use. Degradation of land already in use, due to overgrazing, deforestation, erosion and salinity, associated with poor (non-sustainable) farming practices, has been a global concern for ages. Where will the needed food supply increase come from?

Feeding the world's population will require that research is focussed on food sources that provide sufficient protein and nutrients. Although it is believed that cereals, particularly wheat, rice and maize, will continue to be the major source for the supply of increased food, throughout history, all major civilizations have depended upon the co-cultivation of a cereal and a legume as a food source. In many places of the world, legumes complement cereals or root crops, the primary source of carbohydrates, in terms of amino acid composition. Whereas cereal seed proteins are deficient in lysine, legume seed proteins are deficient in sulphur-containing amino acids (methionine and cysteine) and tryptophan (Wang et al., 2003). However, combined with cereals they result in a balanced diet of energy and protein.

Legumes constitute a large plant family that presents humans with a treasure trove of resources for a variety of uses. They are a diverse and important family of angiosperms. With more than 650 genera and 18,000 species, legumes are the third largest family of higher plants and are second only to grasses in agricultural importance (Singh et al, 2002). All over the world, legumes provide important sources of protein, oil, mineral nutrients, and nutritionally important natural products (Graham and Vance, 2003). Grain legume species, including cowpea (*Vigna unguiculata*), account for over 33% of human dietary protein as seeds of this class of legumes contain at least 20% to 40% protein, while other legumes, like clovers (Trifolium spp.) are widely used as animal fodder. In some developing countries, especially in sub-Saharan Africa, where people do not have access to animal protein and nitrogen fertilizer, legumes may provide up to

two-thirds of the nutritional requirements. Refined oils, such as soybean (*Glycine max*) oil, have industrial applications in paint, diesel fuel, electrical insulation, and solvents. Legumes also accumulate phytochemicals, including isoflavonoids, which impact human health through pharmaceutical use and as dietary supplements (Dixon and Sumner, 2003).

An important feature of legumes is their ability to obtain nutrients via symbioses with soil microbes. Approximately 80% of the atmosphere is nitrogen gas (N_2). Unfortunately, N_2 is unusable by most living organisms. Biological nitrogen fixation is the process that changes inert N_2 to biologically useful ammonia (NH_3). Legume nitrogen fixation starts with the formation of a nodule. A common soil bacterium, *Rhizobium*, invades the root and multiplies within the cortex cells. The nitrogen fixation process between the legume plant and rhizobia bacteria is a symbiotic or mutually beneficial relationship. Rhizobia bacteria provide the legume plant with nitrogen in the form of ammonia whilst the legume plant provides the bacteria with carbohydrates as an energy source. Drought is one of a range of environmental stresses, which can cause considerable reductions in N_2 fixation (Lodeiro et al., 2000). However, it is not obvious which particular physiological process of the stressed plant actually affect nodule growth and metabolism (Gonzålez et al., 1998). The relationship between plant water status, photosynthesis and N_2 fixation, under water stress and the changes in nodule morphology have been studied for some temperate legumes (Venkateswarlu et al., 1990). However, tropical legumes grown in arid regions, have not received such adequate attention.

1.2. Botany of Cowpea

1.2.1. Centre of origin, domestication and distribution

Cowpea (*Vigna unguiculata* [L.] Walp.) (Vercourt, 1970; Maréchal et al., 1978; Pasquet, 1993b) is one of the most ancient human food sources which have probably been used as a crop plant since Neolithic times (Summerfield et al.

1974). Determination of the exact location of the centre of origin of any given species is difficult and is usually a source of debate and contradictions. Botanical and cytological proofs, information on geographical distribution and cultural practices, historical records and presence of wild relatives have been used to speculate on the origin, evolution, dissemination and domestication of the crop (Ng and Maréchal, 1985; Ng, 1995). A lack of archaeological evidence has resulted in contradicting views supporting Africa, Asia, and South America as the origin (Johnson, 1970; Summerfield et al. 1974; Coetzee, 1995). According to Allen (1983), Ethiopia and India are primary centres of origin for cowpeas, and China is considered as a secondary centre. Despite the fact that cowpea was known in India during the first millennium B.C. (Steele and Mehra 1980) and despite its wide distribution in Asia, all evidence points to its origin in Africa, although where the crop was first domesticated is uncertain. Ethiopia (Steele 1976; Allen, 1983), West Africa (Maréchal et al. 1978; Vaillancourt and Weeden 1992; Ng 1995) and Eastern and Southern Africa (Baudoin and Maréchal 1985) have all been considered probable centers of domestication, while a "diffuse" domestication in the savanna after the dispersal of cereals has also been hypothesized (Steele, 1976). The species includes cultivated forms, i.e. *V. unguiculata* ssp. *unguiculata* var. *unguiculata*, wild annual forms, i.e. ssp *unguiculata* var. *spontanea* (Pasquet, 1993b), and ten wild perennial subspecies (Pasquet 1993a, 1993b, 1997). This classification is based on results from morphological (Pasquet 1993a; Padulosi, 1993), allozyme (Panella and Gepts 1992; Vaillancourt et al. 1993; Pasquet 1993b, 1999) and cpDNA studies (Vaillancourt and Weeden, 1992).*V. unguiculata* ssp. *unguiculata* var. *spontanea* is the likely progenitor of the cultivated cowpea (Pasquet 1999). Although cultivated cowpea classification was based on three groups for a long time (Pasque, 1996), it is now based on five cultivar-groups (cv.-gr.) (Pasquet 1999):

1. cv.-gr. Textilis (long inflorescence peduncle, usually 40 cm–1 m) in West Africa;

2. cv.-gr. Sesquipedalis (fleshy pod, wrinkled when ripe, longer than 30 cm, kidney-shaped seeds spaced within the pod, more than 17 ovules), chiefly in East Asia;
3. cv.-gr. Melanophthalmus (seed testa thin and often wrinkled, flower and seed partly white, fewer than 17 ovules, plant able to flower quickly from the first nodes under inductive conditions), originally in West Africa;
4. cv.-gr. Biflora (seed testa thick and shiny, flower and seed most often coloured, fewer than 17 ovules, plant able to flower quickly from the first nodes under inductive conditions);
5. cv.-gr. Unguiculata (seed testa thick and shiny, flower and seed most often coloured, more than 16 ovules, plant flowering late, even under inductive conditions).

Two main groups can be separated on the basis of their physiology and their number of ovules: cv.-gr. Biflora and cv.-gr. Melanophthalmus display a low number of ovules and can flower quickly under inductive conditions, while cv.-gr. Unguiculata and cv.-gr. Sesquipedalis display a high number of ovules and cannot flower quickly under inductive conditions. Each main group is subdivided into a primitive subgroup and an evolved subgroup. The primitive cultivars (from cv.-gr. Unguiculata and especially cv.-gr. Biflora) are characterized by pods more or less dehiscent and small seeds with thick testa and wild colours (tan, gray, mottled and speckled), and their morphological diversity is low. In contrast, cv.-gr. Melanophthalmus and cv.-gr. Sesquipedalis look like the outcome of two divergent lineages and are characterized by the expression of several recessive genes (Pasquet 1999). However, these hypotheses inferred from morphological data, have never been examined using molecular data. Singh and Matsui (2002) also agree that cowpea may have originated from Africa, although they were not certain about where the crop was first domesticated. Ng and Maréchal (1985) have also reported that southern Africa could be the centre of genetic diversity because the most primitive of the wild cowpea species occurs in Namibia from the west, across Botswana, Zambia, Zimbabwe and Mozambique to the east, and the Republic of South Africa and Swaziland to the south. The former

Transvaal (Northern Province) in South Africa is depicted as the most probable centre of speciation of cowpea due to the presence of wild varieties such as var. *rhomboidea*, var. *protracta*, var. *tenuis*, and var. *stenophylla* (Singh et al. 1997). Presently cowpea is grown throughout the tropic and sub-tropic areas around the whole world.

During the process of evolution of V. *unguiculata* according to Ng (1995), there was a change of growth habit from perennial to annual breeding and from predominantly outbreeding to inbreeding, while cultivated cowpea (sub-sp. *unguiculata*) evolved through domestication and selection of the annual wild cowpea (var. *dekindtiana*). Then also during the process of domestication, and after the species were brought under cultivation through selection, there was loss in seed dormancy and pod dehiscence, corresponding with an increase in seed and pod size. The precise location of origin of where cowpea was first domesticated is also still under speculation. The wide geographical distribution of var. *dekindtiana* throughout sub-Sahara Africa suggests that the species could have been brought under cultivation in any part of the region. However, maximum diversity of cultivated cowpea is found in West Africa (Ng and Maréchal, 1985). Carbon dating of wild cowpea remains, found from the Kintampo rock shelter, a town in the Brong-Ahafo region in Ghana, has been carried out by Flight (1976). The results have proved that the remains are the oldest archaeological evidence of cowpea found in Africa. This could show that there was existence of gathering, if not cultivation, of cowpea by African hunters or food gatherers as early as 1500 BC. It is likely that cowpeas were dispersed from Africa to Asia around 1500 BC or about 200 BC to India (Flight, 1976). The crop was also brought to Europe probably through north-eastern Africa around 300 BC and was introduced to the tropical Americas in the 17th century by the Spanish through slave trade. In the southern USA, cowpeas or black-eye peas have been grown since the early 18th century (Padulosi and Ng, 1997).

1.2.2. Taxonomy and cytogenetics

Cowpea is a *Dycotyledonea* according to Maréchal et al. (1978), belonging to the order *Fabales,* Family *Fabaceae*, subfamily *Faboideae,* tribe *Phaseoleae*, sub-tribe *Phaseolinae*, genus *Vigna* and section *Catiang*. The genus *Vigna* is pan-tropical which comprises several species whose exact number varies according to authors from 84 to 184 (Padulosi and Ng, 1997).*Vigna unguiculata* (L.) Walp.,(syn. *Vigna sinensis* (L.) Savi ex Hassk) as already indicated has five cultigroups: *biflora, Sesquipedalis, Melanophthalmus, textilis* and *unguiculata* (Baudoin and Maréchal, 1985). Earlier studies by Rawal (1975) had indicated that subspecies of *Vigna* are easy to hybridize and transfer of genes occurs in nature between weed forms and cultivars with a continuous variation among them in several traits. Despite these observations, recent evaluations of genetic diversity based on chloroplast DNA (Vaillancourt and Weeden, 1992), on isozymes (Panella and Gepts, 1992) and on seed protein (D'Urzo et al. 1990; Panella and Gepts, 1992) revealed that there is a high degree of relationship among several cultivated cowpea genotypes. This high level of genome homology may be partly due to the low level or non-exploitation of the crop's wild relatives and also to its self-pollination habit (Fatokun et al. 1997). Investigation based on isozyme markers and their relationships within and among *Vigna* spp., by Sonnante et al. (1996) showed low levels of similarity among species, whereas variability among accessions differed within each species. Flavonoid HLPC fingerprints have been used to study the intra- and interspecific relationships among *Vigna* species. A greater variability in flavonoid aglycone class (quercetin, kaemferol, and isorhamnetin) and glycosylation pattern has been observed in cultivars of V. *unguiculata* (L.) Walp., as compared to their wild relatives (Cardinali et al. 1996; Lattanzio et al. 1997).

Cowpea is a diploid and possesses 2n = 2x = 22 chromosomes. Pachytene bivalents, cells in mitotic pro-metaphase and metaphase, were used in the development of the karyotype (Barone and Saccardo, 1990; Pignone et al. 1990). The karyotype of cowpea as described by Barone and Saccardo (1990), is

composed of one very long and a very short chromosome, with the remaining nine chromosomes being allocated to three size groups of four long, four medium and one short. There is a great deal of confusion on the proper classification of the cowpea, because of the large number of distinct forms which exist and the fact that hybridization is readily achieved, so that it is probable that some, if not all, of the cultivated forms are in fact hybrids. Numerous specific names have been given to the cowpea and its various forms. However, it is now widely accepted that there should be no distinction between these so called species and that there should be one species *Vigna unguiculata*, with *V. sesquipedalis* and *V. sinensis* as synonyms (Lattanzio et al. 1997).

1.2.3. Vegetative and reproductive morphology

Cowpea (*Vigna unguiculata* (L) Walp) is an annual legume of various morphological characters. Its diverse growth habits vary from erect, semi-erect, shrubby, trailing, prostrate, to climbing. These attributes of growth are generally due to genetic factors but may be also influenced by crop density, soil fertility, water stress, and the interaction of genotypes with day length and night temperatures (Steele and Mehra, 1980). The strong taproot has laterals near the soil surface with clusters of spherical nodules. Germination is epigeal, but cotyledons do not persist and may lose as much as 90% of their dry matter by the time seedlings emerge (Steele and Mehra, 1980). At the seedling stage, the first leaves above the cotyledons are simple, entire and opposite. Subsequent leaves are alternate and trifoliate with the terminal leaflet often bigger and longer than the two asymmetrical laterals. Leaflets are 5-18 cm long, 3-16 cm wide and are described as linear, lanceolate, or broadly or narrowly ovate, entire or obscurely toothed, broadly cuneate or rounded at the base and gradually tapering to a pointed tip. The petiole is stout, grooved, and 5-25 cm long (Duke, 1981). The stems are striate, smooth or slightly hairy and sometimes tinged with purple. The flowers are arranged in racemose or intermediate inflorescence at the distal ends of 5-60 cm long peduncles.

Flowers are borne in alternate pairs, with usually only two flowers per inflorescence. They are conspicuous, self-pollinating, borne on short pedicels and the corollas may be white, yellow, mauve, pink, pale blue or purple in colour. Flowers open in the early day and close at approximately midday. After blooming (opening once) they wilt and collapse. Fruit are pods that vary in size, shape, colour and texture. They may be erect, crescent-shaped or coiled. They are usually yellow when ripe, but may also be brown or purple in colour. There are accessions with determinate and others with indeterminate growth habit. Pod length ranges from 4 cm in the wild subspecies to more than 1 m in subsp. *sesquipedalis*. Most cultivated species produce usually non-dehiscent, brittle or soft, curved, straight or coiled, and pendant, often constricted and distinctly beaked pods 12-20 cm long with about 10-15 seeds per pod. Colour varies from brown, red or black to variously mottled with anthocyanic pigment which may be confined to the sutures and beak (Steele and Mehra, 1980). Pods of wild species are straight, scabrous, slightly pubescent, black, erect, dehiscent and in some subsp. like *cylindrica* never constricted between the seeds. Cowpea seeds have a diverse shapes, texture and colours. They are 2-12 mm long, kidney-shaped, oblong or cylindrical. Cowpea seeds may be smooth or wrinkled, red, black, brown, green, buff or white as dominant full coloured, spotted, marbled, speckled, eyed, or blotched (Duke, 1981). The weight of 100 seeds varies from 1 g in some wild species to 34 g in rare cultivars (Steele and Mehra, 1980). Cowpea is highly self-pollinated crop in most production environments (Williams and Chambliss, 1980) although significant out-crossing associated with insect activities can occur in some environments (Ehlers and Hall, 1997).

1.2.4. Ecology

Cowpeas are adapted to a wide range of soils, especially those with adequate drainage, from sandy to heavy, well drained clays. The lighter soils allow for good rooting. Cowpeas also tolerate a pH condition within the acidic range of pH 4.0 to 6.5 (Davis et al. 1991). Cowpea is used as a silage crop and a green manure for soil improvement (Hall et al. 1997; Terao et al. 1997; Singh et al. 2002). Cowpea

also tolerates moderate drought conditions but excessive soil moisture leading to flooding is harmful, reducing growth and favouring fungal diseases. Nevertheless, the crop is well adapted to a wide precipitation range (650-2000 mm). It also tolerates moderate shading.

Cowpeas have been reported to have greater adaptation to high temperatures than any other crop species. Germination greater than or equal to 80% has been observed in cowpea at temperatures ranging from 10˚C to 40˚C (Wolk and Herner, 1982; Ismail et al. 1997; Ismail et al. 1999). Temperatures outside this range adversely affect germination of cowpea. Variation in germination percentage and rate of germination under stress temperatures have been observed among cowpea cultivars (Marsh, 1993; Craufurd et al. 1996a,b and c).

1.2.5. Areas of cowpea production

Cowpea (*Vigna unguiculata*) is an important food and fodder legume cultivated in the tropics and sub-tropics covering 65 countries in Africa, Asia and Oceania, the Middle East, Southern Europe, southern USA and Central and South America (Singh et al., 1997). Based on the available information from FAO it is estimated that globally cowpea is cultivated on about 14.5 million hectares with an annual production of over 4.5 million tons (Singh et al., 2002). World cowpea production was estimated at 3 319 375 MT and 75% of that production (Fig. 1) is from Africa (FAOSTAT 2000).

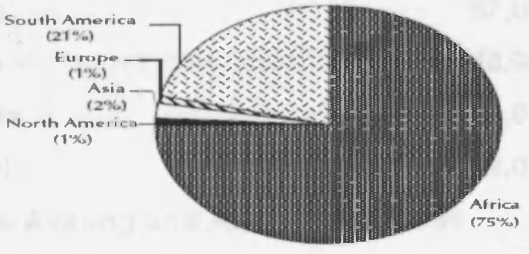


Figure 1. Share of Africa in world cowpea production.
Source: Adapted from FAOSTAT (2000).

West Africa is the key cowpea producing zone, mainly in the dry savannah and semi-arid agroecological zones. The principal cowpea producing countries are Nigeria, Niger, Senegal, Ghana, Mali, and Burkina Faso (FAOSTAT 2000). It is difficult to obtain the reliable statistics on cowpea area and production because most countries do not maintain separate records on cowpea. Probably because of these difficulties, that the Food and Agricultural Organisation (FAO) suspended formal publication of cowpea production data several years ago. In 1981, the world cowpea production area was estimated at 7.7 million hectares with an annual grain production of 2.27 million tonnes (Aveling and Adandonon, 1999) as indicated by Table 1.1. The other important cowpea growing countries in Africa are Togo, Benin, Cameroon, Chad, Sudan, Somalia, Kenya, Malawi, Uganda, Tanzania, Zambia, Zimbabwe, Botswana, Mozambique and Republic of South Africa. Additionally cowpea is produced in other parts of the world like India, Bangladesh, Nepal, Sri Lanka, Indonesia, China, Philippines, Brazil, Cuba, Haiti, USA, and West Indies.

Table1.1: Major cowpea producing countries and estimated production

Country	Production (t)
Nigeria	**850,000**
Brazil	**600,000**
Niger	**271,000**
Burkina Faso	**95,000**
USA	**60,000**
Ghana	**57,000**
Kenya	**48,000**
Uganda	**42,000**
Malawi	**42,000**

Source Aveling and Adandonon, 1999

1.2.6. General cowpea production systems

In West Africa, cowpea is grown mostly in subsistence farming systems and on a small scale in the lowland dry savannah and Sahelian regions. Traditionally, cowpea is grown in association or in relay cropping with cereals such as sorghum, millet, and maize (Ajeigbe et al. 2006). However, cowpea cropping systems are moving towards monocropping as the crop's economic importance increases. The increase in cowpea production is linked to the use of improved crop protection and production practices. Fertilizers and pesticides are generally not used due to the fact that they are too expensive or not accessible to the small scale farmers. In Europe both fodder and grain type varieties are grown mostly as a pure crop. The production of cowpea in USA is highly mechanised and is on purely commercial basis. Of the developed countries, only the United States is a substantial producer and exporter of cowpea (Imrie, 2000).

1.2.7. Cowpea production in Ghana

1.2.7.1. Geography, climate and population of Ghana

Ghana is situated on the west coast of Africa with a total area of 238 540 km^2. The country has a north-south extent of about 670 km and a maximum east-west extent of about 560 km. It shares borders with Côte d'Ivoire (Ivory Coast) to the west, Burkina Faso to the north, and Togo to the east. To the south are the Gulf of Guinea and the Atlantic Ocean. The country is divided into 10 administrative regions. The topography is predominantly undulating and of low relief with slopes of less than 1 percent. Despite the gentle slopes, about 70 percent of the country is subject to moderate to severe sheet and gully erosion. There are five distinct geographical regions:

- The low plains, stretching across the southern part of the country.
- The Ashanti Uplands, stretching from the Côte d'Ivoire border in the west to the elevated edge of the Volta Basin in the east.
- The Akwapim-Togo Ranges in the eastern part of the country consist of a generally rugged complex of folded strata, with many prominent heights

composed of volcanic rocks. The ranges begin west of Accra and continue in a northeasterly direction, finally crossing the border into Togo.

- The Volta Basin occupies the central part of Ghana and covers about 45 percent of the nation's total area. The basin is characterized by poor soil, generally of Voltaian sandstone.
- The high plains in the northern and northwestern part of Ghana, outside the Volta Basin, consist of a dissected plateau. Soils in the high plains are considered to be more arable than those in the Volta Basin.

Ghana has a warm, humid climate. Mean annual rainfall of the country is estimated at 1187 mm. Mean annual temperatures range from 26.1 °C near the coast to 28.9 °C in the extreme north. Annual potential open water evaporation has been estimated as ranging between 1350 mm in the south to about 2000 mm in the north (FAO, 1998). The actual amount of evaporation depends on a number of factors including water availability, vegetation cover and prevailing weather conditions among others. There are six agro-ecological zones defined on the basis of climate, reflected by the natural vegetation and influenced by the soils (Table 1.2). Rainfall distribution is bimodal in the forest, transitional and coastal zones, giving rise to a major and a minor growing season. In the remaining two agro-ecological zones, the unimodal rainfall distribution gives rise to only one growing season. Irrigation is mostly applied for agricultural purposes and only in some minor parts of the country is the climate favourable for non-irrigated agriculture (GIDA, 2001). Rainfall exceeds potential evaporation during relatively short periods. Even in the southern forest zone where rainfall is at its highest, irrigation is essential for short season crops during the dry period. The unreliability of rainfall is a cause of concern (Akagbor, 2002). Complete crop failures can be expected in most northern areas in about one in every five years. This risk can rise to one in every three years during low rainfall periods. The cultivable area is estimated to be 10 million ha, which is 42 percent of the total area of the country and this (the sum of arable land and permanent crops) was about 6.33 million ha in 2002 (Table 1.3).

Table 1.2: Characteristics of agro-ecological zones in Ghana

Characteristics of agro-ecological zones in Ghana

Zone	Rainfall	Portion of total area	Length of growing season	Dominant land use systems	Main food crops
	(mm/yr)	(%)	(days)		
Rain forest	2 200	3	Major season: 150-160 Minor season: 100	forest, plantations	roots, plantain
Deciduous forest	1 500	3	Major season: 150-160 Minor season: 90	forest, plantations	roots, plantain
Transition zone	1 300	28		annual food and cash crops	maize, roots, plantain
Guinea savannah	1 100	63	180-200	annual food and cash crops, livestock	sorghum, maize
Sudan savannah	1 000	1	150-160	annual food crops, livestock	millet, sorghum, cowpea
Coastal savannah	800	2	Major season: 100-110 Minor season: 50	annual food crops	roots, maize

Source: Ministry of Food and Agriculture (MoFA), Ghana (2002).

Table 1.3: Basic statistics and population of Ghana

Basic statistics and population			
Physical area			
Area of the country	2002	23 854 000	ha
Cultivated area (arable land and area under permanent crops)	2002	6 331 000	ha
• as % of the total area of the country	2002	27	%
• arable land (annual crops + temp. fallow + temp. meadows)	2002	4 181 000	ha
• area under permanent crops	2002	2 150 000	ha
Population			
Total population	2004	21 377000	inhabitants
• of which rural	2004	54	%
Population density	2004	90	inhabitants/km²
Economically active population	2004	10 773 000	inhabitants
• as % of total population	2004	50	%
• female	2004	50	%
• male	2004	50	%
Population economically active in agriculture	2004	6 021 000	inhabitants
• as % of total economically active population	2004	56	%
• female	2004	46	%
• male	2004	54	%

Source: Ghana statistical service (2006)

The country's population as surveyed by the Ghana statistical service in 2004 was about 21.4 million (GSS, 2006), of which 54 percent leave in rural environment (Table 1.3). The annual population growth rate is 1.7 percent. Population density is 90 inhabitants per km^2 in Ghana, but varies from 26 inhabitants per km^2 in the Northern region to 896 inhabitants per km^2 in the Greater Accra region.

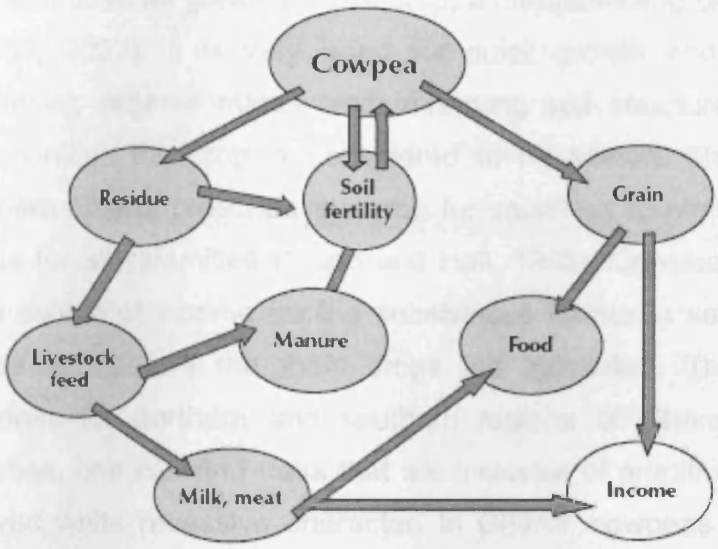
1.2.7.2. Cowpea production systems in Ghana

Cowpea is cultivated throughout Ghana. In general the farming practices vary from one agro-ecological zone to the other. The cowpea planting season is variable and depends on the areas, the objectives of the farmers and the varieties to be sown (MoFA, 2002). In the agro-ecological savanna zones especially the Sudan savanna zone where the bulk of the Ghanaian cowpea is produced, the crop is sown in June-July. If the objectives of the farmers are to produce cowpea as a fodder for their livestock, then the crop is sown anytime during the rainy season, even between some cereal crop rows regardless of the age of the cereal plant (MoFA, 2002). In other parts of Ghana where the farmers enjoy two rainy seasons, the crop is sown in May (major rainy season) and in August (minor season). Cowpea is sown at 2-4 seeds per drill. Spacing between plants varies and depends on the area and other crops being inter-cropped with the cowpea. In Ghana the most common agricultural practice is subsistence farming. Cereals and legumes are cultivated in less populated areas where access to irrigation is difficult. Cowpea production is more important in the three northern regions of Ghana and is generally grown as a sole crop but can be mixed or in rotation with maize or millet. In the central and forest zones cowpea is grown in the early development stage of oil palm plantations where the soil is poor and the land is constrained with little or no fallow period (Marfo and Hall, 1992). Farmers rarely fertilize a cowpea field. Soil preparations only consist of cutting weeds and making holes in the soil where the seeds are directly sown.

1.2.8. Importance of cowpea

Cowpea is the most important food legume in the West and Central African region, particularly in the dry savannas. Estimates of world hectarage of cowpea is in the range of 12.5 million, with about 8 million in West Africa, the majority of these being in Nigeria and Niger (Singh et al. 1997). Current FAO estimates for 1998 are lower than these figures, although the proportions are similar (FAO, 2000). Cowpea contains about 25% protein and so is a cheap source of protein in the daily diet of the rural and urban populations and is widely considered as the "poor man's meat" in Africa. Its haulms are also an important source of nutritious fodder for the livestock in the dry savannas (Bressani 1985; Singh et al. 1997; Tarawali et al. 1997a, b). The dry savannas consist of the drier part of the northern Guinea savannah, plus the Sudan savannah representing more than 50% of the total land area of the sub-Saharan Africa, with a significant proportion located in West Africa. Annual rainfall is less than 1000 mm with a growing period of 180 days or less meaning that much of the region experiences a long (7-9 months) harsh dry season. The growing period shortens on a south-north axis. The sandy soils are generally poor, with low organic carbon, and cation exchange capacity, and are deficient in nutrients, especially nitrogen and phosphorus. As a legume, cowpea can contribute to soil fertility, mainly through its nitrogen fixing abilities. Part of the nitrogen fixed will remain in the soil in the roots, and thereby contribute to the soil fertility for subsequent crops. Some fixed nitrogen will eventually return to the soil as manure after residues are fed to livestock. Thus cowpea plays a very important role in promoting food and feed production as well as sustainable agriculture in the West African sub-region especially in Ghana as summarized in Fig.2 below.

Figure 2: Schematic representation of the important contributions by cowpea



1.2.9. Uses of cowpea in Ghana

Cowpea has a wide variety of uses namely as a nutritious component in the diet of humans as well as nutritious livestock feed (MoFA, 2002). The crop can be used at all stages of growth as a vegetable. The tender green leaves are used in the preparation of pot herb like spinach by some tribes in Ghana. The immature snapped pods are also used in the same way as snap beans, often mixed with other foods. In southern Ghana, the green cowpea seeds are boiled as a fresh vegetable and eaten as snack. Mostly the dry mature seeds are suitable for cooking and have various usages depending upon the cultural practices of that particular area in Ghana. In the urban areas consumers of cowpea are prepared to pay a premium for black-eye varieties (Langyintou et al. 2003). They are also ground into flour and fried with lots of spices and considered a delicacy in most Ghanaian homes.

In many areas especially in the three northern regions of Ghana, cowpea is used as legume hay for livestock feed. It is either used as a green fodder or dry fodder. It is also used as green manure crop, a nitrogen-fixing crop or for erosion control (MoFA, 2002). It is very good for quick growth and establishment and for increasing organic matter and improving soil structure and fertility. In some communities the crop is considered to be sacred. The Dagara tribesmen in northern Ghana prescribe the crop for sacrifices to abate evil and to pacify the spirits for all calamities (Marfo and Hall, 1992). Cowpea production also serves as a source of income for the subsistence farmer to support the upkeep of the house-hold before the main crops are harvested. There are adaptations in cowpeas for northern and southern regions of Ghana. In the West African varieties, one can find traits that are inclusive of primitive wild color or the more derived white recessive character. In Ghana, cowpeas are typically uniform in their morphology, with white and black-eyed most commonly used. These are the same varieties as those used in the U.S.A. In Ghana about 80% of the red ones are consumed at the local level where the grains were produced. These have slightly smaller sized seeds than the white ones.

1.3. Constraints to cowpea production

Cowpea production is affected by a wide range of biotic and abiotic constraints, some of which are discussed below.

1.3.1. Biotic factors

Important biotic constraints to cowpea production include insect pests, (Thrips, Maruca, pod bugs, Aphids and Bruchid), diseases and parasitic weeds. In general the pest spectrum on legumes is large and extremely diverse. Several probable reasons have been given for the large number of major and minor pests on legumes. These could be the fact that legume crops are grown world wide, both as large monocultures and as important components of traditional multiple-cropping systems. Secondly, leguminous plants offer a wide range of feeding

niches for insects and mites, particularly in their reproductive structures. Among the biotic constraints of cowpea production, insect pests rank first and can cause total yield failure in cases of severe attack (Singh, 1999a). In general the pest problem is more severe in Africa than the other areas of cowpea production (Singh et al. 1997). Probable reasons for this are that (a) many insects are considered indigenous to the continent and/or have had ample time to co-evolve with the crop in its centre of origin and domestication (Ng and Maréchal, 1985) or (b) different views from the origin of cowpea pests, that is the interactions between the prevailing environmental conditions and the state of the crop (Tamo' et al. 1997). Insects attack all parts of cowpea plants from seedling emergence to storage (Singh et al. 1997; Jackai and Adalla, 1997). It is very common to find four or more pests on the crop at the same time.

Cowpea diseases induced by various pathogenic groups (fungi, bacteria, viruses, and nematodes) constitute one of the most important constraints to cowpea production in all agroecological zones where the crop is grown. The common diseases associated with cowpea production are anthracnose, cercospora, smut, rust, septoria, scab, ascochyta and bacterial blight. Most of these diseases cause yield losses which can be as high as 90% (IITA, 2000). These diseases can be very destructive and attack the crop from the seedling stage to the reproductive stage (Hall et al. 1997).

Striga gesnerioides also known as witch weed is a parasitic plant that attacks cowpea fields. Striga spp. is widely distributed (Singh et al. 1997) throughout the cowpea production areas and a major contributing factor to low cowpea production in the sub-Saharan Africa and India (Singh et al. 1997). Yield losses from damage by Striga range from 10% to 70% depending on the cultivar and the degree of infestation (Hall et al. 1997). The damage is particularly severe when the crop is under drought and nutrient stress (Omanya et al. 2004). The control of weeds, like Striga, involves the use of chemicals, and mechanical removal using

hoes or cutlasses. Improper use or careless handling of chemicals might result in degradation or pollution of the land and water supplies.

Improving disease and insect pest resistance through breeding and selection can reduce the use of pesticides with accompanying cost and environmental benefits. Even though the effects of biotic factors on cowpea production are very important, they are outside the scope of this study.

1.3.2. Abiotic factors

The effects of the environment on plant growth may be divided into enforced damage effects (stress), caused by the environment, and adaptive responses, controlled by the plant (resistance) (Gowda et al. 2003). Damage which may manifest as death of all or part of the plant, or merely as reduced growth rate due to physiological malfunction, is a common phenomenon and the agents are various; temperature, water availability, soil chemistry, physical properties and others such as air pollution, and wind. However, the most important environmental agents affecting plant growth in the semi-arid tropical zone is drought (Ashley, 1993; Singh et al. 1997).

Cowpea is mostly grown under rain-fed conditions in semi-arid areas. Although cowpea has an impressive ability to survive drought, low rainfall, high irradiance, excessive temperatures and periodic drought make cultivation of the crop challenging (Singh, 1999b). These factors are largely due to the climate, weather and soil of the region where it is grown and the plant must adapt to these conditions to produce good yield. The cowpea growing areas in Africa and Asia are characterised by low and erratic rainfall with dry spells occurring at any time during the growing season (Singh, 1999b). Conversely, excessive rain near to harvest can be particularly damaging causing grain rot and fungal diseases.

In an attempt to reduce the effects of drought stress there is a temptation for the subsistence farmers to adopt inappropriate production practices, such as digging

of well for irrigation when drought sets in, which may degrade the environment in the long term and cause a decline in production and productivity. Irrigation requires large supplies of water to replace evapotranspiration (Akagbor, 2002) and thus is not an option for much of the developing world. When even it is considered as an option, salinisation would lead to degradation of the land and reclamation would be too expensive for any subsistence farmer. There is therefore the need to improve efficiency of water use in agricultural systems by breeding for drought tolerant plants with greater water use efficiency. Recent reviews (Ashley, 1993; Subbarao et al. 1995; Boyer, 1996) have brought together the available knowledge on different aspects of drought tolerance in crop plants and options to minimize yield losses due to drought.

1.4. Drought stress

Since agriculture began, drought has been one of the major plagues limiting crop production worldwide. Although it reaches front pages of the media only when it causes famine and death, drought is a permanent constraint to agricultural production in many areas of the world. Drought stress is especially important in countries where crop agriculture is essentially rain-fed. In sub-Saharan Africa, drought years have a devastating effect on regional food security. It causes marginalization and poverty, and its negative effects are likely to increase as water resources decline globally. While irrigation is the method of choice in averting drought stress in many areas of the world, alternative low-input approaches are being explored, and molecular biology offers a promising array of tools that may be useful in achieving drought tolerance in plants.

Drought can be defined as the absence or deficit of rainfall for a period of time long enough to result in the depletion of soil water and consequent plant water deficit (Munné-Bosch and Alegre, 2004). Katz and Glantz (1997) have earlier indicated that there are two types of drought by definition, meteorological and agricultural drought. A meteorological drought could be defined as that time period when the amount of precipitation is less than some designated percentage

of the long term mean. An agricultural drought, on the other hand, could be defined in terms of seasonal vegetation development. It occurs when there is not enough moisture available at the right time for the growth and development of crops and as a result, yields and/or absolute production decline. In this study drought stress refers to water limiting conditions. Drought reduces the amount of water available for plant growth and leads to water deficit. Plant water deficit starts as soon as the demand exceeds the supply, that is, a situation in which the rate of transpiration exceeds the rate of water uptake. The subsequent cellular water loss is referred to as dehydration.

Water is a fundamentally important component of the metabolism of all living organisms, facilitating many vital biological reactions by being a solvent, a transport medium and evaporative coolant (Bohnert et al., 1995). In plants and other photoautotroph, water plays the additional role of providing the energy necessary to drive photosynthesis. Water molecules are split, in a process termed photolysis, to yield the electrons that are used to drive the energy yielding photosystem II reaction centre (Salisbury and Ross, 1992a). One of the major consequences of drought stress is the loss of protoplasmic water leading to the increasing concentration of ions such as [Cl^-] and [NO_3^-]. At high concentrations these ions effectively inhibit metabolic functions (Hartung et al., 1998). Additionally, the concentration of protoplasmic constituents and the loss of water from the cell lead to the formation of what is termed a glassy state. In this state whatever liquid is left in the cell has a very high viscosity, increasing the chances of molecular interactions that can cause protein denaturation and membrane fusion (Hartung et al., 1998; Hoekstra et al., 2001). Plants thus require the genetically encoded ability to ensure the maintenance of cellular turgor and metabolic functions.

Drought stress is exacerbated by high irradiance, excessive temperatures and high vapour pressure deficits (Jagtap et al. 2001a). Secondary components of drought include high temperatures caused when leaves have low transpiration in

bright light. High temperatures result in high respiration, which uses up sugar reserves and can kill sensitive tissues (Thomas et al. 1992). In Ghana, where cowpea cultivation is largely rain-fed, as is typical of many cowpea growing areas, these conditions affect crop growth and yield. The frequency and severity of drought may increase in future as global warming intensifies.

Furthermore drought stress is highly variable in time (over seasons and years) and space (between and within sites), and is extremely unpredictable. This makes it difficult to identify a representative drought stress condition (Visser, 1994). The unpredictable and variable forms in which drought stress will manifest itself, makes selection of promising individual plants and breeding for drought tolerance extremely difficult.

1.4.1. Effects of drought on plant growth and development

At the whole plant level drought primarily affects phenology, phasic development, carbon assimilation, assimilate partitioning and reproductive processes (Blum, 2004). Growth depends on cell division and expansion. Cell expansion is known to be more sensitive to drought than cell division. It is dependent on turgor, cell wall extensibility, and other factors pertaining to ABA signalling. Reduced cell expansion allows the plant to reduce its water use; however it leads to a reduction in plant productivity. If the reduction in total plant water use is not sufficient to sustain turgor, then transpiration is further reduced by stomatal closure. Stomatal closure initially reduces transpiration more than it reduces CO_2 assimilation, but as the stress becomes more pronounced both are drastically reduced. Turgor loss leads to wilting and eventual demise of the plant. Reduced cell expansion also impacts on meristematic development of yield components, such as inflorescence in legumes resulting in small reproductive organs and reduced yields. This condition unfortunately is an irreversible effect which is difficult to alleviate even by re-watering. Drought stress can also cause both advanced and delayed flowering. A delay of up to 50 days has been observed in rice subjected to pre-flowering drought (Blum, 2002).

1.4.2. Consequences of drought on grain yield

Drought stress conditions cause extensive losses to agricultural production worldwide (Bruce et al. 2002; Rubio et al. 2002; Salvi and Tuberosa, 2005). The amount of the reduction in grain yield depends on the stage of plant development at which drought occurs, its severity and duration and the drought susceptibility of the cultivar (Lorens et al. 1987). Its effects can be particularly severe during flowering in plants like rice where it causes sterility (Lafitte, 2002) and reduced embryo fertilization and growth in maize (Bruce et al. 2002; Earl and Davis, 2003). Drought stress during the vegetative phase can also adversely affect yield. The reduction in yield as a result of drought stress at the vegetative stage is caused by reduced leaf area, reduced intercepted photosynthetically active radiation (PAR), reduced radiation use efficiency and reduced harvest index (Earl and Davis, 2003). Yield losses in crops due to drought result, primarily, from its adverse effects on photosynthetic activity. Drought causes a reduction in photosynthesis thus reducing the availability of photosynthates for grain-filling. Leaf photosynthesis declines during grain-filling due to the onset of senescence, which leads to the breakdown of the photosynthetic apparatus. This situation can be made worse by stress factors like drought, high temperatures and excessive irradiance (Martinez et al. 2003). Drought, for example during grain-filling, reduces the photosynthetic activity of leaves by causing stomatal closure and enhancing the rate of senescence (De Souza et al., 1997). Both morphological and physiological adaptations that enhance water uptake reduce water loss and stimulate photosynthesis under drought stress, combine to maintain yields in resistant crop cultivars.

1.5. Drought stress resistance mechanisms

Drought is a major production constraint, reducing crop yields in water-limited areas where many of the world's poorest farmers live. Development of drought tolerant crops will enhance food production and the livelihoods of farmers in

these areas. Moreover, as the world population continues to grow and water resources for crop production decline, development of drought tolerant cultivars and water-use efficient crops is a global concern. Breeding for drought tolerance has produced improved cultivars for drought-prone environments, but progress has been slow due to the complex physiological responses to drought, various environmental factors, and their interactions.

1.5.1. Drought escape and dehydration avoidance mechanisms

Whole-plant and cellular mechanisms can contribute to avoidance of drought stress and responses expressed depend on the length and severity of drought, the age and development stage of the plant, and the organ, cell type and sub-cellular compartment considered (Bray, 1997).

Plants that escape drought exhibit a high degree of developmental plasticity, being able to complete their life cycle before physiological water deficits occur. Escape strategies rely on successful reproduction before the onset of severe stress. This is important in arid regions, where native annuals may combine short life cycles with high rates of growth and gas exchange, using maximum available resources while moisture in the soil lasts (Maroco et al. 2000a). Improved reproductive success also includes better partitioning of assimilates to developing fruits. This is associated with the plant's ability to store reserves in some organs (stems and roots) and to mobilise them for fruit production, a response well documented in crop plants, such as cereals (Gebbing and Schnyder, 1999; Bruce et al. 2002) and some legumes (Rodrigues et al. 1995; Chaves et al. 2002). This ability to mobilise reserves is increased in droughted plants (Yang et al. 1994). However, longer growth duration is often associated with a high yield potential, so using drought escape as a solution may involve a cost in terms of reduced yield potential. This is particularly a problem if the severity and timing of drought varies from year to year. Then again, some yield is always better than no yield, particularly in subsistence agriculture. Nevertheless, drought escape mechanisms are outside the scope of the current study.

Dehydration avoidance is defined as the plant capacity to sustain high plant water status or cellular hydration under the effect of drought. Hence, by this mechanism the plant avoids being stressed because plant functions are relatively unexposed to tissue dehydration. Crop plants avoid dehydration by enhanced capture of soil moisture, by limited crop water loss, and by retaining cellular hydration despite the reduction in plant water potential.

Much has been written and debated about roots and soil moisture uptake (Price et al. 2002a). All said and done, the essence of the matter is that where deep soil moisture is available a long root to reach this moisture is as effective as a long rope in a deep well. Genetic variation exists in potential root length. However, when plants are exposed to a drying soil, root morphology and growth can change to the extent that the potential root length, whether it is short or long, becomes irrelevant. In cereals, drying hard topsoil resists the penetration and establishment of adventitious roots while existing roots receive all transient assimilates and grow deeper (Blum and Ritchie, 1984; Asseng et al. 1998). It is not clear whether the capacity for developing longer roots under stress is compatible with a high yield potential phenotype. According to Price et al. (2002b), the possession of deep and thick root system allows access to water at deep soil layers and is potentially one of the most promising traits for improving drought avoidance. The ability to extract water from deeper soil layers by plants possessing such traits enable them to maintain their tissue water potential under drought conditions thereby avoiding drought stress. When all their requirements are effectively supplied, plants do not need a large root. However, under conditions of unsecured soil resources, a potentially large root is required to ensure capture of resources under erratic conditions. This form of insurance may pose a load on yield potential if a large root is expressed in large root mass.

Plants are developmentally and physiologically designed by evolution to reduce water use (WU) under drought stress (Ramanjulu and Bartels, 2002). Since plant

production is a function of WU the issue for the breeder is how to reduce WU under stress while minimising the associated reduction in production. Drought has the ability to delay developmental events due to the inhibition of growth by water. As drought progresses, growth is retarded in inverse proportion to the degree of avoidance by tissues or the whole plant (Blum, 2005). Morphological adaptations in the leaf and root contribute to efficient water use and avoidance of drought. For example, the development of hairy, waxy and shiny leaf surfaces decrease transpiration, while the reduction of leaf area and development of sunken stomata allow for efficient use of water per plant (Ramanjulu and Bartels, 2002; Blum, 2004). Physiological acclimations and adaptations like cell turgor and cell volume maintenance also lead to dehydration avoidance. This is through an increased water uptake by an extensive root system or reduced stomatal cuticular transpiration (Sanchez et al. 2002). In addition, shedding of older leaves that contribute to water saving can be viewed as a recycling programme within the plant, allowing the reallocation of nutrients stored in older leaves to the stem or younger leaves.

An increasing number of reports provides evidence on the association between high rate of osmotic adjustment (OA) and sustained yield or biomass under water-limited conditions across different crop plants, birdseed millet (Karyudi and Fletcher, 2003); chickpea (Moinuddin and Khanna-Chopra, 2004); pigeonpea (Subbarao et al. 2000) and wheat (Blum et al. 1999; Morgan, 2000). Since OA helps to maintain higher leaf relative water content (RWC) at low leaf water potential (LWP), it is evident that OA helps to sustain growth while the plant is meeting transpirational demand by reducing its LWP. Thus, OA sustains turgor maintenance and hence the yield-forming processes during moderate and severe water stress as indicated by Ali et al. (1999). Increased deep-soil moisture extraction has been found to be a major contribution of OA in sorghum (Blum, 1999). Beyond the effect on cellular hydration, other putative roles of OA have been assembled under the term osmoprotection (Rontein et al. 2002). This indicates a possible role for cell compatible osmolytes in protecting enzymes

against intensive heat inactivation. Associations between OA and cellular membrane stability under drought stress have been suggested recently by Rontein and his group (2002).

1.5.2. Drought (dehydration) tolerance mechanisms

Whereas drought avoidance is the ability to maintain relatively high tissue water potential despite a shortage of soil moisture, drought tolerance is the ability to withstand water deficit. Dehydration tolerance results from the coordination of both physiological and biochemical changes at the cellular and molecular levels. Cellular water deficit can result in the build up or concentration of solutes, changes in cell volume and membrane shape, disruption of water potential gradients, loss of turgor, disruption of membrane integrity and denaturation of protein (Bray, 1997). Drought tolerance mechanisms are controlled by the expression of many drought-induced genes (Bray, 1997). The products of these genes function in stress tolerance as well as in the regulation of other gene expression and signal transduction pathways involved in stress responses (Shinozaki and Yamaguchi-Shinozaki, 1996). Drought tolerance involves maintaining metabolism even at low tissue water potential and is influenced by osmotic adjustment and antioxidant capacity (Shinozaki and Yamaguchi-Shinozaki, 1996; Sanchez et al. 2002).

Osmotic adjustment (OA) has been considered as one of the crucial processes in plant adaptation to drought, because it sustains tissue metabolic activity and enables regrowth upon rehydration but varies greatly among genotypes (e.g. it is more important in rice or wheat than in maize; Morgan, 1984). However, in terms of crop yield there are not many field studies showing a consistent benefit from OA (Quarrie et al. 1999), presumably because turgor maintenance in cells is often associated with slow growth (Serraj and Sinclair, 2002). Nevertheless, OA is important in roots, enabling its sustained growth under decreasing water availability in the soil. It is normally a slow process and is triggered above a

certain threshold of cell water deficit. The osmotic compounds synthesised include proteins and amino acids (like proline, aspartic acid and glutamic acid) (Samuel et al. 2000; Hamilton and Heckathorn, 2001), methylated quaternary ammonium compounds (glycine betaine and alanine betaine) (Rathinasabapathi et al. 2001; Sakamoto and Murata, 2000), hydrophilic proteins (late embryogenesis abundant, LEA), carbohydrates (fructans and sucrose) (Anderson and Kohorn, 2001) and cyclitols (D-pinitol, mannitol) (Anderson and Kohorn, 2001). In addition to decreasing cell osmotic potential, thus allowing the maintenance of water absorption and cell turgor under water deficit, these solutes may protect the cell membrane and the metabolic machinery under dehydration. Compatible solutes exert their protective activity by interacting with water molecules, rather than by interacting with proteins. Osmoprotection mechanisms are probably not functional until severe dehydration occurs, which implies that OA may be critical to survival rather than to increase plant growth and crop yield under drought.

Oxidative stress is a general term used to describe a state of damage caused by reactive oxygen species (ROS), which are free radicals and peroxides derived from metabolism of oxygen (Blum, 2004). A common aspect of most adverse environmental conditions is the increased production of reactive oxygen species within several subcellular compartments of the plant cell (Van Breusegem et al., 2001). Drought stress leads to the disruption of electron transport systems and thus under water deficit conditions the main sites of ROS production in the plant cell are organelles with highly oxidizing metabolic activities or with sustained electron flows: chloroplasts, mitochondria and micro-bodies. Within the photosynthetic apparatus, photosystem II (PS II) is affected most by drought stress, particularly within the oxygen-evolving complex and the reaction centers (Price et al., 1994). In general, ROS (particularly superoxide and hydroxyl radicals) are damaging to essential cellular components such as DNA, proteins and lipids. Lipid peroxidation disrupts the membrane integrity of the plant cell. As a result, essential solutes leak out of organelles and from the cell, causing

disruption in membrane function and metabolic imbalances. DNA is the blueprint for both future form and function. Any damage to its integrity could mean that proteins that would have been essential for optimal function of the plant will not be synthesized. Similarly, denaturation of important proteins essential for biochemical reactions leads to the whole plant being negatively affected and unable to cope. Plants have evolved complex protective mechanisms to prevent the damage initiated by free radicals. The primary constituents include antioxidant enzymes such as superoxide dismutase, catalases and peroxidases, and free radical scavengers such as carotenoids, ascorbate, tocopherols and oxidized and reduced glutathione (GSSG and GSH, respectively) (Price et al., 1994). Superoxide dismutase regulates the cellular concentration of O_2^- and H_2O_2. The latter is broken down by catalases and peroxidases. Under moderate stress conditions, the radicals are efficiently scavenged by this antioxidant defense system. However, in periods of more severe stress in desiccation-sensitive plants, the scavenging system becomes saturated by the increased rate of radical production, and damage is inevitable.

1.6. Adaptations to drought by cowpea in the Sahelian region.

Rain-fed crops growing in the semiarid tropical Sahelian zone of Africa are subjected to extremely dry and hot conditions. Droughts have occurred for many years in the drier part of this zone, and have been so severe that local landraces and modern cultivars of drought hardy crops like pearl millet, sorghum and peanut have generally failed to produce significant quantities of grain. Also, virtually all cowpea landraces that had evolved in the Sahel have not produced significant quantities of grain in the years and locations with the most severe droughts due to the likely climate change. However, a number of cultivars and crop management practices have been developed by researchers that have enabled cowpeas to produce significant grain in most recent years in the Sahel (Singh et al., 2002).

1.6.1. Cowpea phenology and adaptation.

The initial attempt was to select plants with optimal numbers of days from sowing to first flowering and maturity, and appropriate plant habit. However, rainfall in the Sahel was so variable and droughts have been so extreme that this approach only was partially successful (Cisse et al., 1995). Consequently, cultivars that flower early and mature faster were considered. This was achieved by selecting plants that began flowering early and had synchronous flower production. They have an erect habit and produce their first floral buds on low main stem nodes and subsequent floral buds on the main stem and the first nodes on branches (Elawad and Hall, 2002). Cisse et al., (1995) also cultivated lines that were erect; begin flowering 30–35 days from sowing, and reach maturity within 55–64 days from sowing with the shortest cycles occurring with late-season drought. These erect plants exhibit less ground coverage than traditional Sahelian landraces that have a better spreading habit (Elawad and Hall, 2002). Another type of cultivar developed, begins flowering quite late and has a more spreading plant habit that provides it with more sequential rather than synchronous flowering and a medium cycle from sowing to maturity. According to Cisse et al., (1995), it begins flowering in about 38 days and reaches maturity 70 days from sowing. Cultivars that are more spreading and reach maturity about 75 days from sowing have been shown to be effective in the Sahel. In Ghana, a type of cultivar called 'Asontem'(matures early) exhibits greater resistance to midseason drought but has less ability to escape late-season drought and also has greater yield potential, presumably due to its longer reproductive period than the early erect cultivars (Akagbor, 2002). In years when late-season drought occurs, the early erect cultivar produces abundant grain, while the medium cycle spreading cultivar produces abundant hay but little grain. Farmers in the Sahel appreciate both the grain and the hay of cowpea, and are beginning to grow different types of cultivars (Hall et al., 2003), but, the concept of varietal intercropping has not yet been embraced by them.

1.6.2. Drought, pest and disease resistance in cowpea

There are cowpea cultivars that have substantial resistance to vegetative stage drought, like the 'California Black-eye No. 5' ('CB5') (Elawad and Hall, 2002). This cultivar is known to exhibit the ability to survive a vegetative stage drought that would have killed most other annual crop species and to recover when re-watered and produce very high grain yields of about 4000 kg/ha that were similar to a weekly irrigated control treatment (Turk et al., 1980). The vegetative-stage drought is imposed by sowing seed into a dry soil profile, providing a small amount of water with sprinklers to permit the seedlings to emerge, and then growing plants under hot sunny conditions for 43 days with no further irrigation or rain. In the Sahel where severe vegetative-stage drought occurs, cowpea plants are observed to survive, while pearl millet and peanut plants that are being grown in the same fields and had emerged at about the same time die (Singh and Matsui, 2002). The mechanisms for the resistance to vegetative-stage drought of cowpea compared with the other species have been studied. When the species were subjected to drought, using the simple box method, cowpeas survived 8–12 days longer than either pearl millet or sorghum or peanut (Singh and Matsui, 2002). The authors hypothesized that the resistance mechanism may reside in the plant shoot. Under severe droughts, cowpea exhibits greater dehydration avoidance and maintains leaf water potentials above −1.8MPa, while pearl millet, sorghum and peanut can develop leaf water potentials as negative as −4 to −9MPa under these conditions. Petrie and Hall (1992) found that pearl millet developed more negative leaf water potentials than cowpea, even at predawn with plants of the two species growing in the same pot. Petrie et al. (1992) also hypothesized that these species differences in leaf water potential may be due to pearl millet having a root system that is less effective on a micro-scale in taking up water during soil drying, even though it has more roots than cowpea. They argued that the numerous high-density clumps of roots in pearl millet would develop internal dry soil layers, such that they would only take up water at the ends of the roots. In contrast, cowpea has a more uniform root system and was hypothesized as using more of the root surface and thus being more effective in

taking up water from drying soil than pearl millet. These hypotheses were consistent with results from simulation modeling, but have not been adequately tested. Apparently, the reasons for the high level of vegetative-stage drought resistance in some cowpea cultivars are largely unknown. This indicates that many cowpeas may have substantial resistance to vegetative stage drought. A simple screening technique has been developed that uses visual observations of young plants subjected to drought and recovery while growing in shallow soil in boxes, which may be useful for selecting cowpea genotypes with the ability to survive vegetative-stage drought (Singh et al., 1999; Singh and Matsui, 2002). The research on improving drought resistance by Singh and Matsui (2002) provides a promising avenue for approaching this difficult problem; however, additional information is needed on the extent of genetic variation, mechanisms and types of resistance to vegetative-stage drought present in cowpea cultivars and accessions. In general, cowpeas are very sensitive to drought during pod set and pod filling (Turk et al., 1980). A delayed-leaf-senescence (DLS) trait has been discovered in cowpea that confers some resistance to reproductive-stage drought in erect cowpea cultivars. It begins flowering in about 35 days and produces about 2000 kg/ha of grain by 60 days, followed by a second flush of pods with the potential to produce an additional 1000 kg/ha by 100 days from sowing (Hall et al., 2003). Farm families in Africa and especially in Ghana typically harvest cowpea pods by hand and do not uproot plants such that they can make multiple harvests with individual crops. An early erect DLS cultivar might be adapted to locations in the wetter boundary of the Sahelian zone or the wetter Savanna zone where there is sufficient rainfall in most years to support a cropping season of at least 100 days but a tendency for mid-season droughts to occur.

Farmers are not likely to adopt a new cultivar simply because it has improved adaptation to drought. They are also concerned about the damage readily seen that is caused to cowpea by several pests and diseases that are prevalent in the Sahelian zone (Hall et al., 1997). Ideally one should breed cultivars that are

adapted to drought and resistant to all of the major pests and diseases that can occur in the target production zone, but this is not easy and will take several more years to accomplish for the Sahel and is beyond the scope of this study.

1.7. Genetic diversity and challenges in drought tolerance selection

The development of cultivated species and the breeding of new varieties have always relied on the availability of biological diversity, issuing from the long-term evolution of species. Modern plant breeding methods focusing on wide adaptation and high crop yield and intensive selection on crop species have raised the question of the amount of genetic variation still available in breeding pools for sustainable improvement (Baranger et al. 2004). Genetic diversity refers to the variation of genes within species, that is, the heritable variation within and between populations of organisms. Thus all variation resides in the sequence of the four base pairs that compose the DNA molecule and, as such, constitute the genetic code (Chen et al. 2002). The generation of new genetic variation occurs continuously in individuals through chromosomal and gene mutations and is also influenced by selection. These phenomena lead to changes in gene and allele frequencies that account for the evolution of populations and similar situations can occur through artificial selection such as breeding. Together with the need for biodiversity conservation, the need for an assessment of crop diversity and a better understanding of impact of breeding on this diversity has emerged. Traditionally, diversity within and between populations have been determined by assessing differences in morphology. These measures have the advantage of being readily available, do not require sophisticated equipment and are the most direct measure of phenotype and are therefore available for immediate use. However, morphological dimensions are subject to changes due to environmental factors which may vary at different developmental stages and their number is also limited.

In order to overcome the limitations of morphological traits, other markers have been developed at both the protein level (phenotype) and the DNA level (genotype). Molecular markers have allowed the study of diversity through DNA sequence variation, thus facilitating;

The understanding of crop species domestication (White and Doebley, 1998)

The deciphering of elite cultivar breeding history (Narvel et al. 2000; Russell et al. 2000)

The assessment of genetic diversity within germplasm and/or cultivated types for various species (Prasad et al. 1999; Liu et al. 2001; Métais et al. 2002; Steiger et al. 2002), and,

The identification of cultivars (Russell et al. 1997)

Recently, molecular markers have been used to examine the effect of modern plant breeding methods on genetic diversity in cereals (Russell et al. 2000; Lu and Bernardo, 2001; Chen et al. 2002; Soleimani et al. 2002; Koebner et al. 2003) and the general conclusion was that the level of genetic variability within the cultivated pools had been maintained during modern selection, either through the differentiation of heterotic groups or through the maintenance of independent breeding programmes. Cowpea breeding is facing new challenges to increase its acreage and to develop diversified products like drought tolerant varieties, disease resistant varieties and enhanced seed quality varieties. Drought, salinity and phosphorus deficiency illustrate the range of abiotic stresses that are faced by farmers in developing countries. Most crop species show considerable genetic variation in tolerance to the major climatic and chemical stresses. Plant breeding is therefore a viable option for improving productivity, reducing farmers' risks and bringing marginal land into use.

Breeding for drought tolerance is difficult because of its unpredictable nature. In drought prone areas, crop production may be stabilized and enhanced through the development of genotypes that avoid or tolerate water deficit (Grover et al.

2001). Selection for drought tolerance while maintaining overall productivity has also proved difficult due, primarily, to the lack of a simple and effective screening procedure for the required genotypes (Betran et al. 2003). Grain yield has been used to quantify drought tolerance, but according to Singh et al. (1999), this cannot be a very efficient method. This is because grain yield results from responses over the entire crop season and may not effectively discriminate between drought tolerant and susceptible genotypes. Measurement of yield stability or other traits across drought and non-drought environments has also been proposed by Blum et al. (2001). However, selection for yield stability can lead to stable but poor yielding lines under optimal conditions. Results of field tests under dry-land conditions are also difficult to interpret due to variation in drought conditions between years, while genotypes selected for one environment may show poor adaptation in another dry environment (Khanna-Chopra and Sinha, 1998; Grover et al. 1999; Khush and Baenziger, 1998). This shows that drought tolerance is influenced by both environmental and genetic variation, as well as the genetic by environment interactions. Selection for drought tolerance should therefore integrate high yield potential in good environments and the highest possible yield under drought conditions (Blum et al. 2001).

The difficulty of quantifying drought tolerance has led physiologists and plant breeders to search for specific mechanisms that are associated with tolerance or susceptibility to drought (Betran et al. 2003). If components for drought tolerance can be identified and selected independently of yield, the progress would be more rapid. Use of contrasting selections based on analyses of likely characters is inherent in this approach. However, many traits proposed as indicators of drought tolerance have little direct evidence supporting their value for drought tolerance (Blum et al. 2001). For example, high proline accumulation has been advocated as a drought resistance trait in cereals because water-stressed plants with high proline contents have high yields (Stewart and Hanson, 1980). Subsequent studies, however, have shown that the accumulation is mainly in

dead leaves, which make no contribution to plant survival or yield (Ludlow and Muchow, 1990).

Deep and dense rooting systems with high osmotic adjustment, which maximize water extraction from the soil, may not be useful under terminal drought stress under subsistence agriculture since soil moisture could be exhausted before completion of grain-filling. Maintenance of green leaf area may also lead to reduced yields under terminal drought stress, because it could promote water loss thereby increasing the probability of the crop depleting the soil water before grain-filling is completed (Singh and Matsui, 2002). It was further argued that traits which promote water uptake, like deep and early rooting, OA, early vigour, and those which increase water loss, such as large leaf-air temperature difference and green leaf area maintenance, may be useful under intermittent rather than terminal drought stress since they tend to exhaust soil moisture before completion of grain-filling.

The complex expression of drought tolerance thus makes its study difficult using traditional genetic and physiological methods alone. There is therefore the need to explore and have a better understanding of drought tolerance responses at whole-plant, physiological, biochemical, cellular and molecular levels. Plant molecular biology is a fast-expanding research frontier of our times. This important branch of science has given several clues in understanding how plants respond under stressful regimes.

1.8. Use of molecular genomics in crop improvement

Drought resistance has been established as a complex phenomenon involving drought escape, drought (dehydration) avoidance and drought (dehydration) tolerance, and desiccation tolerance mechanisms (Blum 1988 and 2002; Zhang et al. 1999; and Chaves et al. 2002). Drought resistance has also been defined based on the relative yield or survival of a genotype, compared with other genotypes subjected to the same drought where drought escape is not a major factor (Singh et al. 2002). This definition of drought resistance involves genotypic

comparisons and is, therefore, useful in the context of plant breeding in which plant productivity is the primary aim. Conventional breeding methods have depended mainly on plant performance such as yield or secondary traits highly associated with yield (e.g., anthesis-silking interval in maize or stay green in sorghum) under stress environments as a selection criterion. This approach has produced crop cultivars with improved adaptation and performance under stress

In the past, breeding efforts to improve drought tolerance were hindered by its quantitative genetic basis and the poor understanding of the physiological basis of yield in water-limited conditions (Blum, 1988; Passioura, 2002). Further complexity derived from the occurrence of other abiotic stresses that often amplify the negative impact of drought on growth and metabolism (Mittler, 2005). From an application point of view, it is crucially important to select genotypes able to optimize water harvest and water-use efficiency while maximizing yield in relation to the dynamics of the drought episodes prevailing in each target environment (Mittler, 2005). Compared to conventional approaches, genomics offers unprecedented opportunities for dissecting quantitative traits into their single genetic determinants, that is, quantitative trait loci (QTL), thus paving the way for other manipulations.

Genomics is the applications involved in the determination of a DNA sequence and the identification of the location and function of all the genes contained in the genome of an organism. The advent of large scale sequencing of entire genomes of organisms as diverse as bacteria, fungi, plants, and animals, has led to the identification of the complete complement of genes found in many different organisms. The discipline of functional genomics emerged largely in response to the challenge posed by complete genome sequences. This challenge is to understand the biochemical and physiological function of every gene product, and the complex interplay between them all. Global analyses of various levels of molecular organization have been facilitated by remarkable developments in high throughput technologies. These methods and recent developments in this area

have recently been reviewed by Colebatch and his collaborators (2002). Large scale expressed sequence tags (EST) and genome sequencing projects in different legumes have provided huge datasets for the study of genome organization and transcription in these plants. Until a few years ago, gene expression analysis was confined to one or a few genes at a time, and it was virtually impossible to identify entire sets of genes involved in a common process that are coordinately regulated. Increased numbers of publicly available nucleotide sequences together with development of technologies for high-throughput measurement of gene transcript levels have profoundly changed the way in which molecular biologists approach biological questions. Now it is possible to survey the expression level of thousands of genes in parallel, which not only enables the identification of 'candidate' genes involved in a given biological phenomenon, but also reveals sets of genes and the corresponding biochemical processes that act in concert to achieve a specific biological outcome.

1.8.1. Drought-related traits

Some of the most difficult tasks of plant breeders relate to the improvement of traits that show a continuous range of values. Among such quantitative traits are important traits like yield, plant length and days to flowering (speed of plant development). Selection for quantitative traits is difficult, because the relationship between observed trait values in the field and the underlying genetic constitution is not straightforward. These traits are typically controlled by many genes that each contributes only a small part to the observed variation. Given that the morpho-physiological traits that affect the tolerance of crops to drought are quantitatively inherited (Blum, 1988), the discovery of QTLs plays a central role in their improvement through marker assisted selection (MAS). One major approach to understand and simplifying the genetics of drought tolerance focuses on mapping QTL that condition drought-related physiological traits. Many of the drought-related traits studied at the level of QTL analysis relate to root behaviour. Key QTL have been mapped for root morphology, root distribution

and drought avoidance (Champoux et al. 1995; Price and Tomos, 1997; Yadav et al. 1997; Ali et al. 1999; Courtois et al. 2000; Kamoshita et al. 2000), root penetrating ability (Ray et al. 1974; Price et al. 2000; Zheng et al. 1999a), osmotic adjustment and dehydration tolerance (Lilley et al. 1996; Zheng et al. 1999b), cell-membrane stability (Tripathy et al. 2000) and ABA accumulation (Quarrie et al. 1997).

QTL for drought-related traits have also been reported for other crops, especially cereals. These traits include osmotic adjustment in wheat and barley (Thompson et al. 1998), and the stay-green trait in sorghum (Xu, K. et al. 1998). In spite of changes in the chromosome number, the genomes of the cereals display a high degree of synteny, or conservation of gene order, along homologous chromosomes (Gale et al. 2001). As a result it is sometimes possible to predict the genomic location of gene in one cereal from its known location in another cereal. Orthologous genes for osmotic adjustment in barley, rice and wheat have been located in syntenic regions of the respective genomes by QTL mapping (Zhang et al. 1999).

1.8.2. Candidate genes for drought tolerance

Candidate gene analysis starts with selection of some target genes based on biological pathway or genome location relative to a known QTL identified for the target trait (Byrne and McMullen 1996; Rothschild and Soller 1997). A drought-related EST database, microarray analysis, and the mutagenesis approach will yield a large number of valuable candidates for verification of their association with the drought tolerance traits. Alternatively, searches can be conducted for orthologs in existing literature and databases for information on drought and related abiotic stress genes. In recent years many drought-responsive genes have been identified in plants, especially in *Arabidopsis thaliana*. These genes have been identified using approaches like;

- Studies on the anabolic and catabolic pathways for metabolites that accumulate in drought-stressed plants (e.g. proline, glycine, betaine, trehalose, ABA).
- Analysis of other mechanisms of drought tolerance, such as water use efficiency.
- Analysis of protein or mRNA changes in response to drought.
- Analysis of signal-transduction pathways.
- Mapping of QTL for drought tolerance, using segregation populations.

All accessions of a particular crop species are expected to contain essentially the same genes (Mullet et al. 2001), although evidence from maize may suggest some locational hyper variability exist. Differences in agricultural performance between accessions are thought to be due to allelic differences within the same gene set. Thus, achieving a high level of drought tolerance depends on finding the most appropriate alleles of key genes and combining or pyramiding them together.

1.8.3. Molecular markers for genome analysis

Since the advent of molecular markers, a new generation of markers has been introduced over the last two decades, which has revolutionized the entire scenario of molecular biology. Due to the limitations associated with the use of morphological traits as pointed out before, other markers have been developed at both the protein level (phenotype) and the DNA level (genotype). Protein markers are usually referred to as biochemical markers but, more and more, they are mistakenly considered as a common class under molecular markers. Protein markers like seed storage proteins and isozymes are generated through electrophoresis, taking advantage of the migrational properties of proteins and enzymes, and revealed by histochemical stains specific to the enzymes being assayed. Detecting polymorphisms in protein markers is a technique that shares some of the advantages of using morphological traits. However, protein markers

are also limited by being influenced by the environment and changes in different developmental stages (Piepho and Koch, 2000). DNA polymorphisms can be detected in nuclear and organellar DNA, which is found in mitochondria and chloroplasts. Molecular markers concern the DNA molecule itself and as such, are considered to be objective measures of variation. They are not subject to environmental influences and tests can be carried out at any time during plant development, although if they involve restriction enzymes, they can be subject to methylation variation and epigenetic change. Most importantly, they have the potential of existing in unlimited numbers, covering the entire genome.

Various types of molecular markers are utilized to evaluate DNA polymorphism and are generally classified as hybridization-based markers and polymerase chain reaction (PCR)-based markers. In the former, DNA profiles are visualized by hybridizing the restriction enzyme-digested DNA, to a labeled probe, which is a DNA fragment of known origin or sequence. PCR-based markers involve *in vitro* amplification of particular DNA sequence or loci, with the help of specifically or arbitrarily chosen oligonucleotide sequences (primers) and a thermostable DNA polymerase enzyme. Ideally markers should be economical, independent of each other, robust, frequently occurring and polymorphic. The experimental requirements are such that they should be easy to assay, rapid, show high reproducibility, be exchangeable between laboratories and have the potential to be automated. Co-dominant inheritance is preferable to dominant, since it allows the heterozygous to be identified from the homozygous state (Piepho and Koch, 2000).

Restriction fragment length polymorphisms (RFLP) are generally simply inherited naturally occurring Mendelian characters. They have their origin in the DNA rearrangements that occur due to evolutionary processes, point mutations within the restriction enzyme recognition site sequences, insertions or deletions within the fragments, and unequal crossing over (Schlotterer and Tautz, 1992). RFLP markers were used for the first time in the construction of genetic maps by

Botstein et al. (1980). Being co-dominant markers, they can detect coupling phase of DNA molecules, as DNA fragments from all homologous and orthologous chromosomes are detected. They are very reliable markers in breeding, linkage analysis and can also easily determine whether a linked trait is present in a homozygous or heterozygous state in an individual (Winter and Kahl, 1995). However the use of RFLPs has been hampered due to the large amount of DNA required for restriction digestion and Southern blotting. The usual requirement of radioactive isotope makes the analysis relatively expensive and hazardous. The inability of RFLPs to detect every single base changes also restricts their use in detecting point mutations within the regions at which they are detecting polymorphism. To overcome some of these challenges in genome analysis RFLP markers have been converted into PCR-based markers like sequence-tagged sites (STS), which are useful for studying the relationship between various species (Bruce et al. 2002), allele-specific associated primers (ASAP) which tag specific alleles in the genome and are more or less similar to sequence characterized amplified region (SCAR) markers (Guo et al. 1995), and expressed sequence tag (EST) based markers obtained by partial sequencing of random cDNA clones (Akkaya and Buyukunal-Bal, 2004). Once generated, they are useful in cloning specific genes of interest and synteny mapping of functional genes in various related organisms.

A major step forward in genetic identification is the discovery that about 30-90% of the genome of virtually all the species is constituted by regions of repetitive DNA, which can be highly polymorphic in nature (Kidwell, 2002). These regions contain genetic loci comprising several to hundred of thousands alleles, differing from each other with respect to length, sequence or both and they are interspersed in tandem arrays ubiquitously. The markers belonging to this class are both hybridization-based and PCR-based. Microsatellite based genetic markers, which are distributed across genomes of most of the eukaryotes, fit into this category of molecular markers. Microsatellites also known as simple sequence repeats (SSRs) are short stretches of DNA which consist of an array of

simple tandemly repeated mono, di-, tri-, tetra-, penta or hexanucleotide repeats such as $(A)_n$, $(CA)_n$, $(GA)_n$, $(GTA)_n$, $(ATT)_n$, $(GATA)_n$, $(ATTTT)_n$, $(ACGTCG)_n$. The term microsatellites was coined by Litt and Lutty (1989) and are multi-locus probes creating complex banding patterns. They are ubiquitous in prokaryotic and eukaryotic genomes and are randomly distributed, both in protein coding and non-coding regions. A unique oligonucleotide on each side of the repeat region is chosen for the production of a primer pair for each of the microsatellite loci. PCR products of different lengths can be amplified using primers flanking the variable microsatellite region. Allelic variation among individuals is based mostly on differences in the number of tandem repeats in a microsatellite array providing a ready source of polymorphism. Thus, the only way in which alleles can be distinguished is by measuring the total length of the microsatellite allele. This is most readily accomplished through PCR amplification of the microsatellite itself along with a short stretch of defined flanking sequences on both sides with designed primer pairs for each locus.

A large number of transposable repeat elements have also been studied as part of the repetitive DNA type markers in plants. The major repetitive DNA sequences are divided into two classes – tandem repeat non-coding sequences and dispersed sequences. The former include telomeric repeats, satellites, minisatellites and microsatellites, whereas the latter are primarily composed of transposable elements (TEs) (Kumar and Bennetzen, 1999; Heslop-Harrison, 2000). However, only a few have been exploited as molecular markers. In evolutionary terms, they have contributed to genetic differences between species and individuals by playing a role in retrotransposition events promoting unequal crossing over. Of the various techniques available, inter-retrotransposon amplified polymorphism (IRAP) and REMAP (Kalender et al. 1999) detect high levels of polymorphism without the need of DNA digestion, ligations or probe hybridization to generate marker data, thus increasing the reliability and robustness of the assay. More recently, markers based on PCR have been developed, including random amplified polymorphic DNA (RAPD) (Williams et al.

1990), inter simple sequence repeat (ISSR) (Zietkiewicz et al. 1999), amplified fragment length polymorphism (AFLP) (Vos et al. 1995), single nucleotide polymorphism (SNP) and cleaved amplified polymorphic sequences (CAPs). The genetic similarity in cultivated cowpea has been assessed on the basis of morphological and physiological traits (Ehlers and Hall, 1997), allozymes (Panella and Gepts, 1992; Pasquet, 1993, 1999; Vaillancourt et al., 1993), RFLP (Fatokun et al., 1993), AFLP (Fatokun et al., 1997), and RAPD (Mignouna et al., 1998).

1.8.4. Applications of molecular markers for selection and breeding

Molecular markers have been looked upon as tools for a large number of applications ranging from localization of a gene to improvement of plant varieties by marker-assisted selection. They have also become extremely popular markers for phylogenetic analysis adding new dimensions to the evolutionary theories. Plant improvement, either by natural selection or through the efforts of breeders, has always relied on creating or combining, evaluating and selecting the right combination of alleles. The manipulation of a large number of genes is often required for improvement of even the simplest of characteristics (Flavell, 1998). With the use of molecular markers it is now easy to trace valuable alleles in a segregating population and mapping them. These markers once mapped enable dissection of the complex traits into component genetic units more precisely, as indicated by Hayes (1995), thus providing breeders with new tools to manage these complex units more efficiently in breeding programme. Similar to specific markers like RFLPs, arbitrary markers like RAPDs have also played an important role in saturation of the genetic linkage maps and gene tagging. Apart from mapping and tagging of genes, an important utility of RFLP markers has been observed in detecting gene introgression in a backcross breeding programme (Jena and Kush, 1990), and synteny mapping among closely related species (Ribaut et al. 1997).

Most of the early theories of evolution were based on morphological and geographical variations between organisms. However, it is now evident that the techniques from molecular biology hold a promise of providing detailed information about the genetic structure of natural populations. Various molecular markers like RFLPs, SSRs, IRAPs, and REMAPs have proved to be extremely valuable in the analysis of gene pool variation of crops during the process of cultivar development, and classification of germplasm (Yang et al. 1994). Following domestication, genetic variation in crop plants has continued to narrow due to continuous selection pressure for specific traits like yield, thus rendering them more vulnerable to disease and insect epidemics and jeopardizing the potential for sustained genetic improvement over a long term (Mignouna et al., 1998). It is therefore extremely important to study the genetic composition of the germplasm using molecular markers and such studies are necessary to understand the genetic variations between the existing cultivars, for effective planning of crosses and breeding for the trait of interest.

1.8.5. Current advances in cowpea genomics

The development and use of biochemical-based analytical techniques and molecular technologies, such as analysis of RFLPs, RAPDs, AFLPs, SSRs, IRAP and many others have greatly facilitated the analysis of the structure of plant genomes and their evolution and have contributed significantly to the understanding of cowpea genome organization. Using RFLP analysis, Fatokun et al. (1993) analyzed 18 *Vigna* species including five of the subgenus *Ceratotropis* to determine the taxonomic relationship between the subgenus *Ceratotropis* and other subgenera. The results indicated that a high level of genetic variation exists within the genus, with a remarkably higher amount of variation associated with *Vigna* species from Africa relative to those from Asia. Genetic variation in the subgenus *Ceratotropis* was reinvestigated by Kaga et al. (1996a) using RAPD analysis. They examined 23 accessions of five species within the subgenus to determine possible polymorphisms, and identified approximately 404 amplified fragments capable of providing comparative

information. Based on the degree of polymorphism at these informative loci, they were able to separate the accessions into two main groups differing by about 70% at the molecular level. Statistical analysis was carried out using NTSYS-pc software and a dendrogram was generated using Dice's similarity coefficients.

Sonnante et al. (1996) examined isozyme (a biochemical marker) variation between *V. unguiculata* and other species in the subgenus *Vigna* and showed that *V. unguiculata* was more closely related to *V. vexillata*, a member of the subgenus *Plectotropis*, than to any other species belonging to the section *Vigna.* Polymorphisms in 21 different enzyme systems were used by Pasquet (1999) to evaluate the relationship among 199 accessions of wild and cultivated cowpea differing in breeding system and growth characteristics (annual versus perennial growth habit). Based on these allozymes data, perennial subspecies of cowpea (spp. *unguiculata* var. *unguiculata*) were shown to form a coherent group closely related to annual forms (spp. *unguiculata* var. *spontaea*). Among the 10 subspecies studied, V. *unguiculata* var. *spontanea* and ssp. *pubescens* were the closest taxa to be cultivated into cowpea.

Repetitive DNA sequences have been shown to represent a substantial fraction of the nuclear genome of all higher plant species and to account for much of the variation in genomic DNA content observed among species (Flavell et al. 1998). Many of the repeat sequences found in plant genomes appear to have originated through the activity of transposable elements (transposons), that move either by first forming an RNA intermediate (i.e., retrotransposons [Boeke et al. 1985]) or by direct DNA transposition intermediates (i.e., transposons [Federoff 1989]). To gain insight into the genomic organization and evolution of species within *Vigna*, Galasso et al. (1997) examined the genomic organization and distribution of Ty1-copia type retrotransposons in seven different species and subspecies of *Vigna* and several related leguminous plants. Gel blot analysis of genomic DNA from *V.*

unguiculata, *V. luteola, V. oblongifolia, V. ambacensis*, and *V. vexillata* probed with radioactively-labelled probes to the reverse transcriptase gene amplified from *V. unguiculata* subsp. *unguiculata, V. unguiculata subsp. dekindtiana, V. luteola*, and *V. vexillata*, showed variable hybridization patterns and intensities generally correlating with their previously defined taxonomic position. Fluorescent in situ hybridization (FISH) analysis of the distribution of the Ty1-copia type sequences showed that these elements represented a major fraction of the cowpea genome and were dispersed relatively uniformly over all the chromosomes. Comparisons of retrotransposon structural similarity between *Vigna* and other genera of legumes generally supported the subdivision of the tribes *Phaseoleae* and *Vicieae*, with greater homology seen between members of the *Cicereae* and *Phaseoleae* than *Cicer* species and those from the *Vicieae* (Galasso et al. 1997).

In addition to providing insight into phylogenetic relationships, molecular marker technologies have also been used in the construction of genetic maps for most of the important crop species, including cowpea. The first attempt to generate a comprehensive linkage map for cowpea was by Fatokun et al. (1993) who used polymorphisms detected by 87 random genomic DNA fragments, five cDNAs, and RAPDs to generate a map consisting of ten linkage groups spanning 680 cM. Menacio-Hautea et al. (1993a, b), Kaga et al. (1996b), Menéndez et al. (1997), and many other researchers have contributed in the construction of the genetic map of cowpea using different approaches. Among the most recent developments in understanding cowpea genome organization is the report by Li et al. (1999) who used DNA amplification fingerprinting (DAF) and AFLP analysis to identify additional molecular markers segregating in the F_8 recombinant inbred population derived from a cross between two related species. The considerable progress made in recent years on the development of genomic maps for cowpea and related species is reflected in the ever increasing number of growth, yield, and resistance trait loci that have now been located within the various genomes

(Fatokun et al. 1992, 1997; Menéndez et al. 1997; Myers et al. 1998; Ouédraogo et al. 2001; Gowda et al. 2003).

Significant progress has been made at the International Institute of Tropical Agriculture (IITA), based in Nigeria, in an attempt to develop cowpea drought tolerant genotypes. For example early-maturing cowpea varieties that escape terminal drought have been developed (Singh, 1999b). Two major different drought tolerant lines have also been identified. Those that cease growing as soon as drought stress is imposed, probably to conserve moisture and survive for 2-3 weeks and those that mobilize moisture from lower leaves and remain alive for a longer time (Singh et al. 1999). Consequently, these varieties have a better regeneration potential than others do. A simple technique, using wooden boxes, has been developed to screen germplasm lines at seedling stage, and test their field performance at mature stages under conditions of water deficit. This technique has been found to be more appropriate for breeding programmes in developing countries. Efforts are also being made to combine deep root systems with drought tolerance, to enhance adaptation of cowpeas to low rainfall areas (Watanabe, 1993).

1.9. Objectives and general outline of the study

Cowpea (*Vigna unguiculata*) is an important tropical legume with a high protein content of about 25%, a cheap source of protein for the poor in the West African region, where more than 70% of the total production is grown. However, production is constrained by low and variable grain yields, grain quality and susceptibility to diseases and pests, drought and temperature stresses. Although yields of 2500 kg/ha are achievable, farmer's yields are consistently low at levels between 350 kg/ha and 700 kg/ha. Progress with genetic improvement, particularly for drought tolerance traits, is limited by the lack of knowledge on genetic diversity of the indigenous and cultivated germplasm. Moreover, cowpea is a single crop species, but the varietal requirements in terms of plant type, maturity date, seed type (colour preference), and use pattern are extremely

diverse from region to region, making breeding programmes for cowpea more complex than for other crops. From the review thus far, it is clear that drought stress is a major cause of yield losses in many crops including cowpeas in rain-fed agricultural ecosystems world-wide. There is competition for the limited sources of fresh water for domestic, industrial and agricultural uses, which requires the development of crops with improved productivity for small amounts of water used.

It was therefore hypothesized that the solutions to these problems could be found in the gene pool of the cowpea plant due to the fact that genes for tolerance to most stresses are present in some cowpea accessions or in other species, and also only plant adaptation through the gene pool can meet the challenges in breeding for drought tolerance. The problem was to find and exploit the genetic variation in the cowpea varieties sampled from Ghana. Molecular biology plays a major role in finding, measuring and using genetic variation in speeding up screening processes for breeding of traits of interest and also allows the genes of interest to be transferred. Therefore, both physiological and molecular technological approaches were employed in this study.

The principal objective of the study was to identify DNA polymorphisms linked to drought stress that could be used in breeding programmes for drought tolerance (avoidance and escape) in cowpea. The specific objectives were to:

- screen and identify drought tolerant cowpea varieties from accessions obtained from Ghana,
- establish the pattern of diversity and phylogenetic relationship amongst the cowpea varieties using microsatellite and other PCR-based molecular markers,
- identify and evaluate candidate abiotic-stress related genes targeted at drought tolerance in cowpea,
- determine gene expression under drought that would confer some degree of drought tolerance in cowpea.

Thus emphasis for this study has been on four areas of research as:

- Screening of the cowpea varieties from Ghana for drought tolerance using the wooden box technique and other morphological sampling methods.
- Application of multi locus PCR-based DNA molecular markers to determine genetic diversity and phylogenetic relationships in the accessions from Ghana and other areas.
- Identification and evaluation of candidate abiotic stress-related genes targeted at drought tolerance in cowpea varieties using genes from established drought resistance crops.
- Identification of genes that are differentially expressed in droughted cowpea and non-stressed counterparts for comparative analysis.

If poorly resourced farmers in Ghana have access to cultivars with enhanced tolerance of abiotic stresses, they would reduce their economic risks, improve the livelihood and nutrition of their families, put their marginal land to work, and protect the environment by providing good farming practices. This is what the current study hopes to achieve in the long term.

In chapter two the materials used for the study were thoroughly described and discussed. The methods used for the collection of physiological and molecular data for all the experiments in this study have also been described here. In chapter three a greenhouse experiment using cowpea accessions is described, aimed at screening for drought tolerant and susceptible variants within the accessions using the wooden box technique. Various physiological and morphological parameters were measured and the results used in the determination of the tolerant and susceptible variants. Chapter four involved the molecular study to determine the molecular genetic diversity within and among the Ghanaian accessions of cowpea and those from Nigeria and United Kingdom. In this chapter multi locus PCR-based DNA molecular markers were used to determine polymorphisms and the results were compared with the results

in chapter three to determine any linkages and associations to drought tolerance. In chapter five the identification and evaluation of candidate abiotic stress-related genes targeted at drought tolerance in cowpea varieties using genes from established drought resistance crops was pursued. Specific primer pairs were designed from the sequences of these selected genes for cloning and characterisation. The aim was to ascertain whether these genes were expressed constitutively or otherwise. Chapter six involved the comparative analysis of gene expression in drought stressed and unstressed cowpea plants. The aim was to use the differential display technique to identify and characterise the various genes expressed in these conditions for comparative analysis. In chapter seven an attempt has been made to correlate all the results from physiology, geographical origin, polymorphisms from molecular markers, candidate genes and novel genes from differential display experiments of the cowpea accessions from Ghana to ascertain any linkages in terms of drought tolerance and other biotic stresses. Finally, chapter eight has been devoted for the general discussions of the study, conclusions and recommendations for further research work.

CHAPTER TWO

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CHAPTER TWO

2.0 General materials and methods

The materials and methods used for the collection of the physiological and molecular data for all the experiments in the study are described in this chapter.

2.1 Seed Material

Cowpea seeds were obtained from the germplasm collections of the Plant genetic resources centre (PGRC), which is under the Centre for scientific and industrial research (CSIR), at Bunso, in Ghana, as well as from the open markets and farmers across the length and breadth of Ghana, to reflect farmer and consumer preference of the crop. The seed material obtained from the PGRC is part of the core collection being held for conservation and breeding purposes. All the seed materials obtained from the open market and from farm holdings were properly identified at the PGRC as being part of the collection available to the institute.

In chapter three, experiment one was conducted where 106 cowpea cultivars all from Ghana, were screened for drought tolerance, whilst a total of 121 samples comprising 106 cultivars from Ghana, 10 DNA samples of cowpea cultivars obtained from the International institute for tropical agriculture (IITA) in Nigeria and 5 cowpea accessions bought from the open market in Leicester, United Kingdom, were used for the microsatellite, IRAP and REMAP analysis for polymorphisms and diversity analysis in experiment two, chapter four. For experiment three in chapter five, 30 samples of which 24 were from Ghana, 4 from Nigeria and 2 from Leicester, UK, were selected randomly for the candidate genes and known drought gene structure analysis. For chapter six where experiment four was discussed, 6 samples from Ghana were also selected randomly for the differential display and gene expression experimentations.

Table 2.1 Cowpea accessions used for the study from Ghana

Accession No.	collection No.	Locality	Accession No.	collection No.	Locality
GH 3665	GJ 93/015	Srekpo	GH 5345	OAA 96/135	Tanfeano
GH 6048	KD 98/010	Kumasi	GH 5049	AMO 96/164	Okumaning
GH 4767	GA 96/023	Wa	GH 2313	87/91	Wa
GH 2289	87/41	Tuwuwa	GH 4778	GA 96/137	Siriyiri
GH 4026	SO 96/003	Owusukrom	GH 2342	87/157	Abene
GH 3701	GJ 93/244	Kpong	GH 5045	AMO 96/107	Sutapong
GH 2328	87/135	Akora Darko	GH 4526	BTB 96/042	Kasseh
GH 2314	87/94	Kampaha	GH 4530	BTB 96/055	Kasseh
GH 4773	GA 96/063	Lawra	GH 3685	GJ 93/163	Fukuokrom
GH 2326	87/133	Akora Darko	GH 4083	SO 96/060	Pimpimso
GH 4537	BTB 96/150	Ziope	GH 2302	87/66	Navrongo
GH 2312	87/90	Sombo	GH 2329	87/136	Akora Darko
GH 2291	87/49	Ziong	GH 3671	GJ 93/031	Tedafenu
GH 3678	GJ 93/105	Takunya	GH 3675	GJ 93/087	Sench-Ferry
GH 5048	AMO 96/131	Suminakese	GH 623O	AOA 96/061	Gyankoma
GH 5346	OAA 96/150	Ayakomaso	GH 4024	SO 96/001	Owusukrom
GH 5046	AMO 96/128	Suminakese	GH 3667	GJ 93/026	Juapong
GH 4527	BTB 96/043	Kasseh	GH 2334	87/142	Akora Darko
GH 4772	GA 96/052	Serekpere	GH 5042	AMO 96/062	Ahabasu Gyaesu
GH 4532	BTB 96/091	Dabala	GH 3683	GJ 93/116	Govinakrom
GH 3674	GJ 93/075	Jame Anyinawas	GH 5038	AMO 96/030	Akuni No.2
GH 4770	GA 96/046	Wa	GH 4025	SO 96/002A	Owusukrom
GH 5040	AMO 96/060	Ahabaso Gyaesu	GH 3706	GJ 93/294	Tanina
GH 4529	BTB 96/054	Kasseh	GH 2316	87/103	Ga
GH 4542	BTB 96/213	Kpando	GH 3668	GJ 93/027	Juapong
GH 2338	87/150	Bepong Kwahu	GH 3687	GJ 93/219	Babato
GH 3673	GJ 93/074	Jame Anyinawas	GH 6047	KD 98/009	Fumesua
GH 3710	GJ 93/311	Nabori	GH 4528	BTB 96/048	Kassah
GH 4534	BTB 96/106	Nyinguto	GH 4548	BTB 96/261	Agormanya
GH 3689	GJ 93/243	Defaa	GH 3679	GJ 93/105	Takunya
GH 2284	87/34	Tua	GH 4546	BTB 96/238	Sanga
GH4028	SO 96/005	Owusukrom	GH 3703	GJ 93/250	Loagri
GH 4769	GA 96/045	Wa	GH 2285	87/35	Sanga
GH 2279	87/24	Tianjeni	GH 5043	AMO 96/084	New Tafo
GH 2306	87/73	Tumu	GH 3669	GJ 93/030	Juapong
GH 2332	87/139	Akora Darko	GH 2315	87/96	Manweh
GH 6045	KD 98/007	Fumesua	GH 4774	GA 96/074	Lwara
GH 2347	87/220	Nkurakan	GH 5039	AMO 96/038	Abonse
GH 5044	AMO 96/105	Sutapong	GH 2310	87/83	Nandom
GH 2337	87/147	Ahomahomaso	GH 1608	82/163	Abene
GH 3666	GJ 93/023	Juapong	GH 2321	87/121	Sawla
GH 5344	OAA 96/046	Juaben	GH 3670	GJ 93/031	Tedafenu
GH 2318	87/113	Nasoyiri	GH 6046	KD 98/008	Fumesua
GH 3711	GJ 93/315	Kunkunde	GH 6061	BAG 96/008	Nsawam
GH 4027	SO 96/004	Owusukrom	GH 4765	GA 96/001	Siriyiri
GH 5041	AMO 96/061	Ahabaso Gyaesu	GH 3704	GJ 93/266	Kandiga
GH 4549	BTB 96/262	Agormenya	GH 4535	BTB 96/129	Vidadakope
GH 4541	BTB 96/154	Ziope	GH 1001	EP 05/120	Takoradi
GH 2281	87/27	Labaraga	GH 1002	EP 05/121	Mankesim
GH 3708	GJ 93/303	Grupe	GH 1003	EP 05/122	Obuasi
GH 2336	87/145	Ahomahomaso	GH 1004	EP 05/123	Sunyani
GH 2275	87/08	Kintampo	GH 1005	EP 05/124	Bechem
GH 5050	AMO 96/204	Kokoben	GH 1006	EP 05/125	Cape Coast

Table 2.2 Cowpea samples from Nigeria-IITA and United Kingdom

Accession No.	Country of Collection
IT91K-118	IITA-Nigeria
IT86D-716	IITA-Nigeria
IT89KD-374	IITA-Nigeria
IT85F-1380	IITA-Nigeria
IT93K-699	IITA-Nigeria
IT93K-596	IITA-Nigeria
IT92KD-267	IITA-Nigeria
IT93K-2309	IITA-Nigeria
IT88D-643	IITA-Nigeria
IT810-1228	IITA-Nigeria
Leicester-001	United Kingdom
Leicester-002	United Kingdom
Leicester-003	United Kingdom
Leicester-004	United Kingdom
Leicester-005	United Kingdom

2.2 Plant culture and treatment

All plants were raised from seeds. Three seeds were sown in a 3.0 litre pot filled to the rim with a mixture of enriched Levington seed and modular compost, produced and supplied by Scotts UK Professionals, peat and perlite in a ratio of 7:2:1 respectively. The pots had perforations underneath them for effective drainage. The plants for each genotype were grouped but randomly positioned in the glasshouse.

For experiment one, each genotype had two sets of three replications, with one set serving as a control for the drought treatment. Seedlings were exposed to a night temperature of 15˚C increasing to a maximum of 35˚C during the day with a 10-hour supplementary light to stabilise the temperature regimes. The plants were kept well watered until the first trifoliate leaves were fully established after which watering was terminated in the drought treated plants whilst the controls were watered throughout the experimental period for genotype variability analysis. Trays holding the pots were rotated daily to minimize variation caused

by microclimates in the greenhouse. Days taken to permanent wilting (DPW) were recorded at 7 days interval for each genotype by observing the shoot for any phenotypic changes until 35 days after the drought treatment has began, then watering was resumed and data on percent recovery for each line recorded seven days after re-irrigation. Based on the days taken to permanent wilting and percent recovery the lines were classified into drought tolerant and drought susceptible whilst the International Board for Plant Genetic Resources (IBPGR) descriptors for cowpea was used for the qualitative and quantitative trait evaluation.

In experiments two (chapter four) and three (chapter five), the plants were raised from seeds as earlier described and after the plants were well established, fresh young leaves were harvested, wrapped in aluminium foil and flash-frozen in liquid nitrogen and the genomic DNA extracted from these young leaves following a modified cetyltrimethylammonium bromide (CTAB) method (Gawel et al., 1991).

For experiment four (chapter six) the plants were raised as before, but in this case two sets were used with one set serving as a control. The plants were watered until the seedlings were well established, then watering was stopped in the experimental set. Leaves were then harvested at 5 days interval, wrapped in aluminium foil, flash-frozen in liquid nitrogen and kept in -80°C freezer. The harvesting of leaves continued until the plants were almost dying, then watering was resumed though the harvesting of leaves continued as scheduled. Total RNA was extracted from the harvested leaves and kept at -80°C for the differential display gene expression analysis.

2.3 DNA extraction

Total genomic DNA was extracted from fresh leaves of cowpea using the following cetyltrimethylammonium bromide procedure (Gawel et al., 1991) with minor modifications.

1. Grind frozen leaf material (1 g) to fine powder in a mortar and pestle under liquid nitrogen to prevent enzymatic degradation and the release of phenolic compounds from wounded tissue. Transfer into a 50 ml Falcon tube
2. Add 20 ml preheated CTAB extraction buffer (Table 2.2) and 20 μl mercaptoethanol, then incubate at 60°C for two hours with continuous gentle shaking in a water-bath.
3. Add equal volume of Chloroform/ iso-amyl alcohol (24:1) to the mixture and mix thoroughly for five minutes by gentle inversion of tubes at room temperature.
4. Centrifuge at 5000 rpm for ten minutes. Transfer the supernatant into a new 50 ml Falcon tube.
5. Repeat steps 3 and 4.
6. Precipitate DNA with 0.6 volume of cold (-20°C) isopropanol. Allow the precipitation stage to stand for thirty minutes on ice after mixing thoroughly by inverting the tubes gently for three minutes. Then centrifuge at 4090 rpm for five minutes.
7. Transfer the DNA pellets from step six into 5 ml wash buffer and allow it to stand on ice for twenty minutes before centrifuging at 5000 rpm for five minutes.
8. Dry the precipitate thoroughly in 37°C incubator and re-suspend in about 50 -100 μl of T.E. buffer.
9. Add 1 μl (10 ng/ml) RNase to each 1 ml T.E/DNA mixture and incubate for thirty minutes at 37°C.
10. Re-precipitate the DNA by adding 2 volumes of T.E. buffer, 0.1 volume of 3 M sodium acetate (pH 8), and 2 volumes of cold (-20°C) 100% ethanol.
11. Centrifuge at 14000 rpm at 4°C for twenty minutes.

12. Discard supernatant and add 70% ethanol. Allow it to stand for ten minutes on ice and centrifuge at 14000 rpm at 4°C for ten minutes.
13. Discard the ethanol and dry the DNA pellets thoroughly before adding 50 -100 μl of T. E buffer and store at -20°C.

Table 2.2 CTAB Buffer (1 Litre) pH 7.5-8.5

Material / Reagent	**Weight / Volume**
2% CTAB	20 g CTAB
100 mM Tris-Cl (pH 8.0)	100 ml Tris-Cl stock (1M)
20 mM EDTA	40 ml EDTA stock (0.5 M)
1.4 M NaCl	280 ml NaCl stock (5 M)

2.3.1. DNA quantification

The stored DNA samples were thawed; mixed thoroughly and 5 μl added to 995 μl of sterile distilled water (SDW) in a 1.5 ml micro-centrifuge tube and mixed well before reading the absorbance at 260 and 280 nm using a diode array scanning spectrophotometer, Hewlett Packard model. DNA quantity was calculated as;

[DNA] μg / μl = [A_{260} x 50], where A_{260} is the absorbance at 260 nm.

Thus, the concentration of DNA in μg / ml was calculated as;

[DNA] μg / ml = [A_{260} x 50] x DF, where DF is the dilution factor.

From the quantities of DNA calculated, the appropriate volume was pipetted into sample tubes and topped up with SDW to make concentrations of 20-50 ng / μl used for polymerase chain reaction (PCR) amplifications. The ratios of the absorbance at 260 nm to that at 280 nm were used to determine the purity of the DNA. Samples with ratios of 1.8 or greater were used for PCR amplification.

The DNA samples were also run on 1.5% agarose gels stained with 0.5 µg / ml ethidium bromide to assess their integrity. A mixture of 5 µl DNA, 10 µl of SDW and 2 µl of loading buffer were made and loaded into each well. The gels were run with 1x TAE buffer from the cathode to the anode with a constant voltage of 100 V for 1 hour. After the electrophoresis, the gels were visualized with GeneFlash gel documentation system (Syngene, UK) (Plate2.1) and photomicrographs taken. The samples with no visible shearing were selected for subsequent PCR amplification.

Plate 2.1 Gene Flash gel documentation system (Syngene, UK)

2.3.2 Primer design

Specific oligonucleotide primers were designed for the conserved ortholog set and candidate gene experiments in chapters 5 and 6 using Primer3 software (Rozen and Skaletsky, 1998, http://www-genome.wi.mit.edu/cgi-bin/primer) aiming to generate the longest possible amplicons. DNA and mRNA sequences for the regions of interest used for the design of the primers were obtained from GenBank and EMBL database and have been described in chapters 5 and 6. The specific primers were designed considering certain important factors as the melting temperature, significant product length

relative to the particular region of interest, GC content, 3' stability, predicted secondary structure, primer-dimer formation between primers and primer length. All the primers were ordered and synthesized from Sigma.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 PCR optimization

Since PCR is very sensitive and require only a few DNA molecules for amplification across several orders of magnitude, adequate measures were taken to avoid any contamination from any DNA present in the laboratory environment. Thus the DNA sample preparation, reaction mixture assemblage and the PCR process as well as the subsequent reaction product analysis for all the reactions performed in all the experiments were done in separate areas. The amount of genomic DNA or complimentary DNA (cDNA) and primer pairs used for the all PCR in the study are described in their respective chapters.

2.4.2 PCR reaction

Generally the PCR amplification was carried out using 20-50 ng/μl of template genomic DNA in 15 μl reaction volume containing 10x (Bioline/York Bio) PCR buffer, 2.5 mM $Mgcl_2$, 0.25 μM dNTPs (Bioline), 0.4 μM of each primer and 0.5 U of Taq polymerase (Bioline) in a T-Gradient Thermocycler (Biometra, Goettingen) 96-well plate as shown below in Plate 2.2.

2.4.3 PCR for DNA/ gene amplification

The amplification or cycling conditions were: 94°C for 5 minutes (initial denaturation), followed by 30-35 cycles of denaturation for 30 seconds at 94°C, the annealing temperature (50-65°C) dependent on the primer combinations for 30-50 seconds, elongation by heating 72°C for 60 seconds and a final elongation

step by heating 72°C for 5 minutes. The PCR products were electrophoresed on a mixture of 1% high resolution agarose gel and 1.5% high gel strength agarose in 1x TAE buffer with 0.5 µg/ml of ethidium bromide

Plate 2.2 T-Gradient Thermocycler (Biometra)

2.4.4 PCR for inter retroelement amplified polymorphism (IRAP)

The inter retroelement amplified polymorphism (IRAP) PCR was performed in a 25 µl reaction mixture containing 50 ng DNA, 10X PCR buffer (Promega cat. No. M1861), 2 mM $MgCl_2$, 5 pmol of each primer, 200 µM dNTP mix, 1 U Taq polymerase (Promega, cat. No.1861). Amplification was performed using a T gradient thermocycler (Biometra, Goettingen). The PCR programme was as follows; 95°C for 2 minutes for initial denaturation followed by 30 cycles of 95°C for 1 minute, annealing at a specified Ta depending on the specific primer

combination used for 1 minute, extension at 72°C for 2 minutes and a final extension at 72°C for 10 minutes. The PCR products were then electrophoresed on 2% agarose gel and the bands detected by 0.5 μg / ml ethidium bromide staining.

2.5 Polyacrylamide gel electrophoresis (PAGE)

2.5.1 Reagents and equipment used

Polyacrylamide gel electrophoresis (PAGE) apparatus for 20 cm x 20 cm gels; accessories (glass plates, spacers, shark tooth or saw tooth combs); and power supply.

40% acrylamide/bis-acrylamide (29:1) solution

10% ammonium persulphate (APS)

N, N, N', N'-Tetramethylethylenediamine (TEMED)

Urea

10x TBE buffer

1.25x formamide loading buffer

Hyperladder

Preparation of reagents

- **40% acrylamide/bis-acrylamide (29:1) solution**

Ready-to-use solution of acrylamide/bis-acrylamide commercially available was procured to avoid handling of harmful acrylamide powder.

- **10% ammonium persulfate (APS)**

Dissolve 0.1 g of ammonium persulfate (also known as ammonium peroxodisulfate) in 1 ml of SDW. Aliquots were stored at -20°C.

- **10x TBE buffer**

1 liter of 10x TBE stock was prepared by dissolving 108g of Tris base, 55 g of boric acid and 40 ml of 0.5 M EDTA (pH 8.0) in SDW up to the 1 liter mark. 1x TBE concentration prepared from the stock solution was used for the PAGE by taking 100 ml of 10x TBE and topping it up with 900 ml of SDW.

- **1.25x formamide loading buffer**

1 ml of the formamide loading buffer was prepared by mixing 900 μl of formamide, 22.2 μl of 0.5 M EDTA (pH 8.0), 26.5 μl of 7.5% orange G and 51.3 μl of SDW.

2.5.2 Denaturing 6% polyacrylamide gels

2.5.2.1 Preparation of the plates

The upper surface of the square plate was cleaned with a laboratory detergent and rinsed with warm water. Then it was thoroughly cleaned again with 100% ethanol using blue rolls of tissue paper, polishing until a squeaky sound was heard. A few drops of repel-silane were applied to the cleaned top surface of the plate and spread evenly using a fresh blue roll tissue paper. The top surface was wiped once more with warm water and 100% ethanol to remove excess repel-silane and allowed to dry.

In the same manner the eared shaped plate was washed cleaned and polished with laboratory detergent, warm water and 100% ethanol. Then about 20 μl of bind-silane was applied to the upper surface, spread evenly with blue roll tissue paper and the excess bind-silane removed with warm water and 100% ethanol and allowed to dry.

Clean and dried 1.5 mm spacers were aligned and sandwiched between the square plate and the eared plate and firmly secured with a tape and bulldog clips.

2.5.2.2 Preparation of 6% polyacrylamide gel

For the preparation of 30 ml of the denaturing polyacrylamide gel, 14.4 g of Urea was weighed into a 100 ml beaker, 4.5 ml of the acrylamide solution added and 3.0 ml of 10x TBE buffer also added to the mixture. Then deionized water was added to make up to the 30 ml mark. The mixture was de-gassed in a vacuum desiccator. Then after, 15 μl of TEMED was added to the de-gassed acrylamide solution and also 150 μl of freshly prepared APS was added and mixed thoroughly by swirling the beaker gently.

The de-gassed acrylamide solution was quickly transferred into the prepared plates using a 50 ml syringe in a gentle manner to avoid introduction of bubbles. Then the comb was inserted with the flat side against the top of the gel and more of the mixture added to avoid any breaks on the upper surface of the gel. The plate was allowed to stand for one hour to allow for effective polymerization of the gel. After the polymerization of the gel, the comb was removed, washed well to remove unpolymerised acrylamide and crystallized urea with distilled water, and the tapes removed, and the bottom of the gel plate thoroughly washed to remove any unpolymerised acrylamide. Then the comb was re-inserted with the toothed end just touching the surface of the polymerized gel.

2.5.2.3 Running of the polyacrylamide gel

1x TBE buffer was poured into the top and lower reservoir of the gel apparatus and pre-run at 25W for 30 minutes to help clean and pre-heat the gel. As the pre-heating was in progress, the SSR PCR products were prepared taking 5 μl and adding 5 μl of formamide loading buffer to it. The samples were then heated at 95°C for 5 minutes and immediately chilled by transferring samples onto ice. The wells of the gel were rinsed by gently aspirating buffer in the wells using a syringe until all bubbles and unpolymerised material have been removed. Then 6 μl of the samples were loaded onto the gel, and also a separate well was loaded with 6 μl of a mixture of hyperladder and formamide loading buffer which has

been denatured as the samples. The gel was allowed to run 85W for two hours or until the dark blue dye just run off the bottom of the gel, then the plates were removed ready for the silver staining.

2.5.2.4 Detection of Alleles by silver staining

The oligonucleotide products were visualized by silver staining using a modified version of the method described by Bassam et al. (1991). The gel attached to the plate with the bind-silane was fixed in 10% acetic acid for 30 minutes agitating the solution gently on a shaker. The fixer was poured off and saved for later use. Then the plate was washed in water kept on the shaker for 15 minutes. The plate was rinsed once again with water before being impregnated with $AgNO_3$ (1 g/l) and left for 30 minutes on the shaker. After this step, the gel was rinsed thoroughly in water and developed in a pre-chilled aqueous solution containing 30g/l of $NaCO_3$, sodium thiosulphate (0.1N) and 37% formamide (1.5 ml/l). The gel was agitated in the developing solution until the band development progressed sufficiently and then the reaction was stopped by adding the fixer saved initially. The gel plate was left standing vertically until it was well dried before scanning with a computer flatbed scanner and photographed for further analysis.

2.6 Total RNA extraction and Differential display

Due to the sensitive nature of RNA extraction with regard to contamination, additional care was taken in its extraction and storage. Gloves were worn all the time and only freshly prepared and autoclaved materials as well as sterile plastic pipettes were used. DEPC (diethylpyrocarbonate) was considered to be highly carcinogenic, thus materials were not DEPC- treated, instead corex glass tubes, pestle and mortar had to be pretreated with H_2O_2 for 1 minute, rinsed several times with water and baked for 6 hours at 180°C in an oven.

2.6.1. RNA Extraction Procedure

Solutions

RNA extraction buffer

4 M Guanidine thiocyanate

20 mM EDTA

20 mM MES

Add RNase-free water to a final volume of 400 ml and adjust the pH to 7.0, filtrate and autoclave

Add 1.7 ml (the final concentration being 50 mM) of 2-mercaptoethanol to the 400 ml stock solution and store at 4°C

RNA re-suspension buffer

2 M Lithium Chloride (LiCl)

10 mM Sodium Acetate (NaOAc)

Add RNase-free water to a final volume of 250 ml, pH 5.2, filter, autoclave and store at 4°C

Procedure

RNA was extracted from the leaves of irrigated and non-irrigated cowpea plant as described:

5 g of frozen leaves were ground in liquid nitrogen, transferred into a 50 ml falcon tube containing 10 ml of the RNA extraction buffer for homogenization, and then RNA was purified by phenol:chloroform:Isoamyl alcohol (12:12:1) and acid phenol: chloroform (2:1) extractions, followed by one chloroform extraction. An equal volume of isopropanol was added and preparations stored at -20°C for 2 hours. The RNA was precipitated by centrifugation, and the pellets dissolved in the RNA re-suspension buffer and stored overnight at 20°C. Again, an equal volume of isopropanol was added, allowed to stand for 2 hours at -20°C and

RNA precipitated by centrifugation. Pellets were re-suspended in a freshly prepared 75% ethanol solution, centrifuged and air dried for 15 minutes. Then the RNA pellets were dissolved in 150 μl of RNase-free distilled water. The RNA samples were finally treated with RNase free DNase and stored at -80°C.

2.6.2 Differential display

Differential display of cDNA was performed (Liang and Pardee, 1992) using Delta Differential Display Kit following the manufacturer's protocol (Clontech, UK). The first-strand cDNA was generated by using SuperScript™ III CellsDirect cDNA synthesis system according to the manufacturer's instructions (invitrogen, Catalog Nos. 18080-200 and 18080-300). This was performed in a total volume of 20 μl. The reaction mixture consisted of 2 μg DNA-free total RNA, 2 μl of Oligo(dT)$_{20}$ (50 μM) primer, 1 μl of 10 mM dNTP mix, 6 μl of 5X RT buffer, 1 μl of RNaseOUT (40 U/ μl), 1 μl of SuperScript ™ III RT (200 U/ μl) and 1 μl of 0.1 M DTT.

Differential display PCR (DD-PCR) was performed in a 20 μl reaction mixture, using two dilutions of the first strand cDNA (dilution A-consisted of 4 ng, and dilution B-consisted of 1 ng cDNA). Each reaction mixture contained 2 μl of 10X buffer, 0.5 μl of $MgCl_2$ (50 mM), 0.5 μl of dNTP mix, 1 μl of each anchored oligo-dT and arbitrary primers (20 μM), 2 μl of cDNA, and 0.4 μl of Taq polymerase. The reaction was performed using a T-Gradient Thermocycler (Biometra, Goettingen) 96-well plate, programmed to 94°C for 5 min, followed by annealing at 42°C for 5 min, extension at 68°C for 5 min, then 94°C for 2 min, 42°C for 5 min and 68°C for 5 min and finally, 25 cycles of 94°C denaturing step for 1 min, annealing at 60°C for 1 min, extension at 68°C for 2 min, then a further extension at 68°C for 7 min. DD-PCR products were electrophoresed on a mixture of 2% high resolution agarose gel in 1x TAE buffer with 0.5 μg/ml of ethidium bromide. Differentially expressed cDNAs were excised from the gel, recovered and re-amplified using the same primer set that produced it. The annealing temperature

was dropped from 60°C to 50°C and the dNTP final concentration increased to 0.2 μM. The PCR products were excised again and purified using Qiagen gel extraction kit following the manufacturer's protocol. The purified cDNA fragments were ligated in pGEM-T Easy vector prior to transformation into competent bacterial cells.

2.7 Cloning of genomic DNA PCR products and cDNA fragments

2.7.1 Competent cells

Solutions and Buffers

- **2M $MgCl_2$ (MW:203.31)**

Dissolve 10.17g $MgCl_2$ in 20 ml dH_2O, and top up to 50 ml

- **50 mM $CaCl_2$ (MW:203.31)**

Dissolve 1.1g $CaCl_2$ in 80 ml dH_2O, and top up to 100 ml

- **250 mM KCl (MW:74.56)**

Dissolve 1.86g KCl in 80 ml dH_2O, and top up to 100 ml

- **50% Glycerol (100 ml)**

Dilute 50 ml Glycerol in 50 ml dH_2O, mix well and autoclave

- **SOB Medium**

Dissolve in 80 ml dH_2O:
2.0 g Tryptone
0.5 g Yeast extract
0.05 g NaCl
Add 1 ml 250 mM KCl; adjust to pH 7.0 with NaOH, top up to 100 ml and aliquot in 50 ml portions into 250 ml flask. Add 0.5 ml sterile 2M $MgCl_2$ to each flask before use

- **LB Medium**

Dissolve in 80 ml dH_2O:

2.0 g Tryptone
0.5 g Yeast extract
0.05 g NaCl
Adjust to pH 7.0 with NaOH, top up to 100 ml, aliquot into 10 ml portions in 30 ml vial and autoclave. For plate preparation, add 1.5 g agar, autoclave in 250 ml Schott Bottle

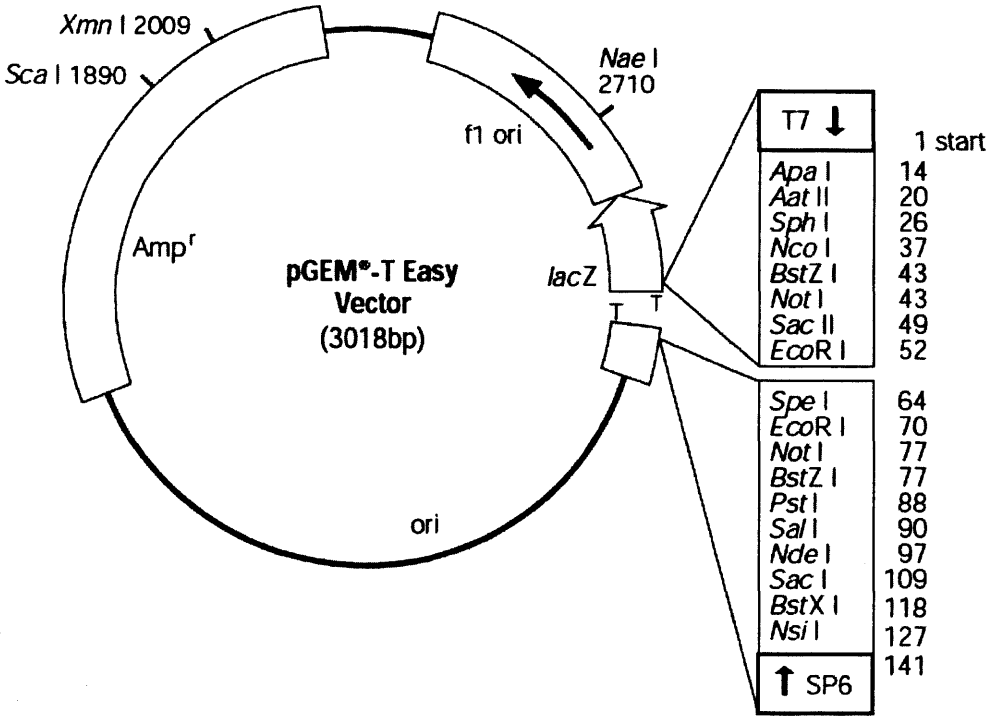
Procedure

Competent cells (DH5α) were cultured by overnight incubation at 37°C in 10 ml antibiotic free LB medium. Then the cultured cells were plated out and single colonies isolated by picking them and being inoculated in a fresh 10 ml antibiotic free LB medium and re-cultured overnight at 37°C in an orbital incubator (shaking at 220 rpm/min). The overnight culture was then divided into two 5 ml portions. Each 5 ml portion of the overnight culture was then inoculated in a 250 ml conical flask containing 50 ml antibiotic free SOB medium supplemented with 10 mM $MgSO_4.7H_2O$ and 0.2% glucose and incubated again in a 37°C orbital incubator (shaking at 220 rpm/min) for about 90-100 minutes until the OD_{600} reached 0.6. The culture was then centrifuged at 3000 rpm for 10 minutes at room temperature (RT) to pellet the bacteria cells, the supernatant was carefully discarded and the pellets gently re-suspended in 25 ml cold (-20°C) 50 mM $CaCl_2$ in a 50 ml Falcon tube. The cells were left standing on ice for 30 minutes before precipitating by centrifuging at 3000 rpm for 10 minutes at 4°C. The cells were again suspended in 2.1 ml ice-cold (-20°C) 50 mM $CaCl_2$ on ice and 900 μl ice cold 50% glycerol was added. Aliquots of 100 μl of cells were pipetted into a 250 μl micro centrifuge tube on ice, flash-frozen in liquid nitrogen and stored at -80°C.

2.7.2 Ligation and Transformation

Purified PCR fragments were always used for ligation purposes. Ligation was performed in a 15 μl volume using the pGEM-T Easy Vector system 1 kit (Promega), consisting of 7.5 μl of 2X Rapid ligation buffer (60 mM Tris-HCl, pH 7.8; 20 mM $MgCl_2$; 20 mM DTT; 2 mM ATP; 10% PEG), 0.9 μl pGEM-T Easy vector, 1.2 μl T4 DNA ligase, 5.4 μl DNA or cDNA and the mixture incubated at 4°C overnight.

Figure 2.1: pGEM®-T Easy Vector circle map.



Transformation process was accomplished by first adding the 15 μl ligation mixture to 100 μl thawed competent cells (DH5α) in a 1.5 ml micro centrifuge tube. Then the mixture was allowed to stand on ice for 30 minutes, and heat-shocked in a water-bath incubator at exactly 42°C for 60 seconds. The transformed cells were then allowed to stand on ice for 10 minutes immediately after the heat-shocking procedure. 500 μl of pre-warmed antibiotic-free SOB

medium was added to the transformed cells and incubated in an orbital incubator at 37°C for 90 minutes shaking at 220 rpm/min. After the incubation, the cells were centrifuged briefly, 400 μl of the supernatant pipetted and discarded, whilst the cells were gently mixed in the remaining supernatant and 100 μl each of the cells cultured on duplicate plates of LB agar medium supplemented with ampicillin antibiotic (100 μg/ml), X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase; 40 μg/ml) and IPTG (isopropyl-β-D-thiogalacto-pyranoside; 0.5 mM) and incubated overnight at 37°C.

2.7.3 Clone screening and storage

Recombinant clone selection was based on screening for white colonies which were indicative that they contain a pGEM-T Easy vector with an insert. A single white colony was picked and grown in a 10 ml LB medium with 10 μl ampicillin added to it. The culture was incubated overnight at 37°C in an orbital incubator (shaking at 250 rpm/min). PCR was used to confirm the recombinant status of the clone by the use of the universal M13 forward and reverse primer pair which anneal at either side of the multiple cloning regions. A 15 μl PCR mixture generally contains PCR buffer, 5 μM of M13 primer pair, 0.1U Taq DNA polymerase, 5 μM dNTP, 5 μM $MgCl_2$, and 0.5 μl of the overnight culture containing a putative recombinant clone. The PCR amplification programme was 94°C for 5 minutes; then 30 cycles of [94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds] and finally one cycle of 72°C for 7 minutes. The PCR product was assessed by electrophoresis on a mixture of 1.5% high resolution agarose gel in 1x TAE buffer with 0.5 μg/ml of ethidium bromide.

Recombinant plasmid DNA isolation was performed from minipreps of the positive clones using the Qiagen miniprep-kit, according to the manufacturer's protocol. The quality of the DNA was ascertained on 1% agarose gel electrophoresis in 1X TAE buffer. Then the selected clones were characterized by restriction enzyme analysis in a 15 μl mixture containing 0.1U *Eco*RI enzyme, 100 ng/ μl plasmid and 1X *Eco*RI reaction buffer, which allowed the size of the

insert to be confirmed. For long term storage, 500 μl of the overnight selected and confirmed cultures were mixed with 500 μl of sterilized 50% glycerol and kept in -80°C.

2.7.4 Clone sequencing and analysis

Clones which have been verified to have the DNA insert of interest as described above were sequenced commercially at John Innes centre (JIC), at Norwich, U.K., using M13 forward and reverse primers according to the requirements of the ABI 3700 capillary sequencing system.

The sequenced data were received in the ABI format and were imported for analysis by Chromas software. DNA and the deduced amino acid sequences were aligned and phylogenetic trees were constructed in CLC Free Workbench 2.2.5 edition and with BioEdit software. Default settings were applied to both multiple alignments (full alignment, gap open- 10, gap extension-1, ends gap-as any other) and sequence trees, based on pairwise distances of the UPGMA (unweighted pair group method using arithmetic averages) clustering algorithm (Michener and Sokal, 1957; Sneath and Sokal, 1973).

Bootstrap was used to evaluate the reliability of the constructed phylogenetic tree (Felsenstein, 1985). Estimates were made by allocating and positioning a non-member sequence at the lowest average dissimilarity for clustering. The reliability of the tree was evaluated and supported with 1000 bootstrap replicates. Low bootstrap values correspond to a significant grouping of samples and branch lengths are proportional to the average amino acid substitution per site.

2.8 Southern hybridization

2.8.1 Restriction enzyme digestion

Genomic DNA samples were digested with the restriction enzymes *Hae*III, *Bam*HI, *Hin*dIII, *Eco*RI and *Sau*3A. Optimization test on pilot was carried out to

finalize the DNA and enzyme concentrations. Each digestion required 6-8 μg of genomic DNA per 25 μl reaction, and restrictions were obtained from an overnight incubation at 37°C. Digested products were separated by 1% agarose gel electrophoresis in 1X TAE buffer for 2-3 hours at 5 V/cm.

2.8.2 Southern blotting

To transfer the separated DNA fragments from the agarose gel to a nylon membrane, the gel was initially depurinated in 0.25 N HCl for 20 minutes, denatured in 0.5 M NaOH, 1.5 M NaCl for 20-30 minutes and neutralized in 0.5 M Tris-HCl (pH 7.5), and 1.5 M NaCl for 30 minutes. The gel was then rinsed twice with SDW.

The transfer apparatus consisted of a tray filled with 10X SSC, and a platform to support the gel and the filter paper (Whatman 3 mm) wicks. The gel was placed up-side-down on the platform, followed by; a soaked nylon membrane (Hybond N+, Amersham), a further three pieces of filter paper soaked with 10X SSC, and a stack of paper towels. The whole set-up was stabilized with a weighted glass plate. The edges of the gel and the tray were sealed with cling film. The transfer was carried out for about 15-20 hours to complete the process. After the transfer, the membranes were rinsed in 2X SSC, air dried and exposed to ultra violet (UV) light to promote DNA cross-linking and stored at room temperature (RT) before use.

Buffers:

- **Buffer I: Depurination (1000 ml)**

Measure 21.6 ml of 11.6 M HCl [0.25 N]. Make up to 1000 ml, autoclave and store at RT.

- **Buffer II: Denaturation (500 ml)**

Weigh 43.9 g NaCl [1.5 M] and 10 g NaOH [0.5 M]. Dissolve in 100 ml of SDW and make up to 500 ml, autoclave and store at RT.

- **Buffer III: Neutralization (1000 ml)**

Weigh 87.7 g NaCl [1.5 M], dissolve in 500 ml of 1 M Tris-HCl, pH 7.4 [0.5 M]. Make up to 1000 ml, autoclave and store at RT.

2.8.3 Membrane hybridization

Pre-hybridization of the prepared membrane was carried out by 3-4 hour rotation in a Hybaid hybridization tube at 60°C in a Hybaid hybridization oven. The pre-hybridization mixture was made up of 50% formamide (v/v), 5X SSC, 2% blocking reagent (Roche) (w/v), denatured salmon sperm DNA (1 μg/μl), 100 mM EDTA and 0.02% SDS (sodium dodecyl sulphate; w/v). The volume of the hybridization solution required was 5 ml per 100 cm^2 membrane. Overnight hybridization with a labeled probe was performed in the same tube at 60°C with the same formulation as in the pre-hybridization plus 50% dextran sulphate (w/v).

After hybridization, the solution was stored at - 20°C for possible re-use. The membranes were rinsed at RT and washed twice each with 12.5 ml 2X SSC and 1% SDS at 60°C for 30 minutes in a rotating hybridization oven and then washed twice with SDW. After the final rinse, the membranes were dried with tissue paper, wrapped in Saran-wrap and placed in an auto-radiographic cassette, along with sheet of X-ray film (FUJI Medical X-ray film) on each side of the membrane.

The cassette was stored at -20°C and the exposure time required depended on the signal strength, varying from overnight (> 500 counts/second) to a week or more (< 100 counts/second). The exposed X-ray film was processed in a photographic developing machine, scanned with an EPSON 1600 Pro scanner and the image imported into Adobe Photoshop.

CHAPTER THREE

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CHAPTER THREE

3.0. Screening of Ghanaian Cowpeas for Drought Tolerance

3.1. Introduction

Water is the most important factor limiting crop productivity. The demand for drought tolerant genotypes has become a matter of great concern as water resources and the funds to access them have become more limited. The difficulties include the identification of physiological characteristics that are correlated with drought stress that could be used as indicators of drought tolerance. Physiologists are able to measure various plant characteristics that correlate with drought tolerance, such as water use efficiency (Quisenberry et al., 1981; Wright and Dobrenz, 1970; Ray et al., 1974), root characteristics (Pace et al., 1999; Basal et al., 2003; Ball et al., 1994; Cook and El-Zik, 1992), detached leaf water loss (Basal et al., 2005; Roark et al., 1975), leaf water potential (Quisenberry et al., 1985; Kaul, 1969), stomatal characteristics (Quisenberry et al., 1982; McDaniel, 2000), and osmotic adjustment (Nepomuceno et al., 1998; Oosterhuis and Wullschleger, 1987).

Water use efficiency (WUE) is defined as the ratio of dry matter production to water use (Hubick et al. 1986). It has also been observed that WUE might not provide much information about the competitive or yield advantage of one particular variety over another because improved WUE may actually restrict growth (Hubick et al. 1986). However, it is one trait that has been studied a great deal because it can give an idea of the variation amongst genotypes in ability where water is limiting. The use of the WUE trait in crop improvement programmes is limited by the volume of work involved with its direct measurement, especially on large numbers of lines under field conditions (Hall et al., 1997). Most research has, therefore, been directed to seeking surrogate traits

that can provide a cheap and rapid measure of WUE. Measuring drought tolerance of individual plants or established genotypes under field conditions is difficult due to variation in weather conditions and between and within field soil type variation. This has led to efforts of drought simulation studies in greenhouses and growth chambers.

Cowpea (*Vigna unguiculata*) is widely grown in the semiarid tropics where drought is a major production constraint (Ashley 1993; Singh 1999b; Singh et al. 1997). Due to the erratic rainfall in the beginning and towards the end of the rainy season, crops are often subjected to drought stress in both seedling and terminal growth stages, which causes a substantial reduction in grain yield as well as biomass production. Early-maturing varieties escape terminal drought (Singh, 1999b) but if exposed to intermittent moisture stress during the vegetative or reproductive stages, they perform very poorly. Cowpea is inherently more drought tolerant than other crops but it still suffers considerable damage due to frequent drought in the Sahelian region where rainfall is scanty and irregular (Singh et al. 1999a). Therefore, there is the need to breed cowpea varieties with enhanced drought tolerance.

Reviews in the past by Ashley 1993, Subbarao et al. 1995 and Boyer 1996 have brought together the available knowledge on different aspects of drought tolerance in crop plants and options to minimize yield losses due to drought. Major differences among and within crop species have been reported and different strategies to breed drought-tolerant varieties have been suggested (Blum 1988; Walker and Miller 1986; Arraudeau 1989; Acevedo and Ceccarelli 1989). However, the success in breeding for drought tolerance has not been as pronounced as for other traits. This is partly due to lack of simple, cheap, and reliable screening methods to select drought-tolerant plants and progenies from the segregating populations, and partly due to the complexity of factors involved in drought tolerance.

Several methods have been used to estimate drought tolerance and WUE as I have already indicated. These involve the measurement of water potential, relative turgidity, diffusion pressure deficit, chlorophyll stability index, and carbon isotope discrimination (Turk and Hall 1980; Morgan 1984; Yadav et al, 1997; Hall et al. 1997, 2003). However, most of these methods are expensive and time consuming and are therefore not very efficient or practical for screening large numbers of plants in segregating populations. Also, screening for drought tolerance in the off-season using controlled watering is often not relevant to the environment of the real growing season, particularly when temperature and photosensitivity play important roles in crop growth and productivity. Most studies in the past have dealt with screening for drought tolerance as a whole and not individual components involved in drought tolerance (Lawan 1983, Watanabe et al. 1997). This could also contribute to variable results, depending on which factors were operational during screening.

Traditionally, drought tolerance is defined as the ability of the plant to live, to grow, and produce satisfactorily yield with limited soil water supply or under periodic water deficiencies (Ashley 1993). Since several factors and mechanisms (in shoots and roots) operate independently or jointly to enable plants to cope with drought stress, drought tolerance appears as a complex trait (Krishnamurthy et al. 1996). However, if the factors and mechanisms contributing to drought tolerance can be separated and studied individually, the components leading to drought tolerance will appear less complex and may be easier to manipulate by breeders. Breeding for early maturity, photosensitivity, indeterminacy, epicuticular wax, pubescence, and awns, which indirectly affect the ability of plants to cope with drought is easy because these traits are inherited and can easily be screened and incorporated in improved varieties as indicated above. For other traits such as osmotic adjustment and stomatal regulations, which directly control the drought tolerance of plants, the ideal approach would be to study the shoot drought tolerance and root characteristics separately and identify gene(s) responsible for stomatal behavior, osmotic adjustment, and root

architecture, and combine them in improved varieties. Depending on timing and the magnitude of the water deficit, cowpea responds by stomatal regulation of water loss, leaf area reduction, hastening or delaying its reproductive cycle or by developing a deep root system (Gwathmey and Hall, 1992).

Only a few studies have been reported, in the selection of cowpea varieties, on the basis of their tolerance to water stress. The first strategy developed to screen cowpea varieties according to their tolerance to water stress was based on the assessment of shoot dry matter and leaf area under well-watered and drought treatments in field conditions. These studies allowed the identification of cowpea varieties showing tolerance to water stress (Hall and Grantz, 1981; Gwathmey and Hall, 1992). The second strategy was based on the intrinsic water-use efficiency, which should be associated with differences in the extent to which C3 plants discriminate against ^{13}C (carbon isotope) compared with ^{12}C during CO_2 fixation. Measurements of plant composition of stable isotopes ($^{13}C/^{12}C$) in field conditions suggest a correlation between ^{12}C and genotype in cowpea (Hall et al., 1997; Hall et al., 2003). These strategies developed in order to screen cowpea varieties tolerant to water stress are very difficult to apply in field conditions and they are time consuming, whilst the easier methods like carbon isotope discrimination analysis is expensive.

Screening techniques to determine whether a crop species is tolerant or susceptible to drought should be rapid and capable of evaluating plant performance at a critical developmental stage in both small and large populations. However, the available selection methods/criteria are not very suitable to adopt on a single plant basis in a segregating generation. Furthermore, field screening is difficult due to uncertain rainfall. A simple method of screening germplasm lines using seedling survivability, which accurately discriminates between drought tolerance and susceptibility under artificial moisture stress conditions (Singh et al. 1999b), was adopted as a selection

criterion in wheat. Such screening methods must be incorporated into plant breeding programs to facilitate meaningful genetic improvement.

The key to an efficient screening method is the ability to balance the accuracy of screening with the need to screen large amounts of plant material in the shortest time possible. Though drought tolerance is always considered to be a complex trait, a recent wooden box screening method for characterizing cowpea and other crops has been described by Singh et al. (1999a; 1999b) and Singh and Matsui (2002). This simple method eliminates the effect of roots and measures only the shoot dehydration tolerance indicated by the number of days it survives after watering has been stopped. This trait is highly reproducible and simply inherited in cowpea (Mai-Kodomi et al., 1999a,b).

The pot screening or wooden box method of varieties at seedling and flowering stages is a reliable method to identify drought tolerance. It is practical because of the ease with which the screening is carried out under a controlled environment and the possibility of a large number of varieties being screened. Watanabe *et al.* (1997) confirmed the suitability of pot screening techniques for drought tolerance and indicated a variety of differences in plant response to drought stress in cowpea. They also indicated that the phenomenon responsible for drought tolerance in the seedling stage is also manifested at the reproductive stage.

In this study, a pot evaluation method for one hundred and six cowpea accessions from Ghana at the seedling stage was carried out. The main aim of the study was to evaluate the drought tolerance of Ghanaian cowpeas grown in environments where the productivity of the crop is influenced by limited water supply.

The objectives of this study were to:

- screen and discriminate among the cowpea accessions for drought tolerance at the seedling stage;

- identify physiological traits that are correlated with drought stress and could be used as indicators of drought tolerance;
- determine the relationship between the cowpea accessions using qualitative and quantitative morphological traits.

3.2. Materials and methods

3.2.1. Materials

Plant materials used in this study were obtained from the germplasm collections of the Plant genetic resources centre (PGRC), which is under the Centre for scientific and industrial research (CSIR), at Bunso, in Ghana, as well as from the open markets and farmers across the length and breadth of Ghana. A total of one hundred and six cowpea accessions were used as shown in Table 2.1.

3.2.2. Methods

An adopted method of Watanabe *et al.* (1997) was used to conduct this study. The accessions were screened for survivability which indicates drought tolerance at the seedling stage. The general methods used in the sowing, management and collection of data have been described in chapter two. The experiments were performed in the greenhouse of the Norman Borlaug Institute for Plant Science Research, De Montfort University. The screening experiment was began on 25th March 2004, and lasted for 64 days. I initially performed a preliminary investigation to determine the best parameters to be adopted for the actual study of which the planting was done in January 2004 and lasted for eight weeks. The preliminary data obtained (not shown) indicated that the optimum temperature extremes for maximum germination of the cowpea seeds could be from 15°C to 35°C, which was subsequently adopted for the study as described in chapter two. Phenotypic observations of the leaves were used as the basis for data collection and percent wilting at various intervals and days taken to permanent wilting were recorded for each line until the genotypes were wilted, many beyond recovery,

before re-irrigation. The data on percent recovery for each line was recorded seven days after re-irrigation. Then based on the days taken to permanent wilting and percent recovery, the lines were classified into drought tolerant and susceptible groups.

3.2.3. Qualitative and quantitative traits evaluation.

The qualitative and quantitative traits were evaluated using different scoring scales.

1. **Growth pattern**

1 = Determinate (apical bud of main stem reproductive)

2 = Indeterminate

2. **Plant pigmentation** (recorded for stem, branches and petioles in the 4th week after planting).

0 = None

1 = Very slight

3 = Moderate at the base and tips of petioles

5 = Intermediate

7 = Extensive

9 = Solid

3. **Terminal leaflet shape** (recorded for the terminal leaflet of a young, mature leaf in the 4th week after planting).

1 = Globose

2 = Sub-globose

3 = Hastate

4 = Sub-hastate

4. **Twining tendency**

0 = None

3 = Slight

5 = Intermediate

7 = Pronounced

5. **Plant hairiness** (of stems and leaves)

3 = Glabrescent

5 = Short hairs

7 = Pubescent to hirsute

6. **Seed shape**

1 = Kidney

2 = Ovoid

3 = Crowder

4 = Globose

5 = Rhomboid

7. **Testa texture**

1 = Smooth

3 = Smooth to rough

5 = Rough (fine reticulation)

7 = Rough to wrinkled

9 = Wrinkled (coarse folds on the testa)

8. **Leaf colour** (intensity of green colour)

3 = Pale green

5 = Intermediate green

7 = Dark green

9. **Plant height (cm)**

Mean of 3 randomly selected plants recorded in the 6^{th} week after sowing.

10. **Seed weight (g)**

Weight (g) of 100 seeds

3.2.4 Data analysis

The PowerMarker software package version 3.25 (Liu and Muse, 2005) was used to analysis the data obtained and to calculate similarity coefficients among the genotypes. A Dendrogram and phylogenetic tree were constructed from the similarity coefficient matrix using the UPGMA method of cluster analysis.

3.3. Results

3.3.1. Physiological Screening of Cowpeas

The seed germination and initial growth of most of the 106 cowpea varieties were normal. About 12 of the cowpea varieties used for the study did not germinate at all. These plants were therefore excluded in the determination of the drought status of the varieties used. In subsequent evaluations these plants were grown using a temperature range of 20°C to 35°C leading to all of them germinating for further use. In Table 3.1 their readings have been designated as **(-)**. In some of the varieties too, since the experiment was performed in triplicate, not all the seeds germinated and emerged leading to the percentage emergence ranging from 33% to 100%. Generally all the seeds that germinated did so within a range 3 to 6 days (DAP) with the mean of 4.56 days as shown in Table 3.1. When the plants were well established the experimental ones were subjected to drought stress by terminating watering and visual observations of the shoot were made at 7 days interval for any signs of wilting (DT) as compared to their respective controls. Those looking normal visually with no signs of wilting have been recorded as **N** and the wilted ones as **W** (Table 3.1).

About 15 days after the commencement of drought treatment, stress effects started appearing in the seedlings of the susceptible varieties, and differences among varieties became visible and progressively more pronounced with advancing days of moisture stress. The effect of the stress manifested on the plants in different ways. In some varieties the unifoliate leaves were the first to become wilted, followed by the emerging trifoliates, and finally the growing tip dried. Some accessions like GH 2289, GH 5046, GH 2338, GH 3673, GH 6045, GH 5041, GH 5050, GH 4526, GH 4530, GH 3675, GH 3706, GH 2316, GH 2285, GH 3669, GH 2315, GH 6046, GH 1002, and GH 1003, were the first to show signs of wilting, though some of them were able to recover after rewatering as shown in Table 3.1. Interestingly, the unifoliate leaves of GH 5346, GH 3685, GH 3667, GH 3687, GH 5050 and GH 2316 turned deep yellow in response to

moisture stress and then dried, whereas other varieties showed different shades of yellow, brown, and green.

The data on days taken to permanent wilting (DPW) and percentage recovery (%R), where the surviving plants were defined as having at least one turgid trifoliate leaves and a live apical growing tip, indicated that some varieties could be considered as susceptible to drought (Table 3.1) whilst others have different levels of drought tolerance. Based on DPW the cowpea varieties were put into three categories. In the first category, the plants started showing signs of wilting 20-23 days after drought treatment began, and then the second category started showing signs of wilting 24-27 days after drought treatment and the third category 28-35 days after drought treatment. In all situations those plants that scored 50% and above in percent recovery (%R) were considered to be drought tolerant and those that scored below 50% were deemed to be susceptible to drought as shown in Table 3.2. The observed (%R) scores of 0%, 33%, 66%, and 100% and in some cases 50% (Table 3.2) were based on the fact that the seeds were sown in triplicates. Thus when none of the three germinated seedlings survived after rewatering, the %R value was scored as 0%, 1 as 33%, 2 as 66% and 3 as 100%. In certain accessions only two seeds germinated out of the three sown and therefore if 1 out of the 2 seedlings that germinated recovered after rewatering, then the %R was scored as 50%. It was also observed that most of the plants that showed signs of wilting 20-23 days after the drought treatment were considered to be drought susceptible whilst the members of categories two and three formed the drought tolerant group. The wilting process began from the lower leaves and progressively to the upper leaves, though the loss of the lower leaves was common for all accessions.

Figure3.1: Pot valuation of cowpea accessions for drought tolerance.

Fig.3.1 (1): Showing both the control and the experimental pots 7 days after the imposition of the drought stress.

Fig.3.1 (2): Showing the control and experimental pots 15 days after drought stress had been imposed. The experimental pot had then started showing some level of leaf discoloration.

Fig.3.1 (3): 21 days after watering of the experimental plants had ceased and there were clear signs of wilting.

Fig.3.1 (4): This was 28 days after the imposition of drought stress and the experimental plants were showing advanced state of wilting.

3.3.2. Morphological traits evaluation.

Scores of ten qualitative and quantitative characters of one hundred and six accessions of cowpea are presented in Table 3.3.

1. **Plant pigmentation**: The pigmentation generally observed varied as 40% of the accessions had no pigmentation. The accessions with very slight pigmentation on the stem, branches, peduncles, and petioles as well as those with moderate pigmentation at the base and tips of the petiole were 24% each. The rest of the accessions forming 12% had either extensive or solid pigmentation on their branches and stem.
2. **Growth pattern**: Most of the accessions used for the study exhibited an indeterminate growth pattern forming 80% with the rest of the accessions being considered to exhibit determinate growth pattern.
3. **Terminal leaflet shape**: Four different leaflet shapes was observed in the accessions used for the study. 20% had globose shape, 46% had sub-globose shape and those having either hastate or sub-hastate together were 34%.
4. **Twining tendency**: 34% of all the accessions used for the study had no twining tendency with 42% of the accessions exhibiting slight twining tendency. The rest of the accessions forming about 24% of the population showed either intermediate or pronounced twining tendency.
5. **Plant hairiness**: Two types of hairiness were observed with 70% of the accessions showing glabrescent plant hairiness and 30% having short appressed hairs.
6. **Seed shape**: The various seed shapes exhibited by the accessions used for the study ranged from Kidney shape, Ovoid, Crowder and Rhomboid with their respective percentages being 49%, 13%, 7% and 31%.

   Testa texture: 38% of the accessions had a smooth testa texture, whilst 33% had their testa being smooth to rough in texture. 29% of the

accessions had either rough or rough to wrinkle testa texture. None of the accessions showed exclusively wrinkle testa texture.

7. **Leaf colour**: The leaf colours observed during the study were pale green, intermediate green and dark green. Those accessions that had pale green leaf coloration were 44%, whilst 40% of the accessions showed intermediate green colour and 16% having dark green coloration.
8. **Seed weight**: The size of the individual seeds used for the study varied extensively with the seed weight of the accessions having a range of 12.7-26.1 g. GH 6048 had the highest seed weight of 26.1 g, whilst GH 2275 had the lowest weight of 12.7 g and the mean weight being 20.03 g.
9. **Plant height**: According to the values of the plant height recorded GH 2326 had the highest plant height of 34.6 cm, whilst GH 4769 had the least plant height of 17.5 cm. The mean plant height was 26.8 cm.

Table3.1. Accession number, Source, Days after planting (DAP) to emergence, percent emergence (E), Days after commencement of drought treatment (DT), days for permanent wilting to occur (DPW), percent recovery of wilted plants.

Code	Accession	Source	DAP	% E	7DT	15DT	21DT	28DT	35DT	DPW	% R
1	GH 3665	SE	4	100	N	N	N	W	W	21-35	66
2	GH 6048	MB	5	100	N	N	N	N	W	29-35	66
3	GH 4767	NW	4	100	N	W	N	W	W	25-35	100
4	GH 2289	NE	4	66	N	N	W	W	W	21-35	100
5	GH 4026	MB	5	100	N	N	N	N	W	29-35	100
6	GH 3701	SE	4	100	N	N	N	N	W	28-35	100
7	GH 2328	MB	6	100	N	N	N	N	W	30-35	66
8	GH 2314	NE	5	100	N	N	N	W	W	26-35	100
9	GH 4773	NW	4	100	N	N	N	N	W	30-35	66
10	GH 2326	MB	3	100	N	N	N	W	W	27-35	66
11	GH 4537	NW	-	-	-	-	-	-	-	-	-
12	GH 2312	NE	-	-	-	-	-	-	-	-	-
13	GH 2291	NW	4	66	N	N	N	W	W	26-35	100
14	GH 3678	MB	-	-	-	-	-	-	-	-	-
15	GH 5048	MB	4	100	N	N	N	N	W	30-35	100
16	GH 5346	SW	5	100	N	N	N	W	W	27-35	0
17	GH 5046	SW	4	100	N	N	W	W	W	21-35	0
18	GH 4527	NE	4	100	N	N	N	W	W	25-35	100
19	GH 4772	SE	-	-	-	-	-	-	-	-	-
20	GH 4532	SE	4	100	N	N	N	N	W	30-35	66
21	GH 3674	SW	-	-	-	-	-	-	-	-	-
22	GH 4770	NW	4	100	N	N	N	W	W	28-35	100
23	GH 5040	SE	-	-	-	-	-	-	-	-	-
24	GH 4529	SW	-	-	-	-	-	-	-	-	-
25	GH 4542	SE	4	100	N	N	N	W	W	27-35	100
26	GH 2338	SE	5	66	N	N	W	W	W	20-35	100
27	GH 3673	SE	5	66	N	N	W	W	W	21-35	33
28	GH 3710	NE	4	100	N	N	N	W	W	27-35	0
29	GH 4534	SW	4	100	N	N	N	W	W	28-35	0
30	GH 3689	NE	4	100	N	N	N	W	W	25-35	66
31	GH 2284	NW	3	100	N	N	N	N	W	30-35	100
32	GH4028	MB	3	100	N	N	N	N	W	30-35	66
33	GH 4769	SE	4	100	N	N	W	W	W	21-35	33
34	GH 2279	NE	-	-	-	-	-	-	-	-	-
35	GH 2306	NW	-	-	-	-	-	-	-	-	-
36	GH 2332	MB	-	-	-	-	-	-	-	-	-
37	GH 6045	MB	4	66	N	N	W	W	W	20-35	0
38	GH 2347	SE	3	100	N	N	N	N	W	30-35	66
39	GH 5044	SE	4	100	N	N	N	N	W	30-35	100
40	GH 2337	MB	4	100	N	N	N	W	W	28-35	100
41	GH 3666	SE	4	66	N	N	N	W	W	26-35	50
42	GH 5344	MB	5	100	N	N	N	N	W	30-35	100
43	GH 2318	NW	5	100	N	N	N	W	W	28-35	66
44	GH 3711	NW	4	100	N	N	N	W	W	25-35	66
45	GH 4027	MB	4	100	N	W	N	W	W	28-35	66
46	GH 5041	SE	4	100	N	N	W	W	W	21-35	0
47	GH 4549	SW	4	66	N	N	N	W	W	28-35	100
48	GH 4541	SE	5	100	N	N	N	N	W	30-35	33
49	GH 2281	NE	5	100	N	N	N	W	W	27-35	100
50	GH 3708	NW	4	100	N	N	N	W	W	26-35	66
51	GH 2336	SW	3	100	N	N	N	N	W	30-35	100
52	GH 2275	NW	3	100	N	N	N	W	W	28-35	100
53	GH 5050	MB	4	100	N	N	W	W	W	21-35	33

Table3.1. (Continued)

Code	Accession	Source	DAP	% E	7DT	15DT	21DT	28DT	35DT	DPW	% R
54	GH 5345	MB	-	-	-	-	-	-	-	-	-
55	GH 5049	MB	4	100	N	N	N	W	W	28-35	100
56	GH 2313	NW	4	66	N	N	N	W	W	26-35	100
57	GH 4778	SW	4	66	N	N	N	W	W	26-35	0
58	GH 2342	NE	5	100	N	N	N	N	W	30-35	100
59	GH 5045	NE	3	100	N	N	N	W	W	27-35	66
60	GH 4526	NE	3	100	N	N	W	W	W	20-35	66
61	GH 4530	MB	4	100	N	N	N	W	W	27-35	0
62	GH 3685	MB	4	66	N	N	W	W	W	21-35	0
63	GH 4083	SE	3	66	N	N	N	W	W	28-35	100
64	GH 2302	SE	4	100	N	N	N	N	W	30-35	66
65	GH 2329	SW	5	66	N	N	N	W	W	28-35	100
66	GH 3671	NE	3	66	N	N	N	W	W	28-35	0
67	GH 3675	NW	4	100	N	N	W	W	W	20-35	0
68	GH 623O	NW	4	100	N	N	N	W	W	27-35	100
69	GH 4024	MB	5	100	N	N	N	W	W	26-35	66
70	GH 3667	MB	5	100	N	N	N	N	W	30-35	66
71	GH 2334	NE	5	100	N	N	N	W	W	28-35	66
72	GH 5042	MB	3	100	N	N	N	W	W	26-35	100
73	GH 3683	NW	-	-	-	-	-	-	-	-	-
74	GH 5038	MB	4	66	N	N	N	W	W	27-35	50
75	GH 4025	MB	4	100	N	N	N	N	W	30-35	100
76	GH 3706	MB	5	100	N	N	W	W	W	22-35	66
77	GH 2316	SE	3	33	N	N	W	W	W	21-35	0
78	GH 3668	MB	4	100	N	N	N	N	W	30-35	100
79	GH 3687	SE	3	100	N	N	N	W	W	28-35	66
80	GH 6047	SW	4	100	N	N	N	W	W	27-35	33
81	GH 4528	NW	4	100	N	N	N	N	W	30-35	66
82	GH 4548	MB	5	66	N	N	N	W	W	28-35	50
83	GH 3679	NE	5	66	N	N	N	W	W	25-35	100
84	GH 4546	SW	3	100	N	N	N	W	W	28-35	66
85	GH 3703	SE	4	66	N	N	N	W	W	27-35	50
86	GH 2285	SW	3	100	N	N	W	W	W	21-35	33
87	GH 5043	MB	4	100	N	N	N	W	W	27-35	0
88	GH 3669	SE	4	100	N	N	W	W	W	21-35	0
89	GH 2315	SE	3	100	N	N	N	N	W	30-35	66
90	GH 4774	SE	4	33	N	N	W	W	W	21-35	0
91	GH 5039	SW	3	100	N	N	N	W	W	28-35	100
92	GH 2310	NE	4	100	N	N	N	W	W	27-35	100
93	GH 1608	NW	4	100	N	N	N	W	W	28-35	66
94	GH 2321	NE	4	100	N	N	N	W	W	26-35	100
95	GH 3670	NW	3	66	N	N	N	W	W	27-35	100
96	GH 6046	MB	4	100	N	N	N	N	W	30-35	100
97	GH 6061	SW	3	100	N	N	W	W	W	21-35	33
98	GH 4765	NW	4	100	N	N	N	W	W	26-35	66
99	GH 3704	NW	5	100	N	N	N	N	W	30-35	100
100	GH 4535	MB	4	100	N	N	N	W	W	28-35	66
101	GH 1001	MB	5	100	N	N	N	N	W	30-35	100
102	GH 1002	NW	4	100	N	N	W	W	W	21-35	33
103	GH 1003	NW	4	100	N	N	N	W	W	28-35	33
104	GH 1004	NE	5	100	N	N	N	W	W	28-35	66
105	GH 1005	NE	6	100	N	N	N	N	W	30-35	100
106	GH 1006	MB	5	100	N	N	N	N	W	30-35	100

N = Normal; W = Wilting; - = Non-emergence and no score; MB = Middle Belt; NE = North East; NW = North West; SE = South East; SW = South West; GH = Accessions collected from Ghana.

Table3.2: Summary of physiological screening experiment for the determination of drought tolerance in Ghanaian cowpea accessions.

Drought Tolerant Varieties

GH 3665, GH 6048, GH 4767, GH 2289, GH 4026, GH 3701, GH 2328, GH 2314, GH4773, GH2326, GH 2219, GH 5048, GH 4527, GH 4532, GH 4770, GH 4542, GH 2338, GH 3689, GH 2284, GH 4028, GH 2347, GH 5044, GH 2337, GH 3666, GH 5344, GH 2318, GH 3711, GH 4027, GH 4549, GH 2281, GH 3708, GH 2336, GH 2275, GH 5049, GH 2313, GH 5045, GH 4526, GH 4083, GH 2302, GH 6230, GH 4024, GH 3667, GH 2334, GH 5042, GH 5038, GH 4025, GH 3706, GH 3668, GH 3687, GH 4528, GH 4548, GH 3679, GH 4546, GH 3703, GH 2315, GH 5039, GH 2310, GH 1608, GH 2321, GH 3670, GH 6046, GH4765, GH 3704, GH 4535, GH 1001, GH 1004, GH 1005, GH 1006.

Drought Susceptible Varieties

GH 5346, GH 5046, GH 3673, GH 3710, GH 4534, GH 4769, GH 6045, GH 5041, GH 4541, GH 5050, GH 4778, GH 4530, GH 3685, GH 3671, GH 3675, GH 2316, GH 6047, GH 2285, GH 5043, GH 3669, GH 4774, GH 6061, GH 1002, GH 1003.

Non- Emergence

GH 4537, GH 2312, GH 3678, GH 4772, GH 3674, GH 5040, GH 4529, GH 2279, GH 2306, GH2332, GH 5345, GH 3683.

Table3.3: Mean scores of ten qualitative and quantitative traits of cowpea

Accession #	PP	GP	TLS	TwT	Ph	SS	TT	LC	PH (cm)	SW (g)
GH 3665	3	2	2	3	3	5	3	5	32.5	24.7
GH 6048	3	2	2	0	3	1	1	5	27.9	26.1
GH 4767	3	2	3	3	5	1	1	5	24.1	17.8
GH 2289	0	2	2	3	3	1	1	5	33.7	15.7
GH 4026	1	2	3	7	5	5	3	3	25.3	23.6
GH 3701	3	2	4	7	3	5	5	3	28.9	19.4
GH 2328	0	2	1	3	3	3	7	5	28.2	18.5
GH 2314	0	2	1	3	3	1	7	5	26.7	23.7
GH 4773	0	2	2	0	3	3	7	5	25.5	15.9
GH 2326	9	2	2	0	5	5	3	3	34.6	18.9
GH 4537	9	2	2	3	3	1	5	7	31.3	19.7
GH 2312	3	2	2	3	3	1	5	7	26.8	18.1
GH 2291	1	2	1	3	3	1	3	3	25.2	25.7
GH 3678	1	2	3	0	3	5	3	3	24.9	22.9
GH 5048	3	2	2	7	5	3	3	3	25.6	23.8
GH 5346	7	2	4	7	3	1	1	5	27.5	22.6
GH 5046	7	2	2	3	3	1	1	5	28.1	15.5
GH 4527	0	2	2	7	3	1	7	5	27.1	23.1
GH 4772	0	2	2	0	5	5	5	5	26.5	24.8
GH 4532	0	2	4	0	5	2	3	5	28.4	22.9
GH 3674	0	2	2	3	5	2	7	5	19.5	17.5
GH 4770	3	2	3	3	3	1	1	5	31.2	21.9
GH 5040	3	2	1	3	3	2	1	3	27.5	22.7
GH 4529	0	2	1	0	3	2	1	3	18.9	23.7
GH 4542	0	1	3	5	3	2	7	3	25.7	18.9
GH 2338	0	1	1	3	3	2	7	3	27.3	23.4
GH 3673	7	2	2	3	3	1	1	5	26.9	17.3
GH 3710	9	2	2	5	5	1	1	3	26.1	15.8
GH 4534	9	2	1	0	5	5	1	5	31.4	25.7
GH 3689	3	2	2	7	3	2	3	5	29.7	23.2
GH 2284	0	2	2	3	5	3	3	5	28.8	13.5
GH4028	0	2	1	0	3	1	3	7	23.8	16.7
GH 4769	1	2	4	3	5	1	1	7	17.5	13.9
GH 2279	0	2	4	3	3	1	3	3	27.9	23.8
GH 2306	0	2	4	7	3	5	3	7	26.1	17.8
GH 2332	1	2	2	5	3	5	3	7	31.7	22.7
GH 6045	3	2	3	0	3	1	5	3	26.2	24.9
GH 2347	3	2	1	0	5	1	5	3	19.9	21.8
GH 5044	3	2	3	3	3	1	5	3	23.4	23.7
GH 2337	7	2	2	0	3	1	7	3	33.4	24.1
GH 3666	7	2	2	3	3	5	7	5	25.3	19.7
GH 5344	3	1	2	3	3	1	7	5	25.9	24.1
GH 2318	0	1	2	3	3	1	1	5	21.5	15.6
GH 3711	0	1	3	7	3	1	3	5	26.3	14.7
GH 4027	0	2	4	7	3	5	3	5	27.5	23.7
GH 5041	3	2	1	5	3	5	1	3	30	22.8
GH 4549	1	2	2	7	5	1	3	3	30.5	21.9
GH 4541	1	2	1	3	5	5	3	3	27.3	14.5
GH 2281	1	2	4	0	3	1	1	3	26.4	21.1
GH 3708	1	2	4	0	5	2	1	7	27.6	19.6
GH 2336	0	2	2	0	3	2	1	7	28.1	22.9
GH 2275	0	2	3	3	3	2	1	7	29.5	12.7
GH 5050	0	2	2	3	3	5	1	3	19.6	20.7

Table3.3. (continued)

Accession #	PP	GP	TLS	TwT	Ph	SS	TT	LC	PH (cm)	SW (g)
GH 5345	1	2	2	3	3	1	1	3	23.2	23.5
GH 5049	1	2	2	3	3	1	1	3	23.4	20.7
GH 2313	0	2	2	3	3	5	3	3	23.4	15.7
GH 4778	0	2	1	0	3	5	3	5	33.7	19.8
GH 2342	0	1	2	3	3	1	1	5	30.5	16.4
GH 5045	1	2	1	0	3	1	1	5	30.8	17.9
GH 4526	3	1	2	0	3	1	1	3	27.6	15.3
GH 4530	3	2	3	0	5	1	5	3	24.7	21.8
GH 3685	3	2	1	0	5	1	3	3	18.9	20.9
GH 4083	0	1	3	0	3	5	1	3	30.6	23.7
GH 2302	0	2	2	0	5	5	1	5	21.8	19.6
GH 2329	7	2	2	3	3	5	3	5	31.5	21.7
GH 3671	7	1	2	7	3	1	5	7	30.7	17.8
GH 3675	3	2	3	7	3	1	5	7	21.7	23.5
GH 623O	0	2	4	0	5	3	5	3	21.3	20.7
GH 4024	0	2	4	0	5	1	1	7	18.9	19.8
GH 3667	0	2	1	0	3	1	3	7	27.8	22.4
GH 2334	1	2	4	0	3	1	3	3	31.9	17.5
GH 5042	1	2	1	3	5	3	3	5	18.5	21.6
GH 3683	3	1	2	3	5	5	1	5	27.7	23.3
GH 5038	1	1	2	5	5	1	1	3	26.2	18.7
GH 4025	3	2	1	5	5	1	1	5	25.7	19.7
GH 3706	0	1	2	0	3	1	1	5	28.4	17.5
GH 2316	0	1	2	3	3	1	1	3	23.2	20.5
GH 3668	0	2	2	3	3	3	3	5	24.6	23.7
GH 3687	9	2	2	3	3	3	7	5	25.1	24.1
GH 6047	7	2	2	5	5	5	7	7	23.9	18.7
GH 4528	7	2	1	7	3	5	7	7	25.7	13.5
GH 4548	3	2	2	3	3	1	7	7	28.4	16.9
GH 3679	1	2	3	3	3	1	1	5	25.1	16.9
GH 4546	1	2	3	0	3	1	1	5	28.4	17.1
GH 3703	1	2	4	0	3	1	1	3	33.2	15.6
GH 2285	0	2	3	0	3	1	3	3	33.1	15.3
GH 5043	0	1	4	3	3	1	3	3	27.1	14.7
GH 3669	0	1	4	3	3	5	3	3	23.6	17.8
GH 2315	0	2	2	3	3	5	3	3	26.8	17.1
GH 4774	1	2	1	0	5	2	3	3	29.4	24.3
GH 5039	1	1	1	0	3	5	1	3	28.5	15.9
GH 2310	1	2	2	0	5	2	1	3	25.4	21.3
GH 1608	0	2	2	3	5	2	1	5	26.7	17.5
GH 2321	0	1	4	3	3	2	3	5	29.5	16.9
GH 3670	0	2	2	3	3	1	3	5	30.1	17.2
GH 6046	1	1	2	0	3	5	3	5	24.3	22.7
GH 6061	0	1	2	0	5	5	3	3	24.3	18.3
GH 4765	0	2	2	0	5	5	1	3	21.8	24.5
GH 3704	1	1	1	3	5	5	3	3	22.7	23.7
GH 4535	3	2	2	7	5	1	1	5	27.8	16.5
GH 1001	3	2	1	7	3	1	5	3	31.8	15.9
GH 1002	3	2	3	5	3	1	7	3	32.7	21.3
GH 1003	0	2	3	5	3	5	7	5	26.2	17.9
GH 1004	1	1	4	7	3	1	5	3	30.5	22.8
GH 1005	1	2	4	3	5	5	5	5	25.2	23.6
GH 1006	1	2	2	3	3	5	5	3	31.7	21.4

PP= Plant pigmentation, GP= Growth pattern, TLS= Terminal leaflet shape, TWT= Twinning tendency, Ph= Plant hairiness, SS= Seed shape, TT= Testa texture, LC= Leaf colour, PH= Plant height (cm), SW= Seed weight (g).

3.3.3. Cluster analysis of morphological traits

The PowerMarker software was used to analyze the data in Table 3.3. The Dendrogram constructed on the basis of the data generated from the 10 qualitative and quantitative traits on 106 accessions (Table 3.3) divided them into numerous clusters (Fig. 3.2). It was observed that out of the 69 accessions that are drought tolerant, 29 of them were collected from the northern part of Ghana, 22 from the middle belt and 18 from the southern parts of the country. Those that were determined to be susceptible to drought were 25 accessions of which 5 were from the north, 5 from the middle belt and 15 from the south. Interestingly, the clusters obtained did not give any clear pattern purely on geographical grounds or source of the accessions and their determined drought status. The susceptible lines could not group together as a clade and could be found both at the top and the bottom part of the phylogenetic tree indicating that the drought status of the accessions could not be easily scored as being present for drought tolerant and absent for susceptibility.

The statistical analysis on the morphological quantitative traits, plant height (cm) and seed weight (g), of the 106 accessions indicated a negative correlation between the two traits with a correlation coefficient value (r) of 0.0605 and R^2 value of 0.25. The Covariance between the parameters was 0.7705 and a standard deviation value of 4.9436. The line plot analysis of the quantitative traits did not show any clear pattern (Fig. 3.3) but confirmed the fact that the two traits were variably correlated.

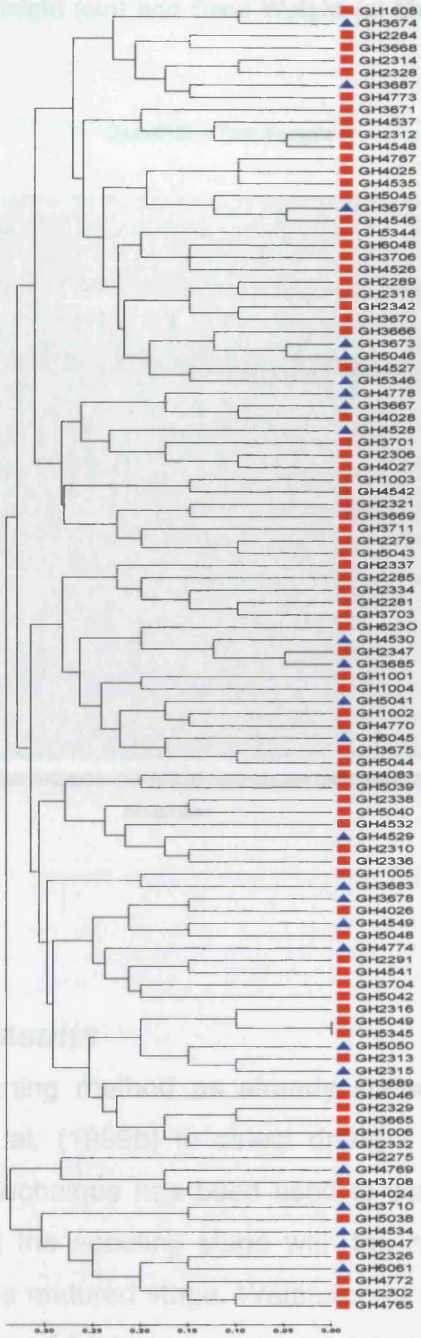
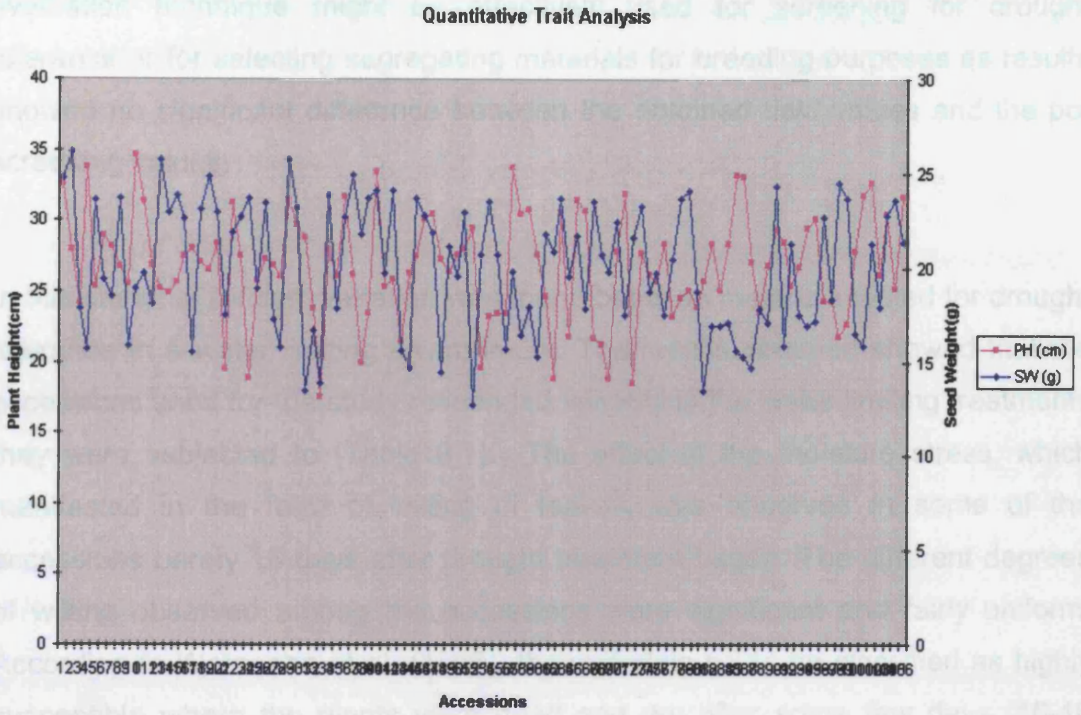


Figure 3.2 UPGMA Dendrogram of 106 cowpea accessions using 10 qualitative and quantitative morphological traits. ■ = Drought tolerant; ▲= Drought susceptible. The scale ranges from 0.00 to 0.30 from right to left, (0.00; 0.05; 0.10; 0.15; 0.20; 0.25; 0.30).

Fig.3.3. Analysis of Plant Height (cm) and Seed Weight (g) Measurements of 106 Cowpea accessions from Ghana:



3.4. Discussion of results

The wooden box screening method as already stated is a simple technique developed by Singh et al. (1999b) to select drought tolerant plants from the susceptible ones. This technique has been used to screen cowpea germplasm for drought tolerance at the seedling stage with the more tolerant lines being evaluated in the field at a matured stage. Watanabe et al. (1997) have used this technique to evaluate drought tolerance in cowpea germplasm, but in their case they used pots rather than wooden boxes as soil containers for the planting of seeds. They observed that the use of pots was simple and labour saving. They could evaluate about 1000 accessions at a time and found it adequate to correctly evaluate drought tolerance provided germination was good and uniform.

They also found a significant correlation (r=0.89) between the pot evaluation results and field evaluation results. These findings suggested that the pot evaluation technique might be effectively used for screening for drought tolerance or for selecting segregating materials for breeding purposes as results showed no significant difference between the obtained field values and the pot screening values.

In this study, significant variation was found between materials tested for drought tolerance in a water limiting environment. The results obtained showed that the accessions used for the study responded variably to the water limiting treatments they were subjected to (Table 3.1). The effect of the moisture stress, which manifested in the form of wilting of leaves, was observed in some of the accessions barely 16 days after drought treatment began. The different degrees of wilting observed among the accessions were significant and fairly uniform. According to Watanabe et al. (1997), the materials could be classified as highly susceptible where the plants were dead and dry after some few days (10-16 days) of the onset of the stress; highly tolerant where most of the lines were still growing with green trifoliate leaves after long spells of drought, and intermediate tolerance where the plants had different degrees of defoliation, and discolouration of their leaves after long period of drought.

Following the same set of criteria, GH 5346, GH 5046, GH3710, GH 4534, GH 6045, GH 5041, GH 4778, GH 4530, GH 3685, GH 3671, GH 3675, GH 2316, GH 5043, GH 3669, and GH 4774 were the most susceptible to drought stress. They showed the highest level of wilted plants 15 days after watering had ceased, and most of the plants were dead and dry 28 days after the commencement of drought stress. They were therefore classified as highly susceptible. About 80% of these lines also happened to have been collected mainly from the southern and the Middle belt regions of Ghana where the rainfall pattern is more reliable. There were other accessions like GH 3673, GH 4769,

GH 4541, GH 5050, GH 6047, GH 2285, GH 6061, GH 1002, and GH 1003, which were classified as susceptible varieties but about a third of their replicates in the experiment were able to recover after re-watering. Some of them did not show signs of wilting until about 28 days after the drought stress has been imposed but very few of the plants in this category were able to recover after watering.

Some of the accessions like GH 3701, GH 5048, GH 2284, GH 5044, GH 2336, GH 2342, GH 3668, GH 6046, GH 3704, GH 1001, GH 1005, and GH 1006, showed very little signs of wilting about 28 days after drought stress had been imposed. They started exhibiting signs of wilting around 29-35 days after drought treatment. When irrigation was introduced after 35 days of drought stress all of them recorded 100% recovery. They were therefore considered as the most tolerant amongst the accessions. The other accessions which showed signs of wilting earlier, from 21-35 days after imposition of drought stress but had a percentage recovery of 50-100% were considered to be drought tolerant. Considering the source of collection of the lines and their deduced drought status, it is clear that most of the drought tolerant varieties originated from the Middle belt region of Ghana right upwards to the dry Northern regions. This pattern is not surprising due to the fact that these areas experience annual rainfall range of about 250-400mm, although the analysis by clustering (fig. 3.2) failed to differentiate the accessions based on their geographical origin. These accessions may have some genetic mechanisms that enable them to withstand drought stress for longer periods than others. The cluster analysis also gave a clear indication that tolerance to drought stress is not a simple trait that could be scored as present or absent (1 or 0), as the tolerant and susceptible accessions identified were interspersed and did not cluster into clade as shown in fig. 3.2.

Morphological traits are known to be influenced by the external environment, resulting in varying relationship patterns (Smith and Smith, 1989). The genetic

diversity analysed using morphological traits showed that there was a high amount of variation for the quantitative traits. In cowpea, seed weight is one of the three most important yield components and large seeds usually command consumer preference. Hence, breeders have endeavoured to develop varieties characterised by larger seeds coupled with other desirable traits. Polygenes controlling important metric traits are usually distributed among several quantitative trait loci (QTLs), which may not be linked to one another. It is therefore likely that tagging useful agricultural genes, such as those for seed weight and plant height, with tightly linked molecular markers could enhance efforts aimed at their improvement. The cluster analysis (fig. 3.2), could not give any meaningful groupings of the accessions, whilst line plot analysis of the two quantitative morphological parameters, seed weight and plant height (Fig. 3.3), was also not very informative thus indicating that in cowpea seed weight and plant height may be governed by several genes as in drought tolerance. For the qualitative traits, the level of variation among accessions observed was very low and most of the traits showed a similar pattern of phenotypic expression. From this study, it has been ascertained that genetic diversity and phylogenetic relationship in the cowpea accessions based on morphological traits could not reflect the real genetic variation and taxonomical relationship among them due to the small number of morphological parameters used for the study as well as the low level of variability amongst the accessions used.

The study also found that qualitative and quantitative morphological traits are valuable tools for cowpea characterisation. Many researchers have carried out studies on cowpea using morphological traits like plant pigmentation, plant growth habit, root traits, grain quality, and yield. These traits were all found to be of great importance to distinguish genetic variability, leading to a better classification of cowpea genotypes (Apte et al., 1987; Emebiri and Obisesan, 1991; Fery et al, 1994; Karkannavar et al., 1991; Drabo et al., 1985, Hall et al., 1997). Emebiri (1989) also characterised cowpea cultivars using their flower size and style length, and reported that both characters were highly heritable. Some

of the morphological traits used in this study like, seed weight, plant length, seed shape and twinning tendency were found to be efficient in discrimination of accessions. The results obtained for a trait like growth pattern indicated that most of the accessions exhibited an indeterminate growth pattern, which means that most of them could have the ability to produce new leaves even after flowering and therefore could lose all its leaves, which may enable the plant even during the harsh environmental conditions to protect its photosynthetic apparatus from destruction, and then produce fresh leaves when conditions are favourable.

A close observation of cowpea plants and its inheritance in boxes by Mai-Kodomi et al (1999a) had shown two types of drought tolerance mechanisms. The type 1 drought-tolerant lines stopped growing and conserved moisture in all plant tissues, stayed alive for over two weeks, and gradually the entire plant parts dried. In the type 2 drought-tolerant lines the trifoliates continued to grow slowly after the introduction of the stress. However, with continued moisture stress, the unifoliates showed early senescence and dropped off but the growing tips remained turgid and alive even longer, suggesting that the moisture was being mobilized from the unifoliates to the growing tips. In this study the type 2 mechanism was largely observed in most of the accessions used. The unifoliates dried and dropped off, whilst it was also observed that the general growth rate of the experimental lines was lower than their respective controls, suggesting that metabolic activities might have been slowed down to compensate for the lack of moisture.

The simplicity of the pot method in this study may be due to its focus on only shoot drought tolerance without involving the contribution of roots and other factors. Most of the earlier studies on drought tolerance have been conducted in the field where different mechanisms contribute to the overall drought tolerance of the plants and make it appear to be a complex trait. Screening for dehydration tolerance of the shoots only in the seedling stage using the pot method should be

related primarily to the stomatal behaviour or osmotic adjustments as other mechanisms would not be operative. Therefore, once the plants sense stress, it is likely that the genes controlling stomatal behaviour or osmotic adjustments would be activated. The opening and closing of stomata, permitting solutes to accumulate in the cells may be simple phenomena, and may be under major gene control.

Difficulties experienced include situations where tolerance scores in the replicates were not consistent. In some accessions some plants grew better than others, indicating that residual soil moisture sometimes differed. It also indicated that the degree of competition for water between plants may have affected plant growth. It is therefore recommended that for further analysis in the future, after germination of plants, only one plant should be kept in each pot to avoid competition.

3.5. Conclusions

- The pot evaluation screening method used in this study, though yet to be confirmed by field tests, has been able to discriminate between drought tolerant and susceptible accessions of the cowpeas used. Although the screening technique used has been successful in a controlled environment, further work on field evaluation basis is necessary to ensure its reliability for large scale screening. The study has also indicated that it is possible to simplify the screening process by separating the shoot drought tolerance from the influence of roots and may lead to faster progress in breeding for drought tolerant cowpeas and other crops based on this mechanism.
- The one hundred and six cowpea accessions are genetically variable and clustered into groups not necessarily based on the source of collection or the drought status of the line as determined by the results of the study.

- The study also showed that qualitative and quantitative morphological traits are valuable tools for cowpea characterisation but a clear cut relationship between these traits and the screening method used could not be ascertained and therefore molecular markers should be used for further analysis.

CHAPTER FOUR

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4.0. MOLECULAR DIVERSITY AND PHYLOGENETIC STUDIES ON GHANAIAN COWPEA ACCESSIONS 106

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CHAPTER FOUR

4.0. Molecular diversity and phylogenetic studies on Ghanaian cowpea accessions.

4.1. Introduction

Diversity can provide one means of sustaining and improving the livelihoods of farmers where or when environmental conditions are unfavorable in production systems. Much of the world's agricultural biodiversity is found in environments marginal for agricultural production. It is in such environments where management of high levels of diversity can become a central part of the livelihood management strategies of farmers and the survival of their communities. Loss of such diversity or genetic choices diminishes farmers' capacities to cope with extreme temperatures, drought and salinity. Thus, in the face of constant vulnerability to loss of key stress-resistant types, the management of appropriate diversity of a specific crop constitutes a significant component of livelihood strategies of farmers in highly stressed production environments.

In this chapter I have considered the genetic relationships between 121 cowpea lines using multi-locus DNA markers. Cowpea (*Vigna unguiculata*) is of major importance in the livelihoods of millions of relatively poor people in less developed countries. From the production of this crop, rural families derive food, animal feed and cash. Due to the high protein content (20-25%), cowpea has been referred to as "poor man's meat" by Nielsen and colleagues (1997). However, cowpea is extensively grown in the Sub-Saharan region of Africa, and in other semi-arid and arid regions of the world, where drought stress is often a major problem, leading to poor cowpea quality and major yield loss. Expanding the genetic resource base of drought tolerant material might improve the development of progenies with high genetic variability for further selection as well

as prevent any future problems associated with selections based on one or few sources.

4.1.1. Importance of genetic diversity studies

Analysis of genetic diversity in crops is important for crop improvement and provides essential information to enable more efficient use of available genetic resources (Vaughan et al. 2007; Mohammadi and Prasanna, 2003). Additionally, it is a platform for stratified sampling of breeding populations (Mohammadi and Prasanna, 2003) by putting them into subgroups with similar genetic characteristics. Even before modern molecular marker methods were available, it was recognized that accurate assessment of the levels and patterns of diversity would be invaluable in the analysis of genetic variability in cultivars (Smith et al. 1997; Sharp et al., 2000; Gale et al. 2001; Menz et al. 2002), identification of diverse parental combinations to create segregating progenies with maximum genetic variability for further selection (Barrett and Kidwell, 1998; Bruce et al. 2002) and in introgressing desirable genes from diverse germplasm into the available genetic base (Thompson et al., 1998). Genetic diversity can be estimated using morphological, biochemical and DNA-based markers. Morphological markers, as observed in chapter three, are influenced by prevailing environmental conditions. DNA-based markers offer consistent results regardless of cropping conditions, type, and age of sampled tissue (Sakiyama, 2000). These characteristics of DNA-based markers make it suitable for cowpea research.

4.1.2. Application of SSRs and LTR retrotransposons as molecular markers

4.1.2.1. SSR markers

Simple sequence repeat (SSR) is a relatively new class of plant DNA marker. It has many advantages including being rapid, reliable (Diwan and Cregan, 1997), abundant, co-dominant (Sanchez et al., 2002), highly heterozygous (Powell et

al., 1996), highly polymorphic, evenly dispersed along the genome, highly reproducible, somatically stable (Rovelli et al., 2000; Hormaza, 2002) and easy to assay using polymerase chain reaction (PCR) (Kuleung et al., 2004). It has been used for construction of genetic linkage maps of maize (Taramino and Tingey, 1996) sorghum (Tarawali et al., 1997b), soybean (Cregan et al., 1999) and rice (Temnykh et al., 2000). It has been used for cultivar identification of potato (Meagher , 2002), peach (Aranzana et al., 2003) and in genetic diversity analysis of different plant species including cowpea (Diwan et al., 1997; Courtois et al., 2000; Li et al., 2000; Rovelli et al., 2000; Smith et al., 1997; Kubik et al., 2001; Li et al., 2001; Manninen et al., 2000; Aasamaa et al., 2001; Hormaza, 2002; Pinto et al., 2003; Akkaya and Buyukunal-Bal, 2004; Bandelj et al., 2004; Li et al., 2004; Vaz Patto et al., 2001).

The primary disadvantage of SSR as a molecular marker is the cost and research effort required for development, since the technique usually demands specific primers for every species of interest (Kuleung et al., 2004). However, transferability, the application of SSR markers developed for one species on another related species, has been proposed but not successfully demonstrated to date. Comparative studies using RFLP, RAPD, AFLP and SSR indicated that SSR markers detected higher levels of polymorphism compared to other markers (Powelll et al., 1996; Pejic et al., 1998; Maguire et al., 2002; Bandelj et al., 2004). The highly polymorphic nature of SSR markers make them particularly useful for genetic diversity analysis in species with a narrow genetic base (Akkaya et al., 1995; Basu et al., 2007).

4.1.2.2. Retrotransposons as molecular markers

The ubiquitous nature of retrotransposons and their activity in creating genomic diversity by stably integrating large DNA segments into dispersed chromosomal loci make these elements ideal for development as molecular markers (Heslop-Harrison et al., 1997). Integration sites shared between germplasm accessions are highly likely to have been present in their last common ancestor. Therefore,

retrotransposon insertional polymorphisms can help establish pedigrees and phylogenies (Shimamura et al., 1997; Roveili et al., 2000) as well as serve as biodiversity indicators. In recent years, several molecular marker methods based on retrotransposons have been developed (Kumar and Hirochika, 2001). All rely on the principle that a joint is formed, during retrotransposon integration, between genomic DNA and the retrotransposon. These joints may be detected by amplification between a primer corresponding to the retrotransposon and a primer matching a nearby motif in the genome. The methods have been named according to the particular motif that provides the second priming site. The Sequence-Specific Amplified Polymorphism (SSAP) method, the first retrotransposon-based method to be described, amplifies products between a retrotransposon integration site and a restriction site to which an adapter has been ligated. In Inter-Retrotransposon Amplified Polymorphism (IRAP) (Fig.4.1), segments between two nearby retrotransposons or long terminal repeats (LTRs) are amplified. The Retrotransposon-Microsatellite Amplified Polymorphism (REMAP) technique detects retrotransposons integrated near a microsatellite or stretch of SSRs (Fig.4.1). The Retrotransposon-Based Insertional Polymorphism (RBIP) marker system, in contrast to the others, detects a given locus in both alternative states, namely, empty and occupied by a retrotransposon, by using both flanking primers and a retrotransposon primer. Although these methods are examples with primers specific to a particular family of retrotransposons, it should be noted that retrotransposon marker methods are generic. Any organism in which retrotransposons are dispersed components of the genome, and in which they have been active over a timescale relevant to the question being asked, can be examined with retrotransposon markers. A couple of direct comparisons of retrotransposon marker methods with AFLP indicate that the retrotransposon markers are some 25% more polymorphic (Waugh et al., 1997; Yu and Wise, 2000).

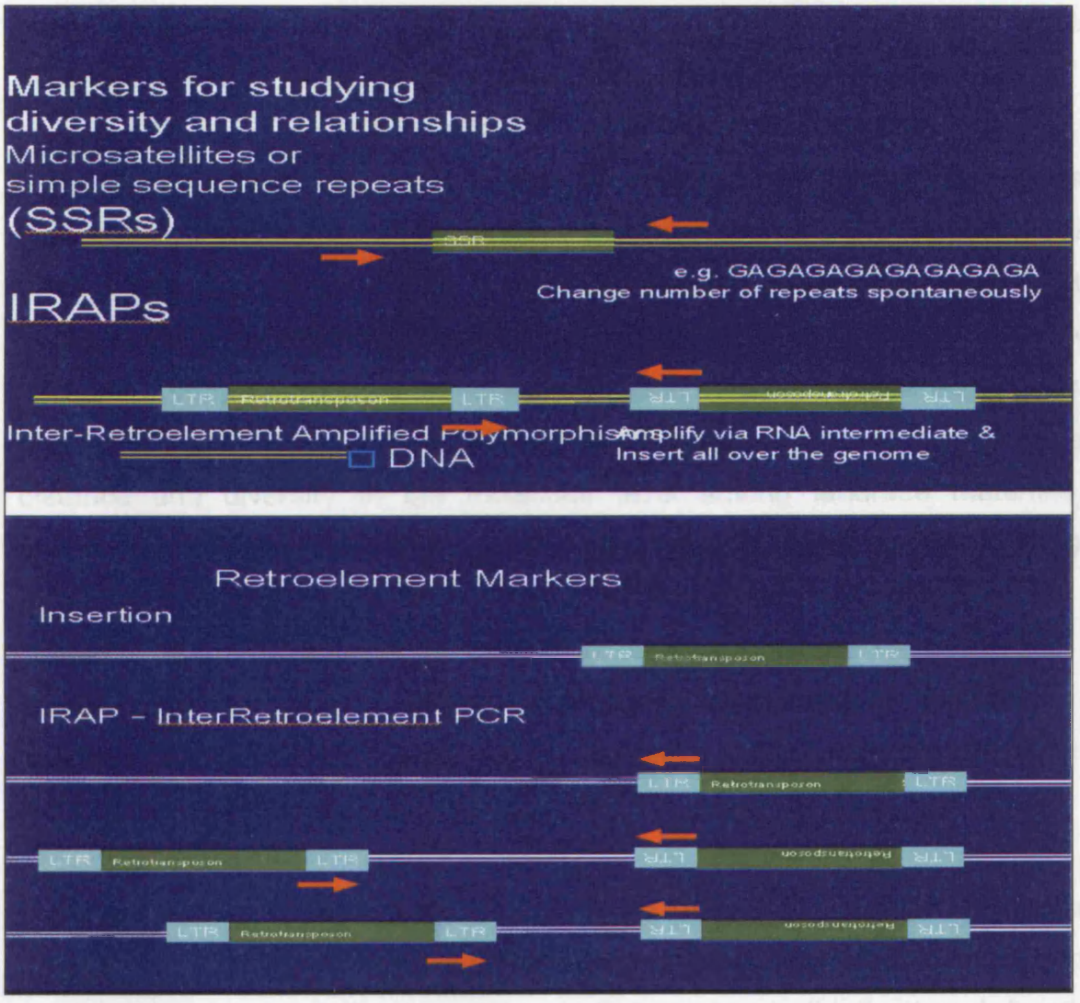
IRAP (Fig.4.1) detects two retrotransposons or LTRs sufficiently close to one another in the genome to permit PCR amplification of the intervening region.

Unlike AFLP or SSAP, the method requires only intact genomic DNA as the template and PCR reagents and apparatus for amplification. There are no restriction enzyme digestion or adapter ligation steps. The amplification products are generally resolved by electrophoresis in wide-resolution agarose gels, but if labeled primers are used, sequencing gel systems may be employed. The amplified fragments range from under 100 bp to over several kilobase pairs, with the minimum size depending on the placement of the PCR priming sites with respect to the ends of the retrotransposon. The IRAP method (Kalendar et al., 1999) has found applications in gene mapping in barley (Manninen et al., 2000) and wheat (Boyko et al., 2005) and in studies of genome evolution in the grasses (Vicient et al., 2001). Even given a large genome and a highly prevalent retrotransposon family, one would not expect the IRAP method to produce very many resolvable PCR products. The method, however, does produce a range of sub-kilobase fragments, in part because the genomes are organized into gene-rich islands surrounded by seas of repetitive DNA. The retrotransposons, which comprise large portions of the repeat seas, tend to be nested, one inserted into another, in barley, maize, and other grasses (Kumar et al., 2006; Heslop-Harrison et al., 1997). The IRAP amplification products can derive, therefore, variously both from nearby solo LTRs and full-length elements interspersed with non-retrotransposon DNA and from nested retrotransposons.

REMAP is conceptually similar to IRAP, but it differs in that it detects polymorphisms in the presence of retrotransposons or LTR derivatives sufficiently near simple sequence repeats (SSRs), often referred to as microsatellites, to allow PCR amplification. Microsatellites are ubiquitous features of eukaryotic genomes, and they have served directly to generate molecular markers in many plants (Saghai Maroof et al., 1994; McCouch et al., 1997). For this reason, researchers became interested in whether retrotransposons were associated with microsatellites in the genome, and to what extent such associational polymorphism could serve as molecular markers. Kalendar and colleagues (1999) reported that for *BARE-1* in barley, retrotransposon insertions

near microsatellites were considerably polymorphic and this finding was later confirmed by Ramsay et al (2000).

Fig.4.1: Marker methods for studying diversity based on SSR and LTR Retrotransposons



The REMAP method combines outward-facing LTR primers of the sort used in IRAP with SSR primers containing a set of repeats and one or more non-repeat nucleotides at the 3' end to serve as an anchor. The anchor is necessary to provide specificity to the PCR amplification; otherwise, the repetitive structure of the primer might cause it to anneal in multiple positions in any given microsatellite. Both IRAP and REMAP consist of PCR carried out on undigested

template DNA and resolve the products on agarose gels. Following the initial publication of the technique by Kalendar et al (1999), and almost simultaneously by Provan et al (1999) under the guise *"copia-SSR,"* REMAP has been used to examine genome evolution in wild barley *(*Kalendar et al., 2000*),* to map a major resistance gene in barley *(*Manninen et al., 2000*),* and as a sensitive method for detecting genomic copies of retrotransposons amidst retrotransposon cDNAs *(*Jääskelainen et al., 1999*).*

It has been demonstrated that the cultivated cowpea have lower genetic diversity than many other crops (Doebley, 1989), especially legume crops (Pasquet, 1993, 1999, 2000). Cowpea is believed to have been domesticated only once (Pasquet, 1999), unlike bean (*Phaseolus vulgaris*) (Singh et al., 1991) or rice (*Oryza sativa*) (Second, 1985). Currently, most characterization data on cowpea accessions in genebanks are based on morphological characters. Knowledge of genetic distance and diversity at the molecular level among landrace materials is important for a more profound characterization and to identify gene flow among populations. While numerous studies have evaluated molecular diversity of common bean, recent reports related to cowpea genetics have focused on linkage map and genome analysis (Menéndez et al., 1997). Information on molecular diversity of cowpea is still limited and data on molecular variation at the DNA level of Ghanaian cowpea is lacking.

Genetic diversity represented in the genepool underpins the improvement and adaptation of crops. After characterization of diversity, plant breeders can produce new crop varieties to resist new disease threats (biotic stress), to adapt to changing environments and abiotic stresses, and breeding can increase the sustainability of crop populations. Loss of genetic diversity may affect population viability and reduce the potential to adapt to environmental changes like drought. DNA-based techniques are essential to assay the variation present in wild and cultivated populations of crops both to ensure that breeders have full range of diversity for assessment and application, and to prevent loss of diversity for

future farmers. In this study, 106 cowpea (Vigna unguiculata) accessions from Ghana have been characterized with 10 other cultivars from IITA, Nigeria and 5 cultivars purchased from food stores in the United Kingdom. The aim of the study was to assess the genetic variability of the 121 accessions of cowpeas used in order to evaluate the potential application of SSR, IRAP and REMAP marker techniques in cowpea breeding programmes.

The objectives of the study were to:

- identify the polymorphisms generated by SSR, IRAP and REMAP techniques;
- determine the pattern and the extent of SSR, IRAP and REMAP marker variations within and among the Ghanaian cowpea accessions used;
- examine the population structure of the cowpea accessions used and determine the degree of genetic relatedness among the accessions used;
- determine whether the results obtained could be related to the drought status and the source of collection of the accessions used for the study.

4.2. Materials and methods

4.2.1. Genetic material and DNA extraction protocol

Seeds of one hundred and six cowpea (*Vigna unguiculata*) accessions collected from across the length and breadth of Ghana and fifteen other genotypes from Nigeria and United Kingdom were used for the study. Their description, collection source and accession number are given in section 2.1 and Tables 2.1 and 2.2. Genomic DNA was extracted from fresh leaves using the CTAB method as described in section 2.3.

4.2.2. SSR primers and analysis protocol

Thirty SSR primer pairs which previously showed clear polymorphisms in cowpea (Li et al., 2001) were used in this study (Table 4.1). SSR assays were performed

according to Colebatch et al. (2002) with minor modifications. Reaction mixtures for PCR amplifications of SSR loci have been described in section 2.4.2. The reactions were performed using a Touchdown amplification procedure depending upon the annealing temperature™ of the primers used. The general amplification cycle consisted of 18 cycles of 94˚C for 1 min (denaturing) and 72˚C for 1 min (extension). The annealing temperatures (30 s) were progressively decreased by 0.5˚C every cycle from 64 to 55˚C. The PCR reaction then continued for 30 additional cycles at 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min. The reaction ended with a 10 min extension at 72˚C. Amplification products were initially separated on 2% agarose gel in 1x TAE buffer stained with 0.5 μg/ml ethidium bromide and those found to be polymorphic were subsequently separated on 6% denaturing Polyacrylamide gels as described in section 2.5. The oligonucleotide products were visualized by silver staining as described under section 2.5.2.4.

4.2.3 SSR data analysis

DNA fragments were scored visually both from the agarose gel as well as from the scanned images. For each gel, the distance travelled by each marker size of the DNA ladder was measured using a ruler in Adobe Photoshop Elements 2.0. The PowerMarker software package version 3.25 (Liu and Muse, 2005) was used to analysis the data obtained and to calculate similarity coefficients among the genotypes. A dendrogram and phylogenetic tree were constructed from the similarity coefficient distance matrix using the UPGMA method of cluster analysis.

Table 4.1 Summary of cowpea microsatellite primer pairs used in the study (mostly obtained from Li et al., 2001)

Primer	Primer	Sequence Repeat	Product Size(bp)
VM 3	5'GAG CCG GGT TCA ATA GGT A 5'GAG CCA GGG CAC AGG TAG T	$(AG)_{27}$	171
VM 4	5'AGT AAA TCA CCC GCA CGA TCG 5'AGG GGA AAT GGA GAG GAG GAT	$(CT)_{20}$	248
VM 5	5'AGC GAC GGC AAC AAC GAT 5'TTC CCT GCA ACA AAA ATA CA	$(AG)_{32}$	188
VM 6	5'GAG GAG CCA TAT GAA GTG AAA AT 5'TCG GCC AGC AAC AGA TGC	$(AG)_{26}$	248
VM 8	5'TGG GAT GCT GCA AAG ACA C 5'GAA AAC CGA TGC CAA ATA G	$(AG)_{16}$	285
VM 11	5'CGG GAA TTA ACG GAG TCA CC 5'CCC AGA GGC CGC TAT TAC AC	$(TA)_4..(AC)_{12}$	195
VM 12	5'TTG TCA GCG AAA TAA GCA GAG A 5'CAA CAG ACG CAG CCC AAC T	$(AG)_{27}$	157
VM 13	5'CAC CCG TGA TTG CTT GTT G 5'GTC CCC TCC CTC CCA CTG	$(CT)_{21}$	135
VM 14	5'AAT TCG TGG CAT AGT CAC AAG AGA 5'ATA AAG GAG GGC ATA GGG AGG TAT	$(AG)_{24}$	144
VM 17	5'GGC CTA TAA ATT AAC CCA GTC T 5'TGT GTC TTT GAG TTT TTG TTC TAC	$(CT)_{12}$	152
VM 19	5'TAT TCA TGC GCC GTG ACA CTA 5'TCG TGG CAC CCC CTA TC	$(AC)_7..(AC)_5$	241
VM 22	5'GCGGGT AGT GTA TAC AAT TTG 5'GTA CTG TTC CAT GGA AGA TCT	$(AG)_{12}$	217
VM 23	5'AGA CAT GTG GGC GCA TCT G 5'AGA CGC GTG GTA CCC ATG TT	$(CT)_{16}$	174
VM 25	5'CCA CAA TCA CCG ATG TCC AA 5'CAA TTC CAC TGC GGG ACA TAA	$(TC)_{18}$	240
VM 26	5'GCC ATC AGA CAC ATA TCA CTG 5'TGT GGC ATT GAG GGT AGC	$(TC)_{14}$	294
VM 27	5'GTC CAA AGC AAA TGA GTC AA 5'TGA ATG ACA ATG AGG GTG C	$(AAT)_5 (TC)_{14}.(AC)_3$	207
VM 28	5'GAA TGA GAG AAG TTA CGG TG 5'GAG CAC GAT AAT ATT TGG AG	$(TC)_{20}$	250
VM 30	5'CTC TTT CGC GTT CCA CAC TT 5'GCA ATG GGT TGT GGT CTG TG	$(TC)_{10}$	140
VM 31	5'CGC TCT TCG TTG ATG GTT ATG 5'GTG TTC TAG AGG GTG TGA TGG TA	$(CT)_{16}$	200
VM 33	5'GCA CGA GAT CTG GTG CTC CTT 5'CAC CGA GCG CGA ACC	$(AG)_{18}.(AC)_8$	270
VM 34	5'AGC TCC CCT AAC CTG AAT 5'TAA CCC AAT AAT AAG ACA CAT A	$(CT)_{14}$	216
VM 35	5'GGT CAA TAG AAT AAT GGA AAG TGT 5'ATG GCT GAA ATA GGT GTC TGA	$(AG)_{11}.(T)_9$	127
VM 36	5'ACT TTC TGT TTT ACT CGA CAA CTC 5'GTC GCT GGG GGT GGC TTA TT	$(CT)_{13}$	160
VM 37	5'TGT CCG CGT TCT ATA AAT CAG C 5'CGA GGA TGA AGT AAC AGA TGA TC	$(AG)_5.(CCT)_3.(CT)_{13}$	289
VM 38	5'AAT GGG AAA AGA AAG GGA AGC 5'TCG TGG CAT GCA GTG TCA G	$(AG)_{10}..(AC)_5$	135
VM 39	5'GAT GGT TGT AAT GGG AGA GTC 5'AAA AGG ATG AAA TTA GGA GAG CA	$(AC)_{13}.(AT)_5.(TACA)_4$	212
VM 40	5'TAT TAC GAG AGG CTA TTT ATT GCA 5'CTC TAA CAC CTC AAG TTA GTG ATC	$(AC)_{18}$	200
VM 68	5'CAA GGC ATG GAA AGA AGT AAG AT 5'TCG AAG CAA CAA ATG GTC ACA C	$(GA)_{15}$	254
VM 70	5'AAA ATC GGG GAA GGA AAC C 5'GAA GGC AAA ATA CAT GGA GTC AC	$(AG)_{20}$	186
VM 71	5'TCG TGG CAG AGA ATC AAA GAC AC 5'TGG GTG GAG GCA AAA ACA AAA C	$(AG)12.(AAAG)_3$	225

To investigate the discriminatory power of each SSR primer, the polymorphic information content (PIC) was calculated. The observed heterozygosity (H_0) for each primer set was also obtained.

4.2.4 IRAP and REMAP primers and analysis protocol

The IRAP amplification reaction was performed according to the protocol described by Kalendar et al. (1999). The primer sequences, retrotransposon source, and orientation are shown in Table 4.2. An additional degenerate IRAP primer, designed from a multiple sequence alignment of chickpea (*Cicer*) retrotransposon sources, was also used and is shown in Table 4.2. PCR for IRAP was performed as described in section 2.4.4. The PCR products were analysed by electrophoresis on 2 % (w/v) agarose gel and detected by ethidium bromide staining.

The primers used for the IRAP amplification were combined with SSR primers (BT-GAC, BT-CTG, and Musa Ty2R) performing 8 LTR-SSR primer combinations as shown in Table 4.2. REMAP amplifications were performed in a final volume of 25 μl, containing 50 ng DNA, 10x (Bioline/York Bio) PCR buffer, 2.5 mM $Mgcl_2$, 0.25 μM dNTPs (Bioline), 0.4 μM of each primer and 0.5 U of Taq polymerase (Bioline/York Bio) in a T-Gradient Thermocycler (Biometra, Goettingen) 96-well plate. The amplification programme consisted of an initial denaturation cycle at 94˚C for 2 min, followed by 30 cycles at 94˚C for 30 s, 52˚C for 2 min, and 72˚C for 2 min for denaturation, annealing and extension, respectively. A final extension step was performed at 72˚C for 10 min. The amplification products were separated on 2 % agarose gel stained with ethidium bromide.

4.2.5. IRAP and REMAP data analysis

The amplification products were scored independently as 1 and 0 for presence and absence of bands, respectively, and the obtained binary data were used for the analysis. Phylogenetic trees were constructed by a distance-based method

and the genetic similarity between individual pairs of genotypes analysed by using neighbour-joining method (Nei et al., 1985;1998) and the MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007).

Table 4.2 Summary of primers used for IRAP and REMAP analysis (from Kalender et al. 1999; Teo et al. 2004)

Name	Retrotransposon Source and orientation	Sequence	Accession position
LTR 6149	BARE-1 (Forward)	CTC GCT CGC CCA CTA CAT CAA CCG CGT TTA TT	Z17327 1993-2012
LTR 6150	BARE-1 (Reverse)	CTG GTT CGG CCC ATG TCT ATC TAT CCA CAC ATG TA	Z17327 418-439
3' LTR	BARE-1 (Forward)	TGT TTC CCA TGC GAC GTT CCC CAA CA	Z17327 2112-2138
5' LTR1	BARE-1 (Reverse)	TTG CCT CTA GGG CAT ATT TCC AAC A	Z17327 1-26
5' LTR2	BARE-1 (Reverse)	ATC ATT GCC TCT AGG GCA TAA TTC	Z17327 314-338 7417-7441
SUKKULA	SUKKULA (Forward)	GAT AGG GTC GCA TCT TGG GCG TGA C	AY054376 4301-4326
NIKITA	Nikita (Forward)	CGC ATT TGT TCA AGC CTA AAC C	AY078073 AY078074 AY078075 1-22
CICER IRAP	CICER	ACT TTG GCW WAA AAG YCT CCG AGC C	14715228 14715227 AJ411814.1 16-41, 58-83
BT-GAC	SSR-GAC [RICH]	GAG AGA GAG AGA GAG AGA C	Randomly designed
BT-CTG	SSR-CTG [RICH]	CTC TCT CTC TCT CTC TCT G	Randomly designed
MUSA Ty2R	SSR-MUSA	GCA TGT CGT CAN CAT ANA RC	Randomly designed

Nucleotide degeneracy: R=A+G; Y=C+T; W=A+T; N=A+G+C+T

Table 4.3 Polymorphic band scorings of 16 SSR primers, source, and drought status of the accessions.

Accession #	Source	Drought Status	VM 35	VM 36	VM 31	VM 39	VM 70	VM 17	VM 26	VM 19
GH 3665	SE	DT	120/120	160/160	200/200	210/210	280/280	150/150	290/290	250/250
GH 6048	MB	DT	130/130	160/160	200/200	210/210	290/290	160/160	290/290	260/260
GH 4767	NW	DT	130/130	150/150	200/200	210/210	300/300	150/150	300/300	240/240
GH 2289	NE	DT	120/120	150/150	190/190	210/210	290/290	140/140	290/290	240/240
GH 4026	MB	DT	120/120	150/150	200/200	190/190	290/290	150/150	290/290	250/250
GH 3701	SE	DT	120/120	150/150	200/200	200/200	280/280	150/150	290/290	270/270
GH 2328	MB	DT	120/120	150/150	200/200	200/200	290/290	160/160	290/290	250/250
GH 2314	NE	DT	120/120	150/150	210/210	210/210	290/290	160/160	310/310	250/250
GH 4773	NW	DT	120/140	150/170	180/210	220/220	280/280	120/150	300/300	250/250
GH 2326	MB	DT	140/140	150/150	190/190	210/210	280/280	150/150	300/300	240/240
GH 4537	NW	DT	140/140	170/170	200/200	210/210	270/270	160/160	300/300	250/250
GH 2312	NE	DT	120/140	140/160	200/210	210/230	280/290	130/130	290/290	250/250
GH 2291	NW	DT	120/120	160/160	220/220	200/200	290/290	160/160	310/310	240/240
GH 3678	MB	DS	140/140	170/170	190/190	210/210	290/290	150/150	290/300	240/260
GH 5048	MB	DT	150/150	170/170	210/210	220/220	310/310	150/150	290/310	250/250
GH 5346	SW	DS	120/120	160/160	200/200	220/220	280/280	150/150	300/300	250/250
GH 5046	SW	DS	120/150	160/180	200/220	190/220	280/280	150/150	290/290	260/260
GH 4527	NE	DT	120/120	150/150	220/220	210/210	300/300	160/160	290/290	250/250
GH 4772	SE	DT	150/150	160/160	200/200	210/210	290/290	140/140	290/290	250/250
GH 4532	SE	DT	130/130	150/160	200/200	210/210	290/290	150/150	290/290	240/240
GH 3674	SW	DS	120/130	150/160	200/210	180/210	290/290	140/160	290/310	240/260
GH 4770	NW	DT	140/140	140/150	200/210	220/220	290/290	160/160	300/300	240/240
GH 5040	SE	DT	120/120	140/150	200/220	220/220	280/280	170/170	310/310	250/250
GH 4529	SW	DS	120/120	150/150	200/200	210/210	260/260	160/160	310/310	250/250
GH 4542	SE	DT	130/130	160/160	210/210	210/210	280/280	160/160	310/310	240/240
GH 2338	SE	DT	120/160	160/160	200/200	210/210	280/280	150/150	290/290	250/250
GH 3673	SE	DS	140/140	150/150	200/200	210/210	290/290	130/130	290/290	250/250
GH 3710	NE	DS	120/120	150/150	210/210	220/220	290/290	150/150	290/290	240/240
GH 4534	SW	DS	130/130	160/160	170/170	210/210	290/290	140/140	290/290	240/240
GH 3689	NE	DT	120/120	170/170	200/200	210/210	290/290	155/155	300/300	250/250
GH 2284	NW	DT	130/130	170/170	200/200	200/200	290/290	150/150	300/300	240/240
GH 4028	MB	DT	120/120	140/170	190/190	210/210	280/280	155/155	310/310	250/250
GH 4769	SE	DS	120/120	150/150	200/200	210/210	280/280	160/160	290/290	250/250
GH 2279	NE	DT	120/120	160/160	190/190	210/210	290/290	160/160	300/300	260/260
GH 2306	NW	DT	150/150	160/160	180/180	220/220	290/290	160/160	310/310	250/250
GH 2332	MB	DS	130/130	160/160	180/180	210/210	290/290	155/155	290/290	240/250
GH 4065	MB	DS	130/130	150/150	190/190	210/210	290/290	150/150	290/290	250/250
GH 2347	SE	DT	130/130	150/150	210/210	210/210	290/290	140/140	300/300	250/250
GH 5044	SE	DT	130/130	150/150	200/200	210/210	290/290	150/150	290/290	240/240
GH 2337	MB	DT	120/120	160/160	200/210	210/210	280/280	150/150	300/300	240/240
GH 3666	SE	DT	130/150	160/180	200/220	170/210	270/300	130/160	300/300	240/250
GH 5344	MB	DT	120/120	150/150	200/200	220/220	280/280	160/160	300/300	250/250
GH 2318	NW	DT	120/120	160/160	210/210	220/220	290/290	170/170	290/290	250/250
GH 3711	NW	DT	120/120	150/150	200/200	210/210	290/290	155/155	300/300	240/240
GH 4027	MB	DT	120/120	160/160	220/220	210/210	290/290	155/155	300/310	240/240
GH 5041	SE	DS	120/120	160/160	190/190	200/200	290/290	150/150	290/290	250/250
GH 4549	SW	DS	120/120	150/150	190/190	210/210	280/280	150/150	290/290	240/240
GH 4541	SE	DT	120/120	170/170	200/200	210/210	280/280	150/150	310/310	240/240
GH 2281	NE	DT	120/120	130/130	210/210	210/210	290/290	150/150	300/300	250/250
GH 3708	NW	DT	130/130	130/130	190/190	210/210	280/280	155/160	300/300	255/255
GH 2336	SW	DT	120/120	140/140	210/210	220/220	290/290	150/150	290/290	240/240
GH 2275	NW	DT	120/140	150/170	170/210	210/210	290/310	140/140	290/300	240/240
GH 5050	MB	DS	150/150	160/160	200/200	210/210	290/290	160/160	290/300	250/250
GH 5345	MB	DT	140/140	150/150	200/200	210/210	290/290	150/150	300/300	250/250
GH 5049	MB	DT	140/140	150/150	200/200	220/220	280/280	150/155	300/300	250/250
GH 2313	NW	DT	120/120	160/160	190/190	210/210	270/270	160/160	300/300	250/250
GH 4778	SW	DS	120/120	150/150	200/200	210/210	300/300	155/155	300/300	250/250
GH 2342	NE	DT	120/120	150/150	200/200	210/210	290/290	155/155	290/290	250/250
GH 5045	NE	DT	140/140	160/160	220/220	230/230	280/280	155/155	290/290	255/255
GH 4526	NE	DT	120/150	140/160	200/210	210/230	290/300	150/150	290/290	250/260

Table 4.3 Continued

Accession #	Source	Drought Status	VM 35	VM 36	VM 31	VM 39	VM 70	VM 17	VM 26	VM 19
GH 4530	MB	DS	140/140	170/170	200/200	210/210	300/300	150/150	310/310	240/240
GH 3685	MB	DS	120/120	160/160	210/210	210/210	290/290	155/155	300/300	250/250
GH 4083	SE	DT	130/130	150/150	230/230	210/210	300/300	160/160	300/300	250/250
GH 5043	SE	DT	120/120	150/150	210/210	220/220	290/290	150/150	290/290	240/240
GH 3669	SW	DT	150/150	170/170	200/200	220/220	280/280	150/155	290/290	250/250
GH 2315	NE	DS	130/130	160/160	200/200	210/210	280/280	155/155	300/300	255/255
GH 4774	NW	DS	120/120	160/160	200/200	210/210	270/270	155/155	300/300	240/240
GH 5039	NW	DT	130/130	150/150	200/200	210/210	290/290	160/160	300/300	250/250
GH 2310	MB	DT	140/140	160/160	200/200	220/220	280/280	150/150	310/310	250/250
GH 1608	MB	DT	120/120	150/150	210/210	220/220	290/290	150/150	310/310	230/230
GH 2302	NE	DT	120/120	150/150	210/210	220/220	280/280	155/155	290/290	240/240
GH 2329	MB	DT	130/130	150/150	200/200	190/190	280/280	155/155	290/290	250/250
GH 3671	NW	DT	120/120	160/160	230/230	190/190	290/290	180/180	300/300	250/250
GH 3675	MB	DT	120/120	160/160	220/220	180/180	280/280	155/155	290/290	250/250
GH 6230	MB	DT	130/130	150/150	190/190	190/190	290/290	160/160	300/300	240/240
GH 4024	MB	DT	150/150	160/160	210/210	200/200	280/280	150/160	310/310	250/250
GH 3667	SE	DS	120/120	150/150	200/200	220/220	300/300	150/150	300/300	245/245
GH 2334	MB	DT	120/140	160/170	200/210	210/230	280/290	150/170	290/290	240/240
GH 5042	SE	DT	130/130	160/160	200/200	190/190	290/290	150/160	290/290	250/250
GH 3683	SW	DS	120/120	150/150	200/200	210/210	260/260	160/160	290/290	250/250
GH 5038	NW	DT	140/140	150/150	190/190	210/210	280/280	155/155	300/300	250/250
GH 4025	MB	DT	140/140	150/150	210/210	220/220	290/290	155/155	300/300	250/250
GH 3706	NE	DT	120/120	150/150	200/200	220/220	290/290	150/150	290/290	250/250
GH 2316	SW	DT	120/130	160/170	200/210	200/220	290/310	150/170	290/310	230/260
GH 3668	SE	DT	120/120	160/160	200/200	210/210	280/280	155/155	300/300	250/250
GH 3687	SW	DS	120/120	150/150	200/200	210/210	290/290	155/155	300/310	250/250
GH 6047	MB	DS	130/130	160/160	190/190	210/210	280/280	150/150	310/310	260/260
GH 4528	SE	DS	120/120	150/150	200/200	200/200	290/290	150/150	310/310	250/250
GH 4548	SE	DT	130/130	160/160	180/180	220/220	280/280	155/155	310/310	250/250
GH 3679	SE	DS	120/120	170/170	200/200	210/210	290/290	150/160	300/300	240/260
GH 4546	SW	DT	120/120	160/160	210/210	210/210	290/290	150/150	290/290	250/250
GH 3703	NE	DT	120/130	170/180	200/220	210/230	290/290	160/160	290/290	250/250
GH 2285	NW	DT	120/140	160/180	200/210	210/220	270/290	160/160	290/290	250/250
GH 2321	NE	DT	120/120	170/170	210/210	220/220	290/290	155/155	290/290	250/250
GH 3670	NW	DT	120/120	150/150	200/200	220/220	280/280	150/150	300/300	240/240
GH 6046	MB	DT	120/120	150/150	220/220	210/210	300/300	155/155	290/290	250/250
GH 6061	SW	DS	120/120	150/150	200/200	190/190	290/290	155/155	290/290	240/240
GH 4765	NW	DT	120/120	160/160	200/200	180/180	290/290	150/150	300/300	250/250
GH 3704	NW	DT	140/140	170/170	190/190	200/200	280/280	160/160	300/300	250/250
GH 4535	MB	DT	140/140	150/150	210/210	210/210	300/300	150/150	290/310	250/250
IT91K-118	IT	NK	140/140	160/160	210/210	210/210	290/290	150/150	300/300	240/240
IT86D-716	IT	NK	120/120	160/160	210/210	200/200	290/290	155/155	290/290	250/250
IT89KD-374	IT	NK	120/120	160/160	200/200	190/190	290/290	155/155	290/290	240/240
IT85F-1380	IT	NK	130/130	170/170	200/200	210/210	310/310	140/140	290/290	250/250
IT93K-699	IT	NK	120/120	160/160	210/210	210/210	300/300	150/150	300/300	260/260
IT93K-596	IT	NK	120/120	170/170	210/210	220/220	310/310	160/160	300/300	260/260
IT92KD-267	IT	NK	140/140	150/150	200/200	210/210	290/290	140/140	310/310	250/250
IT93K-2309	IT	NK	140/140	150/150	200/200	210/210	280/280	155/155	290/290	250/260
IT88D-643	IT	NK	130/130	150/150	190/190	210/210	290/290	155/155	310/310	250/250
IT810-1228	IT	NK	120/120	160/160	210/210	220/220	290/290	160/160	290/290	250/250
LE 001	UK	NK	130/130	150/150	230/230	220/220	310/310	140/140	320/320	260/260
LE 002	UK	NK	150/150	180/190	210/210	170/170	310/310	140/140	310/310	250/250
LE 003	UK	NK	160/160	170/170	230/230	180/180	290/290	140/150	290/290	250/250
LE 004	UK	NK	180/180	190/190	240/240	210/210	270/270	140/140	320/320	250/250
LE 005	UK	NK	150/150	200/200	220/220	210/210	290/290	155/155	290/290	260/260
GH 1001	MB	DT	120/120	160/160	200/200	220/220	290/290	160/160	300/300	240/240
GH 1002	NW	DT	130/130	160/160	210/210	220/220	310/310	160/160	290/290	240/240
GH 1003	NW	DT	130/130	170/170	210/210	180/180	290/290	155/155	310/310	260/260
GH 1004	NE	DT	130/130	160/160	220/220	210/210	280/280	150/150	290/290	250/250
GH 1005	NE	DT	120/120	160/160	210/210	210/210	300/300	160/160	290/290	240/260
GH 1006	MB	DT	150/190	140/170	230/230	210/210	290/290	150/170	300/300	250/250

Table 4.3 Continued

Accession #	Source	Drought Status	VM 22	VM 27	VM 28	VM 30	VM 37	VM 38	VM 40	VM 68
GH 3665	SE	DT	220/220	190/190	260/280	140/140	270/270	130/130	190/190	300/300
GH 6048	MB	DT	220/220	210/210	260/260	140/140	280/280	140/140	190/190	300/300
GH 4767	NW	DT	220/220	210/210	260/260	150/150	290/290	140/140	200/200	290/290
GH 2289	NE	DT	220/220	200/200	260/260	130/130	290/290	130/130	210/210	300/300
GH 4026	MB	DT	220/220	210/210	240/260	140/140	280/280	140/140	190/190	300/300
GH 3701	SE	DT	210/210	210/210	240/240	140/140	290/290	140/140	210/210	270/270
GH 2328	MB	DT	220/220	210/210	230/230	150/150	300/300	140/140	190/200	290/290
GH 2314	NE	DT	220/220	200/200	260/260	150/150	280/280	150/150	190/190	300/300
GH 4773	NW	DT	230/230	200/200	260/260	150/160	290/290	140/140	180/180	280/280
GH 2326	MB	DT	230/230	210/210	250/250	160/160	290/300	130/140	190/200	290/290
GH 4537	NW	DT	230/230	210/210	250/250	140/140	300/300	130/140	210/210	300/300
GH 2312	NE	DT	220/220	210/210	250/250	140/140	290/290	150/150	200/200	270/300
GH 2291	NW	DT	220/220	210/210	250/260	160/160	280/280	130/130	200/200	290/290
GH 3678	MB	DS	220/220	200/200	260/300	150/150	290/290	140/140	200/200	290/290
GH 5048	MB	DT	220/220	210/220	260/260	150/150	290/290	140/140	190/190	300/300
GH 5346	SW	DS	220/230	220/220	300/300	140/140	280/280	140/140	200/200	290/290
GH 5046	SW	DS	210/210	210/210	300/300	160/160	280/280	130/130	200/200	290/290
GH 4527	NE	DT	210/210	190/190	250/250	150/150	280/280	140/140	190/190	290/290
GH 4772	SE	DT	220/220	210/210	260/260	160/160	290/290	140/140	200/200	300/300
GH 4532	SE	DT	220/220	210/210	270/270	160/160	280/280	130/130	210/210	300/300
GH 3674	SW	DS	230/230	220/220	300/300	150/150	280/280	130/130	180/180	290/290
GH 4770	NW	DT	230/230	220/220	260/260	140/140	290/290	130/130	190/190	290/290
GH 5040	SE	DT	210/210	210/210	250/250	140/160	280/290	130/130	190/190	290/290
GH 4529	SW	DS	220/220	230/230	260/260	160/160	270/270	150/150	200/200	290/290
GH 4542	SE	DT	220/220	210/210	250/250	140/140	280/280	140/140	200/200	280/300
GH 2338	SE	DT	230/230	200/210	250/300	140/140	290/290	150/150	190/190	300/300
GH 3673	SE	DS	210/210	190/190	280/280	150/150	280/280	130/130	190/190	300/300
GH 3710	NE	DS	210/210	220/220	260/260	160/160	300/300	130/130	190/190	300/300
GH 4534	SW	DS	210/210	220/220	250/260	160/160	290/290	130/130	200/200	290/290
GH 3689	NE	DT	210/210	220/220	260/260	150/150	300/300	140/140	200/200	290/290
GH 2284	NW	DT	220/220	210/210	260/260	130/130	290/290	150/150	190/190	280/280
GH 4028	MB	DT	220/220	210/220	280/300	140/140	300/300	150/150	190/190	290/290
GH 4769	SE	DS	210/210	210/210	280/280	150/150	300/300	140/140	200/200	290/290
GH 2279	NE	DT	210/210	220/220	260/260	160/160	280/280	130/130	200/200	290/290
GH 2306	NW	DT	210/210	200/200	260/260	140/140	290/290	130/130	200/200	300/300
GH 2332	MB	DS	210/210	200/200	250/250	140/140	300/300	130/130	200/200	290/290
GH 4065	MB	DS	210/210	190/190	250/250	140/140	290/290	140/140	200/200	280/280
GH 2347	SE	DT	220/220	210/210	260/260	130/130	300/300	130/130	200/200	290/290
GH 5044	SE	DT	220/230	200/200	290/290	150/150	280/280	160/160	190/190	290/290
GH 2337	MB	DT	220/220	200/210	280/280	150/150	280/280	140/140	190/190	280/300
GH 3666	SE	DT	220/220	200/200	280/280	160/160	290/290	140/160	210/210	290/300
GH 5344	MB	DT	220/220	200/200	250/250	150/150	300/300	130/130	200/200	290/290
GH 2318	NW	DT	220/220	210/210	250/250	150/160	280/280	140/140	200/200	300/300
GH 3711	NW	DT	210/210	220/220	260/260	140/140	290/290	150/150	200/200	290/290
GH 4027	MB	DT	230/230	210/210	280/280	150/150	290/290	130/130	190/190	300/300
GH 5041	SE	DS	210/210	220/220	250/260	160/160	290/290	130/130	190/190	300/300
GH 4549	SW	DS	230/230	210/210	250/250	140/140	290/290	140/140	200/200	300/300
GH 4541	SE	DT	210/230	210/210	280/280	140/140	300/300	130/130	200/200	290/290
GH 2281	NE	DT	220/220	200/200	260/260	150/150	280/280	140/140	190/190	280/280
GH 3708	NW	DT	220/230	200/210	260/260	150/150	290/290	140/140	190/190	290/290
GH 2336	SW	DT	220/230	200/210	280/300	150/150	290/290	130/130	200/200	290/290
GH 2275	NW	DT	210/210	210/210	300/300	160/160	290/290	130/130	190/190	280/280
GH 5050	MB	DS	210/210	190/190	260/260	140/140	280/280	140/140	200/200	290/290
GH 5345	MB	DT	210/210	210/210	260/260	140/140	290/290	150/150	200/200	290/290
GH 5049	MB	DT	220/220	210/210	280/280	150/150	290/290	140/140	200/200	280/280
GH 2313	NW	DT	210/210	200/200	280/280	150/150	290/290	140/140	190/190	290/290
GH 4778	SW	DS	210/210	200/200	260/260	140/140	300/300	150/150	200/200	290/290
GH 2342	NE	DT	210/210	180/180	210/210	140/140	280/280	130/130	190/190	270/300
GH 5045	NE	DT	210/210	220/220	230/230	150/150	290/290	130/130	190/190	280/280
GH 4526	NE	DT	220/230	210/230	250/250	150/160	290/290	130/130	200/200	290/290

Table 4.3 Continued

Accession #	Source	Drought Status	VM 22	VM 27	VM 28	VM 30	VM 37	VM 38	VM 40	VM 68
GH 4530	MB	DS	210/210	207/210	260/260	140/140	290/290	150/150	200/200	290/290
GH 3685	MB	DS	220/220	210/210	260/260	130/130	300/300	140/140	200/200	300/300
GH 4083	SE	DT	220/220	210/210	240/240	150/150	300/300	140/140	190/190	300/300
GH 5043	SE	DT	200/200	210/210	280/280	150/150	290/290	140/140	190/190	280/280
GH 3669	SW	DT	210/210	220/220	250/250	160/160	290/290	130/130	200/200	280/280
GH 2315	NE	DS	210/210	200/200	250/250	150/150	280/280	130/130	200/200	290/290
GH 4774	NW	DS	200/200	210/210	250/250	150/150	280/280	130/130	200/200	290/290
GH 5039	NW	DT	210/210	220/220	280/280	140/140	290/290	130/130	200/200	280/280
GH 2310	MB	DT	210/210	220/220	260/260	160/160	280/280	140/140	200/200	280/280
GH 1608	MB	DT	210/210	200/200	250/250	140/140	290/290	140/140	180/200	290/290
GH 2302	NE	DT	190/190	210/210	260/260	150/150	300/300	130/130	180/180	290/290
GH 2329	MB	DT	230/230	200/210	280/280	150/150	290/300	130/130	190/190	290/290
GH 3671	NW	DT	230/230	210/210	290/290	140/140	290/290	130/130	190/190	290/290
GH 3675	MB	DT	220/220	200/200	270/270	150/150	290/290	140/150	190/190	290/290
GH 6230	MB	DT	220/220	210/210	260/260	140/140	290/290	130/130	180/180	290/290
GH 4024	MB	DT	220/220	210/210	260/300	160/160	290/290	140/140	190/190	280/280
GH 3667	SE	DS	210/210	210/210	250/250	140/140	300/300	130/160	200/200	290/290
GH 2334	MB	DT	220/230	210/210	250/260	140/150	290/290	130/130	200/200	300/300
GH 5042	SE	DT	230/230	210/210	260/260	160/160	290/290	130/130	200/200	290/290
GH 3683	SW	DS	230/230	200/200	260/260	140/140	290/290	140/140	190/190	290/290
GH 5038	NW	DT	210/210	200/200	250/250	150/150	300/300	140/140	190/190	300/300
GH 4025	MB	DT	210/210	210/210	270/270	160/160	290/290	130/130	180/180	290/290
GH 3706	NE	DT	220/220	210/210	280/280	150/150	280/280	140/140	180/180	300/300
GH 2316	SW	DT	220/220	200/200	260/260	140/140	300/300	140/140	200/200	280/280
GH 3668	SE	DT	220/220	210/210	250/250	150/150	290/290	130/130	190/190	290/290
GH 3687	SW	DS	210/210	220/220	240/240	160/160	280/280	130/130	190/190	290/290
GH 6047	MB	DS	220/220	210/210	250/250	140/140	300/300	140/140	200/200	300/300
GH 4528	SE	DS	220/220	210/210	280/280	150/150	290/290	140/140	200/200	290/290
GH 4548	SE	DT	230/230	210/210	260/260	150/150	290/290	130/150	200/200	290/290
GH 3679	SE	DS	220/230	190/220	250/250	160/160	290/300	150/150	200/200	280/280
GH 4546	SW	DT	220/220	210/210	280/280	150/150	290/290	140/140	190/190	290/290
GH 3703	NE	DT	220/220	210/210	280/280	160/160	300/300	140/140	190/190	280/300
GH 2285	NW	DT	220/220	210/210	260/280	150/160	280/300	150/150	180/190	300/300
GH 2321	NE	DT	230/230	200/200	260/260	160/160	300/300	130/130	200/200	300/300
GH 3670	NW	DT	220/220	200/200	300/300	160/160	290/290	130/130	190/190	300/300
GH 6046	MB	DT	220/220	210/210	280/280	150/150	300/300	130/130	190/190	290/290
GH 6061	SW	DS	220/220	210/220	260/260	160/160	290/290	140/140	190/190	290/290
GH 4765	NW	DT	230/230	210/210	280/280	150/150	280/280	140/140	180/180	290/290
GH 3704	NW	DT	220/220	190/190	250/250	150/150	290/290	130/130	190/190	300/300
GH 4535	MB	DT	210/230	210/210	280/280	140/140	290/290	140/140	190/190	300/300
IT91K-118	IT	NK	220/220	200/200	250/250	140/140	290/290	140/140	200/200	290/290
IT86D-716	IT	NK	220/220	210/210	260/260	150/150	300/300	150/150	190/190	290/290
IT89KD-374	IT	NK	220/220	210/210	280/280	160/160	280/280	150/150	190/190	300/300
IT85F-1380	IT	NK	220/220	220/220	280/280	140/140	290/290	160/160	200/200	290/310
IT93K-699	IT	NK	220/220	200/200	280/280	160/160	290/290	130/130	190/190	290/300
IT93K-596	IT	NK	230/230	210/210	280/280	150/150	280/280	130/130	200/200	290/290
IT92KD-267	IT	NK	220/220	210/210	300/300	150/150	300/300	140/140	200/200	290/290
IT93K-2309	IT	NK	220/220	210/210	250/250	160/160	290/290	140/140	190/190	300/300
IT88D-643	IT	NK	210/210	200/200	260/260	160/160	280/280	140/140	190/190	300/300
IT810-1228	IT	NK	210/210	200/200	250/250	150/150	280/280	150/150	200/200	290/290
LE 001	UK	NK	230/230	180/180	220/220	150/150	290/290	140/140	200/200	280/280
LE 002	UK	NK	240/240	210/210	280/280	140/140	300/300	130/130	200/200	290/290
LE 003	UK	NK	210/210	220/220	210/210	120/120	280/280	140/140	210/210	290/290
LE 004	UK	NK	230/230	220/220	310/310	130/130	290/290	140/140	190/190	300/300
LE 005	UK	NK	220/220	190/190	310/310	160/160	280/280	130/130	200/200	300/300
GH 1001	MB	DT	220/220	230/230	300/300	150/150	280/300	130/130	200/200	280/300
GH 1002	NW	DT	210/210	220/220	250/260	150/150	280/280	140/140	190/190	300/300
GH 1003	NW	DT	230/230	210/210	280/280	140/140	290/290	150/150	180/180	290/290
GH 1004	NE	DT	220/220	200/200	260/260	140/140	290/290	140/140	200/200	290/290
GH 1005	NE	DT	220/220	210/210	250/250	150/150	300/300	130/130	200/200	300/300
GH 1006	MB	DT	210/210	200/200	260/260	140/160	280/280	140/140	200/200	290/290

MB = Middle Belt; NE = North East; NW = North West; SE = South East; SW = South West; DT = Drought Tolerant; DS = Drought Susceptible; NK = Not Known; IT = IITA, Nigeria; UK = United Kingdom

4.3. Results

The SSR, IRAP and REMAP protocols amplified DNA sequences from cowpea accessions with high reliability. Many primer pairs identified extensive polymorphisms between the accessions analyzed. Table 4.3 above shows the results of the polymorphic band scorings of 16 primers used for the study. The Ghanaian cowpea accessions were divided into five groups according to their location of collection or sampling, North-east (NE), North-west (NW), Middle-belt (MB), South-east (SE), and South-west (SW) (Table4.3). Analysis of the data generated from the scoring of the polymorphic bands as shown in Table 4.3 indicated that a total of 101 alleles were amplified among 121 cowpea genotypes from sixteen SSR primers out of a total of thirty SSR primers. However, VM (3,4,5,6,8,11,12,13,14,23,25,33,34, and 71) were either monomorphic or failed to amplify any products and were therefore not considered for further analysis.

4.3.1 Polymorphisms of SSRs in cowpea accessions

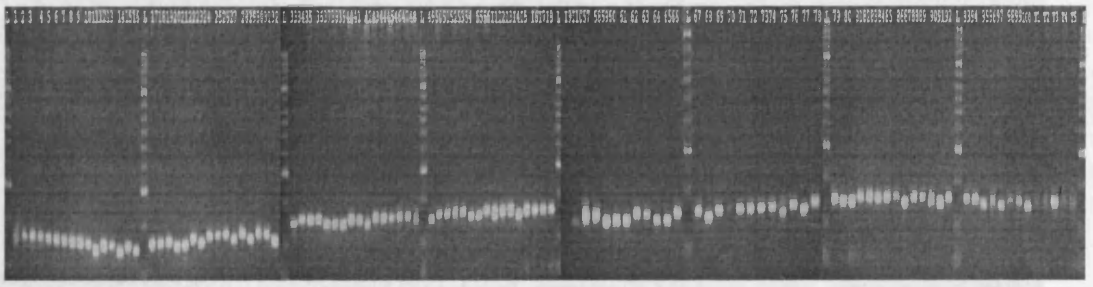
Table 4.4 Summary of genetic information generated by sixteen SSR primers on 121 accessions of cowpeas from Ghana, Nigeria and United Kingdom

SSR Marker	Allele Size Range (bp)	No. of Alleles	Allele Frequency	Gene Diversity	H_o	PIC
VM 17	130-170	8	0.35	0.74	0.11	0.70
VM 19	240-260	7	0.57	0.59	0.07	0.53
VM 22	210-240	6	0.46	0.65	0.07	0.59
VM 26	290-320	4	0.45	0.64	0.07	0.57
VM 27	240-310	7	0.50	0.67	0.09	0.62
VM 28	210-310	11	0.34	0.77	0.11	0.74
VM 30	130-160	5	0.38	0.69	0.06	0.63
VM 31	170-240	8	0.45	0.71	0.12	0.67
VM 35	120-190	7	0.51	0.66	0.11	0.61
VM 36	130-200	8	0.39	0.69	0.14	0.63
VM 37	270-300	4	0.48	0.64	0.05	0.57
VM 38	130-160	4	0.43	0.63	0.05	0.56
VM 39	170-230	7	0.55	0.63	0.07	0.59
VM 40	180-210	4	0.47	0.60	0.03	0.53
VM 68	270-310	5	0.52	0.61	0.07	0.54
VM 70	260-310	6	0.51	0.64	0.06	0.59
MEAN		**6.31**	**0.46**	**0.66**	**0.08**	**0.60**

PIC = Polymorphic information content; H_o = Observed Heterozygosity.

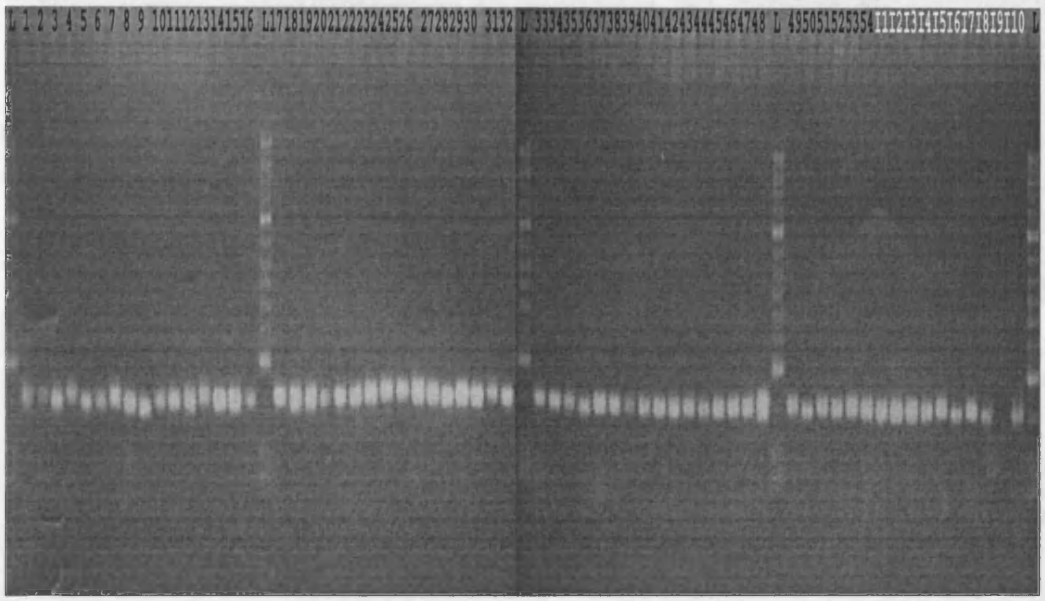
The number of amplified alleles per primer varied from 4 for VM 26,37 and 38 to 11 for VM 28, with a mean allele number per primer being 6.31 (Table 4.4). The allele frequencies for all the primers were generally below 0.95 indicating that they were all polymorphic in character. Gene diversity was high ranging from 0.59 in VM 19 to 0.77 in VM 28. The discriminative power of each SSR primer was assessed by calculating polymorphic information contents. The PIC per primer ranged from 0.53 for VM 19 and 40 to 0.74 for VM 28, with an average of 0.60. PIC values positively correlated (r = 0.65) with the number of amplified alleles per primer. The observed heterozygosity (H_0) calculated for each primer ranged from 0.03 to 0.14 with the mean being 0.08 (Table 4.2). The lowest H_0 value was recorded for VM 40 while the highest value was for VM 36. These low observed heterozygosity values were significant, since they tend to substantiate the homozygote nature of most of the accessions and the fact that cowpea is largely self pollinated. The sizes of amplified alleles ranged from 110 to 310 bp depending upon their respective expected product sizes as shown in Table 4.2 and Figures 4.1(A,B, and C), and except, for VM68 and VM70, was different from the reference (Table 4.4).

Figure 4.1A, B and C: PCR amplification products of microsatellite primers, VM 36, VM 31 and VM 35 respectively, on genomic DNA of cowpea accessions.
Fig.4.1A: Polymorphism patterns for VM 36



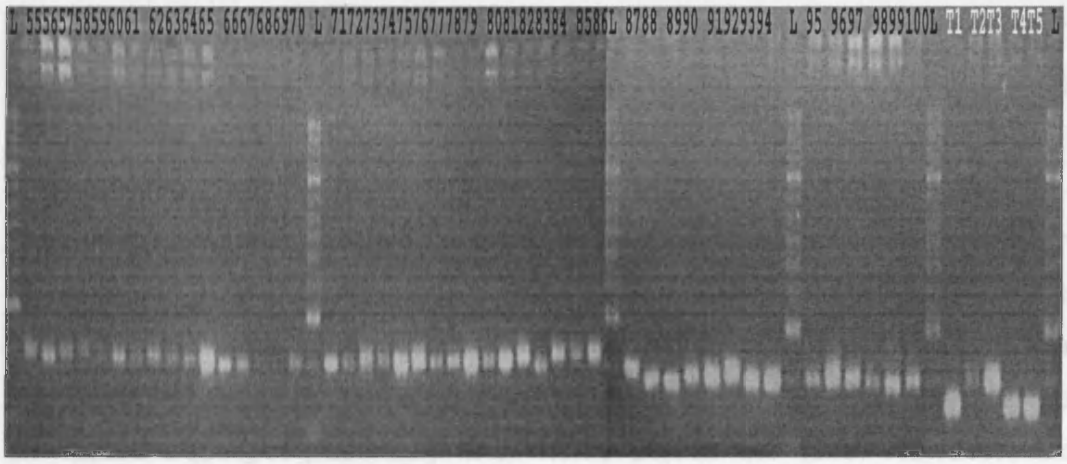
L-Hyperladderl;1-GH3665; 2-GH6048; 3-GH4767; 4-GH2289; 5GH4026; 6-GH3701; 7-GH2328; 8-GH2314; 9GH-4773; 10-GH2326; 11-GH4532; 12-GH4770; 13-GH2338; 14-GH-3673; 15-GH2347; 16-GH2281;17-GH5046;18-GH4527;19-GH4772;20-GH4532;21-GH3674;22-GH4770;23-GH5040;24-GH4529;25-GH4542;26-GH2338;27-GH3673;28-GH3710;29-GH4534;30-GH3689;31-GH2284;32-GH4028;33-GH4769;34-GH2279;35-GH2306;36-GH2332;37-GH6045;38-GH2347;39-GH5044;40-GH2337;41-GH3666;42-GH5344;43-GH2318;44-GH3711;45-GH4027;46-GH5041;47-GH4549;48-GH2281;50-GH3708;51-GH2336;52-GH2275;53-GH58050;54-GH5345;IITA(1-10); 55-GH5049; 56-GH2313; 57-GH4778; 58-GH2342; 59-GH5045; 60-GH4526; 61-GH6230; 62-GH3685; 63-GH4083; 64-GH5043; 65-GH3669; 66-GH2315; 67-GH4774; 68-GH5039;69-GH2310;70-GH1608;71-GH2302;72-GH2329;74-GH3675;75-GH6230;76-GH4024;77-GH3667;78-GH2334;79-GH5042;80-GH3683;81-GH5038;82-GH4025;83-GH3706;84-GH2316;85-GH3668;86-GH3687;87-GH6047;88-GH4528;89-GH4548;90-GH3679;91-GH4546;92-GH3703;93-GH2285;94-GH2321;95-GH3670;96-6046;97-GH6061;98-GH4765;99-GH3704;100-GH4535;T1;T2;T3;T4;T5.

Fig4.1B: Polymorphism patterns for VM 31



L-Hyperladder1;1-GH3665;2-GH6048;3-GH4767;4-GH2289;5GH4026;6-GH3701;7-GH2328; 8-GH2314; 9GH-4773; 10-GH2326; 11-GH4532; 12-GH4770; 13-GH2338; 14-GH-3673; 15-GH2347;16-GH2281;17-GH5046;18-GH4527;19-GH4772;20-GH4532;21-GH3674;22-GH4770;23-GH5040;24-GH4529;25-GH4542;26-GH2338;27-GH3673;28-GH3710;29-GH4534;30-GH3689;31-GH2284;32-GH4028;33-GH4769;34-GH2279;35-GH2306;36-GH2332;37-GH6045;38-GH2347;39-GH5044;40-GH2337;41-GH3666;42-GH5344;43-GH2318;44-GH3711;45-GH4027;46-GH5041;47-GH4549;48-GH2281;50-GH3708;51-GH2336;52-GH2275;53-GH58050;54-GH5345;IITA(1-10)

Fig4.1B: Polymorphism patterns for VM 31



L-Hyperladderl;55-GH5049;56-GH2313;57-GH4778;58-GH2342;59-GH5045;60-GH4526;61GH6230; 62-GH3685; 63-GH4083; 64-GH5043; 65-GH3669; 66-GH2315; 67-GH4774; 68-GH5039; 69-GH2310; 70-GH1608;71-GH2302;72-GH2329;74-GH3675;75-GH6230;76-GH4024;77-GH3667;78-GH2334;79-GH5042;80-GH3683;81-GH5038;82-GH4025;83-GH3706;84-GH2316;85-GH3668;86-GH3687;87-GH6047;88-GH4528;89-GH4548;90-GH3679;91-GH4546;92-GH3703;93-GH2285;94-GH2321;95-GH3670;96-6046;97-GH6061;98-GH4765;99-GH3704;100-GH4535;T1;T2;T3;T4;T5

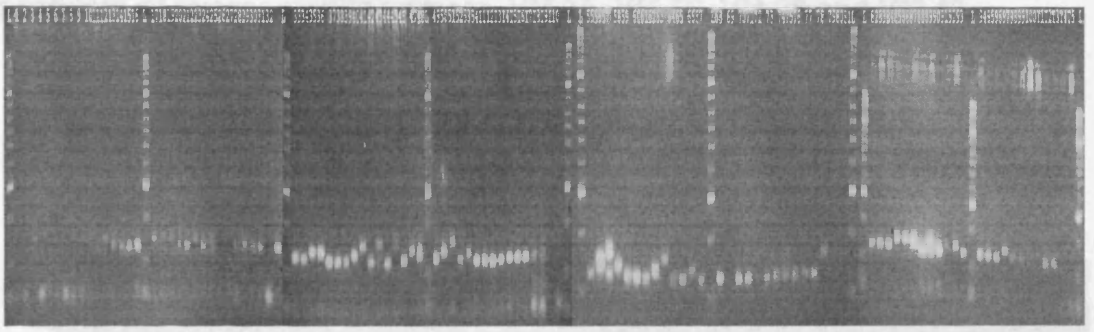


Fig4.1C: Polymorphism patterns for VM 35
L-Hyperladderl;1-GH3665; 2-GH6048; 3-GH4767; 4-GH2289; 5GH4026; 6-GH3701; 7-GH2328; 8-GH2314; 9GH-4773; 10-GH2326;11-GH4532; 12-GH4770; 13-GH2338; 14-GH-3673; 15-GH2347; 16-GH2281;17-GH5046;18-GH4527;19-GH4772;20-GH4532;21-GH3674;22-GH4770;23-GH5040;24-GH4529;25-GH4542;26-GH2338;27-GH3673;28-GH3710;29-GH4534;30-GH3689;31-GH2284;32-GH4028;33-GH4769;34-GH2279;35-GH2306;36-GH2332;37-GH6045;38-GH2347;39-GH5044;40-GH2337;41-GH3666;42-GH5344;43-GH2318;44-GH3711;45-GH4027;46-GH5041;47-GH4549;48-GH2281;50-GH3708;51-GH2336;52-GH2275;53-GH58050;54-GH5345;IITA(1-10); 55-GH5049; 56-GH2313; 57-GH4778; 58-GH2342; 59-GH5045; 60-GH4526; 61-GH6230; 62-GH3685; 63-GH4083; 64-GH5043; 65-GH3669; 66-GH2315; 67-GH4774; 68-GH5039;69-GH2310;70-GH1608;71-GH2302;72-GH2329;74-GH3675;75-GH6230;76-GH4024;77-GH3667;78-GH2334;79-GH5042;80-GH3683;81-GH5038;82-GH4025;83-GH3706;84-GH2316;85-GH3668;86-GH3687;87-GH6047;88-GH4528;89-GH4548;90-GH3679;91-GH4546;92-GH3703;93-GH2285;94-GH2321;95-GH3670;96-6046;97-GH6061;98-GH4765;99-GH3704;100-GH4535;T1;T2;T3;T4;T5

The SSR primers were able to distinguish all the 121 accessions used for the study. In fact, the four most polymorphic primer pairs (VM17, VM31, VM35 and VM36) could distinguish all lines. The scores of microsatellite alleles and calculated genetic distances were used to generate a dendrogram showing relationships between the accessions used for the study. Using PowerMarker software and frequency based distances after Nei and colleagues (1998), a UPGMA tree based on number of differences was obtained (Figure 4.3). The dendrogram generated generally agreed with the nature of the lines used for the study. The cowpea varieties acquired from Leicester, United Kingdom, were found to serve as out group together with other accessions from IITA, Nigeria. Within the 121 accessions used, there were no strongly significant groupings, indicating that the diversity represented by these SSR marker alleles was widely distributed both geographically and widely across the taxa. However, broad groupings, mostly with similarities in both locality and taxon, were evident in the trees, although these had low bootstrap support (data not shown).

Figure 4.2 Polyacrylamide gel images of cowpea accessions using products of microsatellite primers, VM 35, VM 39, VM 28 and VM 40.

Polyacrylamide gel photographs of a cross-section of cowpea accessions used for the study amplified with VM35, VM39, VM28, and VM40 primer pairs.

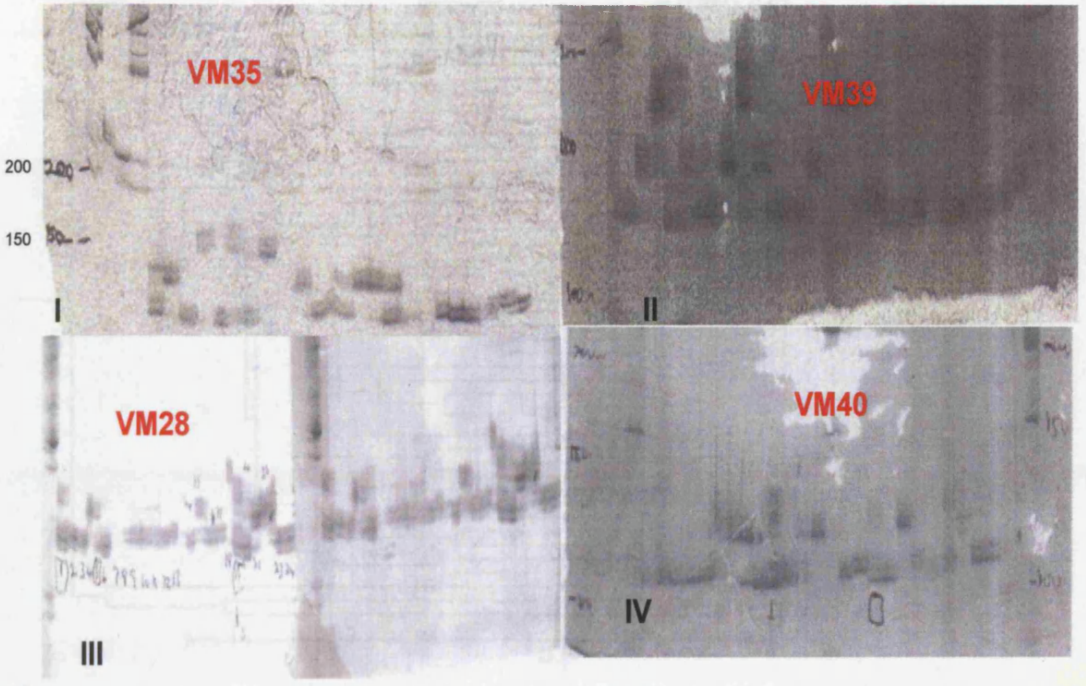
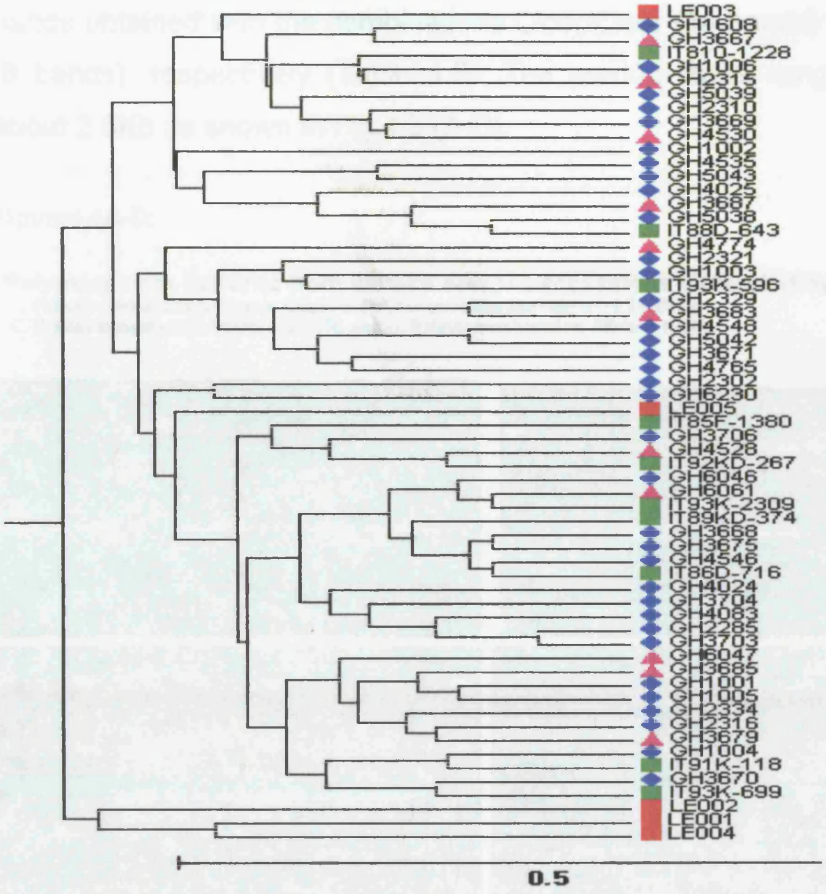


Fig.4.3. Phylogenetic relationship among 61 cowpea lines revealed by cluster analysis (UPGMA) based on genetic similarity (Nei et al., 1998) using 16 microsatellite polymorphic primers.



♦= Drought Tolerant; ▲ = Drought Susceptible; ■ = Leicester Out group; ■ = IITA.

4.3.2. Polymorphisms in IRAP and REMAP in cowpea accessions.

Retrotransposons can potentially integrate in either orientation, enabling the finding of members of a retrotransposon family as head-to-head, head-to-tail and tail-to-tail. To increase the probability of finding bands, one can combine primers from both 5' and 3' LTR ends or combine LTR primers with SSR primers to amplify intervening genomic DNA. As expected, the IRAP analysis produced a high level of polymorphism. All the six IRAP primer combinations (Table 4.5a) generated multiple fragments of defined sizes from the genomic DNA of all the cowpea accessions (Fig.4.4A-D). On average, single Cicer and LTR primer

cowpea accessions (Fig.4.4A-D). On average, single Cicer and LTR primer yielded 8-14 polymorphic bands, with the highest and the smallest numbers of bands obtained with the combinations Cicer/Cicer (14 bands) and 3' LTR/3' LTR (8 bands), respectively (Table 4.5). The product sizes ranged from 100bp to about 2.5Kb as shown in Fig.4.3 (A-D).

Figure4.4A-D:

Polymorphism patterns from sixteen cowpea accessions generated by IRAP.
A. Primer combination Cicer + Cicer; B. Primer combination Nikita + LTR 6149;
C. Primer combination Nikita + 3'LTR; and D. Primer combination Nikita + Nikita

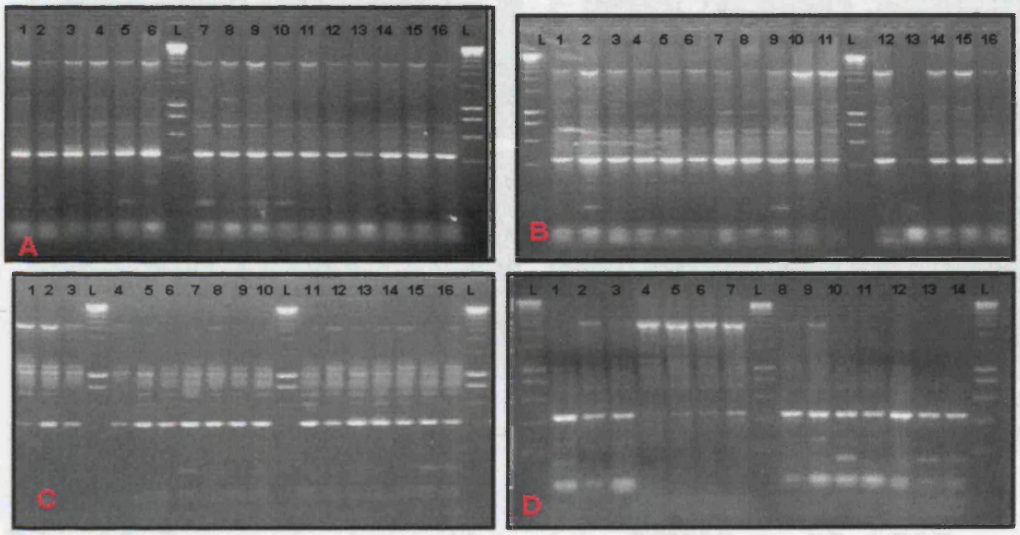
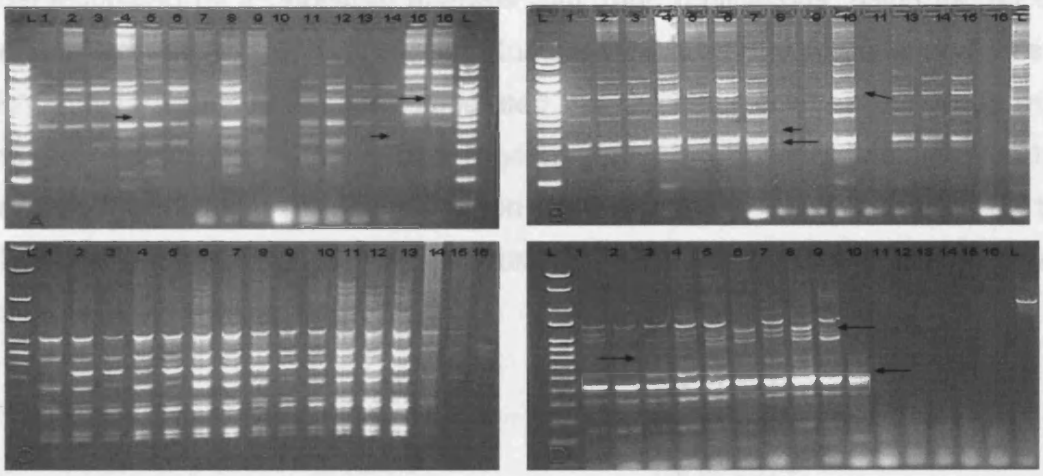


Table 4.5: Degree of polymorphism of the IRAP amplified PCR products within the Cowpea accessions

Primer Combination	Polymorphic Bands
Cicer + Cicer	14
Nikita + 3' LTR	10
Nikita + LTR 6149	12
3' LTR + 3' LTR	8
Nikita + Nikita	12
3' LTR + LTR 6149	10
Total No. of Polymorphic bands	66
Mean	11.0

The REMAP analysis was performed with 8 primer combinations, generating multiple fragments of defined sizes from the genomic DNA of all cowpea accessions. A total of 114 highly polymorphic bands were obtained (Table 4.6) which were highly reproducible. The primer combinations that amplified the highest and lowest number of bands were Cicer/BT-CTG (18 bands) and TY-2R/BT-CTG (10 bands), respectively. The product sizes ranged from 100bp to about 3.5kb as shown in Fig.4.5 (A-D).

Figures 4.4A-D: Polymorphism patterns from sixteen cowpea accessions generated by REMAP.



A. Primer combination Cicer + BT-GAC; B. Primer combination Cicer + BT-CTG; C. Primer combination TY-2R + BT- GAC; and D. Primer combination TY-2R + BT-CTG. The arrowed represents the unique bands identified for future analysis.

Table 4.6: Degree of polymorphism of the REMAP amplified PCR products within the Cowpea accessions

Primer Combination	Polymorphic Bands
Cicer + BT-GAC	16
Cicer + BT-CTG	18
Cicer + Musa Ty2R	14
Nikita + BT-GAC	14
Nikita + BT-CTG	16
Musa Ty2R + BT-GAC	10
Nikita + Musa Ty2R	12
Cicer + VM 35	14
Total No. of Polymorphic bands	114
Mean	14.25

Amplification products for both IRAP and REMAP were scored independently as 1 and 0 for presence and absence of bands, respectively, and the obtained binary data were used for the analysis. The genetic similarity between individual pairs of genotypes was analysed by using the MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007). The average similarity for all genotype pairs was used as a cut-off value for defining the clusters. The statistical stability of the clusters was also estimated by a bootstrap analysis with 1000 replications, using the MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007). The phylogenetic analyses of IRAP and REMAP techniques separated the Leicester cowpea out-group from the accessions from Ghana and Nigeria generally. However not all the 121 cowpea germplasm accessions used for the study were included in the phylogenetic tree, but the results obtained reflected the trend and diversity patterns in the SSR analysis.

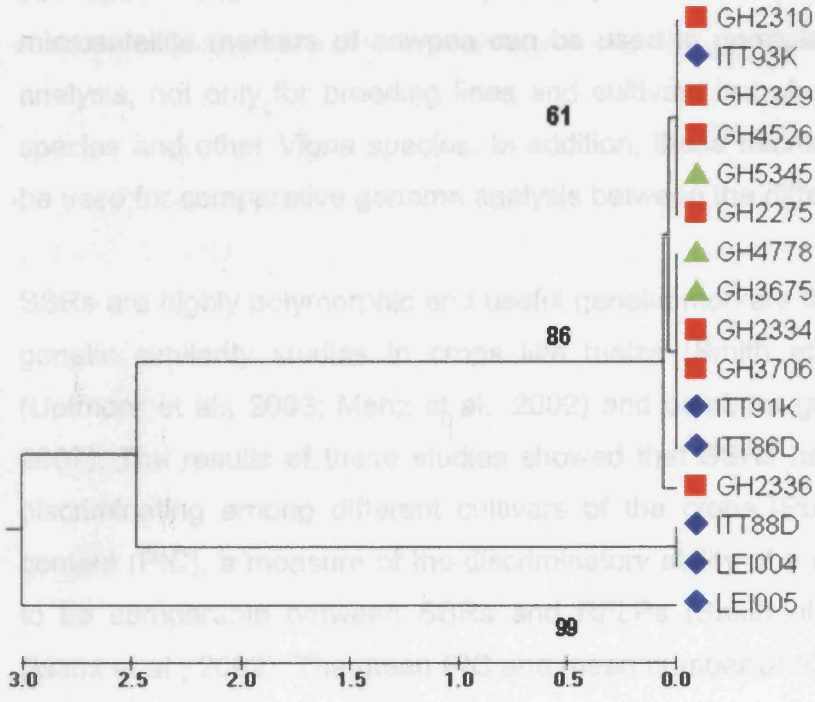


Figure 4.6: Phylogenetic model of the IRAP data based on six primer combinations for 16 cowpea accessions using UPGMA clustering method. ■ = Drought Tolerant; ▲ = Drought Susceptible; ◆ = IITA and Leicester out-groups. The percentage values for groups represent 1000 bootstrap cycles.

4.4. Discussion

Microsatellite markers have shown high level of polymorphism in many important crops including rice (*Oryza sativa* L., Chen et al., 1997), wheat (*Triticum aestivum* L., Devos et al., 1995; Röder et al., 1995), barley (*Hordeum vulgare* L., Liu et al., 1996), oat (*Avena sativa* L., Li et al., 2000), maize (*Zea mays* L., Senior et al., 1998), sorghum [*Sorghum bicolor* (L.) Moench, Brown et al., 1996], soybean [*Glycine max* (L.) Merr., Akkaya et al., 1992], beans (*Phaseolus* and *Vigna*, Yu et al., 1999), *Brassica* (Szewc-McFadden et al., 1996), alfalfa (*Medicago* spp., Diwan et al., 1997), sun-flower (*Helianthus annuus* L., Brunel, 1994), and tomato (*Lycopersicon esculentum* Mill., Smulders et al., 1997). The study in this chapter showed that microsatellite markers were also highly polymorphic in cowpea. They could distinguish cowpea accessions used in the study to a large extent. Twenty two out of the thirty microsatellite primer pairs of cowpea used in the study could successfully amplify DNA from all the cowpea accessions and 16 of these primer pairs were polymorphic. Therefore, microsatellite markers of cowpea can be used in germplasm conservation and analysis, not only for breeding lines and cultivars but also for the wild cowpea species and other *Vigna* species. In addition, these microsatellite primers could be used for comparative genome analysis between the different *Vigna* species.

SSRs are highly polymorphic and useful genetic markers that have been used in genetic similarity studies in crops like maize (Smith et al., 1997), sorghum (Uptmoor et al., 2003; Menz et al., 2002) and bambara groundnut (Basu et al., 2007). The results of these studies showed that SSRs have great potential for discriminating among different cultivars of the crops. Polymorphic information content (PIC), a measure of the discriminatory ability of a locus, has been found to be comparable between SSRs and RFLPs (Smith et al., 1997) or AFLPs (Menz et al., 2002). The mean PIC and mean number of alleles per locus for this study were 0.60 and 6.3 respectively, which compares favorably with results obtained from another studies by Tams and colleagues (2004), where a study of 128 accessions of Triticale with 28 SSR markers gave a PIC of 0.54 and

identified an average of 6.8 alleles per primer pair. Saeidi et al (2006), in a study of 57 Iranian *Aegilops tauschii* accessions with 9 SSRs found a PIC average of 0.66, and an average of 7.3 alleles per primer pair. The observed heterozygosity (H_0) calculated for the 16 primer pairs ranged from 0.03 to 0.14 with the mean being 0.08. The cowpea crop is known to be generally a self pollinated plant and therefore these results are indicative of this fact. It also indicates that the accessions used were mostly homozygote in nature.

Microsatellite markers have been used to investigate genetic diversity of a large number of cultivars in rice (Yang et al., 1994), soybean (Rongwen et al., 1995), wheat (Plaschke et al., 1995), and maize (Senior et al., 1998).The number of alleles amplified per primer pair was from 3 to 25 for rice, 11 to 26 for soybean, 3 to 16 for wheat, and 2 to 23 for maize. In the present study, only 4 to 11 alleles per primer pair were amplified from the 121 cowpea lines. Thus the level of microsatellite polymorphism in cowpea, although relatively high, is much lower than other crops. One possible reason is that the materials used in the present study were mostly from the Ghanaian open market and farmers and thus had a relatively narrow genetic base. Another possible reason for the low level of microsatellite polymorphism is that the cultivated cowpea is relatively low in genetic diversity compared with other crops. Genetic diversity of cultivated cowpea and its wild species have been extensively investigated by means of isozyme markers (Panella and Gepts, 1992; Pasquet, 1993, 1999; Vaillancourt et (Doebley, 1989), especially legume crops (Pasquet, al., 1999). It has been suggested that cowpea was only domesticated once (Pasquet, 1999), unlike *P. vulgaris* (Singh et al., 1997) or rice (Sweeney and McCouch, 2007). The low genetic diversity in cultivated cowpea may be a result of this narrow genetic base. The low level of genetic diversity at the DNA level among cowpea breeding lines and cultivars could be increased by using its wild relatives to broaden the genetic base. Studies by Xu et al., 1998 in Azuki bean had demonstrated that genetic diversity was low and less within the cultigens as compared to their wild relatives, where the genetic diversity was high. The current SSR study has

demonstrated that microsatellite markers are conserved among *Vigna* species. Hence microsatellite markers can provide a simple approach to assaying the introduction of such genetic material.

The results of this study clearly support the fact that IRAP and REMAP techniques have the ability to detect high levels of polymorphism without the need of DNA digestion, ligations, or probe hybridization to generate marker data. The degree of polymorphism of the IRAP and REMAP products in cowpea was high (Table 4.5 and Table 4.6) which are similar to that observed in sixteen species of barley (Kalender et al., 1999), and rice (Boyko et al., 2005). Retrotransposons can potentially integrate in either orientation, enabling the finding of members of a retrotransposon family as head-to-head, head-to-tail and tail-to-tail (Fig.4.1). Table 4.5 shows that all the primer combinations gave quite a high degree of polymorphism with the least from the 3' LTR/3' LTR combination whose orientation was tail-to-tail. This may probably suggest that the integration level of copia-retrotrasposons in tail-to-tail orientation in cowpea is lower than the other two orientations (head-to-tail and head-to-head), but needs further analysis using other combinations of primers. Although retrotransposon markers have previously been applied in the study of cereals and grasses (Boyko et al., 2005; Kalender et al., 1999; Kalender et al., 2000; Manninen et al., 2000) as well as in monocots like banana (Teo et al., 2005), the work reported here is the first application of both the IRAP and REMAP techniques on the Ghanaian cowpeas which are dicots.

The results of the cluster analysis of cowpea accessions in both the SSR and IRAP techniques (Fig. 4.3 and Fig. 4.6) indicated that the marker selection used was efficient in identifying the genetic variability within the collection. In the microsatellite phylogenetic tree (Fig. 4.3), sixteen Ghanaian accessions were observed to have clustered below a branch point at the lower part of the tree and all these accessions have been determined to be drought tolerant. Above this group of clusters are eleven drought susceptible and eighteen drought tolerant Ghanaian accessions. Although the bootstrap values for these branch points

were low and not conventionally significant (results not shown), the results of the cluster analysis is notable in showing that a group of accessions defined by several branch points are all drought tolerant, while other accessions include a mixture of susceptible and tolerant genotypes. Within the top half of the tree both tolerant and susceptible lines were observed but there was little structure evident as some of the most closely related pairs of accessions included both susceptible and drought tolerant lines.

Similarly, the phylogenetic model of the IRAP data based on six primer combinations for 16 cowpea accessions (Fig. 4.6) showed interesting clustering results, indicating very low genetic distances between the groups and non significant bootstrap values. Both the SSR phylogenetic tree and the IRAP tree could cluster most of the Leicester lines as the out-group and therefore were different from the African lines, however, the Leicester lines in both situations did not represent much diversity. The question that arises from the results of this study is "Can parental material for breeding for drought tolerance, selected based on the results of the analysis?" Ideally, if the susceptible lines had clustered together at the bottom part of the trees, the answer to the question would have been a definitive 'yes'. The results obtained with the use of IRAP and REMAP techniques has proven to be as reliable molecular markers as AFLPs, but they have also shown additional information indicating their great potential use in genome assessments for fingerprinting, mapping and diversity studies.

4.5 Conclusions

- The use of microsatellite markers (SSRs) and retrotransposon-based markers like IRAP and REMAP were found to be highly polymorphic and informative, suggesting that genomic fingerprinting has a major role in characterizing populations. The ubiquitous presence of LTR retrotransposons in plant genomes further suggests their usefulness either isolated or in combination.

- Diversity in the 106 Ghanaian cowpea accessions, which spanned the country from the dry sub-Saharan north to the wet central and coastal areas, were unique, substantially different from the UK sources and some of the Nigerian lines. The results indicated that there is much unexplored diversity for future varietal improvement.
- No significant correlation was observed in the results between the geographic origin of the accessions generally and their drought status, though markers used were able to classify lines and showed diversity not previously reported.
- The multi-locus PCR-based markers have potential to be effective tool for diversity analysis in cowpea, which may be useful in identifying promising candidates for interspecific hybridization programmes and marker assisted selection (MAS).

CHAPTER FIVE

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CHAPTER FIVE

5.0. Cloning and characterization of candidate gene fragments for drought tolerance in cowpea.

5.1. Introduction

Research into the plant responses to water stress is becoming increasingly important, as most climate-change scenarios suggest aridity in many areas of the globe (Phelps et al., 2002), and irrigation is recognized as a non-sustainable solution to plant water needs. On a global basis, drought in conjunction with coincident high temperature and radiation, poses the most important environmental constraints to plant survival and to crop productivity or range where a crop can be grown. Agriculture is a major user of water resources in many regions of the world. With increasing aridity and a growing population, water will become an even scarcer commodity in the near future. Even though in viable agriculture severe water deficits will be rare but catastrophic event (Passioura, 2002), a better understanding of the effects of drought on plants is vital for improved management practices and breeding efforts in agriculture and for predicting the fate of natural vegetation under climate change. Rainfed or sustainable agriculture, marginal land where irrigation is impossible due to other demands on limited water availability, as well as salinitization of irrigated lands are major problems to be addressed.

Plants respond to environmental stresses by initiating a number of physiological and developmental changes. Drought is one of the most severe environmental stresses that plants encounter and it affects almost all plant functions, including photosynthesis, growth, and development. Several genes have been described that respond to dehydration at the transcriptional level in a variety of plant species (Shinozaki et al., 2003). Analysis of these genes has indicated that their products might function cooperatively to protect cells from dehydration (Bray

1993, Bartels and Nelson 1994, Bohnert et al. 1995, Shinozaki and Yamaguchi-Shinozaki 1996). The gene products are thought to function in the control of water potential; the accumulation of osmoprotectants, such as sugars, proline and betaine; protein turnover; the protection of macromolecules; signaling pathways; transcriptional control, and so on (Bray 1993, Bohnert et al. 1995, Shinozaki and Yamaguchi- Shinozaki 1996). The existence of complicated mechanisms of drought tolerance in plants is indicated from the results of analysis of the functions of the various gene products.

Abscisic acid (ABA) plays an important role in the responses of plants to drought stress. Many drought-inducible genes are also induced by the application of exogenous ABA (Qin and Zeevaart, 1999; Bray, 1997). It appears that drought stress triggers the production of endogenous ABA, which, in turn, induces the transcription of several genes. Many genes that respond to ABA are also expressed at the late stages of embryogenesis during the development of seeds, and that are thought to function in the protection of cells from dehydration (Aasamaa et al., 2001). The expression of ABA-inducible genes has been analyzed extensively, and *cis-* and *trans-acting* factors involved in ABA-inducible gene expression have been reported (Bray, 2002a; Luchi et al., 2001). There are, however, several genes that are induced by dehydration but not by exogenous ABA (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki 1996). Analysis of these genes has revealed that ABA-independent, as well as ABA-dependent; signal-transduction cascades operate between the initial signal of drought stress and the expression of specific genes. The ABA-independent expression of drought-inducible genes has been analyzed extensively and multiple signal-transduction cascades have been proposed between the initial signal of drought stress and the expression of the genes (Bray 1997; Shinozaki and Yamaguchi-Shinozaki, 1996; Shinozaki et al., 2003).

Drought-inducible genes have also been analyzed in the past in the resurrection plant *Craterostigma plantagineum* (Bartels and Nelson 1994) and in the model

plant *Arabidopsis thaliana* (Shinozaki et al., 2003). By contrast, drought-inducible genes in drought tolerant crops have not been studied. It is obviously important to analyze drought-inducible genes and their expression in drought-tolerant crops if we are to understand the molecular mechanisms of drought tolerance for development of technologies for the molecular breeding of drought-tolerant crops. Outside maize (Xia et al., 2004), compared to disease resistances and yield characteristics, there has been rather less work on germplasm diversity with respect to drought responses, not least because the experiments are hard to control, replicate, evaluate and analyze.

At present, hundreds of genes that are induced under drought have been identified, and a range of tools (gene expression patterns, transgenic plants, and so on) is being used to study their specific function and role in plant acclimation or adaptation to water deficits, particularly in the model species including rice and Arabidopsis. However, because plant responses to stress are complex, the functions of many of these genes are still unknown (Cushman and Bohnert, 2000; Bray, 2002b). Not all stress-responsive genes are involved in cellular adaptive processes; some of them are simply involved in short-term deleterious responses. Many of the traits that explain plant adaptation to drought are those determining plant development and shape, such as phenology, the size and depth of root system, xylem properties or storage reserves. Responses may differ depending on whether drought is chronic (present throughout the life of the plant), acute (starting suddenly), and whether a normal or exceptional climatic characteristic (e.g. end of rainy season) is encountered. The question being asked in this study is "are these traits constitutive or stress-induced?"

Cowpea is a leguminous crop that is widely grown in semi-arid regions of the tropics because of its tolerance to drought and its superiority as a source of protein (Singh et al. 2002). Cowpea is also well adapted to a wide range of growth conditions. Moreover, many cultivars have been selected by farmers and plant breeders for specific environments with different levels of tolerance to

drought. The ability of cowpea to tolerate severe drought conditions makes it ideal for the study of molecular mechanisms of drought tolerance in crops. However, the reports on drought-inducible genes in cowpea are limited. In order to analyze the responses to dehydration stress of such a drought-tolerant plant at the molecular level, Luchi et al (1996) isolated 24 cDNA clones that corresponded to dehydration-induced genes from cowpea by a differential screening method. The cDNA clones were classified into ten groups and collectively named CPRD (**C**ow**p**ea clones **R**esponsive to **D**ehydration). The cloning and initial characterization of these 10 CPRD cDNAs and the expression of the 10 corresponding CPRD genes in response to dehydration and rehydration were subsequently reported.

My main aim of the study in this chapter is to gain a better insight of the molecular mechanisms of drought tolerance in certain Ghanaian cowpea accessions using the drought inducible CPRD family of genes as a basis for cloning, sequencing and comparative analysis of the genomic DNA of these lines.

The objectives of the study were to:

- Ascertain whether primers designed from sequences of drought inducible CPRD genes would amplify genomic DNA of unstressed cowpeas using simple PCR technique.
- Determine the genomic organization of CPRD gene fragments, allelic differences and how they correlate with drought tolerance.
- Find out whether the genes and their linked QTLs to drought tolerance are constitutive or adaptive in nature.

5.2. Materials and methods

5.2.1. Genetic material and DNA extraction protocol

Ten cowpea (*Vigna unguiculata*) accessions (six were drought tolerant lines and two were drought susceptible lines from Ghana, the drought status of the other two lines from Nigeria and UK not determined) selected randomly from the one hundred and twenty one cowpea lines were used for the study. Eight of the lines were from Ghana and one line each from Nigeria and United Kingdom. Genomic DNA was extracted from fresh leaves using the CTAB method as described in chapter two, section 2.3.

5.2.2. Primers designed from candidate genes

The list of candidate genes published by Luchi et al. (1996) for novel drought-inducible genes in drought-tolerant cowpea was used as the core in selecting potential candidate genes for amplification from genomic DNA. Specific primers were designed to facilitate the cloning and characterization of the candidate gene sequences and introns using Primer3 software programme aiming to generate the appropriate lengths of amplicons (http://www.frodo.wi.mit.edu/cgi-bin/primer3.html). Sequences of mRNA for the CPRD family were obtained from the GenBank and EMBL database. Specific primer sets were designed considering certain important factors enumerated in chapter 2 (section 2.3.2). Table 5.1 gives the source, type of gene and the size of the mRNA sequences used for the primer design for the study. From the sources of the sequences for the candidate genes (Table 5.1), 11 primer pairs were designed depending on the size of the coding sites of the gene. The primer sets designed are shown in Table 5.2, and the full length of the sequences with their respective primers has been displayed in Table 5.3.

Table5.1. Candidate Genes for Drought Tolerance from CPRD family

Accession	Gene	Size	CDS
D83970	CPRD 8	1515 bp	16...1170
AB030295	CpABA	2349 bp	185..2023
D88122	CPRD 46	4015 bp	71...2770
AF159804	Dhn 1	1149 bp	31...810
D88121	CPRD 12	1064 bp	76...879
D83971	CPRD 14	1227 bp	42...1019
D83972	CPRD 22	1129 bp	46...816
AB030294	CPRD 86	515 bp	1....246

5.2.3. PCR reaction, cloning and sequencing

The general PCR amplification reactions for the genomic DNA of the selected cowpea lines, using the primers designed and their respective annealing temperatures as shown in Table 5.2 were performed as already stated in chapter two, section 2.4.3. The PCR products confirming the expected product size of the primer being used were excised from the agarose gel and the DNA eluted using MinElute gel extraction kit (Cat. No. 28606; QIAGEN).

The eluted PCR product was ligated using the Promega pGEM-T easy vector and transformed into competent bacterial cells as described in section 2.7.3 of chapter two. The clones which were verified to have the DNA insert of interest as described in section 2.7.4 were sequenced commercially at John Innes Centre (JIC), at Norwich, United Kingdom, using M13 forward and reverse primers according to the requirements of the ABI 3700 capillary sequencing system.

Table5.2. List of Primer sets with their sequences and their respective expected product sizes and annealing temperature.

PRIMER	SEQUENCE	ANNEALING TEMP. & Product Size
CPRD22(a)	5' TCGCAAGATCGACGAGTATG 3' 5' TGAAGCACCCTGCTCTACAA 3'	55°C Exp. Prod. Size -997bp
CPRD22(b)	5' GCAGACACCCGTAGACAACA 3' 5' ACAACTGAAGCACCCTGCTC 3'	55°C Exp. Prod. Size -851bp
CPRD86	5' AGCATCATGGTGAGCACAAG 3' 5' CACACCAACCAAACCACAGA 3'	57°C Exp. Prod. Size -508bp
CPRD14	5' TTGCTTCCTGGGTCGTTAAG 3' 5' ATTCAAGCCCCAAGCTCTTT 3'	55°C Exp. Prod. Size -855bp
CPRD12	5' TCACTCATGGCAAATGGTTC 3' 5' GTCGGAAGCTACGGTTTCTG 3'	55°C Exp. Prod. Size -889bp
Dhn1	5' GTGGTCTTGGGGATGACACT 3' 5' ACAACTGAAGCACCCTGCTC 3'	60°C Exp. Prod. Size -909bp
CPRD46(a)	5' GCTGAGCTTGATCCCAAGAC 3' 5' CCAAATTCTTCGTCCCTCAA 3'	55°C Exp. Prod. Size -881bp
CPRD46(b)	5' AGCGGGTCTTAACCCTTGTT 3' 5' ACGGTTCATCCTTTTTGTCG 3'	57°C Exp. Prod. Size -947bp
CpABA(a)	5' GCAGAGGAGGAAAGTGATGC 3' 5' ATCCCCAAGCAAAGTCACAC 3'	55°C Exp. Prod. Size -982bp
CpABA(b)	5' AGAACCTCCTGGTGGTGTTG 3' 5' TGCCATGTTCACTCCGTAAA 3'	55°C Exp. Prod. Size -884bp
CPRD8	5' GACGAAAGAGCAGGTGGAAG 3' 5' GGATGCTACACCGTTCGATT 3'	60°C Exp. Prod. Size -915bp

Table5.3. Nucleotide sequences of the candidate genes used for the analysis showing the various primer pairs (in colour and underlined) designed from the sequences.

>CPRD8

CCTAACACCACCACAATGGTCACCGCCACCGCCACCGCCGCCACCGCTCCTTCTCACCAGGCAATTCCTCTTCTCACTCCCTACAAGAT
GGGCAAATTCAATCTCTCTCACAGAGTTGTCTTGGCACCGCTCTCCAGAGAGAGGTCCTACAACAACGTTCCGCAACCCCACGCCGTCGTCT
ATTATTCTCAGCGAACCTCCAACGGTGGCCTTCTCATCGCCGAAGCTACCGGAGTTTCCGACACCGCTCAAGGGTACCCCAACACGCCTGG
CATATGGACGAAAGAGCAGGTGGAAGCATGGAAACCCATCGTCGATGCCGTTCATGCCAAAGGCGGTATCTTCTTTTGCCAGATTTGGCAT
GTAGGAAGGGTTTCAGATTCAAGTTATCAGCCGAATGGGCAAGCACCCATTTCTTCTACCGACAAGCCACTGCCACCAACGCCTCGAGCTAA
CGGCCTTGGATATATCGAACACACGCCACCACGGCGCCTAACGACCGAAGAACTTCCTGGTATCGTCAATGACTTCAGAATTGCTGCAAGGA
ATGCCATCGAAGCTGGTTTTGATGGGGTTGAGGTCCATGGGGCACATGGGTACCTACTTGATCAATTTTTGAAAGACCAGGCTAATGACAGA
ACAGATCAATACGGTGGATCCCTCGAGAACCGGTGCAGACTTCCTCTGGAGGTTGTTGAAGCTGTTGTGAATGAGGTTGGGGCTGATAGAG
TTGGAATTAGGTTATCACCTTTTGCAGACTTTAACGATTGTGGTGACTCAAATCCCCTGCAACTGGGGCTTTACATGGTCAATGCACTCAATAA
GTATAACATTCTCTACTGTCACATGGTGGAACCCAGAATGGGAAGTGTTGGAGGTCCAGATGAAAACCTGACGCCAATGAGAAAGGTCTTCA
ATGGCACTTTTATAGTTGCCGGAGGTTATGACCGGGAAGATGGGATCAAAGCCATTGCTGAAGACAGGGCTGATCTTGTTGCCTATGGCCGT
TTGTTCTTGGCCAATCCAGATTTGCCAAAGAGATTTGCTCTCAATGCTCCTCTCAACCAGTATAATCGCAAGACATTCTACCACGAGGATCCT
GATCCGCTTGTTGGGTACATCGATTATCCGTTTCTTGATGAAGAATCGAACGGTGTAGCATCC

>CPRD22 (Two primer pairs, CPRD22(a); CPRD22(b))

CAAAAGTTCTTAAAATCACAACCTATCACAACAACAAGAACAACCATGGCAAGTTACCAGAAGCAGTACGAGGATCAGGGTCGCAAGATCGA
CGAGTATGGCAACGTTGTACAGGAAACTGATGAATATGGCAACCCGGTTCATGCAGCAAGTGTCACCTACATAACCTCCACCACCGGTGGTC
TTGGGGATGACTCTAACAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAACACGGAACTATAGGTGACACCGGTAGA
CAGCACGGAACTACCGGTGGTTTTACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGTTTTACCGGTGACACCGGGAGACAACATG
GGACTACCGGTGGGTTTACCGGTGACACCGGGAGACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAGACAACATGGGACTA
CCGGTGGGTTTACCGGTGACACTGGGAGACAACATGGGACTACCGGCGGCTTTACTGGTGGTGACACTGGTCTGGGAGGCCCTTACGTTG
GAGCCAACACCGCTGATACAGGGACTGGTCCTAGAAGTGGAACCACAGGTGGCAGCGCCTATGGATCGGGTGGCGCTGGTGGTGGTTATG
GAAGTGGAGCTGGAGCTGGTGGAGCTGGGTATGGTATGAACACAGGGGGAGCACACAGTGATGAAAGGTATGGAAGGGAGTATCGTGAGC
ATGATCAGTCTCGTGGAGATCATGACAAGAAAGGGATAGTGGACAAGATTAAGGAGAAGCTTCCCGGAGGACACAGTGACAACAAGTAGAG
ACATCATGGGTGTGCATGCATGCGTATATATACATAGTATAATTAAAGATGTTATATTGTTGTGTTTTTGAATAAGTTTGCTGCATATATACGTA
CTCGTACAGTACTGTCTTGTCGTGTAAATGTGGTGGATCTTGTATATGGTTATAGTGTATGCAAGAGGAATAAAAGGTGCATGCATGCAAGGG
AAACAAAGAAGGCATAGATGTTCTGCCAATGGATATGTAAAACCTACCCTGTTGTAGAGCAGGGTGCTTCAGTTGTGTGTTTTGAATTTTTCA
ATGAAGCAACTTTTTCTTTCAAAAAAAAAA

>CPRD86

GGCACGAGCCATGGTGAACACAGTGAGTACAAAGGAGAGCATCATGGTGAGCACAAGGGTGAATACAAAGGGGAGCACAAACCCGAGCAC
CATGGAGAAGAGCACAAAGAAGGGTTCGTAGAGAAGATCAAGGACAAGCTCCACGGTGAAGGTGGTGAGGGCGAGAAGAAGAAGAAGAAG
GAGAAGAAGAAGAAACATGAAGATGGCCATGGCCATGACAGCAGCAGCAGCAGTGACAGTGATTAGATATCACTGCTGCATCACCTATATAT
ATTTATATATGTTAAGCTAAGGTGTATGTGACCAGAGTAGTGAGGGCTGTGACTTCTTTTCTTGCTTTCTTGTGATCTGAATTTATGAGTGAAA
AAAAAAGTGAGAGAAAATTGTGGTTGGTCATTTCTGTGGTTTGGTTGGTGTGTAATTAACCCCACTGCTTTGGTTGTATATTATCATTATGAAA
TAAAAATTTTAATTATCAGTTTTAGTATTAAAAAAAAAAAAAAAAAAAAAAAA

>CPRD14

CAGAGTATCAGCTCAAAGCAGAACAAACAACATAATCCAGAATGAGCACCGGCGCCGGACAAGTAGTGTGTGTTACCGGAGCTTCCGGTTA
CATTGCTTCCTGGGTCGTTAAGTTTCTTCTCGAACGCGGCTACACCGTGAAGGCCACCGTTCGCGACACAAGTGATCCCAAAAAGGTAGATC
ACTTGCTTAGCCTCGATGGTGCTAAGGAAAGGCTGCATCTCGTCAAGGCGAATCTTCTAGAAGAAGGTTCCTTTGACTCTGCCGTTGAAGGT
TGTCACGCTGTGTTCCACACTGCTTCTCCCTTTTTCGACGATGCCAAGGACCCGCAGACTGAGTTGTTGGATCCAGCTTTGAAGGGGACTCT
GAATGTTCTGAAATCGTGCGTGAACTCGCCCACGCTGAAGCGCGTGGTTGTCACTTCTTCTATTGCTGCAGTTTCGTTCAACGACAGGCCCA
AAAACCCTGATGTAGTGGTTGACGAGACTTGGTATTCCGACCCAGAATACTGTAAGAGAAATGGGATATGGTATAACCTCTCAAAGACTTTGG
CCGAAGATGCTGCCTGGAAATTTGCGAAAGAAAACAATATTGACTTGGTTACAGCCAACCCTGCATTGGTTGTTGGACCTCTCTTGCAACCAG
TTCTTAACACTAGTTCTGCAGCAGTTTTAAATTTAATTAATGGTTCGCCAACATTTAAAAATGTGACTTTGGGATGGGTGGACGTGAGAGATGT
TGCAATTGCCCATGTTCTGGCATATGAGAATGCTTCAGCTAATGGAAGATATTTACTAGTTGAGAGAGTGGCACACTTCGGAGACGTTGTGAA
GATTTTGCATGATTTGTACCCAACATTGCAACTTCCACAGAAGTGTGTAGATGATAGGCCATATGATCCAATATTCCAGGTTTCCAAGGAAAAA
GCAAAGAGCTTGGGGCTTGAATTTACTCCTTTGGAAGTGAGCATCAAGGATACTGTTGAAAGCTTGAAGGAAAAGGGTTTTATCAAATTTTAA
GTTGTTACCCTTTCTTGAAAGAAACCTTCTTTAGTTCTACACTTGGTTTCTTATGTTCATGCTTATGTACAATAAGACTTATATCAGAACTGCTA
AGTACTGAAATAAGTTACTCTTATTGTGTGGTTTGTAAGAGTTTGCATTAAAGACTGTATCCTTGTTATATTATTATGGATTGTTATATTATTATG
GATTAAAAAAAAAAAAA

>CPRD46b

TTAAGGACAGAGGAGAAGACATTCTCCGTTTCGAGATTCCCGCAACAATGGACAAGGATAGATTCTTCTGGTTGAGGGACGAAGAATTTGGA
AGACAAACTCTAGCGGGTCTTAACCCTTGTTGCATCCAGTTGGTCACGGAATGGCCATTGAAAAGCAATCTTGACCCTGCAATTTATGGCCC
TGCGGAATCAGCCATCACCACTGAACTTGTTGAGCAGGAAATCAGAGGATTCCTTACAGTCGAAGAGGCCATAAAACAGAAGAGACTGTTCG
TCTTGGACTACCACGATTTGTTATTGCCATTAGTGGAGGAGGTGAGAAAAATAGAAGGCACAACGCTATACGGATCGAGGGCATTGTTCTTC
CTAACCCGCGACGGCACTTTGAGACCACTGGCCATTGAGCTGGTTCGGCCACCAATCGACGGAAAGCCTCAGTGGAAGAAAGTCTTCGCAC
CCACCTGGCACTCAACCGGTGTCTGGCTATGGCGGCTCGCCAAAATCCATGTCCTCGCTCACGACACCGGTTACCACCAACTCGTGAGTCA
CTGGCTAAGAACTCATTGTGCAACAGAGCCATACATTATAGCAGCAAACCGGCAACTGAGTGCAATGCACCCGATCTACAGATTACTGCATC
CGCATTTCCGTTACACGATGGAGATCAACGCGCTTGCTCGAGAGTCTTTGATAAACGCCGGCGGAATAATAGAGCAGTGTTTTACTCCGCAG
AAACACTCCGTTCTGCTAAGCTCAATCGCCTATGACAAACACTGGCGATTTGATCTCCAATCCCTCCCCAAGGACCTCATCCACAGGGGCTT
GGCCGTGGAGGACCCCACCGCCCCTCACGGCCTCAAACTCACCATCGAAGACTACCCTTACGCCAACGACGGCCTCGACCTCTGGGCGGC
TTTCAAATCGTGGTTCACCGAATACATCGACCACTACTACGCCGACTCCAACGCTGTTCAATCAGACACAGAGCTCCAAGCCTGGTGGGATG
AGGTCATAAACGTTGGACACGCCGACAAAAAGGATGAACCGTGGTGGCCGGCGCTGAAAACCAAAGAGGACCTCGTTGAAGTCGTGACGA
CCATTGCGTGGACAACTTCGGG

Table5.3. (cont'd). Nucleotide sequences of the candidate genes used for the analysis showing the various primer pairs (in colour and underlined) designed from the sequences.

>Dhn1

AAAGTTCTTATAATCACAACAACAACAACCATGGCAAGTTACCAGAAGCAGTACGAGGATCAGGGTTGCAAGATCGACGAGTATGGCAACGT
TGTACAGGAAACTGATGAATATGGCAACCCGGTTCATGCAGCAAGTGTCACCTACATAACCTCCACCACCGGTGGTCTTGGGGATGACACTA
ACAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAGCACGGAACTATAGGTGACACCGGTAGACAGCACGGAACTAC
CGGTGGTTTTACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGCTTTACCGGTGACACCGGGAGACAACATGGGACTACCGGTGGT
TTTACCGGTGACACTGGGAAACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAGACAACATGGGACTACTGGTGGTTTTACCG
GTGACACTGGGAGACAACATGGGACTACCGGTGACACTGGGAGACAACATGGGACTACCGGCGGCTTTACTGGTGGTGACACTGGTCTGG
GAGGCCCTTACGTTGGAGCCAACACCGCTGACACAGGGACTGGTCCTAGAAGTGGAACCACAGGTGGCAGCGCCTATGGATCGGGTGGTT
ATGGAAGTGGAGCTGGAGCTGGGTATGGTATGAACACGGGGGGAGCACACAGTGATGAAAGGTATGGAAGGGAGTATCGTGAGCATGATC
AGTCTCGTGGAGATCATGACAAGAAAGGGATAGTGGACAAGATTAAGGAGAAGCTTCCCGGAGGACACAGTGACAACAAGTAGAGACATCA
TGGGTGTGCATGCATGCGTATATATACGTAGTATAATTAAAGATGTTATATTGTTGTGTTTTTGAATAAGTTTGCTGCATATATACGTACTCGTA
CAGTACTGTCTTGTCGTGTAAATGTGGTGGATCTTGTATATGGTTATAGTATATGCAAGAGGAATAAAAGGTGCATGCATGCAAGAGAAAGAA
GGCATAGATGTTCTGCCAATGGAAAAGTAAAACCTACCCTGATGTAGAGCAGGGTGCTTCAGTTGTGTGTTTTTGAATTTTCCAATGAAGCAA
GTTTTTCTTTC

>CPRD12

GCTCAGAAGAACAATTAAGTTGAACACTTCAAAAACCCTTTTTCCAGGTTTTATATATATCCCAAACGCTCACTCATGGCAAATGGTTCTGTTC
TCTCACCTGTTGTTAAAAGGCTTGAGGGGAAAGTGGCGATTATCACCGGCGGTGCCAGCGGCATAGGTGAGGCCACTGCAAGACTCTTCTC
ACAGCATGGAGCTCACGTGGTGGTGGCTGATATTCAAGACGATGTGGGACTTTCTCTCTGCAATGAGTTGAAATCAGCCATATATGTTCATTG
CGATGTGACAAAGGAAGAAGACATCGAAAAGTGCGTGGACACAGCAGTTTCCAAGTTCGGGAAGCTAGACATCATGTTTAATAACGCCGGTA
CAGGTGATGAGTTCAAAAAAAGCATACTCGACAACACAAAATCTGATTTTGAGAGAGTGATAAGTGTGAACCTGGTTGGTCCATTTCTGGGAA
CAAAGCACGCTGCAAGAGTCATGATTCCTGCTAGAAGAGGGTGCATAATTAACACAGCGAGTGTTGCAGGATGCATAGGTGGAGGAGCTAC
ACATGCCTACACCAGTTCAAAGCATGCCCTAGTGGGACTCACCAAAAACACTGCGGTGGAGCTTGGACAATTCGGTATTCGTGTGAACTGTG
TGTCCCCTTTTGCCATTGTGACACCGTTGTTGAACAAATATTTCAATCTTGATGAAGAAGGAGTTCGCAAGACTTATATGAACCTAAAAGGTTG
GTATCCTGTGCCTAACGATGTGGCAGAAGCTGCTCTTTACTTAGCAAGTGATGAGTCTAAGTTTGTTAGTTCTCATAATCTTGTCATAGACGG
AGGTTTGATCAATTCAAACGTAGGATTTCCTATGTTTGAGATGTAAATATGTTCTGGTTCCTTAAATAGTTATATTGTTGGTGTTATTTGGCTTT
GAGGACAGTTCAGAAACCGTAGCTTCCGACATATGTGAAGAAAGAAGGCAATAGATTTGGTGGATATTACAATCCTCACTCAAATCTAGCCA
TTTTGCTCATTCTTGTGAAAAAAAATAAACATGTGTTACGATGG

>CpABA (Two primer pairs, CpABA(a); CpABA(b))

AATTCGGCACGAGGATTTTCCTACGTGGGCGAGCTTACAACATACACACCACTCCCATCCATGACCACAATTAAGAACCCACTTTTCAGTTCC
CTTGAATAAAGAACTTCCATTGGATGCTTCACTTCTTGTTGGCTATAACTGTCCCCTGGGATGCAGAACCAGGAAGCAGAGGAGGAAAGTGA
TGCTTGTGAAAGGTGCGGTGGTAGAGGCTCCACCAAGTGTTTCACCCTCGTCACAAGGTGGAAGTGGGGCCGCTTCAAAGAAGCAGCTTCG
GGTACTTGTTGCTGGTGGAGGGATTGGAGGGTTGGTCTTTGCGTTGGCTGCGAAGAAAAAGGGGTTTGATGTGGTGGTGTTTGAGAAGGAC
CTGAGTGCTATAAGAGGGGAGGGACAGTATAGGGGTCCAATTCAGATTCAGAGCAATGCTTTGGCTGCTTTGGAAGCTATAGATTCAGAGGT
TGCAGAGGAAGTTATGAGAGTTGGTTGCATCACCGGTGATAGAATCAATGGACTTGTAGATGGGGTTTCTGGTTCTTGGTACGTCAAGTTTG
ATACATTCACTCCAGCTGTGGAACGTGGGCTTCCAGTCACAAGGGTTATTAGTCGAATGGTTTTACAAGAGATCCTTGCCCGCGCAGTTGGG
GAAGATATCATTATGAATGCTAGTAATGTTGTTAATTTTGTGGATGATGGAAACAAGGTAACAGTAGAACTTGAGAATGGTCAGAAATATGAAG
GAGATATATTGGTTGGAGCGGATGGTATATGGTCGAGGTGAGGAAGCAATTATTTGGGCACAAAGAAGCTGTTTACTCTGGCTACACTTGTT
ACACTGGCATTGCAGATTTTGGCCTGCTGACATTGAAACTGTTGGATACCGGGTATTCTTGGGACACAAACAATACTTTGTATCTTCAGATGT
TGGTGCTGGAAAGATGCAATGGTATGCATTCCACAAAGAACCTCCTGGTGGTGTTGATGGCCCCAACGGAAAAAAGGAAAGGCTGCTTAAG
ATATTTGAGGGTTGGTGTGATAATGCTGTAGATCTGATACTTGCCACAGAAGAAGATGCAATTCTAAGAAGAGACATATATGACAGGATACCG
ACATTGACATGGGGAAAGGGTCGTGTGACTTTGCTTGGGGATTCCGTCCATGCCATGCAGCCAAACATGGGCCAAGGAGGGTGCATGGCTA
TTGAGGACAGTTATCAACTTGCATTGGAGTTGGACAATGCATGGGAACAAAGTGTTAAATCAGGGAGTCCAATTGACATTGATTCTTCCCTAA
GGAGCTACGAGAGAGAAAGAAAACTACGAGTTGCCATCATTCATGGAATGGCTAGAATGGCCGCTCTCATGGCTTCAACTTACAAGGCATAT
CTGGGTGTTGGTCTTGGCCCTTTAGAATTTTTGACCAAGTTTCGCATACCACATCCTGGAAGAGTTGGAGGAAGGTTTTTCGTTGACATCATG
ATGCCTTCTATGTTGAGCTGGGTCTTAGGTGGCAATAGCTCCAAACTTGAGGGTAGACCACTAAGTTGCAGGCTCTCAGACAAAGCTAATGA
TCAGTTACGCCAATGGTTTGAAGACGATGAAGCCCTTGAGCGTGCTATTAATGGAGAGTGGATTTTAATACCGCATGGAGATGGAACAAGTC
TTTCAAAGCCTATAGTTTTAAGTCGAAATGAGATGAAACCCTTTATAATCGGGAGTGCACCAGCGGAAGATCATCCTGGCACTTCAGTTACAA
TACCTTCTCCTCAGGTTTCTCCAAGGCATGCTCGAATTAACTATAAGGATGGTGCCTTCTTCTTGATTGATTTACGGAGTGAACATGGCACCT
GGATCATTGACAATGAAGGAAAGCAGTACCGGGTACCCCCTAATTATCCTGCTCGCATTCGCCCATCTGAGGCTATTCAGTTTGGTTCTGAG
AAGGTTTCATTTCGTGTTAAGGTGACAAGATCTGTTCCAAGAATCTCAGAGAATGAAAGGCCTCTAACGTTGCAGGAAGCGTGAGTGGTTCT
GTTCAGTTGCAGTTTGTAAGTAATGGAAAAGTTATACAAAGCAAATTTACATTTGTAGAGCACTATCTGCGTTACTTTAGGGTGGGATATTAAA
CAACGATCCAGTTATCTTAATGTTTATATGGACCTTTAAGAGGGATTGTTGGTTATAAATTCGTTACCCCACTAAAAAACTTTTTGTGTAATAAC
ATTTGTTAGTTAGATAGATTTGTAAAATGACTGAAACTTGCACCACATTAATGTTGAATGGAGTAAGCAATGCTAAGCTGAGAATTTTTTTCACT
TTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

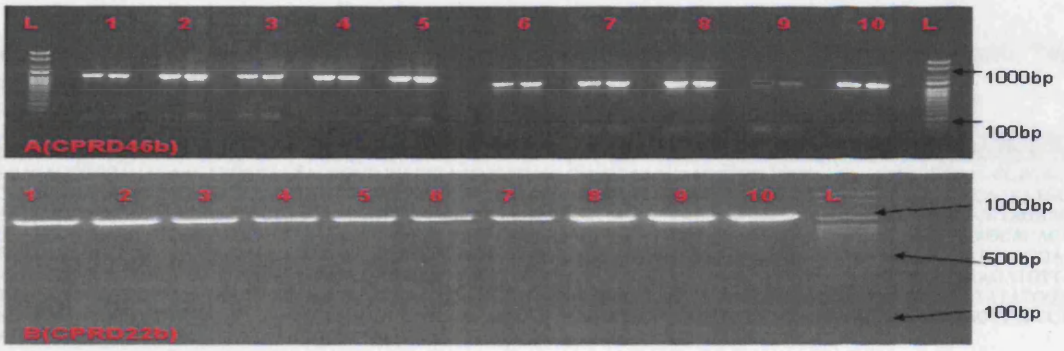
The resulting genomic sequences were received in the ABI format and imported into workable forms using Chromas software package. The nucleotide and the deduced amino acid sequences were aligned using ClustalW and DNASTAR software programmes.

5.3. Results

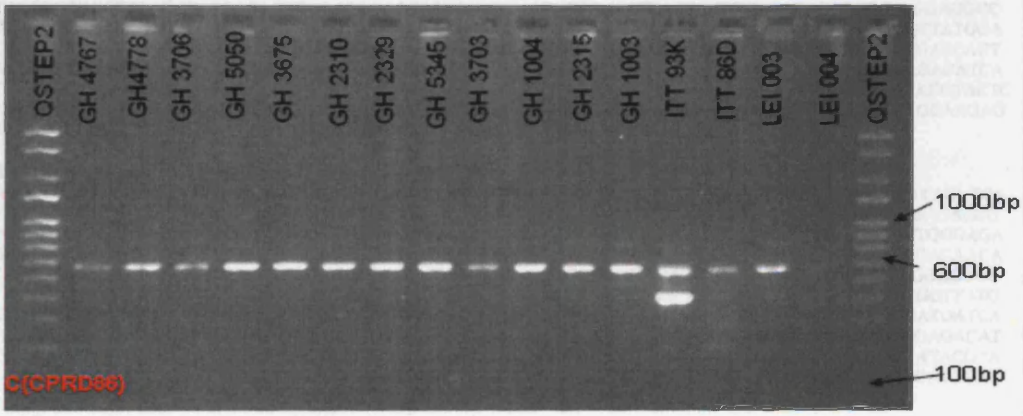
5.3.1. PCR amplification

There were 11 candidate gene primer pairs designed from eight candidate genes selected for this study. The primer pairs were designed from mRNA sources and therefore were not designed from less conserved non-coding regions of the genome, but they may have spanned introns present in the genomic DNA but not present in mRNA. The primers were used to amplify genomic DNA of the cowpea lines which resulted in PCR amplification with a characteristic product size. The primer pairs amplified PCR products that ranged from 550 bp to 1200 bp, compared to the range of 508 bp to 997 bp in the mRNA.

Figure5.1: PCR amplification products of CPRD46b (A), CPRD22b (B) and CPRD86 (C) using genomic DNA of 10 selected cowpea accessions for the cloning and sequencing.



L-Hyperladder2; 1-GH4767; 2-GH4773; 3-GH2310; 4-GH3706; 5-GH2336; 6-GH4774; 7-GH2334; GH1003; 8-GH4535; 9-IT93-K; 10-LEI-003



5.3.2. Sequence analysis of selected clones

Out of the 11 primer pairs designed for the study, seven of them, CPRD8, CPRD12, CPRD14, CPRD22b, CPRD46b, CPRD86, and Dhn1 amplified desired PCR products. The four primer pairs which failed to amplify single PCR products were cpABA(a), cpABA(b), CPRD22a, and CPRD46a. The successful amplified products were extracted from the gel, purified, cloned into pGEM-T Easy vector and sequenced. The sequenced genomic DNA fragments were different in size from the original mRNA fragments used to design the primer pairs but mostly identical in homology outside the introns regions, although some had larger inserts or deletions within coding regions. Some of the sequenced fragments with their original sequence and their respective aligned products have been shown below in Tables (5.4 to 5.8) and figures (5.2 to 5.6).

Table5.4: Sequences obtained from genomic DNA of cowpeas using Dhn1 primer pair. The forward and reverse primers in the sequences have been shown in red colour.

>Dhn1-Ori
TCCACCACCGGTGGTCTTGGGGATGACACTAACAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAGCACGGAA
CTATAGGTGACACCGGTAGACAGCACGGAACTACCGGTGGTTTTACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGCTTTACCG
GTGACACCGGGAGACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAAACAACATGGGACTACCGGTGGTTTTACCGGTGAC
ACTGGGAGACAACATGGGACTACTGGTGGTTTTACCGGTGACACTGGGAGACAACATGGGACTACCGGTGACACTGGGAGACAACATGG
GACTACCGGCGGCTTTACTGGTGGTGACACTGGTCTGGGAGGCCCTTACGTTGGAGCCAACACCGCTGACACAGGGACTGGTCCTAGAAG
TGGAACCACAGGTGGCAGCGCCTATGGATCGGGTGGTTATGGAAGTGGAGCTGGAGCTGGGTATGGTATGAACACGGGGGGAGCACACA
GTGATGAAAGGTATGGAAGGGAGTATCGTGAGCATGATCAGTCTCGTGGAGATCATGACAAGAAAGGGATAGTGGACAAGATTAAGGA
GAAGCTTCCCGGAGGACACAGTGACAACAAGTAGAGACATCATGGGTGTGCATGCATGCGTATATATACGTAGTATAATTAAAGATGTT
ATATTGTTGTGTTTTTGAATAAGTTTGCTGCATATATACGTACTCGTACAGTACTGTCTTGTCGTGTAAATGTGGTGGATCTTGTATATGGT
TATAGTATATGCAAGAGGAATAAAAGGTGCATGCATGCAAGAGAAAGAAGGCATAGATGTTCTGCCAATGGAAAAGTAAAACCTACCCT
GATGTAGAGCAGGGTGCTTCAGTTGTGTGTTTTTGAATTTTCCAATGAAGCAAGTTTTTCTTTC

>Dhn-GH4767
GTGGTCTTGGGGATGACACTAACAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAGCACGGAACTATAGGTGA
CACCGGTAGACAGCACGGAACTACCGGTGGTTTTACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGCTTTACCGGTGACACCG
GGAGACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAAACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAGA
CAACATGGGACTACCGGTGGTTTTACCAGTGACACTGGGAGACAACATGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACA
TGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACATGGGACTACCGGCGGCTTTACTGGTGGTGACACTGGTCTGGGAGGCC
CTTACGTTGGAGCCAACACCGCTGACACAGGGACTGGTCCTAGAAGTGGAACCACAGGTGGCAGCGCCTATGGATCGGGTGGTTATGGA
AGTGGAGCTGGAGCTGGGTATGGTATGAACACAGGGGGAGCACACAGTGATGAAAGGTATGGAAGGGAGTATCGTGAGCATGATCAGT
CTCGTGGAGATCATGACAAGAAAGGGATAGTGGACAAGATTAAGGAGAAGCTTCCCGGAGGACACAGTGACAACAAGTAGAGACATCA
TGGGTGTGCATGCATGCGTATATATACGTAGTATAATTAAAGATGTTATATTGTTGTGTTTTTGAATAAGTTTGCTGCATATATACGTACTC
GTACAGTACTGTCTTGTCGTGTAAATGTGGTGGATCTTGTATATGGTTATAGTATATGCAAGAGGAATAAAAGGTGCATGCATGCAAGAG
AAAGAAGGCATAGATGTTCTGCCAATGGATATGTAAAACCTACCCTGATGTAGAGCAGGGTGCTTCAGTTGT

>Dhn-GH4773
GTGGTCTTGGGGATGACACTAACAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAGCACGGAACTATAGGTGA
CACCGGTAGACAGCACGGAACTACCGGTGGTTTTACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGCTTTACCGGTGACACCG
GGAGACAACATGGGACTACCGGTGGTTTTACCGGTGACACCGGGAAACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAGA
CAACATGGGACTACCGGTGGTTTTACCAGTGACACTGGGAGACAACATGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACA
TGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACATGGGACTACCGGCGGCTTTACTGGTGGTGACACTGGTCTGGGAGGCC
CTTACGTTGGAGCCAACACCGCTGACACAGGGACCTGGTCCTAGAAGTGGAACCACAGGTGGCAGCGCCCTATGGATCGGGTGGTTATG
GAAGTGGAGCTGGAGCTGGGTATGGTATGAACACAGGGGGAGCACACAGTGATGAAAGGTATGGAAGGGAGTATCGTGAGCATGATCA
GTCTCGTGGAGATCATGACAAGAAAGGGATAGTGGACAAGATTAAGGAGAAGCTTCCCGGAGGACACAGTGACAACAAGTAGAGACAT
CATGGGTGTGCATGCATGCGTATATATACGTAGTATAATTAAAGATGTTATATTGTTGTGTTTTTGAATAAGTTTGCTGCATATATACGTA
CTCGTACAGTACTGTCTTGTCGTGTAAATGTGGTGGATCTTGTATATGGTTATAGTATATGCAAGAGGAATAAAAGGTGCATGCATGCAA
GAGAAAGAAGGCATAGATGTTCTGCCAATGGATATGTAAAACCTACCCCTGATGTAGAGCAGGGTGCTTCAGTTGT

>Dhn-GH2275
GTGGTCTTGGGGATGACACTAACAAGCAACATGATACCAGTAATGTCTACGGTGCAGACACCCGTAGACAGCACGGAACTATAGGTGA
CACCGGTAGACAGCACGGAACTACCGGTGGTTTTACTGGTGACACCGGTAGACAATATGGCACTACCGGAGGCTTTACCGGTGACACCG

GGAGACAACATGGGACTACCGGTGGTTTTACCGGTGACACTGGGAAACAACACGGGACTACCGGTGGTTTTACCGGTGACACTGGGAGA
CAACATGGGACTACCGGTGGTTTTACCAGTGACACTGGGAGACAACATGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACA
TGGGACTACCGGTGGGTTTACCGGTGACACTGGGAGACAACATGGGACTACCGGCGGCTTTACTGGTGGTGACACTGGTCTGGGAGGCC
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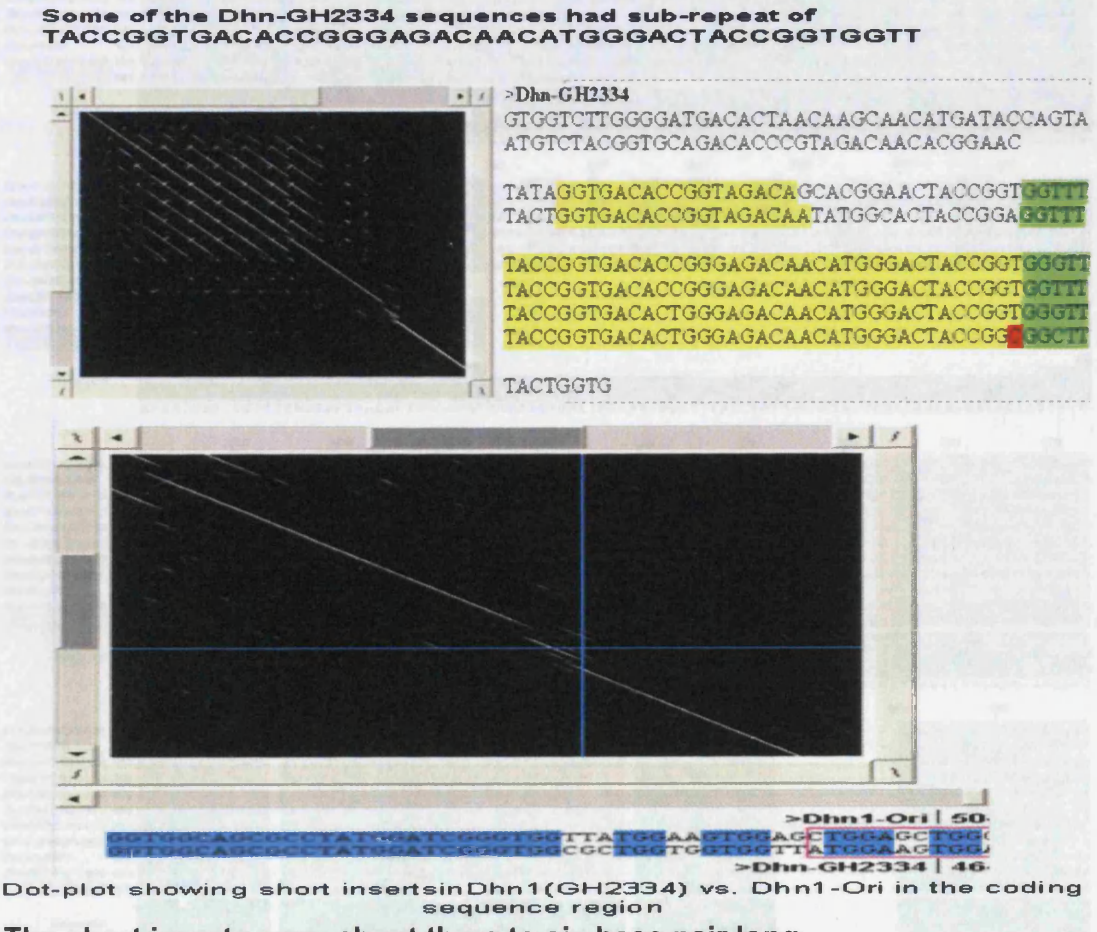
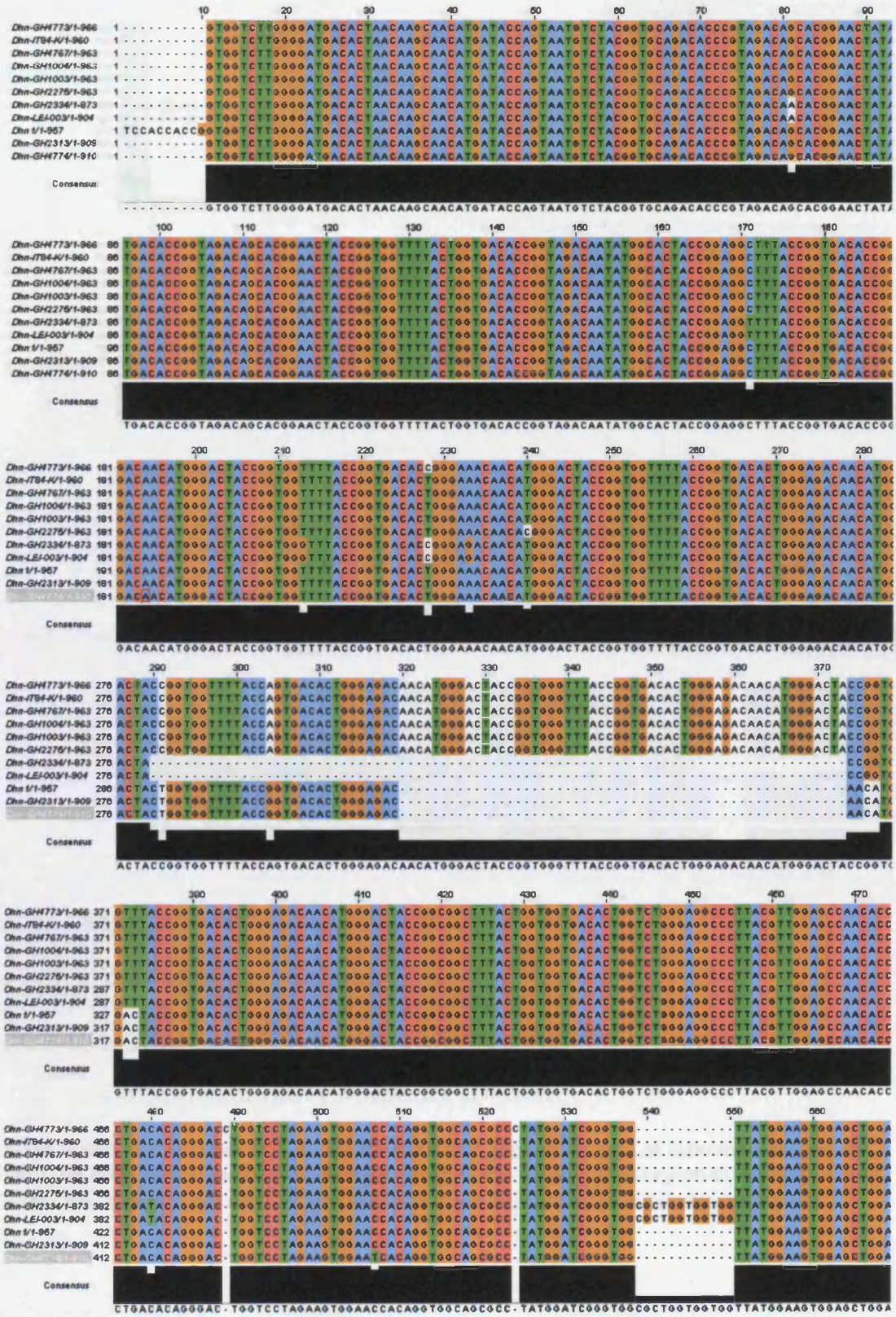
GGTGCTTCAGTTGT

Figure5.2: Dot-plot analysis and multiple alignments of nucleotide sequences of the cowpea accessions using Dhn1 primer pair with the original Dhn1 sequence as reference. Each nucleotide is coloured in a different colour in the alignment (lower panel)



Dot-plot showing short insertsinDhn1(GH2334) vs. Dhn1-Ori in the coding sequence region

The short inserts were about three to six base pair long.


Dhn-GH4773/1-966
Dhn-IT84-K/1-960
Dhn-GH4767/1-963
Dhn-GH1004/1-963
Dhn-GH1003/1-963
Dhn-GH2275/1-963
Dhn-GH2334/1-873
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Dhn t/1-957
Dhn-GH2313/1-909
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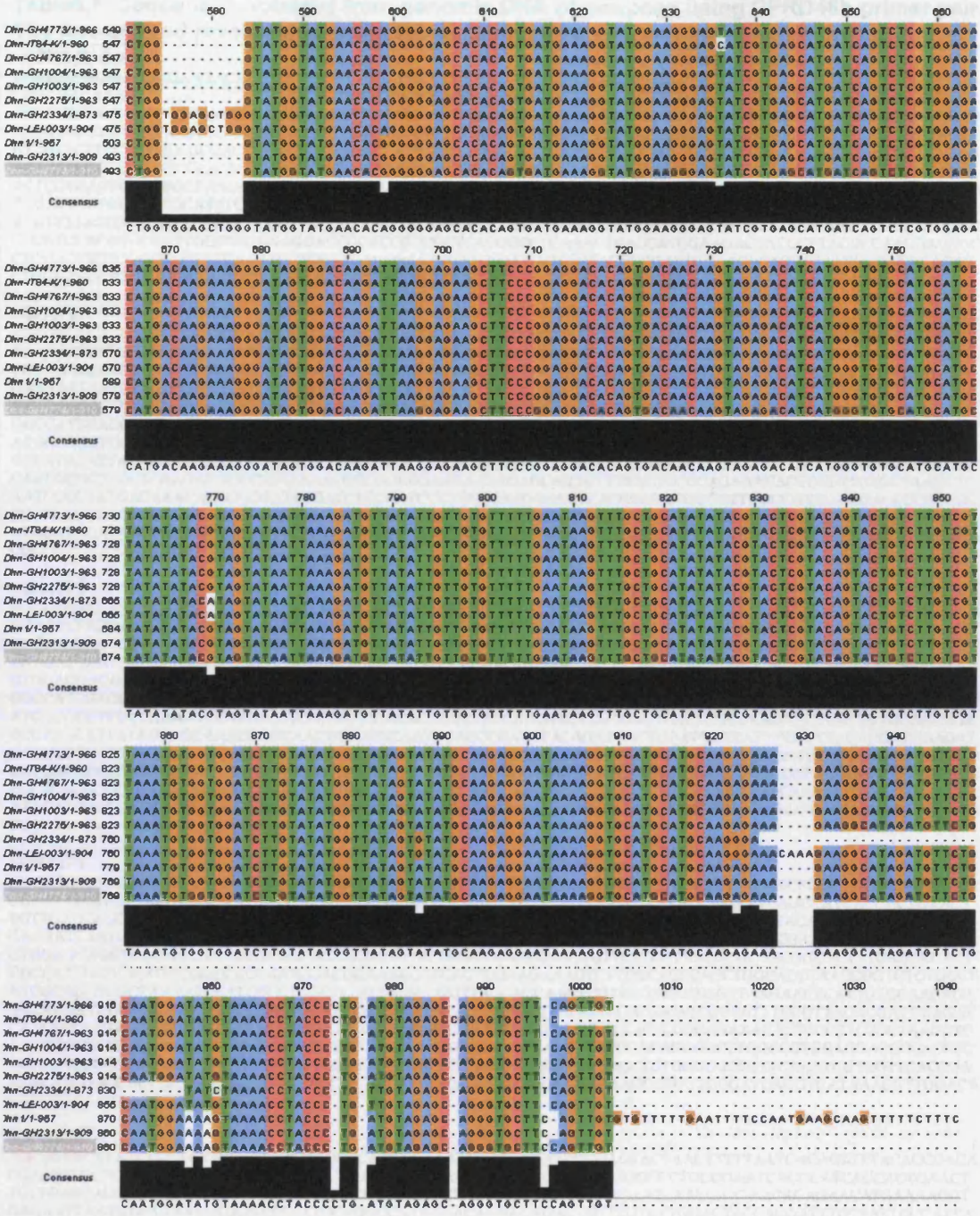
Consensus



Gaps within the sequences indicate deletion and gaps at the end of reference sequences with the other samples showing sequences indicate either intron region or no information available. Some of the dashes also indicate gaps introduced to maximize alignment.

Table5.5: Sequences obtained from genomic DNA of cowpeas using CPRD46b primer pair. The forward and reverse primers in the sequences have been shown in red colour.

>CPRD46-Ori

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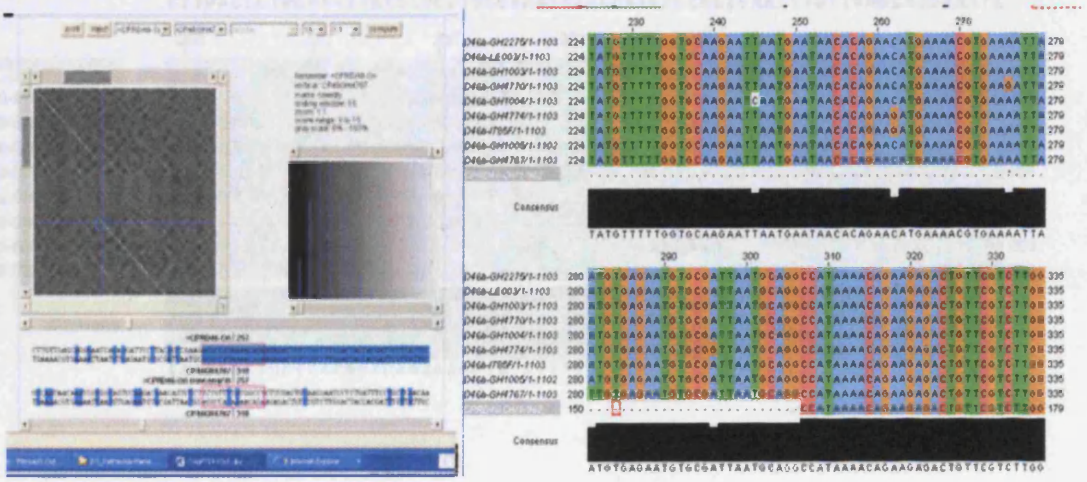
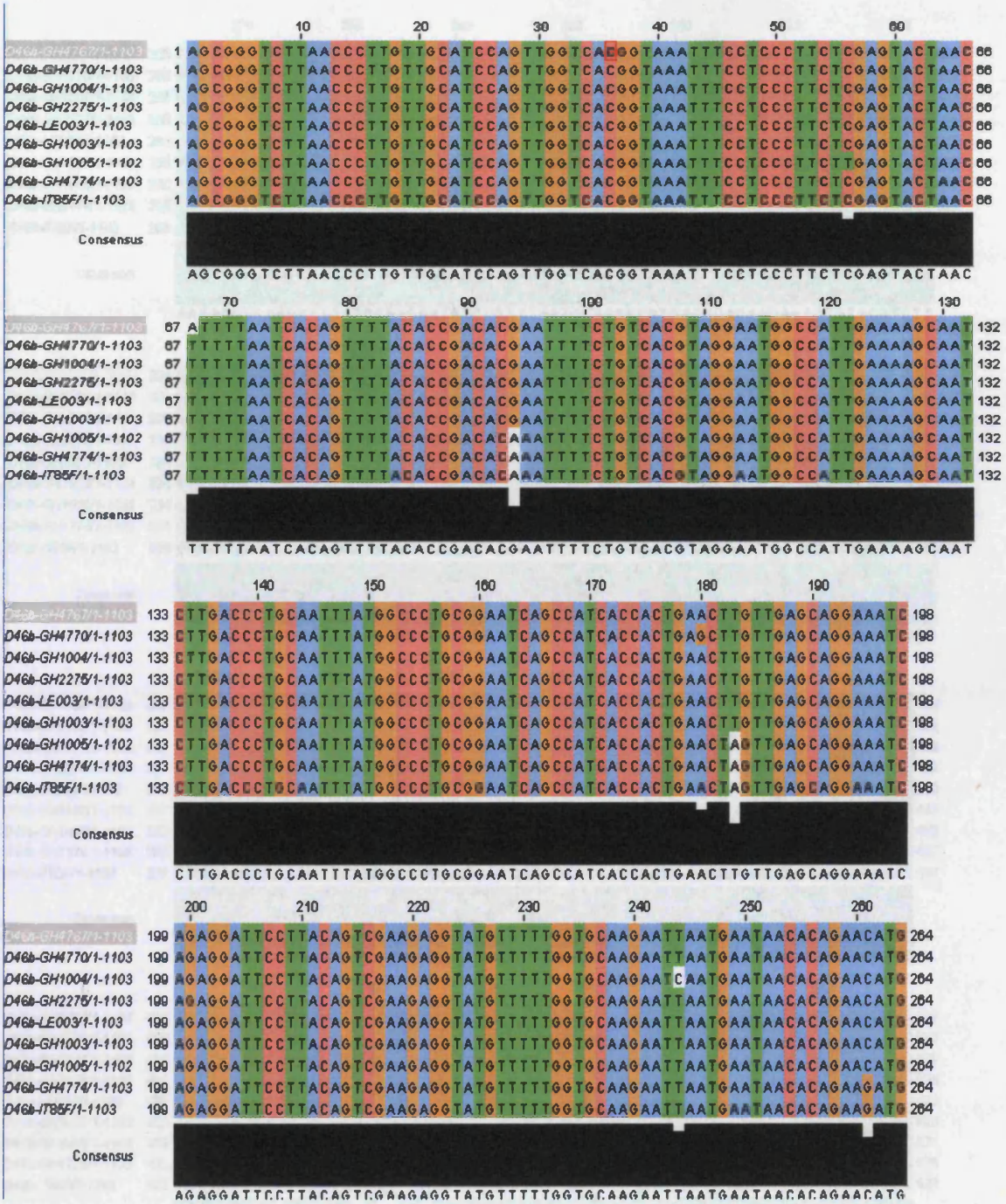
Figure5.3: Dot-plot and multiple alignments of nucleotide sequences of the cowpea accessions using CPRD46b primer pair and the original CPRD46b sequence as reference. The coding sequences show high homology, and an intron of 84bp is evident in dot plot and alignment. Each nucleotide is coloured in a different colour in the alignment (lower panel)

CPRD46b Dot-plot analysis showing alignment of the introns region of the genomic DNA of cowpeas as compared to CPRD46b-original CDNA

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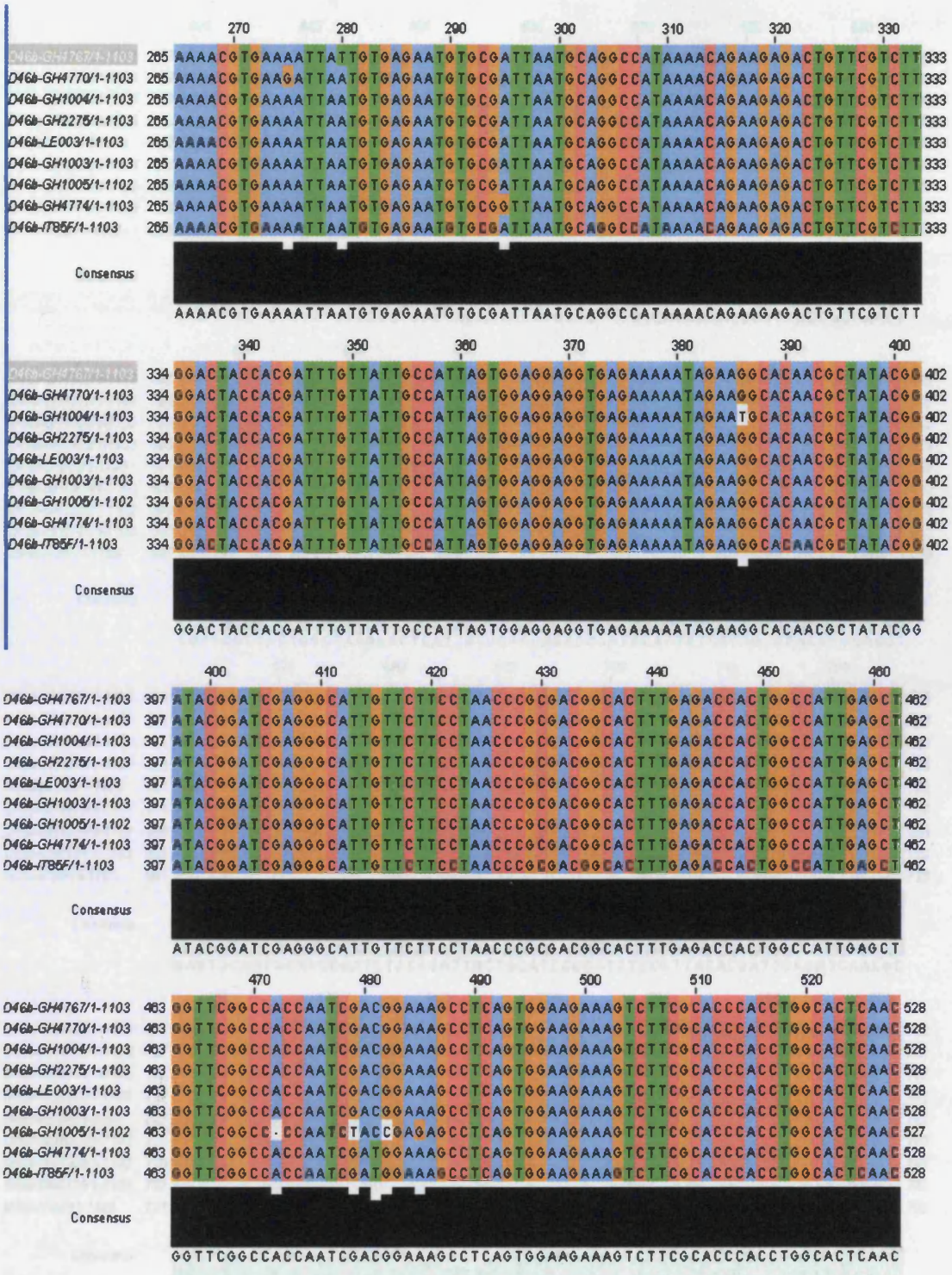
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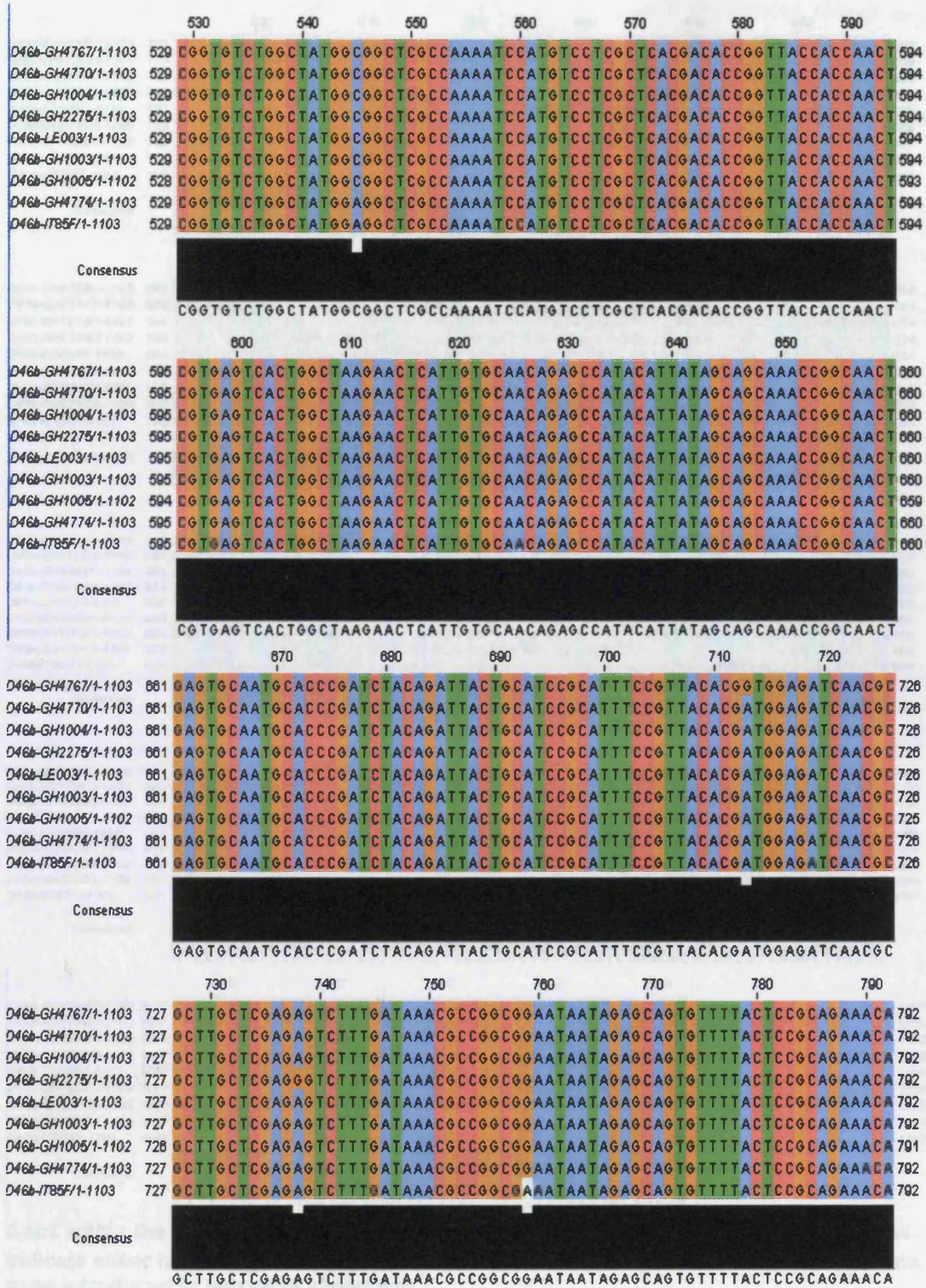
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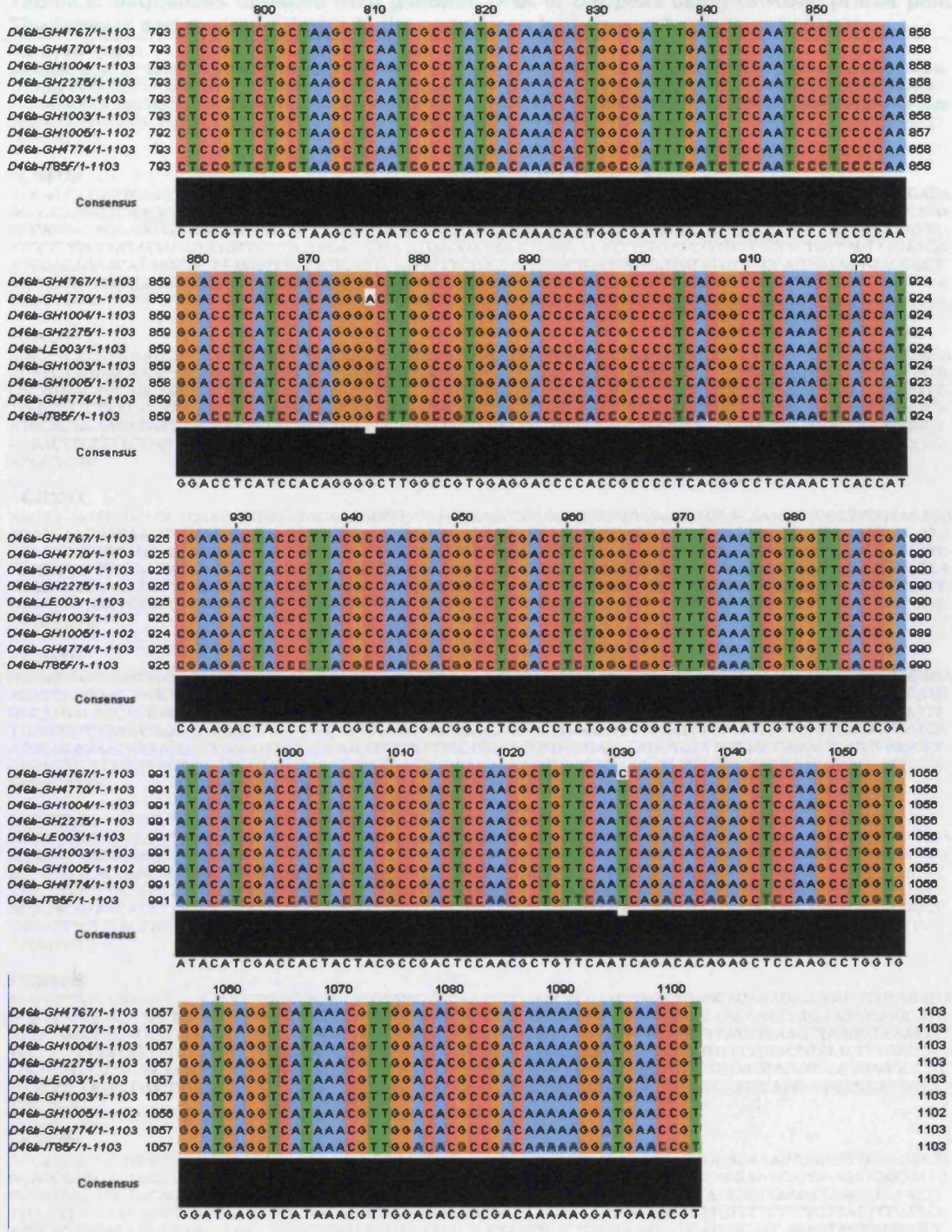

Gaps within the sequences indicate deletion and gaps at the end of reference sequences indicate either intron region or no information available. Some of the dashes also indicate gaps introduced to maximize alignment.

Table5.6: Sequences obtained from genomic DNA of cowpeas using CPRD86 primer pair. The forward and reverse primers in the sequences have been shown in red colour.

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>GH2313
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TTGGTGTG

>GH4774
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GCCATGACAGCAGCAGCAGCAGTGACAGTGATTAGATATCACTGCTGCATCACCTATATATATTTATATATGTTAAGCTAAGGTAAATTT
TTCCTTTTCTTATATTGTGTATGTTCTCTCAACATTCTCCATGAGGATGAACTAGGAAATTTCTGATCTTGTTTTCTTCTGTTACTTTGATCA
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TTGGTGTG

>GH1004
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GCCATGACAGCAGCAGCAGCAGTGACAGTGATTAGATATCACTGCTGCATCACCTATATATATTTATATATGTTAAGCTAAGGTAAATTT
TTCCTTTTCTTATATTGTGTATGTTCTCTCAACATTCTCCATGAGGATGAACTAGGAAATTTCTGATCTTGTTTTCTTCTGTTACTTTGATCA
ATGCACAGAACATAATGACTAAGGTTTGTTCTGATTTTGTTTTTCTCCTTTTTGTCTGATGCAGGTGTATGTGACCAGAGTAGTGAGGGCT
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TTGGTGTG

>GH1005
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TGGTGTG

>GH1006
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TTGGTGTG

>IT85F
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TTCCTTTTCTTATATTGTGTATGTTCTCTCAACATTCTCCATGAGGATGAACTAGGAAATTTCTGATCTTGTTTTCTTCTGTTACTTTGATCA
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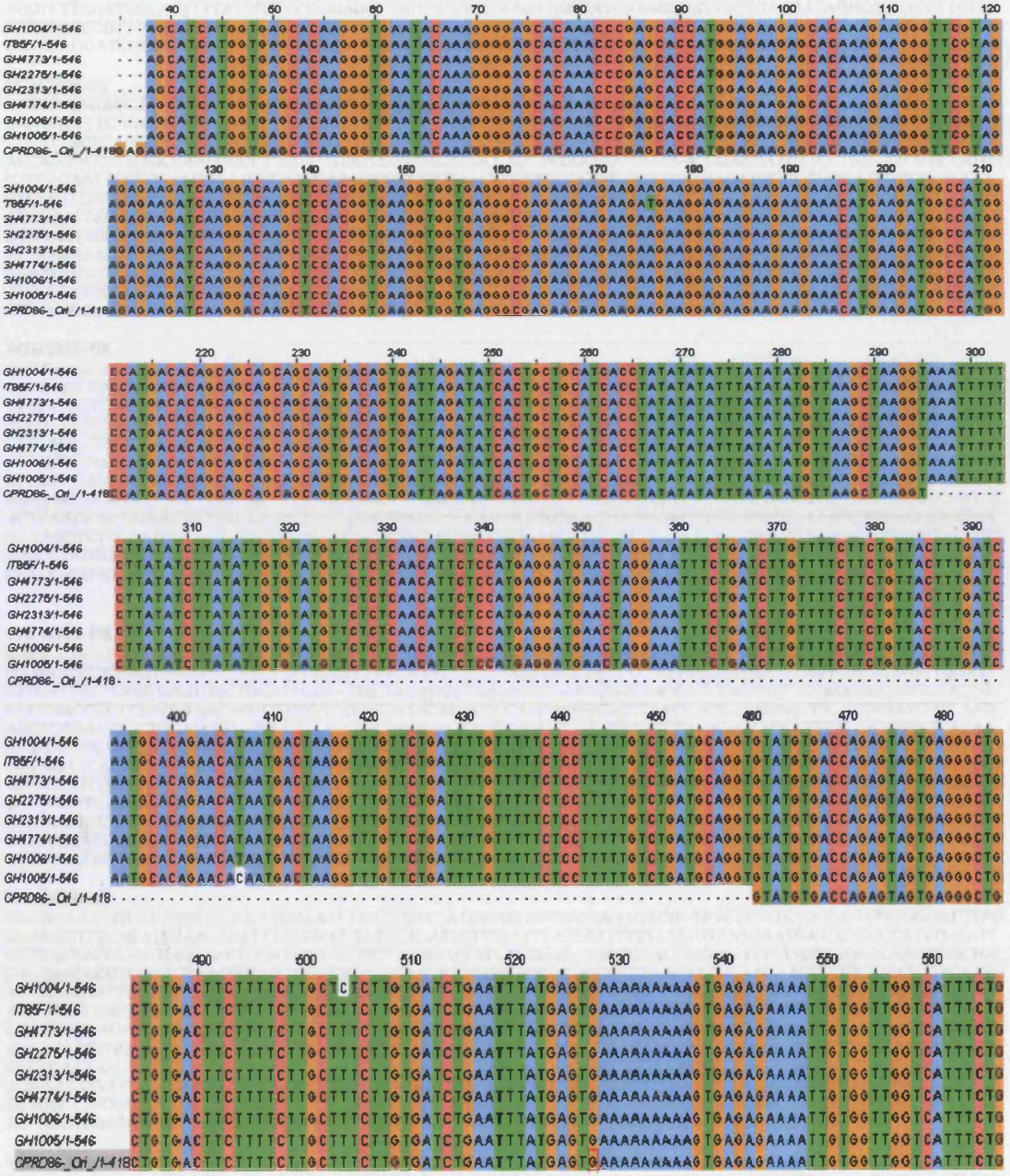
GTGACTTCTTTTCTTGCTTTCTTGTGATCTGAATTTATGAGTGAAAAAAAAAGTGAGAGAAAATTGTGGTTGGTCATTTCTGTGGTTTGG
TTGGTGTG

Figure5.4: Multiple alignments of nucleotide sequences of the cowpea accessions using CPRD86 primer pair and the original CPRD86 sequence as reference. The coding sequences show high homology, and an intron of 164bp is evident in the alignment. Each nucleotide is coloured in a different colour in the alignment



Gaps within the sequences indicate deletion and gaps at the end of reference sequences indicate either intron region or no information available. Some of the dashes also indicate gaps introduced to maximize alignment.

Table5.7: Sequences obtained from genomic DNA of cowpeas using CPRD8 primer pair. The forward and reverse primers in the sequences have been shown in red colour.

>CPRD8-Ori

GACGAAAGAGCAGGTGGAAGCATGGAAACCCATCGTCGATGCCGTTCATGCCAAAGGCGGTATCTTCTTTTGCCAGATTTGGCATGTAG
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GCCTTGGATATATCGAACACACGCCACCACGGCGCCTAACGACCGAAGAACTTCCTGGTATCGTCAATGACTTCAGAATTGCTGCAAGGA
ATGCCATCGAAGCTGGTTTTGATGGGGTTGAGGTCCATGGGGCACATGGGTACCTACTTGATCAATTTTTGAAAGACCAGGCTAATGACA
GAACAGATCAATACGGTGGATCCCTCGAGAACCGGTGCAGACTTCCTCTGGAGGTTGTTGAAGCTGTTGTGAATGAGGTTGGGGCTGATA
GAGTTGGAATTAGGTTATCACCTTTTGCAGACTTTAACGATTGTGGTGACTCAAATCCCCTGCAACTGGGGCTTTACATGGTCAATGCACT
CAATAAGTATAACATTCTCTACTGTCACATGGTGGAACCCAGAATGGGAAGTGTTGGAGGTCCAGATGAAAACCTGACGCCAATGAGAA
AGGTCTTCAATGGCACTTTTATAGTTGCCGGAGGTTATGACCGGGAAGATGGGATCAAAGCCATTGCTGAAGACAGGGCTGATCTTGTTG
CCTATGGCCGTTTGTTCTTGGCCAATCCAGATTTGCCAAAGAGATTTGCTCTCAATGCTCCTCTCAACCAGTATAATCGCAAGACATTCTA
CCACGAGGATCCTGATCCGCTTGTTGGGTACATCGATTATCCGTTTCTTGATGAAGAATCGAACGGTGTAGCATCC

>GH2275-D8

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CTGACTTCACTGATTGTAGGTTTTGATGGGGTTGAGGTCCATGGGGCACATGGGTACCTACTTGATCAATTTTTGAAAGACCAGGCTAATG
ACAGAACAGATCAATACGGTGGATCCCTCGAGAACCGGTGCAGACTTCCTCTGGAGGTTGTTGAAGCTGTTGTGAATGAGGTTGGGGCTG
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>GH2310-D8

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GAAGGGTTTCAGATTCAAGTAATTTTTTTCCCTATTCAGATTCTTTTATTTATATATTTTTTTTGTAATGAATCATTCTCCGTATTTGGATTC
TTGATTACTGAGTGAGTGACTGCATTCTGATTTCTACAGGTTATCAGCCGAATGGGCAAGCACCCATTTCTTCTACCGACAAGCCACTGCC
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>GH4767-D8

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>GH4770-D8

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CACCAACGCCTCGAGCTAACGGCCTTGGATATATCGAACACACGCCACCACGGCGCCTAACGACCGAAGAACTTCCTGGTATCGTCAATG
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GACAGAACAGATCAATACGGTGGATCCCTCGAGAACCGGTGCAGACTTCCTCTGGAGGTTGTTGAAGCTGTTGTGAATGAGGTTGGGGCT
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>GH4773-D8

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ACTTCAGAATTGCTGCAAGGAATGCCATCGAAGCTGGTATCTAACTTCATTCCATTTTGCTTCAACAGATGCTTTTATCATTGTTTCACAA
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CACTCAATAAGTATAACATTCTCTACTGTCACATGGTGGAACCCAGAATGGGAAGTGTTGGAGGTCCAGATGAAAACCTGACGCCAATG
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GTTGCCTATGGCCGTTTGTTCTTGGCCAATCCAGATTTGCCAAAGAGATTTGCTCTCAATGCTCCTCTCAACCAGTATAATCGCAAGACAT
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>GH1003-D8

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TGATTACTGAGTGAGTGACTGCATTCTGATTTCTACAGGTTATCAGCCGAATGGGCAAGCACCCATTTCTTCTACCGACAAGCCACTGCCA
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CAGAACAGATCAATACGGTGGATCCCTCGAGAACCGGTGCAGACTTCCTCTGGAGGTTGTTGAAGCTGTTGTGAATGAGGTTGGGGCTGA
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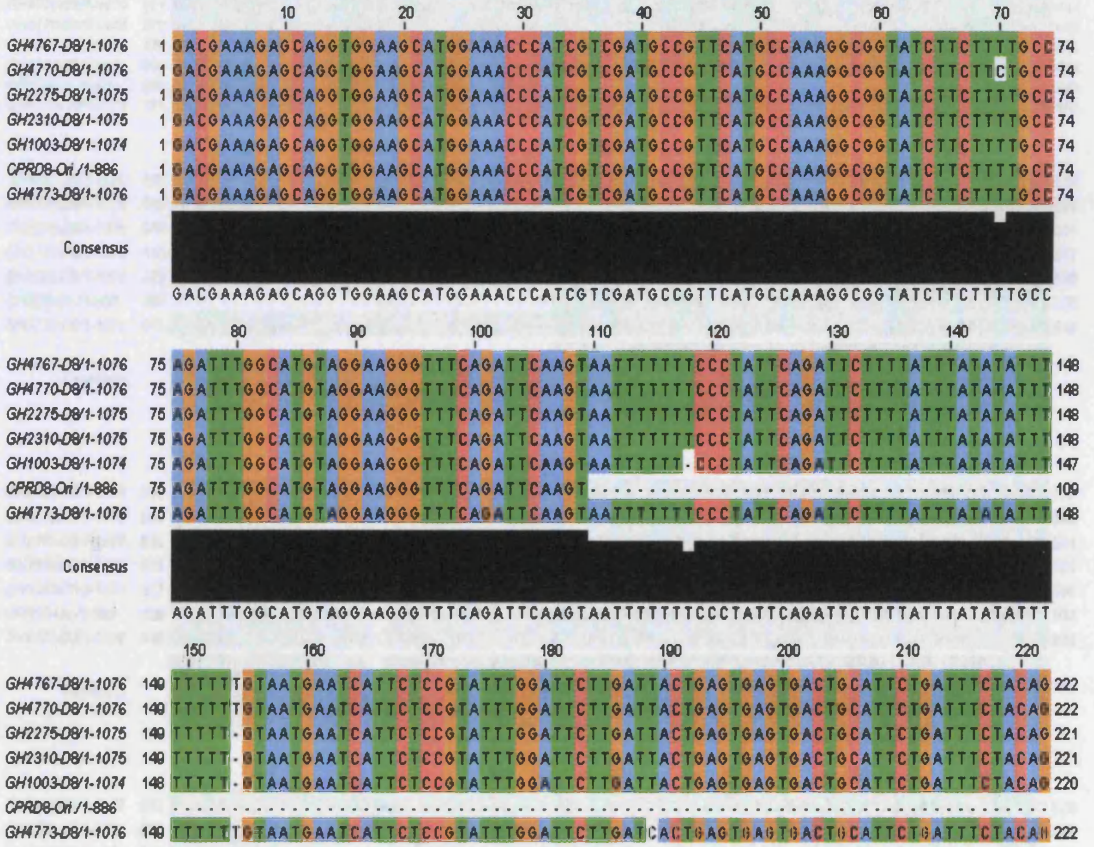
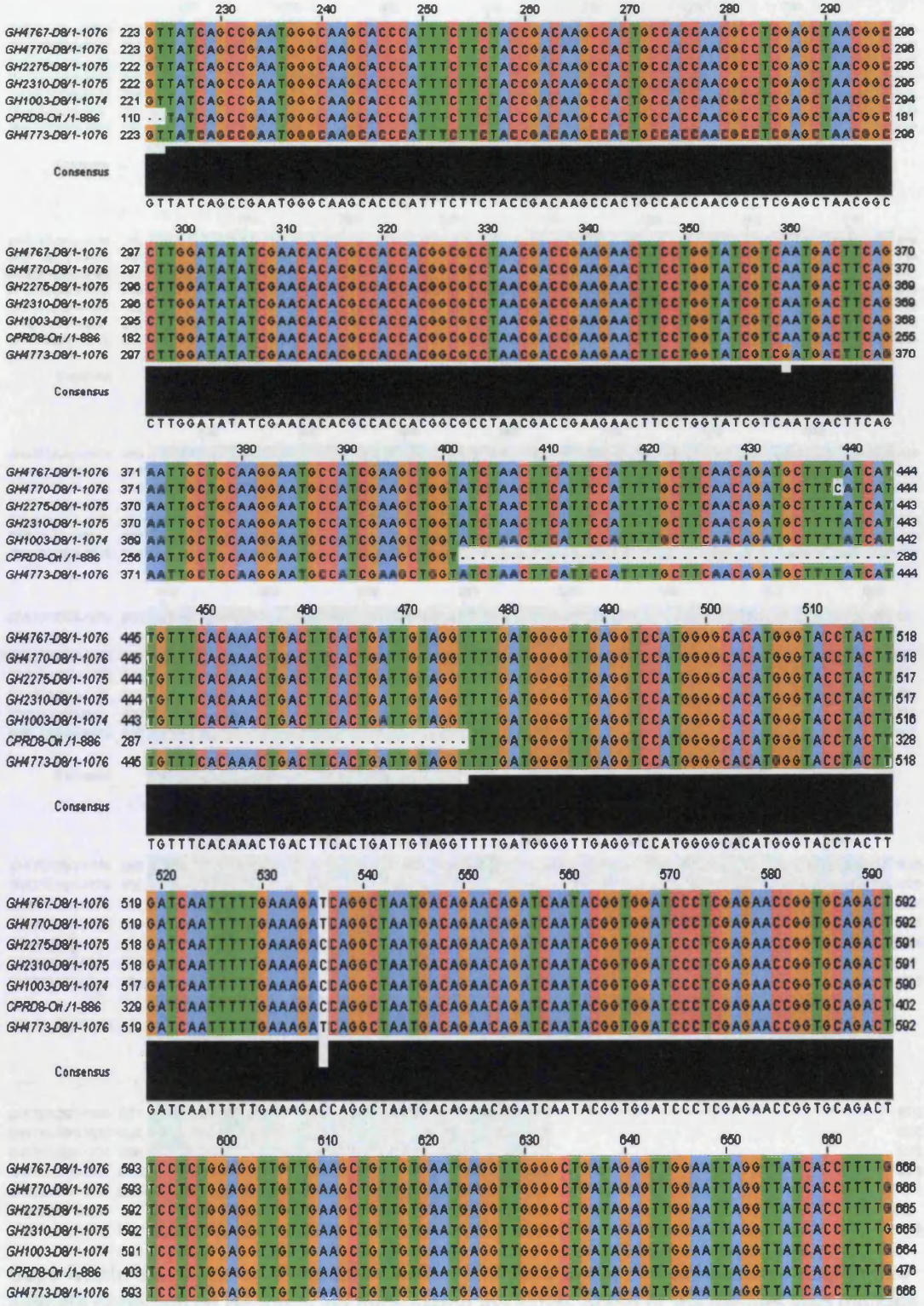
Figure5.5: Multiple alignments of nucleotide sequences of the cowpea accessions using CPRD8 primer pair and the original CPRD8 sequence as reference. The coding sequences show high homology, and two introns of 113bp and 74bp are evident in the alignment. Each nucleotide is coloured in a different colour in the alignment

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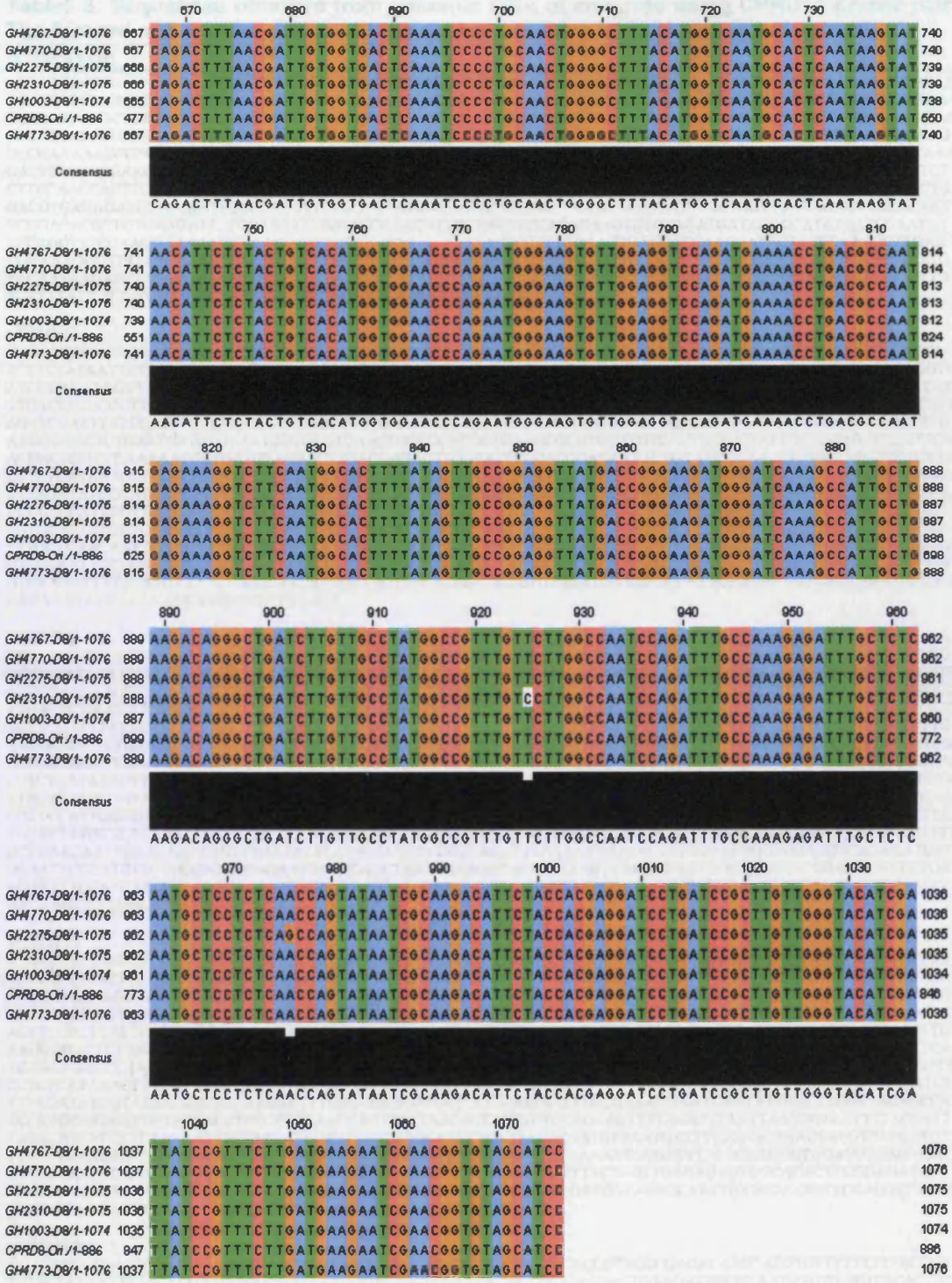
Consensus



Gaps within the sequences indicate deletion and gaps at the end of reference sequences indicate either intron region or no information available. Some of the dashes also indicate gaps introduced to maximize alignment.

Table5.8: Sequences obtained from genomic DNA of cowpeas using CPRD14 primer pair. The forward and reverse primers in the sequences have been shown in red colour.

>CPRD14-Ori

TTGCTTCCTGGGTCGTTAAGTTTCTTCTCGAACGCGGCTACACCGTGAAGGCCACCGTTCGCGACACAAGTGATCCCAAAAAGGTAGAT
CACTTGCTTAGCCTCGATGGTGCTAAGGAAAGGCTGCATCTCGTCAAGGCGAATCTTCTAGAAGAAGGTTCCTTTGACTCTGCCGTTGAA
GGTTGTCACGCTGTGTTCCACACTGCTTCTCCCTTTTTCGACGATGCCAAGGACCCGCAGACTGAGTTGTTGGATCCAGCTTTGAAGGGGA
CTCTGAATGTTCTGAAATCGTGCGTGAACTCGCCCACGCTGAAGCGCGTGGTTGTCACTTCTTCTATTGCTGCAGTTTCGTTCAACGACAG
GCCCAAAAACCCTGATGTAGTGGTTGACGAGACTTGGTATTCCGACCCAGAATACTGTAAGAGAAATGGGATATGGTATAACCTCTCAAA
GACTTTGGCCGAAGATGCTGCCTGGAAATTTGCGAAAGAAAACAATATTGACTTGGTTACAGCCAACCCTGCATTGGTTGTTGGACCTCT
CTTGCAACCAGTTCTTAACACTAGTTCTGCAGCAGTTTTAAATTTAATTAATGGTTCGCCAACATTTAAAAATGTGACTTTGGGATGGGTG
GACGTGAGAGATGTTGCAATTGCCCATGTTCTGGCATATGAGAATGCTTCAGCTAATGAAGATATTTACTAGTTGAGAGAGTGGCACACT
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>GH4767

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GTTGCAATTGCCCATGTCCTGGCATATGAGAATGCTTCAGCTAATGGAAGATATTTACTAGTTGAGAGAGTGGCACACTTCGGAGACGTT
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>GH4773

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>GH2275

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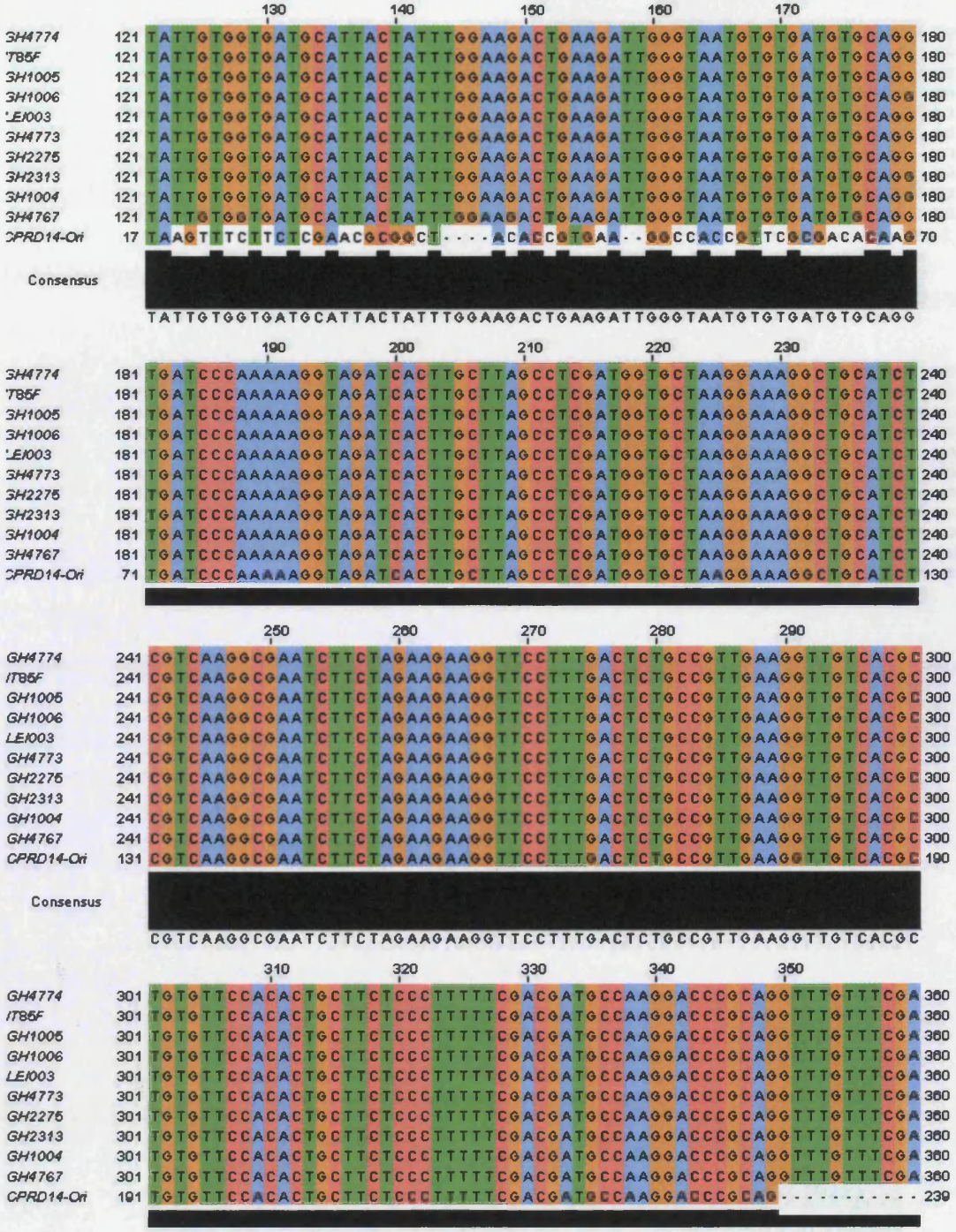
Figure5.6: A. Multiple alignments of nucleotide sequences of the cowpea accessions using CPRD14 primer pair and the original CPRD14 sequence as reference, B. Deduced amino acid sequences of CPRD14 and C. Sequence base traces of part of CPRD14-clone. The coding sequences show high homology, and three introns of 83bp, 114bp and 149bp are evident in the alignment. Each nucleotide is coloured in a different colour in the alignment

A. Multiple alignments-ClustalW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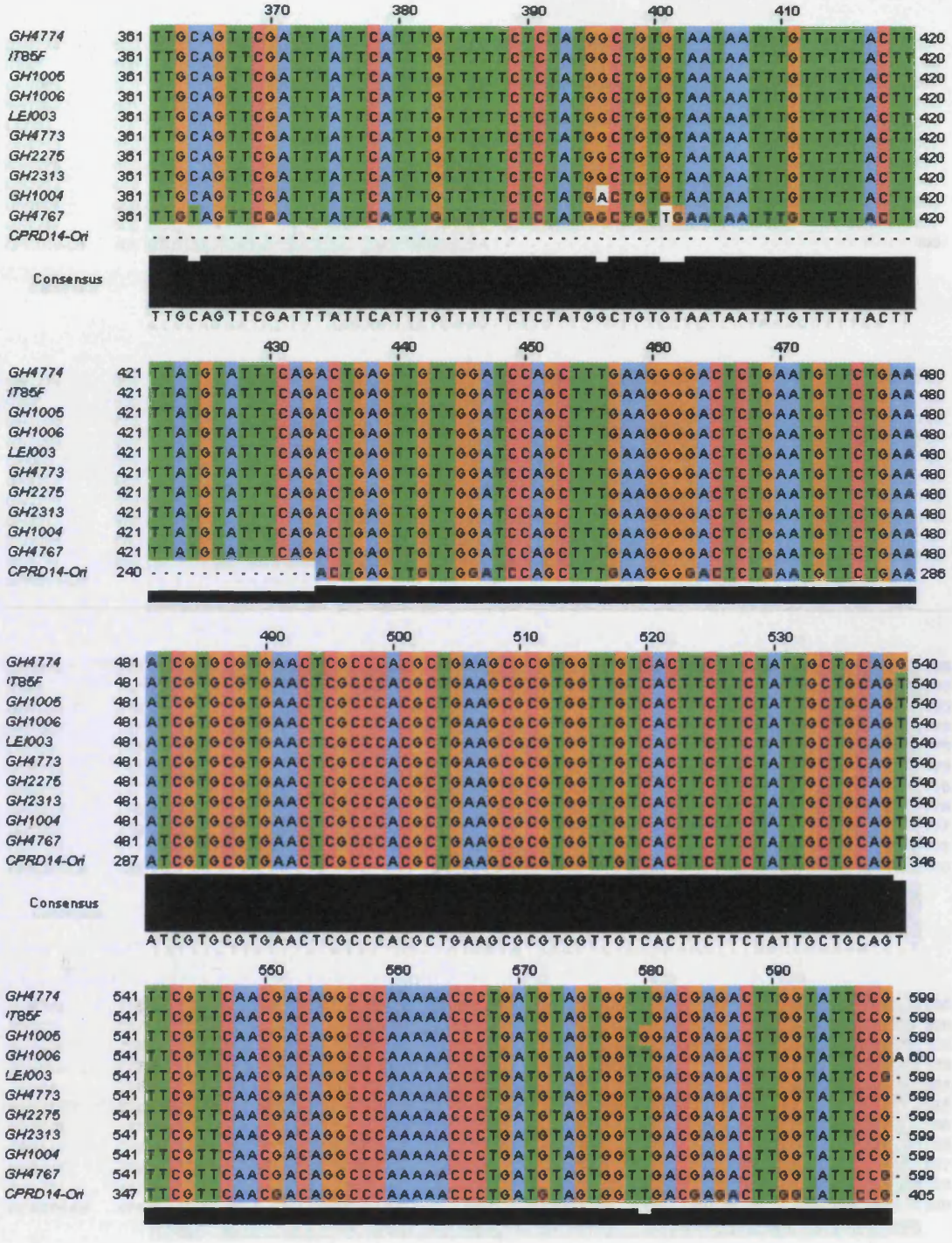
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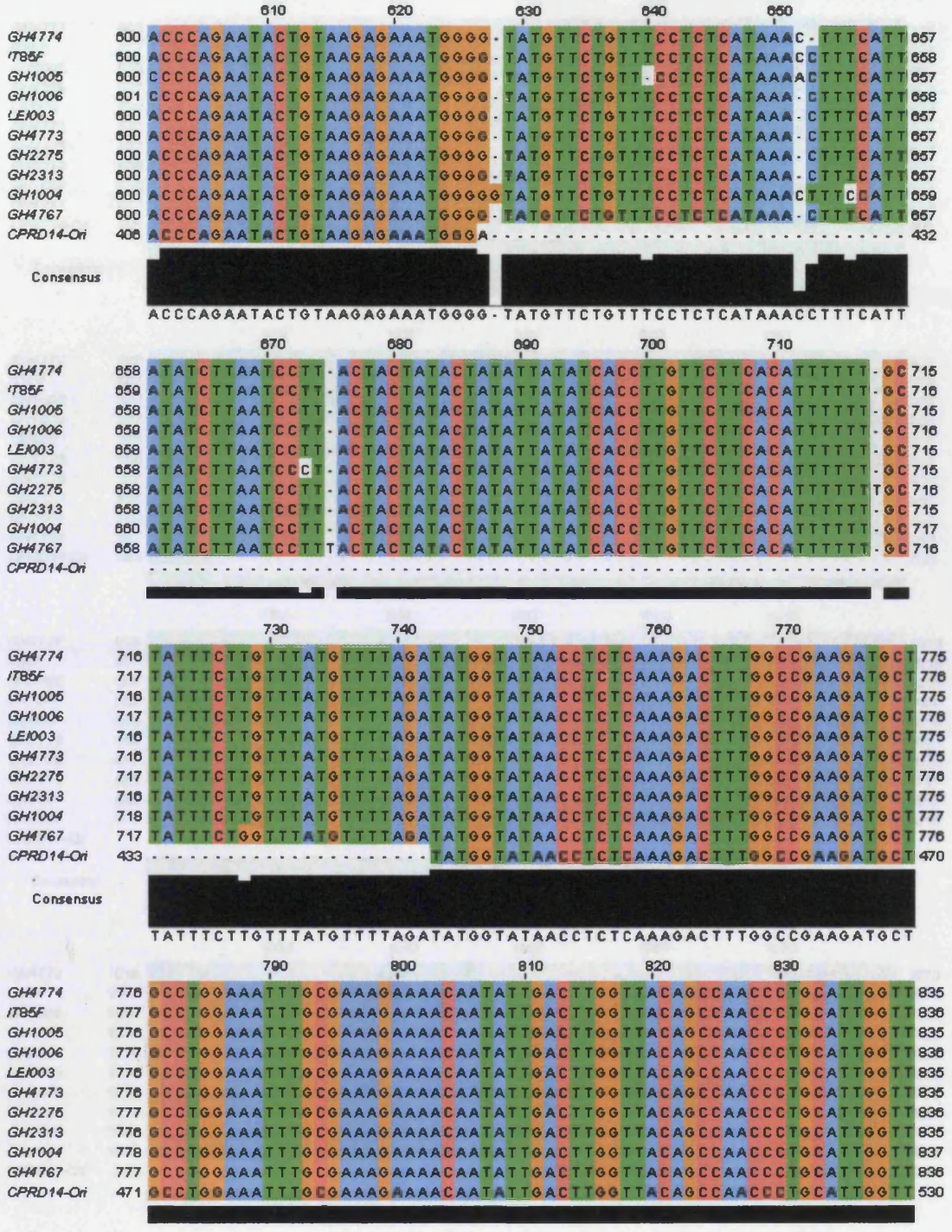
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GH4767 658 ATATCTTAATCCTTTACTACTATACTATATTATATCACCTTGTTCTTCACATTTTTT-GC 716
CPRD14-Ori ----------
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770
GH4774 716 TATTTCTTGTTTATGTTTTAGATATGGTATAACCTCTCAAAGACTTTGGCCGAAGATGCT 775
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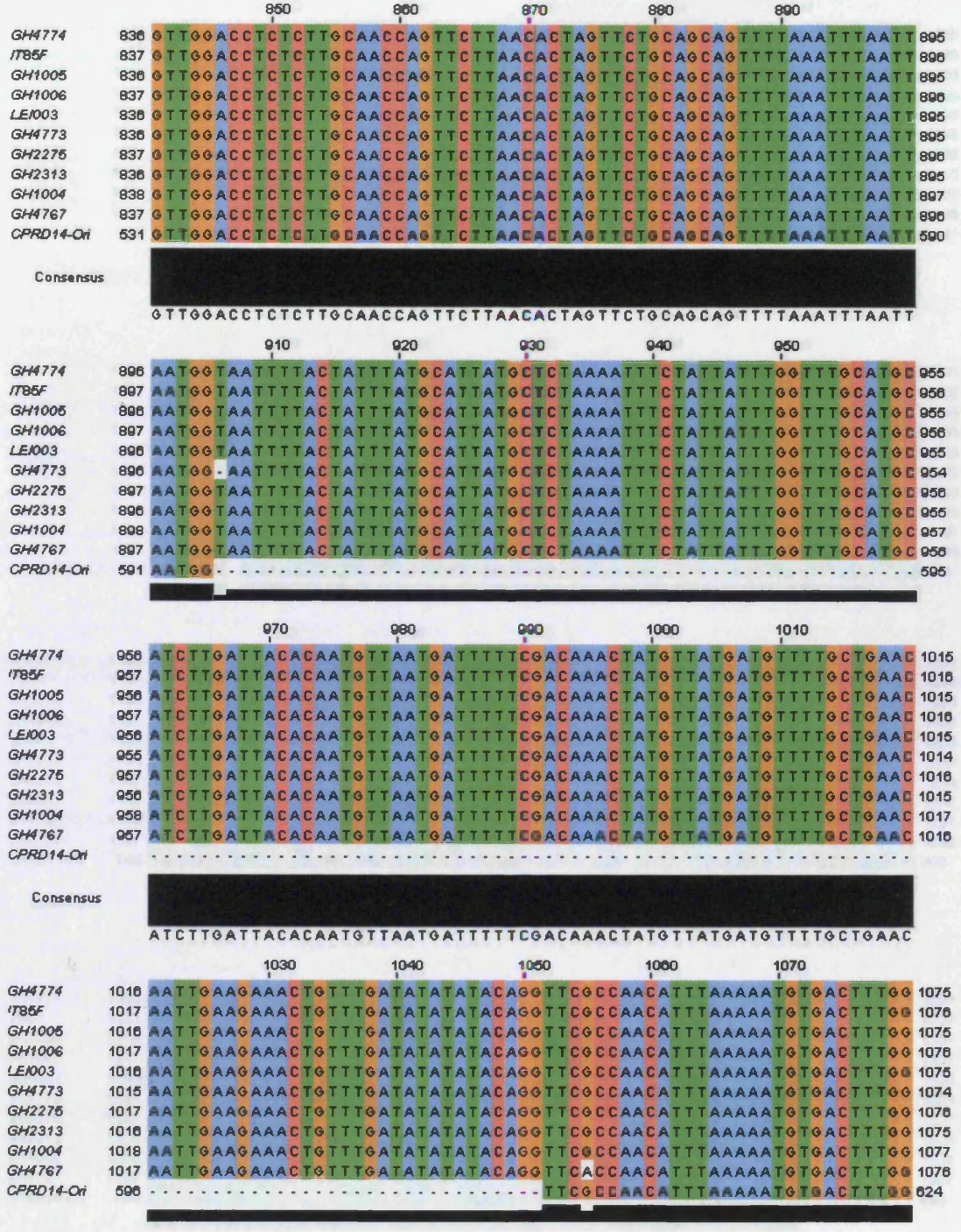
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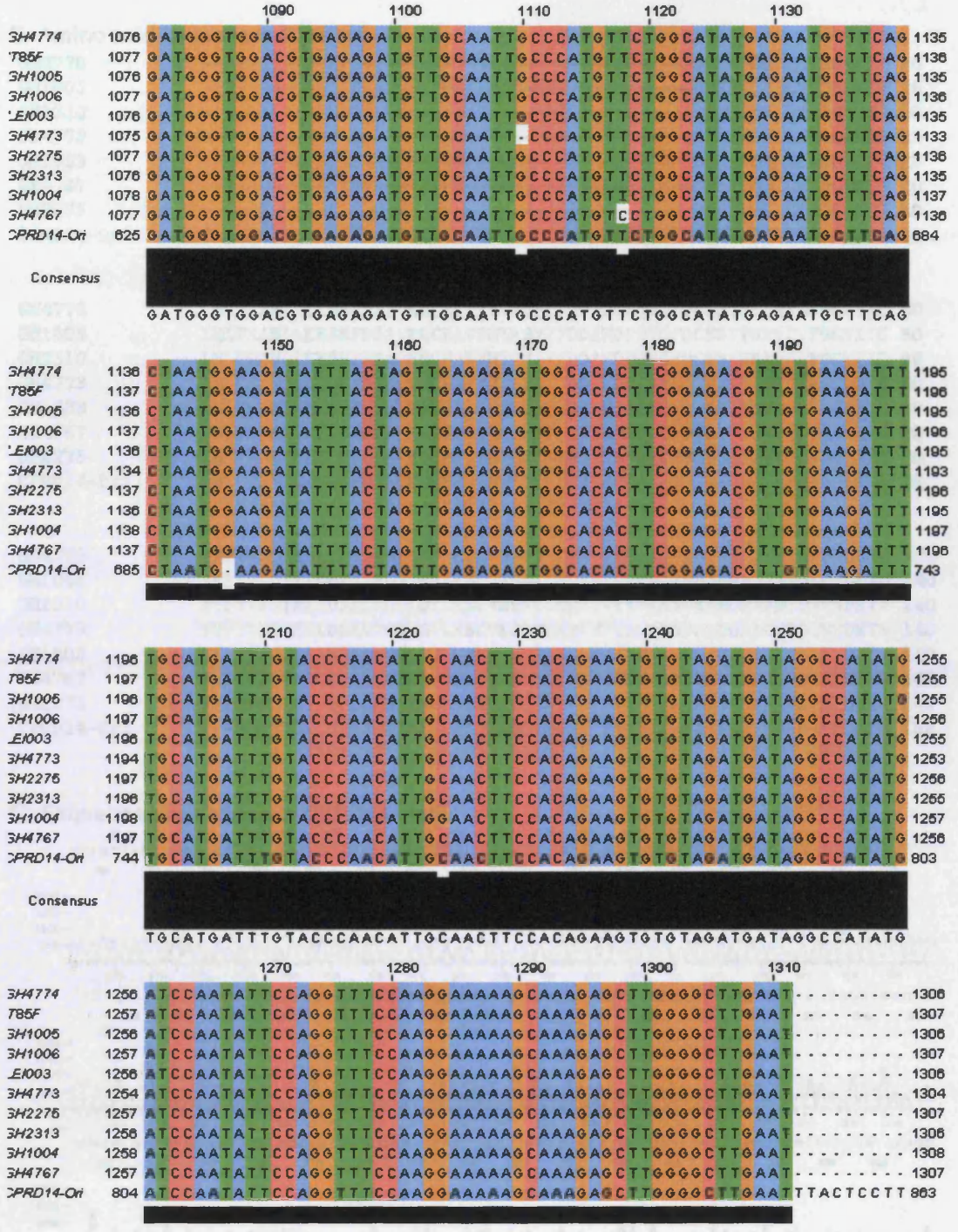
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SH1005 1076 GATGGGTGGACGTGAGAGATGTTGCAATTGCCCATGTTCTGGCATATGAGAATGCTTCAG 1135
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SH4767 1077 GATGGGTGGACGTGAGAGATGTTGCAATTGCCCATGTCCTGGCATATGAGAATGCTTCAG 1136
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Consensus
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1150
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1170
1180
1190
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1290
1300
1310
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B. Amino acid sequences-CPRD14

```
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GH2275          ----------CAG----------------------------DPKKVDHLLSLDGAKER 20
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                          *.                              ******************

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GH1005          LHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFDCSSIYSFVFLYGCVIIC 80
GH2310          LHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFDCSSIYSFVFLYGCVIIC 80
GH4773          LHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFDCSSIYSFVFLYGCVIIC 80
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GH4767          LHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFDCSSIYSFVFLYGCVIIC 80
GH2275          LHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFDCSSIYSFVFLYGCVIIC 80
CPRD14-Ori      LHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDP---------------------- 98
                ****************************************

GH4770          FYFYVFQTELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAGSFNDRPKNPDVVVDETW 140
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GH4767          FYFYVFQTELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAVSFNDRPKNPDVVVDETW 140
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CPRD14-Ori      ------QTELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAVSFNDRPKNPDVVVDETW 152
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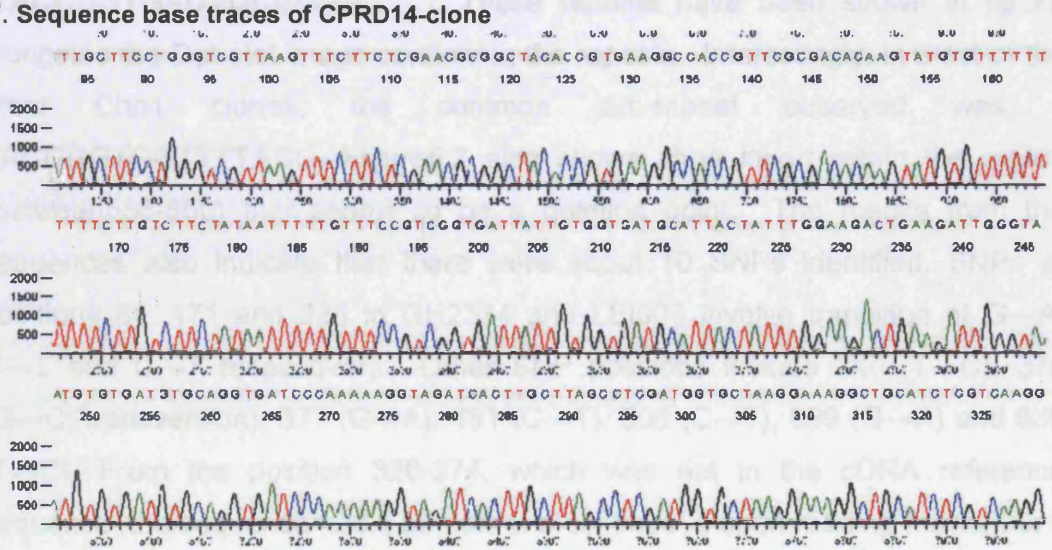
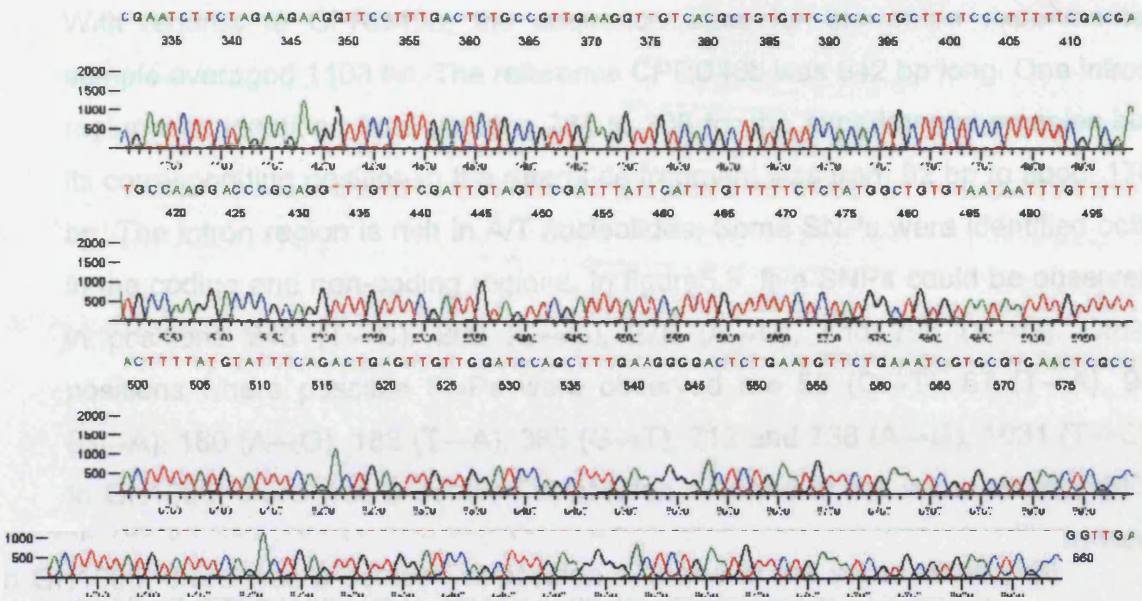
C. Sequence base traces of CPRD14-clone





The sizes of the sequences of Dhn1 clones (Table5.4) ranged from 873 bp (GH2334) to 966 bp (GH4773). The reference fragment with the forward and reverse primers inclusive was 909 bp long, indicating that there could be deletions and insertions as well as the intron regions that are not accounted for from the mRNA source. Unique patterns were observed in all the clones as shown in Fig.5.2. Dhn-GH2334 had about six sub-repeat motifs of largely (**TACCGGTGACACCGGGA**……). These repeats have been shown in fig.5.2 alongside the Dot-plot image confirming the repeats. Interestingly, in most of the other Dhn1 clones, the common sub-repeat observed was – **TACCGGTGGTTTTACC**. Figure5.2 also shows short insert within the region (between538-550) that seems to be a deletion point. The results from the sequences also indicate that there were about 10 SNPs identified. SNPs at positions 81, 171 and 228 in GH2334 and LEI003 involve transition of G→A, C→T and C→T respectively. Other SNP positions include 240 (T→C), 376 (G→C; transversion), 377 (G→A), 481 (C→T), 508 (C→T), 599 (G→A) and 636 (T→C). From the position 320-374, which was not in the cDNA reference sequence, the sequence could be part of a continuous open reading frame, but it was not clear whether this is a coding region present in the Ghanaian accessions or a non-coding intron.

With regards to CPRD46b, the sequence sizes for the entire experimental sample averaged 1103 bp. The reference CPRD46b was 942 bp long. One intron region was identified from position 224 to 308 for the experimental samples but its corresponding position in the reference fragment was from 92 bp to about 178 bp. The intron region is rich in A/T nucleotides. Some SNPs were identified both in the coding and non-coding regions. In figure5.3, five SNPs could be observed in positions 245 (T→C), 262 (C→G), 276 (A→G), and 296 (A→G). Other positions where possible SNPs were observed are 56 (C→T), 67 (T→A), 94 (G→A), 180 (A→G), 183 (T→A), 385 (G→T), 712 and 738 (A→G), 1031 (T→C). In GH1005, there was a deletion at position 472 and in the same clone, SNPs were observed at positions 479 (G→T), 482 (G→C) and 485 (A→G). In total about 28 SNPs could be observed in all the sequences in CPRD46b.

The average length observed in CPRD86 sequences was 546 bp with the reference clone being 418 bp. One intron region was identified from position 296 to 460. The intron region was also predominantly A/T rich in character. There was only one SNP observed within this region at position 407 (T→C) for GH1005 clone. Outside the intron region SNPs were observed at positions 176 (A→T), 279 (A→T) and 504 (T→C).

The average sequence length of the CPRD8 clones was 1036 bp; however, the size of the reference was 847 bp. Two intron regions were observed spanning from positions 110 to 223 and from 402 to 476. Both regions were noted to be A/T rich and SNPs were also found at positions 189 and 439 (T→C). Outside the two intron sites other SNPs were observed at positions 70 (T→C), 360 (A→G), 537 (C→T), 925 (T→C), and 976 (A→G). Deletion of T nucleotide was also observed at positions 118 and 154 all within the intron region.

The size of the CPRD14 was 1306 bp long in the clones and 963 bp long in the reference. Three intron regions were observed from positions 350 to 433, 628 to 742 and 903 to 1052 which were all A/T rich in character. SNPs were observed at positions 91 (T→C), 364 (C→T), 396 (G→A), 401 (G→T), 402 (T→G), 540

(T→G), 580 (T→G), 627 (G→A), 673 (T→C), 728 (T→G), 1055 (G→A), and 1118 (T→C). There were insertions of A at position 610, G at 628, C/A at 652, and T at 675, whilst deletions were also observed at position 640 and 901 of T nucleotide. The translated deduced amino acid sequences when analyzed with dot-plot also showed three intron sites. An intron region has been depicted in fig.5.6 (B), with a SNP of G→D at position 75 of GH1003. The base sequence traces (fig.5.6C) also show clean and clear traces of nucleotides and this was observed in all the various clones used for the study. In general, about 80% of all the SNPs observed within the clones from CPRD14 to Dhn1 were transitions in nature, with transversion SNPs forming about 20%, compared to a random expectation that 66% of changes would be transversions.

In other to determine the relationship of these cloned fragments with other closely related gene products from other sources as well as ascertain whether the various SNPs observed in the study caused functional shifts or were rather silent, Blastx of the sequences obtained for the CPRD fragments were performed. The results obtained are shown in table 5.9 below.

Table5.9: Results of blastx analysis of CPRD (22, 46b, Dhn1, 8, and 14), their close homologues, and E values for five closely related sequences.

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protein

 Score = 92.4 bits (228), Expect = 3e-17
 Identities = 41/41 (100%), Positives = 41/41 (100%)

Query: 523 HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 645
           HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK
Sbjct: 216 HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 256
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Vigna
           unguiculata (Cowpea)
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 Score = 92.4 bits (228), Expect = 3e-17
 Identities = 41/41 (100%), Positives = 41/41 (100%)
 Frame = +1
Query: 523 HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 645
           HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK
Sbjct: 219 HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 259
```

```
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Sbjct: 417 EWPLKSTLDPEIYGPPESAITTELIEKEIGGMISVEE 453

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            +A+KQK+LF+LDYHDLLLP V +V +++G  LYGSR LFFLT DGTLRPLAIEL RPP+
Sbjct: 458  EAVKQKKLFILDYHDLLLPYVNKVNELKGRVLYGSRTLFFLTPDGTLRPLAIELTRPPVH 517

Query: 482  GKPQWKKVFAPTWHSTGVWLWRLAKIHVLAHDTGYHQLVSHWLRTHCATEPYIIAANRQL 661
             KPQWK+V+ PTWH+TG WLW+LAK HVLAHD+GYHQLVSHWLRTHCATEPYIIA NRQL
Sbjct: 518  DKPQWKEVYCPTWHATGSWLWKLAKAHVLAHDSGYHQLVSHWLRTHCATEPYIIATNRQL 577

Query: 662  SAMHPIYRLLHPHFRYTVEINALARESLINAGGIIEQCFTPQKHSVLLSSIAYDKHWRFD 841
            SA+HPIYRLLHPHFRYT+EINALARE+LINA GIIE  F P K+++ LSS+AYD  WRFD
Sbjct: 578  SAIHPIYRLLHPHFRYTMEINALAREALINANGIIESSFFPGKYAMELSSVAYDLEWRFD 637

Query: 842  LQSLPKDLIHRGLAVEDPTAPHGLKLTIEDYPYANDGLDLWAAFKSWFTEYIDHYYADSN 1021
             ++LP+DLI RG+AV+DP AP+GLKLTIEDYP+ANDGL LW     W T+Y++HYY ++
Sbjct: 638  REALPEDLISRGMAVKDPNAPYGLKLTIEDYPFANDGLVLWDILIQWVTDYVNHYYTETK 697
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UR100:UniRef100_Q9SPL8 Cluster: Dehydrin; n=1; Vigna unguiculata|Rep: Dehydrin -
Vigna-Dhn1

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           GLGDDTNKQHDTSNVYGADTRRQHGTIGDTGRQH           RQY           R
Sbjct: 46  GLGDDTNKQHDTSNVYGADTRRQHGTIGDTGRQHGTTGGFTGDTGRQYGTTGGFTGDTGR 105

Query: 183 QHXXXXXXXXXXXKQHXXXXXXXXXXXXRQHGTTGGFTSDTGRQHXXXXXXXXXXXRQHXX 362
           QH           KQH           RQHGTTGGFT DTGRQH           RQH
Sbjct: 106 QHGTTGGFTGDTGKQHGTTGGFTGDTGRQHGTTGGFTGDTGRQH----GTTGDTGRQH-- 159

Query: 363 XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXANTADXXXXXXXXXXXXXXXXXXXXXXX 542
                                        ANTAD
Sbjct: 160 ------------GTTGGFTGGDTGLGGPYVGANTADTGTGPRSGTTGGSAYGSGGYGSGA 207

Query: 543 XXXXXXXXXXXHSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 698
                   HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK
Sbjct: 208 GAGYGMNTGGAHSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 259
```

```
>UR100:UniRef100_P93699 Cluster: CPRD8 protein; n=1; Vigna unguiculata|Rep: CPRD8
protein -
            Vigna unguiculata (Cowpea)
          Length = 384

 Score =  749 bits (1935), Expect = 0.0
 Identities = 362/372 (97%), Positives = 362/372 (97%)
 Frame = +1

Query: 52   PSHQAIPLLTPYKMGKFNLSHRVVLAPLSRERSYNNVPQPHAVVYYSQRTSNGGLLIAEA 231
            PSHQAIPLLTPYKMGKFNLSHRVVLAPLSRERSYNNVPQPHAVVYYSQRTSNGGLLIAEA
Sbjct: 13   PSHQAIPLLTPYKMGKFNLSHRVVLAPLSRERSYNNVPQPHAVVYYSQRTSNGGLLIAEA 72
```

```
Query: 232  TGVSDTAQGYPNTPGIWTKEQVEAWKPIVDAVHAKGGIFFCQIWHVGRVSDSSYQPNGQA 411
            TGVSDTAQGYPNTPGIWTKEQVEAWKPIVDAVHAKGGIFFCQIWHVGRVSDSSYQPNGQA
Sbjct: 73   TGVSDTAQGYPNTPGIWTKEQVEAWKPIVDAVHAKGGIFFCQIWHVGRVSDSSYQPNGQA 132
```

```
>UR100:UniRef100_O23777 Cluster: Old-yellow-enzyme homolog; n=1; Catharanthus
roseus|Rep:
Query: 70   PLLTPYKMGKFNLSHRVVLAPLSRERSYNNVPQPHAVVYYSQRTSNGGLLIAEATGVSDT 249
            PLLTPYK+GKF LSHR+VLAPL+R+RSY NVPQPHAV+YYSQRTS GGLLI+EA GVS+T
Sbjct: 19   PLLTPYKLGKFQLSHRIVLAPLTRQRSYGNVPQPHAVLYYSQRTSKGGLLISEAAGVSNT 78

Query: 250  AQGYPNTPGIWTKEQVEAWKPIVDAVHAKGGIFFCQIWHVGRVSDSSYQPNGQAPISSTD 429
            AQGYP TPGIWTKEQVEAWKPIVDAVHAKGG+FFCQI HVGRVS+ SYQPNGQAPISSTD
Sbjct: 79   AQGYPMTPGIWTKEQVEAWKPIVDAVHAKGGVFFCQIGHVGRVSNYSYQPNGQAPISSTD 138

Query: 430  KPLPPTPRANGLGYIEHTPPRRLTTEELPGIVNDFRIAARNAIEAGFDGVEVHGAHGYLL 609
            K L P  RANG+GY+EHTPPRRLTT+ELPGIVNDFR AA NAIEAGFDGVE+HGAHGYL+
Sbjct: 139  KGLTPLYRANGIGYVEHTPPRRLTTDELPGIVNDFR-AALNAIEAGFDGVEIHGAHGYLI 197

Query: 610  DQFLKDQANDRTDQYGGSLENRCRLPLXXXXXXXXXXGADRVGIRLSPFADFNDCGDSNP 789
            DQFLKDQ ND TD+YGGSLENRCR  L          GADRVGIRLSPFA + + GDSNP
Sbjct: 198  DQFLKDQVNDSTDEYGGSLENRCRFALEIVEAVSNAIGADRVGIRLSPFAGYMESGDSNP 257
```

```
>UR100:UniRef100_A0M0F0 Cluster: Secreted protein; n=1; Gramella forsetii
KT0803|Rep:CPRD86
          Secreted protein - Gramella forsetii (strain KT0803)
         Length = 145

 Score = 35.4 bits (80), Expect = 1.6
 Identities = 17/53 (32%), Positives = 26/53 (49%)
 Frame = -3

Query: 487 HKFRSQESKKRSHSPHYSGHIHLHQTKRRKTKSEQTLVIMFCALIKVTEENKI 329
           H     + +K+  + H+SG  H HQ K+ + KSE  L  +F      T  N+I
Sbjct: 27  HAHHEHQEEKKEIAHHHSGEDHHHQDKKEEGKSEGFLSFLFAMHSHTTTSNEI 79
```

```
>UR100:UniRef100_P93700 Cluster: CPRD14 protein; n=1; Vigna unguiculata|Rep: CPRD14
protein

Query: 182 DPKKVDHLLSLDGAKERLHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFD 361
           DPKKVDHLLSLDGAKERLHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQ
Sbjct: 42  DPKKVDHLLSLDGAKERLHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQ---- 97

Query: 362 CSSIYSFVFLYGC*IICFYFYVFQTELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAV 541
                              TELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAV
Sbjct: 98  ------------------------TELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAV 133

Query: 542 SFNDRPKNPDVVVDETWYSDPEYCKRNGV 628
           SFNDRPKNPDVVVDETWYSDPEYCKRNG+
Sbjct: 134 SFNDRPKNPDVVVDETWYSDPEYCKRNGI 162
```

```
 Score =  112 bits (281), Expect(3) = 4e-95
 Identities = 56/58 (96%), Positives = 57/58 (98%)
 Frame = +3

Query: 738 IWYNLSKTLAEDAAWKFAKENNIDLVTANPALVVGPLLQPVLNTSSAAVLNLINGNFT 911
           IWYNLSKTLAEDAAWKFAKENNIDLVTANPALVVGPLLQPVLNTSSAAVLNLING+ T
Sbjct: 162 IWYNLSKTLAEDAAWKFAKENNIDLVTANPALVVGPLLQPVLNTSSAAVLNLINGSPT 219



 Score = 49.3 bits (116), Expect(3) = 4e-95
 Identities = 23/23 (100%), Positives = 23/23 (100%)
 Frame = +3

Query: 3  ASWVVKFLLERGYTVKATVRDTS 71
           ASWVVKFLLERGYTVKATVRDTS
Sbjct: 19 ASWVVKFLLERGYTVKATVRDTS 41



 Score =  181 bits (458), Expect = 1e-43
 Identities = 87/93 (93%), Positives = 90/93 (96%)
 Frame = +2

Query: 1028 LFDIYTGSPTFKNVTLGWVDVRDVAIAHVLAYENASANGRYLLVERVAHFGDVVKILHDL 1207
           + ++  GSPTFKNVTLGWVDVRDVAIAHVLAYENASANGRYLLVERVAHFGDVVKILHDL
Sbjct: 210  VLNLINGSPTFKNVTLGWVDVRDVAIAHVLAYENASANGRYLLVERVAHFGDVVKILHDL 269

Query: 1208 YPTLQLPQKCVDDRPYDPIFQVSKEKAKSLGLE 1306
           YPTLQLPQKCVDDRPYDPIFQVSKEKAKSLGLE
Sbjct: 270  YPTLQLPQKCVDDRPYDPIFQVSKEKAKSLGLE 302
```

```
>UR100:UniRef100_Q9SQK0 Cluster: Aldehyde reductase; n=1; Vigna radiata|Rep: Aldehyde
          reductase - Vigna radiata
         Length = 325

 Score =  219 bits (559), Expect(3) = 2e-87
 Identities = 113/149 (75%), Positives = 119/149 (79%)
 Frame = +2

Query: 182 DPKKVDHLLSLDGAKERLHLVKANLLEEGSFDSAVEGCHAVFHTASPFFDDAKDPQVCFD 361
           DPKKVDHLLSLDGAKERLHLVKANLLEEGSFDSAVEG HAVFHTASPFF+DAKDPQ
Sbjct: 42  DPKKVDHLLSLDGAKERLHLVKANLLEEGSFDSAVEGVHAVFHTASPFFNDAKDPQ---- 97

Query: 362 CSSIYSFVFLYGC*IICFYFYVFQTELLDPALKGTLNVLKSCVNSPTLKRVVVTSSIAAV 541
                                TELLDPA+KGTLNVLKSCVNSPTLKRVV+TSSIAAV
Sbjct: 98  ------------------------TELLDPAVKGTLNVLKSCVNSPTLKRVVLTSSIAAV 133

Query: 542 SFNDRPKNPDVVVDETWYSDPEYCKRNGV 628
           +F+DRPKNPDVVVDETWYSDPEYCKR G+
Sbjct: 134 AFSDRPKNPDVVVDETWYSDPEYCKRTGL 162



 Score =  103 bits (257), Expect(3) = 2e-87
 Identities = 49/55 (89%), Positives = 52/55 (94%)
 Frame = +3

Query: 738 IWYNLSKTLAEDAAWKFAKENNIDLVTANPALVVGPLLQPVLNTSSAAVLNLING 902
           +WYNLSKTLAEDAAWKFAKENNIDLVT NPALVVGPLLQPVLNTS+A VL L+NG
```

```
Sbjct: 162 LWYNLSKTLAEDAAWKFAKENNIDLVTMNPALVVGPLLQPVLNTSAAIVLGLVNG 216


 Score = 47.0 bits (110), Expect(3) = 2e-87
 Identities = 21/23 (91%), Positives = 23/23 (100%)
 Frame = +3

Query: 3  ASWVVKFLLERGYTVKATVRDTS 71
          ASW+VKFLLERGYTVKATVRDT+
Sbjct: 19 ASWLVKFLLERGYTVKATVRDTN 41


 Score =  151 bits (382), Expect = 8e-35
 Identities = 69/90 (76%), Positives = 80/90 (88%)
 Frame = +2

Query: 1037 IYTGSPTFKNVTLGWVDVRDVAIAHVLAYENASANGRYLLVERVAHFGDVVKILHDLYPT 1216
            +  G+ TFKN +LGWVDV+DVA+AH+LAYENASANGRYLLVERVAHFGD  KIL DLYPT
Sbjct: 213  LVNGAKTFKNASLGWVDVKDVALAHILAYENASANGRYLLVERVAHFGDAAKILRDLYPT 272

Query: 1217 LQLPQKCVDDRPYDPIFQVSKEKAKSLGLE 1306
            LQ+P KC DD+P +PIFQVSKEKAKSLG++
Sbjct: 273  LQIPDKCEDDKPLEPIFQVSKEKAKSLGID 302
```

5.4. Discussion

As a first step towards the understanding of the responses of drought-tolerant plants to dehydration stress at the molecular level and the diversity of genes involved, the CPRD family of candidate genes induced by dehydration stress was selected and primers designed from them to amplify genomic DNA regions of some cowpea accessions from Ghana. The genes from the CPRD family have been confirmed to be induced by drought stress and so the aim of this study was to determine whether primers designed from drought inducible genes could amplify genomic DNA from unstressed cowpea plants, and detect any polymorphisms within the gene pool of the germplasm used for the study.

The results from the PCR amplification reactions showed that all the primers designed for the study amplified products within the expected range of the anticipated product sizes with the exception of cpABA(a) and cpABA(b) primers which could not amplify any product. The results further indicated that polymorphisms abound within the Ghanaian cowpea in the form of SNPs, deletions and insertions of single or group of nucleotides. In some instances especially in CPRD86, SNPs detected were not as high as the others. These

SNPs are still discussed as they present potential candidates, but need to be verified by future analyses.

In this study I performed a blastx for sequences homologous to that of CPRD8 protein and found the putative protein only to show weak similarities to bacterial and fungal proteins in contrast to the other sequences where many homologies were found in cowpea, legumes and other plants. The CPRD8 weak homologies were to morphinone reductase (morB) of *Pseudomonas putida* and old yellow enzyme of *Saccharomyces cerevisiae* with 47% and 30% homology, respectively (French and Bruce 1994, Stott et al. 1993) (Table5.9). The sequence of the putative CPRD8 protein was also similar to that of the estrogen-binding protein of *Candida albicans* (Madani et al. 1994) and bile acid-inducible protein C of *Eubacterium* sp. VPI 12708 (Mallonee et al. 1990). Notably and perhaps related to its function in plants, the putative CPRD8 protein contained conserved amino acids that are involved in the active site (His 191- Asnl94) and flavin mononucleotide-binding (FMN-binding) site (Thr37, Glnll4 and Arg243, but not Arg348) or old yellow enzyme (Fox and Karplus 1995).

The deduced nucleotide and amino acid sequences of Dhn1 and CPRD22 protein revealed structural features homologous to those of group 2 LEA proteins. These proteins are thought to function in the maintenance of protein structure (Bray, 2002b). Recently, it was shown that two LEAs can prevent protein aggregation during water stress (Goyal *et al.*, 2005b) and the ability of plant LEAs to confer increased tolerance to water deficit stress on yeast and other plants (Swire-Clark and Marcotte, 1999; Xu *et al.,* 1998; Sivamani *et al.,* 2000) also suggests LEAs play an important role in protecting tissues from the effects of water loss. The CPRD22 protein might also function to protect cowpea cells from dehydration. Group 2 LEA proteins usually contain a conserved sequence, a lysine-rich conserved sequence, KIKEKLPG. The putative CPRD22 protein contained the KIKEKLPG sequences as shown below with the conserved motif underlined and coloured red. The query is the fragment from the study;

```
Query: 523 HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 645
           HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK
Sbjct: 219 HSDERYGREYREHDQSRGDHDKKGIVDKIKEKLPGGHSDNK 259
```

A stretch of 69 amino acids with five tandemly repeated Gly-rich sequences are found in the amino-terminal half of the polypeptide. The consensus sequence of these repeats is GTTGGFTGDTGRQ, and the function of such sequences remains to be elucidated. In the case of the CPRD22 fragment a SNP (G→S) was identified between positions 181-261 in the fragment as compared to its corresponding position 123-149 within this consensus sequence as shown below. The query is the fragment from the study;

```
Query: 181 XXXXXXXXXXRQHGTTGGFTSDTGRQH 261
                    RQHGTTGGFT DTGRQH
Sbjct: 123 TTGGFTGDTGRQHGTTGGFTGDTGRQH 149
```

The amino acid SNP observed G→S, which is Gly→Ser is equivalent to a G→A nucleotide SNP observed in the analysis of the nucleotide sequences already discussed in the results section.

Another notable motif was the RHQ- related sequence which was repeated up to eight times and showed polymorphisms is spacing between the accessions under analysis and the reference sequences.

```
Query: 3   GLGDDTNKQHDTSNVYGADTRRQHGTIGDTGRQHXXXXXXXXXXXRQYXXXXXXXXXXXR 182
           GLGDDTNKQHDTSNVYGADTRRQHGTIGDTGRQH          RQY          R
Sbjct: 46  GLGDDTNKQHDTSNVYGADTRRQHGTIGDTGRQHGTTGGFTGDTGRQYGTTGGFTGDTGR 105

Query: 183 QHXXXXXXXXXXXKQHXXXXXXXXXXXRQHGTTGGFTSDTGRQHXXXXXXXXXXXRQHXX 362
           QH           KQH           RQHGTTGGFT DTGRQH           RQH
Sbjct: 106 QHGTTGGFTGDTGKQHGTTGGFTGDTGRQHGTTGGFTGDTGRQH----GTTGDTGRQH-- 159
```

The amino acid sequence of the putative CPRD14 protein was similar to those of dihydroflavonol-4-reductase (DFR). DFR is involved in the biosynthesis of medicarpin, which is a major phytoalexin in alfalfa. The secondary metabolites produced by medicarpin are involved in protection of cells from UV light, defense against attack by pathogens, legume nodulation and pollen viability (Li et al.,

2000). The CPRD 14 protein revealed homology to DFR from *Vitis vinifera* (39%; Sparvoli et al. 1994) and *Gerbera hybrida* (35%; Helariutta et al. 1993) and to vestitone reductase from alfalfa (36%; Guo and Paiva 1995). The product of the CPRD14 gene might be involved in the synthesis of such compounds, which protect plant cells from stress conditions. The deduced sequences of all the other candidate genes used for the study were found to be involved in the protection of the plant cells from environmental stress and the maintenance of cell integrity as shown in detail analysis in Table5.9.

To address the main question of whether the genes for drought tolerance are constitutive or adapted to the imposed stress on a drought tolerant plant like cowpea, the comparative analysis of the drought induced gene products and the unstressed products were determined. The assumption was that if they are constitutive, then as and when the stress is imposed, the perception of the stress will be communicated through the signalling system for the plant to react to the stress by up-regulating or down-regulating certain genes to mitigate the effect imposed. The results of the multiple alignment of the sequenced products in comparison with the original drought induced gene sequences (fig.5.2-5.7), showed minimal discrepancies between the induced state and the unstressed state. This may probably suggest that these genes may be constitutive in nature, but their mode of operation as to when a particular inducible gene is up-regulated or down-regulated to combat the effect of an imposed stress need further analysis. According to Chaves and colleagues (2002), many of the traits that explain plant adaptation to drought like, phenology, root size and depth, hydraulic conductivity and storage of reserves, are associated with plant development and are constitutive rather than stress induced, which seems by and large to support the findings of this study.

5.5. Conclusions

- Primers designed from drought inducible gene sequences were able to amplify genomic DNA extracted from unstressed cowpea plants using simple PCR technique.
- The deduced nucleotide and amino acid sequences obtained included the non-coding regions of the gene products, showing a depth of information within the entire region and the presence of some SNPs in both the coding and non-coding regions that need further analysis.
- Results showed that the observed genes in the drought tolerant plant like cowpea may be constitutive in nature which necessitates further analysis, possibly differential display analysis of the gene expression of both stressed and unstressed states for comparative analysis to further accelerate our understanding of the mechanisms of drought tolerance in cowpea.

CHAPTER SIX

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CHAPTER SIX

6.0. Comparative analysis of gene expression in stressed and unstressed cowpea plants.

6.1. Introduction

Water deficit elicits a complex of responses beginning with stress perception, which initiates signal transduction pathway(s) and is manifested in changes at the cellular, physiological, and developmental levels. Physiological and in the last decade molecular studies have demonstrated that plants react to drought with a series of accurately controlled and well defined responses induced by detection of the onset of drought or environmental features indicating drought is likely. The set of responses observed depends upon nature, severity and duration of the stress, plant genotype, developmental stage, and environmental factors providing the stress. Cellular water deficit may result from stresses such as drought, salt, and low temperature. This complexity makes it difficult to uncover the responses to water deficit that enhance stress tolerance, particularly since some responses anticipate stress. In recent years efforts have turned toward isolation of genes that are induced during water deficit in order to study the function of drought-induced gene products, the pathways that lead to gene induction and the earliest events leading to drought tolerance. While some short-term changes – stomatal closure for example – are physiological, changes in gene expression are fundamental to the responses that occur during water deficit, and they control many of the medium, long and even short-term responses.

Studies on the molecular responses to water deficit have identified multiple changes in gene expression leading to different proteins using a two dimensional polyacrylamide gel electrophoresis (2D-PAGE), and many genes that are water-deficit-induced have been isolated by differential screening of cDNA libraries using either laboratory-based methods or informatics studies of EST collections.

Functions for many of these gene products have been predicted from the deduced amino acid sequence of the genes and comparison with data from known genes characterized in model species. Genes expressed during stress are anticipated to promote cellular tolerance of dehydration through protective functions in the cytoplasm, alteration of cellular water potential to promote water uptake, control of ion accumulation, and further regulation of gene expression. Although these studies are promising, it continues to be difficult to ascertain the actual function of drought-induced gene products. Expression of a gene during stress does not guarantee that a gene product promotes the ability of the plant to survive stress. The expression of some genes may result from injury or damage that occurred during stress. Other genes may be induced, but their expression does not alter stress tolerance. Yet others are required for stress tolerance and the accumulation of these gene products is an adaptive response.

Complex regulatory and signaling processes control the expression of genes during water deficit; although they are not fully understood, systems biology approaches to investigate signalling networks are beginning to provide useful information, at least in simpler systems. Multiple stresses may connect into the same or a similar transduction pathway, which is evidenced by the involvement of ABA in the induction of genes induced by a number of different stresses. In addition to induction by stress, the expression of water-deficit-associated genes is controlled with respect to tissue, organ, and developmental stage and may be expressed independently of the stress conditions. For example, some genes expressed during drought stress are also expressed during the maturation and desiccation phases of seed development. The regulation of specific processes will also depend upon the experimental conditions of stress application. Stress conditions that are applied in the laboratory may not accurately represent those that occur in the field. Frequently, laboratory stresses are rapid and severe, whereas stress in the field often develops over an extended period of time (Chaves et al., 2002). Further more, plant root systems, even from relatively small plants in the field, may extend for many metres, while pot-grown plants are

constrained to few centimeters. These differences must also be evaluated when studying the adaptive value of certain responses. The function of the gene products and the mechanisms of gene expression are intertwined, and both must be understood to fully comprehend the molecular response to water deficit.

The numerous responses to water deficit are controlled by an array of genes with many different functions. As water is lost from the cell, regulatory processes are initiated that adjust cellular metabolism to the new cellular conditions. At the same time, growth inhibition and alterations of developmental pathways will result in changes in gene expression. Many of the water-deficit-induced genes also encode gene products predicted to protect cellular function (Liu and Baird, 2003). Genes that function during changes in metabolism, regulation, signalling, and recognition of stress are also expected to be induced, but fewer of these classes of genes have been identified. A number of water-deficit-induced gene products are predicted to protect cellular structures from the effects of water loss. These predictions are derived from the deduced amino sequence and expression characteristics. These genes, frequently called late embryogenesis abundant genes (*lea*), were first identified as genes that are expressed during the maturation and desiccation phases of seed development (Baker et al., 1988). It has since been recognized that these genes are also expressed in vegetative tissues during periods of water loss resulting from water, osmotic, and low-temperature stress. At least *six* groups of *lea* genes have been identified, based on amino acid sequence similarities among several species (Dure, 1993b). The majority of the *lea* gene products are predominantly hydrophilic biased in amino acid composition, and lacking in Cys and Trp, and are proposed to be located in the cytoplasm. The individual amino acid sequences and predicted protein structures have been used to propose specific functions for each group of LEA proteins (Dure, 1993b). These predicted functions include sequestration of ions, protection of other proteins or membranes, and renaturation of unfolded proteins. It is critical to determine if plant responses to stress are adaptive or merely a consequence of stress.

Responses that are triggered by environmental cues as well as developmental signals are promising as responses that are important for adaptation to stress. However, knowing that these genes are induced is not sufficient to conclude that specific gene products are required for stress tolerance. Therefore, experimental means to evaluate the adaptive nature of specific gene products are required. A correlation between organ survival and LEA protein accumulation during recovery from severe dehydration has been identified. In wheat seedlings, shoots and scutellum resume growth during recovery from 90% water loss, whereas the roots are killed. There is a correlation between dehydration survival and LEA protein accumulation, but not mRNA accumulation (Ortiz et al, 2007). These results support the hypothesis that LEA proteins function in stress tolerance. However, mRNA accumulation alone certainly cannot be used to demonstrate that a response is adaptive. In addition, these results highlight an important role for posttranscriptional regulation of gene expression during stress.

The molecular response of plants to water deficit defines a very interesting puzzle: how does a physical phenomenon, the loss of water from the cell, cause a biochemical response, the induction of specific genes? Or, how does the cell recognize the loss of water and respond to it? The answer to these questions is not known. Currently, it is thought that loss of turgor or change in cell volume resulting from different environmental stresses permits the detection of loss of water at the cellular level. One or both of these changes may activate stress-activated channels, alter conformation or juxtaposition of critical proteins, or cause alterations in the cell wall plasma membrane continuum (Li et al., 2004), thereby triggering a signal transduction pathway(s) that induces gene expression. Therefore, several different stresses may trigger the same or similar signal transduction pathways. The induction pathway(s) is also poorly understood, although there is evidence that there is more than one pathway. There may be a direct pathway, or additional signals may be generated that, in turn, alter the pattern of gene expression. The plant hormone ABA also accumulates in

response to the physical phenomena of loss of water caused by different stresses, and elevation in endogenous ABA content is known to induce certain water-deficit-induced genes (Shinozaki and Yamaguchi-Shinozaki, 1996). Despite these studies, relatively little is known about the fundamental differences and cross-talk between drought and other abiotic response pathways in plants, especially in cowpea.

Differential display-polymerase chain reaction (DD-PCR) (Liang and Pardee, 1992) is a simple, sensitive and powerful method for screening cDNAs, and is useful in characterizing tissue-, organ- or development-specific cDNAs (Cushman and Bohnert, 2000). DD-PCR has been used successfully to isolate a number of differentially expressed genes from plants (Martin-Laurent et al., 1997; Roux and Perrot-Rechenmann, 1997; Deleu et al., 1999; Wei et al., 2004). In this chapter two sets of cowpea plants, each set comprising of three accessions, identified in chapter three as being tolerant or susceptible to drought, have been used for gene expression analysis for comparative studies.

The objectives of the study were to:

- exploit whether plants exposed to different water regimes induce different responses to gene expression.
- determine whether the identified clones could be cloned, sequenced, and characterized.
- ascertain whether the identified differentially expressed genes could be linked to the physiological and morphological results already obtained.

6.2. Materials and methods

6.2.1. Plant materials and drought treatments

Seeds of six cowpea (*Vigna unguiculata*) accessions, GH4767, GH2313, GH3679, GH 3710, GH4778 and GH3687 were used for this study. These are both drought tolerant and susceptible accessions identified in Chapter three. The

plants were grown from seeds and the seedlings subjected to drought treatment as has been described in chapter two, section 2.2. The leaves were harvested at five days interval after the imposition of the drought treatment for a period of 30 days. Total RNA was extracted from the harvested leaves and kept at -80°C for the differential display gene expression analysis.

6.2.2. RNA isolation

Total RNA from the stressed and unstressed cowpea leaves harvested was extracted using the procedure described in chapter two, section 2.6.1. The extracted RNA samples were then treated with RNase-free DNase to remove any DNA contaminants and stored at -80°C.

6.2.3. Differential cDNA display

Differential display of cDNA was performed (Liang and Pardee, 1992) using Delta Differential Display Kit following the manufacturer's protocol (Clontech, UK). The procedure has been described in section 2.6.2 in chapter two. Then differential display PCR (DD-PCR) was performed in a 20 μl reaction mixture, using two dilutions of the first strand cDNA (dilution A-consisted of 4 ng, and dilution B-consisted of 1 ng cDNA), and a primer pair combination of one arbitrary primer and one Oligo (dT) primer as described in section 2.6.2. The primers from which the combinations were selected have been shown below.

DD-PCR products were electrophoresed on a mixture of 2% high resolution agarose gel in 1x TAE buffer with 0.5 μg/ml of ethidium bromide. Differentially expressed cDNAs identified visually by comparison of banding patterns were excised from the gel, recovered and re-amplified using the same primer set that produced it. The annealing temperature was dropped from 60°C to 50°C and the dNTPs final concentration increased to 0.2 μM. The PCR products were excised again and purified using Qiagen gel extraction kit following the manufacturer's protocol. The purified cDNA fragments were ligated in pGEM-T Easy vector prior to transformation into competent bacterial cells.

PCR primers for Delta Differential Display

• 10 arbitrary primers (20 mM):

P1: 5'-ATTAACCCTCACTAAATGCTGGGGA-3'

P2: 5'-ATTAACCCTCACTAAATCGGTCATAG-3'

P3: 5'-ATTAACCCTCACTAAATGCTGGTGG-3'

P4: 5'-ATTAACCCTCACTAAATGCTGGTAG-3'

P5: 5'-ATTAACCCTCACTAAAGATCTGACTG-3'

P6: 5'-ATTAACCCTCACTAAATGCTGGGTG-3'

P7: 5'-ATTAACCCTCACTAAATGCTGTATG-3'

P8: 5'-ATTAACCCTCACTAAATGGAGCTGG-3'

P9: 5'-ATTAACCCTCACTAAATGTGGCAGG-3'

P10: 5'-ATTAACCCTCACTAAAGCACCGTCC-3'

• 9 Oligo (dT) primers (20 mM):

T1: 5'-CATTATGCTGAGTGATATCTTTTTTTTTAA-3'

T2: 5'-CATTATGCTGAGTGATATCTTTTTTTTTAC-3'

T3: 5'-CATTATGCTGAGTGATATCTTTTTTTTTAG-3'

T4: 5'-CATTATGCTGAGTGATATCTTTTTTTTTCA-3'

T5: 5'-CATTATGCTGAGTGATATCTTTTTTTTTCC-3'

T6: 5'-CATTATGCTGAGTGATATCTTTTTTTTTCG-3'

T7: 5'-CATTATGCTGAGTGATATCTTTTTTTTTGA-3'

T8: 5'-CATTATGCTGAGTGATATCTTTTTTTTTGC-3'

T9: 5'-CATTATGCTGAGTGATATCTTTTTTTTTGG-3'

6.2.4. Cloning and sequencing

The reamplified products were cloned into pGEM-T Easy vector according to the manufacturer's protocol (Promega) as described in section 2.7.3. The clones which were verified to have the cDNA insert of interest as described in section 2.7.4 were sequenced commercially at John Innes Centre (JIC), at Norwich, United Kingdom, using M13 forward and reverse primers according to the requirements of the ABI 3700 capillary sequencing system.

6.2.5. Dot blot analysis of candidate differential expression

A Northern Dot Blot analysis was carried out as described in chapter two (section 2.8). This analysis was intended to confirm the differential expression of gene fragments identified in the DD-PCR analysis. However, due to some cDNA contamination and the lack of RNA for the repetition of this process, as well as limitation of time, this part of the experimentation could not be followed to its end. It has therefore been proposed that this part of the work should be repeated in the near future.

6.3. Results

6.3.1: Physiological parameters of the water-stressed plants

Water was withheld from cowpea plants for a 30-day period to simulate gradual development of a field-like water stress. Early during the stress process, plants were phenotypically indistinguishable from well-watered plants. However, plants mid-way through the stress period displayed leaf wilting, leaf curling, yellowing and reduced leaf length. Over the 30-day period, the leaf relative water content (RWC) could be observed to have decreased to a large extent but the drought tolerant plants were able to recover when watered.

6.3.2: Molecular analysis of the cowpea plants for response to water stress

Cowpea gene fragments whose expression was regulated by drought stress were studied by differential mRNA display. Each experiment was repeated by

using total RNA from at least two independent preparations as results are only accepted if identical profiles were obtained.

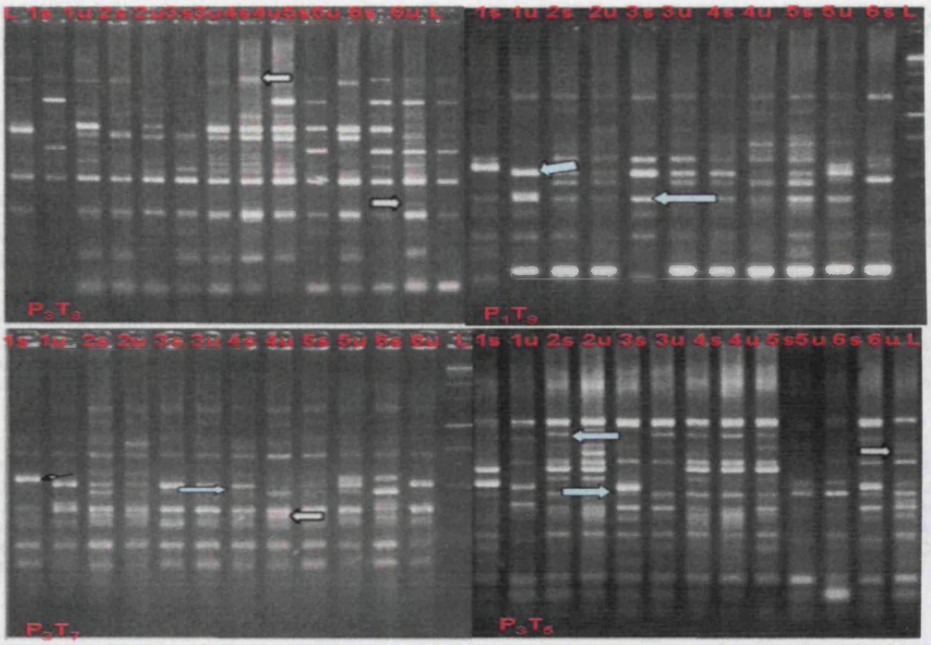


Figure 6.1: Amplification profiles of P_3T_8, P_1T_9, P_3T_7, and P_3T_5. The cDNAs were synthesized from leaves of cowpea plants; 1-GH4767, 2-GH2313, 3-GH3679, 4-GH3710, 5-GH4778, 6-GH3687;S-Plants subjected to drought treatment; U-Plants watered during experimental period; L-Hyperladder1; Arrows in the ethidium bromide stained gels show candidate bands with differential expression.

By screening 16 primer-pair combinations of arbitrary and Oligo (dT) primers in cowpea leaves of both stressed and unstressed samples partial cDNAs potentially differentially expressed in response to drought stress (Fig. 6.1) were observed and selected for cloning and sequencing analysis. In all 14 differentially expressed bands were isolated and cloned with nine of them being successfully transformed and sequenced. These nine selected clones were named as cowpea differential display products (CDDP). Six of the cloned fragments were isolated from drought challenged plants which were CDDP1, CDDP2, CDDP3, CDDP5, CDDP8, and CDDP9. The other three clone fragments were isolated from unstressed plants and comprised CDDP4, CDDP6, and CDDP7. Table 6.1 below is the results of the sequencing of the selected clones obtained from the study. The length of the sequences ranged from 52 bp (CDDP7) to 821 bp (CDDP9).

Table 6.1: Nucleotide sequences of the clones selected as having been expressed differentially. Those up-regulated by drought are shown in red captions.

>CDDP1
CATTATGCTGAGTGATATCTTTTTTTTTCAAACTTGGTGAAGAAGGGGAGAGGTTGAATGGGGGTAGGAAAGTACTTCCAGAAGGAACA
GTTCTCAGGGAATATATCATAGGGGACAGAGGTTTTCCTCTTTTGCCATGGCTTCTTACACCTTACGAAGGTAAAGGACTCTCAAATGTA
CAAGTTGAGTTCAATAGAAGGGTTGTTGAAACTCAAATGTTGGCCAAGAAAGCATTGGCTAGGCTGAAGGAGATGTGGAGGATAATCC
AAGGTGTGATGTGGAAACCTGACAAGCACAAGTTACCAAGAATTATTCTTGTCTGCTGCATACTGCATAATATAGTTATCGATATGAAGG
ATGAAGTACTGAATGATATGTCCTCTTGCCACCAGCATTTAGTGAGGGTTAAT

>CDDP2
ATTAACCCTCACTAAATGCTGGTGGCTGTGGAATCTCTTCTTCCTCTTCATCCTCTTCCTCATCCTCATCTTCATCTTCATCTTCATCTTC
ATCTTCTTCATCACCATCATCACCATCTTCGCCATCTTCACCATCTTCACCATCTTTTCCACCAGCATTTAGTGAGGGTTAAT

>CDDP3
ATTAACCCTCACTAAATGCTGGTGGATACCTCGCCAGACGCTGATGTCATAACTATTAACTCGCACCCCAATTCTCGCATCATAAGTCC
GAGCTTCCTCAAACATTCATTGGATAAAAATGAATGGGTAGCTTCTGAGTCATACAGCACAACCACACTGTTACCAAAAAGCAAGTATTC
TACACCAGGTTACCTGACTGAGTCGCCTCGGTGGTCGTCAGTGCAAACACACGCCCTAATGCTCTAGGTCACTCTCCAACAGGTTTCT
TAGCTAGCGCACCACCAGCATTTAGTGAGGGTTAAT

>CDDP4
ATTAACCCTCACTAAATGCTGGTGGCAAAAATCCTAAAAGACGAGTATATGGTGTTGGAAAACTGAATGAAAATTATCTTTGTTGGGAAG
TCTTCACACAACAACCATTCAGTTCTACAAGTATGGATTCACAAAAGATTCTAAGGTTGGAAGAAGAAATTCGTCAATCTAGGGAGGAGT
TTCGTCAATCTAGGGAGGAGAATCAACGGCTGCAGAGAAAATTAGAATCCCTAGTAATTGTTGTTCTCTTCCTCCCGTCGCACAAACCA
TTTTGCAAGACGTCAATGAACAACCACAAAATGAGGATCAAAACCAAGATGATGCTCGGGAGAAGGACCACCAGCATTTAGTGAGGGT
TAAT

>CDDP5
ATTAACCCTCACTAAATGCTGGTGGGGCTGGGAAATAGGAGCCACCCCAATGATAAGTATTGTGAAGGACATAGTGAACAACATGAAG
GCGATGGAAGAGGAAGAAGGGAGCAACATAGAGGAGGGAAGCCCACAGAAAAGAAATGGTGTGAACAAGTTTAACACTAGGAGGGCT
TACTTCTATTGGGTGACAAGGGAGCAAGGTTCTTTTGACTGGTTTAAAGGGGTGATGAATGAGGTTGCTGAAGAGGATCACAAGGGTG
TCATTGAACTCCACAACTATTGCACCAGTGTGTATGAAGAGGGTGATGCTCGTTCTGCTCTCATTGCCATGTTGCAATCTCTAAACCATG
CTAAGAATGGCGTCGACATTGTCTCTGGCACGAGTGTCAAGTCACACTTTGCCAAACCCAATTGGCGCAGTGTCTACAAACGCATTGC
GCTCACTCATCCAGGGGCTCGTGTTGGGGTGTTTTACTGTGGGCCACCAGCATTTAGTGAGGGTTAAT

>CDDP6
ATTAACCCTCACTAAATGCTGGTGGCCCACAGTAAAACACCCCAACACGAGCCCCTGGATGAGTGAGCGCAATGCGTTTGTAGACACT
GCGCCAATTGGGTTTGGCAAAGTGTGACTTGACACTCGTGCCAGAGACAATGTCGACGCCATTCTTAGCATGGTTTAGAGATTGCAAC
ATGGCAATGAGAGCAGAACGAGCATCACCCTCTTCATACACACTGGTGCAATAGTTGTGGAGTTCAATGACACCCTTGTGATCCTCTTC
AGCAACCTCATTCATCACCCCTTTAAACCAGTCAAAAGAACCTTGCTCCCTTGTCACCCAATAGAAGTAAGCCCTCCTAGTGTTAAACTT
GTTCACACCATTTCTTTTCTGTGGGCTTCCCTCCTCTATGTTGCTCCCTTCTTCCTCTTCCATCGCCTTCATGTTGTTCACTATGTCCTTC
ACAATACTTATCATTGGGGTGGCTCCTATTCCCAGCCCCACCAGCATTTAGTGAGGGTTAAT

>CDDP7
ATTAACCCTCACTAAATGCTGGTGGAAAAAAAAGATATCACTCAGCATAATG

>CDDP8
ATTAACCCTCACTAAATGCTGGTGGGGCTGGGAATAGGAGCCACCCCAATGATAAGTATTGTGAAGGACATAGTGAACAACATGAAGG
CGATGGAAGAGGAAGAAGGGAGCAACATAGAGGAGGGAAGCCCACAGAAAAGAAATGGTGTGAACAAGTTTAACACTAGGAGGGCTT
ACTTCTATTGGGTGACAAGGGAGCAAGGTTCTTTTGACTGGTTTAAAGGGGTGATGAATGAGGTTGCTGAAGAGGATCACAAGGGTGT
CATTGAACTCCACAACTATTGCACCAGTGTGTATGAAGAGGGTGATGCTCGTTCTGCTCTCATTGCCATGTTGCAATCTCTAAACCATG
CTAAGAATGGCGTCGACATTGTCTCTGGCACGAGTGTCAAGTCACACTTTGCCAAACCCAATTGGCGCAGTGTCTACAAACGCATTGC
GCTCACTCATCCAGGGGCTCGTGTTGGGGTGTTTTACTGTGGGCCACCAGCATTTAGTGAGGGTTAAT

>CDDP9
AATAACCCTCACTAAATCTGGTGGATGGCGGATGCAAGGCGTGGAAGATCGCGGTGTGATGACGGCGTTTATGCAGGCGCTGGGGTT
TTAGGGCACAAACAGGGCGGCTGGGAGAAGGTGTTATCGTCAAATTTGATTTCAAAAGTGATATTGCCTGATGCGCTACGCTTATCAG
GCCTACAATGTGTGTTGCAATTTACTGATTTCTTTGGATCTTGTAGGCCGGATAAGGCGTTTACGCCGCATCCGGCATGAAGCAACGTA
CTCGATATTAGCAATTTGGCGGCAACCCAAAGTTGCCGATTAATGATTAATTCGCATCGGTCGCCGTGCCGTTGGCGTGCCAGTCAAAT
ACGCCGAACTCAAAGCCTTTCAGATCACCTTTCTCATCCCAGGTCAGCGGTCCCATTACGGGATCCACGGAGTTCGCTTTCAGGTATTT
GGCGATTTCAGCCGGATCGTCAGACTGATTCAGGCCCGCCTGCAAAGAATGCAGCGCGGCGTAGGTGGTCCAAACGAATGCGCCCAT
TGGGTCCTGTTTTTTCGCTTTGATCGCGTCAACAAAGGGTTTGTTCGCCGGAACCTGATCGTAGTTCTTCGGCTTGGTCACCAGCAGCC
CTTCCGCTGATTCGCCCGCAATGTAAGACAGCGAAACGTTAGCCACACCTTCCGGCCCCATAAACTGAGTTTTCAGCCCTGCCGCGCG
TGCCTGACGCAGGATTTGCCCCAATTTCCGGGTGATAACCGCCGTAGTAAACGAAGTCGATATTCTCTTTTTTCAGACGCGCCGCCCA
GCCATTTCAGTTGAAAGAGGGGGGGGG

The 9 CDDP sequences were analyzed by being protein translated to hypothetical proteins and alignment of the sequences was performed by using the molecular analysis software ClustalW (http://www.ebi.ac.uk). Sequence

similarity was also analyzed with blastx based on GENBANK (Altschul et al., 1997). Nucleotide blastx search showed that the isolated cDNA fragments (CDDP1, CDDP3, CDDP5, CDDP8, and CDDP9) shared some sequence homology with genes encoding proteins, transcription factors, protein kinases, heat shock proteins, LEAs, etc as shown in Tables 6.2 – 6.6.

Table 6.2: Homology matches of CDDP1 fragment with their respective E-values, and multiple alignments of deduced amino acid sequences of other related putative proteins.

>CDDP1

CATTATGCTGAGTGATATCTTTTTTTTTCAAACTTGGTGAAGAAGGGGAGAGGTTGAATGGG
GGTAGGAAAGTACTTCCAGAAGGAACAGTTCTCAGGGAATATATCATAGGGGACAGAGGTTT
TCCTCTTTTGCCATGGCTTCTTACACCTTACGAAGGTAAAGGACTCTCAAATGTACAAGTTGA
GTTCAATAGAAGGGTTGTTGAAACTCAAATGTTGGCCAAGAAAGCATTGGCTAGGCTGAAGG
AGATGTGGAGGATAATCCAAGGTGTGATGTGGAAACCTGACAAGCACAAGTTACCAAGAATT
ATTCTTGTCTGCTGCATACTGCATAATATAGTTATCGATATGAAGGATGAAGTACTGAATGAT
ATGTCCTCTTGCCACCAGCATTTAGTGAGGGTTAAT

1:	UNIPROT:A5AZB6_VITVI	A5AZB6	Putative uncharacterized protein.	159	4e-38
2:	UNIPROT:Q9M2U3_ARATH	Q9M2U3	Putative uncharacterized protein T22...	144	1e-33
3:	UNIPROT:A4PRM7_MEDTR	A4PRM7	Trp repressor/replication initiator.	138	1e-31
4:	UNIPROT:A4PRM9_MEDTR	A4PRM9	Trp repressor/replication initiator.	137	2e-31
5:	UNIPROT:A2Q269_MEDTR	A2Q269	Trp repressor/replication initiator ...	137	2e-31
6:	UNIPROT:Q943M0_ORYSJ	Q943M0	Putative uncharacterized protein P0O...	130	2e-29
7:	UNIPROT:A2WWT6_ORYSI	A2WWT6	Putative uncharacterized protein.	130	2e-29
8:	UNIPROT:A2ZZE4_ORYSJ	A2ZZE4	Putative uncharacterized protein.	130	2e-29
9:	UNIPROT:Q0D898_ORYSJ	Q0D898	Os07g0175100 protein (Fragment).	127	2e-28
10:	UNIPROT:Q8H572_ORYSJ	Q8H572	Putative uncharacterized protein OJ1...	127	2e-28
11:	UNIPROT:A3BH29_ORYSJ	A3BH29	Putative uncharacterized protein.	127	2e-28
12:	UNIPROT:A2YIN1_ORYSI	A2YIN1	Putative uncharacterized protein.	127	2e-28
13:	UNIPROT:A2ZH02_ORYSI	A2ZH02	Putative uncharacterized protein.	125	6e-28
14:	UNIPROT:Q53NM6_ORYSJ	Q53NM6	Putative uncharacterized protein (Os...	125	1e-27
15:	UNIPROT:A3CDY9_ORYSJ	A3CDY9	Putative uncharacterized protein.	125	1e-27
16:	UNIPROT:Q2QZ53_ORYSJ	Q2QZ53	Expressed protein.	125	1e-27
17:	UNIPROT:Q94K49_ARATH	Q94K49	Putative uncharacterized protein At3...	113	3e-24
18:	UNIPROT:Q9M1W3_ARATH	Q9M1W3	Putative uncharacterized protein F16...	113	3e-24
19:	UNIPROT:A4PTU1_MEDTR	A4PTU1	Putative uncharacterized protein.	72	1e-11
20:	UNIPROT:Q9SZQ1_ARATH	Q9SZQ1	Putative uncharacterized protein F27...	64	2e-09

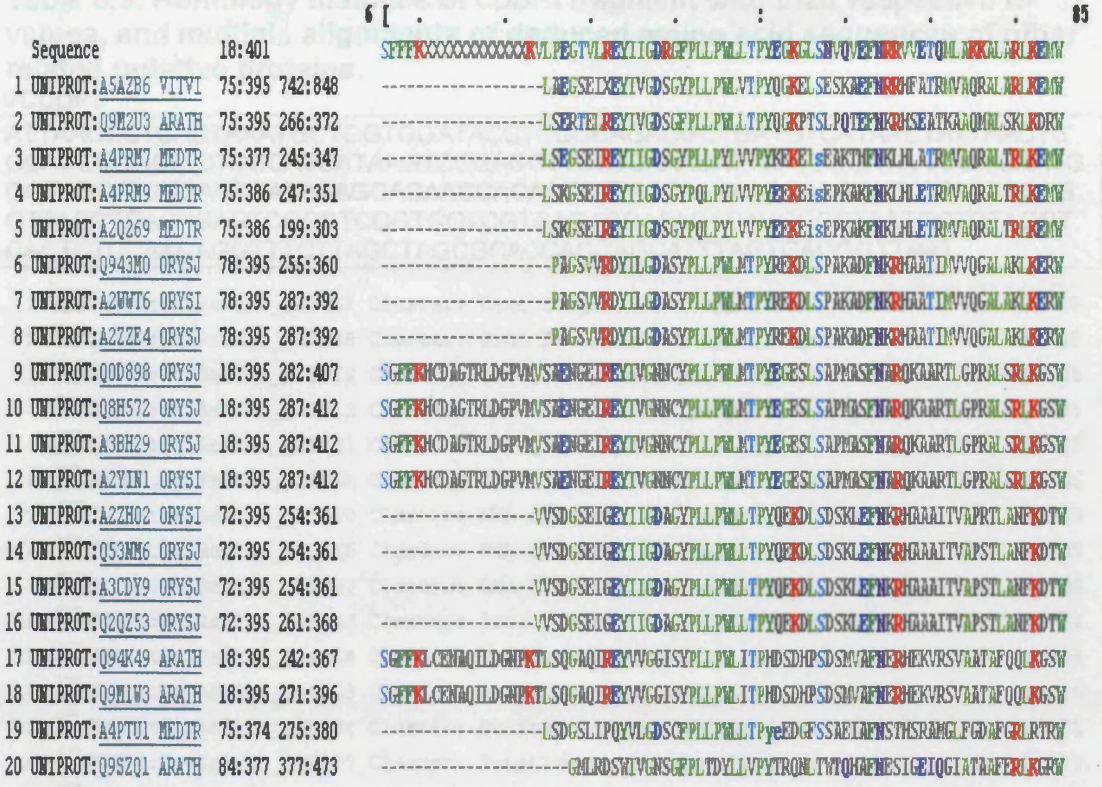


Table 6.3: Homology matches of CDDP3 fragment with their respective E-values, and multiple alignments of deduced amino acid sequences of other related putative proteins.

>CDDP3

ATTAACCCTCACTAAATGCTGGTGGATACCTCGCCAGACGCTGATGTCATAACTATTAACTC
GCACCCCAATTCTCGCATCATAAGTCCGAGCTTCCTCAAACATTCATTGGATAAAAATGAATG
GGTAGCTTCTGAGTCATACAGCACAACCACACTGTTACCAAAAAGCAAGTATTCTACACCAG
GTTACCTGACTGAGTCGCCTCGGTGGTCGTCAGTGCAAACACACGCCCTAATGCTCTAGGT
CACTCTCCAACAGGTTTCTTAGCTAGCGCACCACCAGCATTTAGTGAGGGTTAAT

1:	UR100:UniRef100_Q1SUX9 Cluster: Zinc finger, CCHC-type; Peptidas...	42	1e-05
2:	UR100:UniRef100_Q1S9A0 Cluster: Zinc finger, CCHC-type; Peptidas...	43	1e-05
3:	UR100:UniRef100_Q1SIT2 Cluster: Integrase, catalytic region; Zin...	44	1e-05
4:	UR100:UniRef100_Q1SV13 Cluster: RNA-directed DNA polymerase (Rev...	44	2e-05
5:	UR100:UniRef100_Q1T121 Cluster: Integrase, catalytic region; Zin...	41	3e-05
6:	UR100:UniRef100_Q1S9A1 Cluster: Zinc finger, CCHC-type; Peptidas...	44	3e-05
7:	UR100:UniRef100_Q1S988 Cluster: RNA-directed DNA polymerase (Rev...	42	4e-05
8:	UR100:UniRef100_A4Q4X5 Cluster: RNA-directed DNA polymerase (Rev...	42	4e-05
9:	UR100:UniRef100_Q2HW87 Cluster: RNA-directed DNA polymerase (Rev...	42	4e-05
10:	UR100:UniRef100_Q1T546 Cluster: Zinc finger, CCHC-type; Peptidas...	42	2e-04
11:	UR100:UniRef100_Q949L4 Cluster: Putative polyprotein; n=1; Cicer...	40	3e-04
12:	UR100:UniRef100_Q949L3 Cluster: Putative polyprotein; n=1; Cicer...	40	6e-04
13:	UR100:UniRef100_A5BRB4 Cluster: Putative uncharacterized protein...	33	0.011
14:	UR100:UniRef100_A5BBP7 Cluster: Putative uncharacterized protein...	33	0.025
15:	UR100:UniRef100_A5BZM7 Cluster: Putative uncharacterized protein...	33	0.032
16:	UR100:UniRef100_A5AXA5 Cluster: Putative uncharacterized protein...	36	0.15
17:	UR100:UniRef100_Q1SUX8 Cluster: Zinc finger, CCHC-type; Peptidas...	33	0.16
18:	UR100:UniRef100_A5C3Z5 Cluster: Putative uncharacterized protein...	38	0.17
19:	UR100:UniRef100_A5BMS5 Cluster: Putative uncharacterized protein...	30	0.20
20:	UR100:UniRef100_UPI00006A13BC Cluster: UPI00006A13BC related clu...	38	0.22

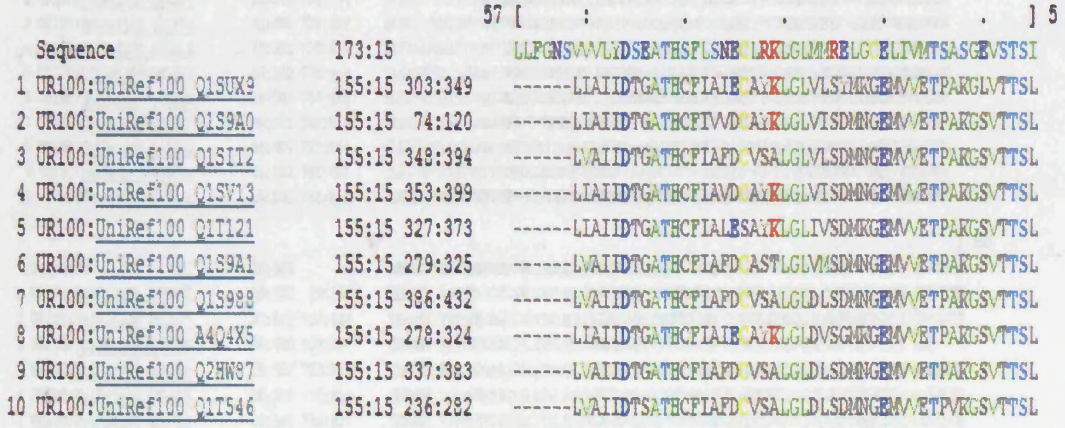


Table 6.4: Homology matches of CDDP5 fragment with their respective E-values, and multiple alignments of deduced amino acid sequences of other related putative proteins.

>CDDP5

ATTAACCCTCACTAAATGCTGGTGGGGCTGGGAAATAGGAGCCACCCCAATGATAAGTATTG
TGAAGGACATAGTGAACAACATGAAGGCGATGGAAGAGGAAGAAGGGAGCAACATAGAGGA
GGGAAGCCCACAGAAAAGAAATGGTGTGAACAAGTTTAACACTAGGAGGGCTTACTTCTATT
GGGTGACAAGGGAGCAAGGTTCTTTTGACTGGTTTAAAGGGGTGATGAATGAGGTTGCTGA
AGAGGATCACAAGGGTGTCATTGAACTCCACAACTATTGCACCAGTGTGTATGAAGAGGGTG
ATGCTCGTTCTGCTCTCATTGCCATGTTGCAATCTCTAAACCATGCTAAGAATGGCGTCGAC
ATTGTCTCTGGCACGAGTGTCAAGTCACACTTTGCCAAACCCAATTGGCGCAGTGTCTACAA
ACGCATTGCGCTCACTCATCCAGGGGCTCGTGTTGGGGGTGTTTTACTGTGGGCCACCAGCA
TTTAGTGAGGGTTAAT

Sequences producing significant alignments:	Score (bits)	E Value
EM_EST:CX710384; CX710384 gmrtDrNS01_18-D_M13R_H08_050.s2 Water ...	461	e-126
EM_EST:CX710088; CX710088 gmrtDrNS01_15-D_M13R_F01_005.s2 Water ...	461	e-126
EM_EST:EV266544; EV266544 GLLB833TF JCVI-SOY1 Glycine max cDNA 5...	419	e-113
EM_EST:AW703751; AW703751 sk23h08.y1 Gm-c1028 Glycine max cDNA c...	417	e-113
EM_EST:AW703752; AW703752 sk23h09.y1 Gm-c1028 Glycine max cDNA c...	414	e-112
EM_EST:DY617867; DY617867 AC4064 NOLLY Medicago truncatula cDNA ...	407	e-109
EM_EST:BE475645; BE475645 sp79h05.y1 Gm-c1044 Glycine max cDNA c...	401	e-108
EM_HTG:AP007863; AP007863 Lotus japonicus genomic DNA, chromosom...	398	e-107
EM_PL:AP004933; AP004933 Lotus japonicus genomic DNA, chromosome...	390	e-105
EM_EST:BU080562; BU080562 saq26b11.y1 Gm-c1045 Glycine max cDNA ...	379	e-101

Table 6.5: Homology matches of CDDP8 fragment with their respective E-values, and multiple alignments of deduced amino acid sequences of other related putative proteins.

>CDDP8

ATTAACCCTCACTAAATGCTGGTGGGGCTGGGAATAGGAGCCACCCCAATGATAAGTATTGT
GAAGGACATAGTGAACAACATGAAGGCGATGGAAGAGGAAGAAGGGAGCAACATAGAGGA
GGGAAGCCCACAGAAAAGAAATGGTGTGAACAAGTTTAACACTAGGAGGGCTTACTTCTATT
GGGTGACAAGGGAGCAAGGTTCTTTTGACTGGTTTAAAGGGGTGATGAATGAGGTTGCTGA
AGAGGATCACAAGGGTGTCATTGAACTCCACAACTATTGCACCAGTGTGTATGAAGAGGGTG
ATGCTCGTTCTGCTCTCATTGCCATGTTGCAATCTCTAAACCATGCTAAGAATGGCGTCGAC
ATTGTCTCTGGCACGAGTGTCAAGTCACACTTTGCCAAACCCAATTGGCGCAGTGTCTACAA
ACGCATTGCGCTCACTCATCCAGGGGCTCGTGTTGGGGTGTTTTACTGTGGGCCACCAGCA
TTTAGTGAGGGTTAAT

1:	UR100:UniRef100_A4PRP2 Cluster: Cytochrome b-245, heavy chain; n...	266	5e-70
2:	UR100:UniRef100_Q9LDD7 Cluster: Whitefly-induced gp91-phox; n=1;...	254	1e-66
3:	UR100:UniRef100_Q15FD4 Cluster: NOX3; n=1; Striga asiatica|Rep: ...	253	3e-66
4:	UR100:UniRef100_Q2HXL0 Cluster: NADPH oxidase; n=1; Solanum tube...	253	4e-66
5:	UR100:UniRef100_Q8LRN5 Cluster: NADPH oxidase; n=1; Nicotiana ta...	252	5e-66
6:	UR100:UniRef100_Q8RVJ9 Cluster: NADPH oxidase; n=1; Nicotiana ta...	252	5e-66
7:	UR100:UniRef100_Q84KK7 Cluster: Respiratory burst oxidase homolo...	252	5e-66
8:	UR100:UniRef100_Q1SIX3 Cluster: Calcium-binding EF-hand; Ferric ...	242	7e-63
9:	UR100:UniRef100_Q8GZ82 Cluster: Putative respiratory burst oxida...	239	4e-62
10:	UR100:UniRef100_Q9FI42 Cluster: Respiratory burst oxidase protei...	239	4e-62
11:	UR100:UniRef100_O81210 Cluster: Respiratory burst oxidase protei...	239	4e-62
12:	UR100:UniRef100_Q5ENY4 Cluster: Respiratory burst oxidase 1; n=1...	238	1e-61
13:	UR100:UniRef100_Q5VI40 Cluster: Respiratory burst oxidase protei...	236	3e-61
14:	UR100:UniRef100_Q2R351 Cluster: Respiratory burst oxidase protei...	236	5e-61
15:	UR100:UniRef100_Q9FIJ0 Cluster: Respiratory burst oxidase protei...	234	1e-60
16:	UR100:UniRef100_O81212 Cluster: Respiratory burst oxidase protei...	234	1e-60
17:	UR100:UniRef100_Q2HXK9 Cluster: NADPH oxidase; n=1; Solanum tube...	234	2e-60
18:	UR100:UniRef100_Q1RSM3 Cluster: Calcium-binding EF-hand; Ferric ...	230	3e-59
19:	UR100:UniRef100_A5CBK9 Cluster: Putative uncharacterized protein...	228	8e-59
20:	UR100:UniRef100_UPI0000196DB3 Cluster: respiratory burst oxidase...	224	2e-57

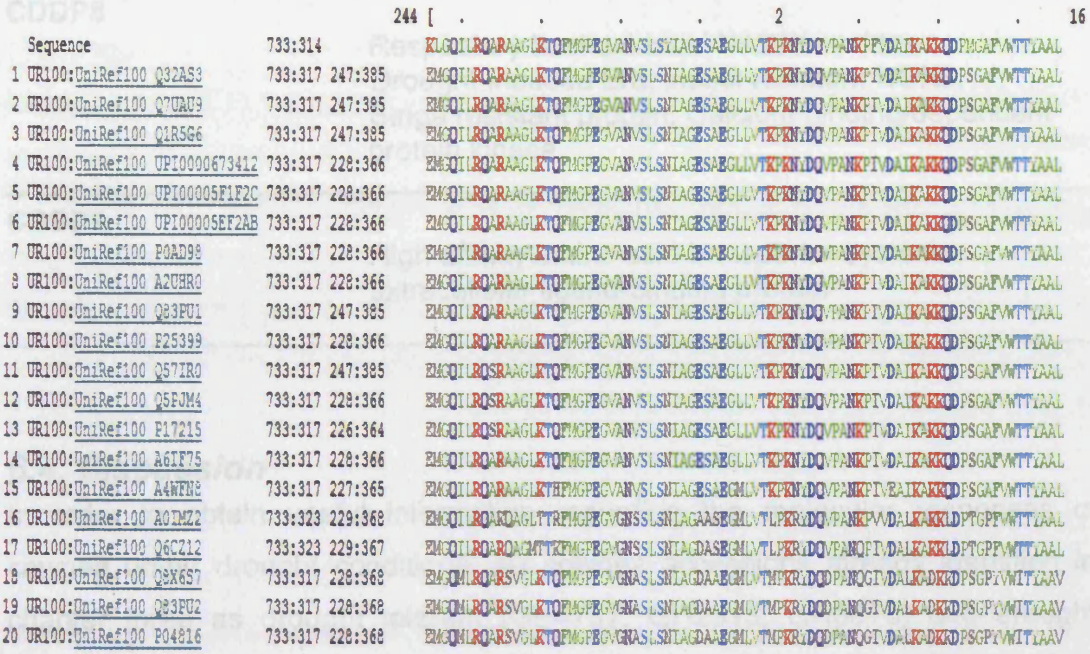

Sequence 16:501
UR100:UniRef100_A4FRF2 16:501 25:194
UR100:UniRef100_Q9LDD7 16:501 735:914
UR100:UniRef100_Q15FD4 16:489 330:497
UR100:UniRef100_Q2HXL0 16:501 735:914
UR100:UniRef100_Q8LRN5 16:501 735:914
UR100:UniRef100_Q8RVJ9 16:501 736:915
UR100:UniRef100_Q84KK7 16:501 736:915
UR100:UniRef100_Q1SIX3 16:486 709:869
UR100:UniRef100_Q8GZ82 16:501 719:881
UR100:UniRef100_Q9FI42 16:501 719:881
UR100:UniRef100_O81210 16:501 681:843
UR100:UniRef100_Q5ENY4 16:486 743:903
UR100:UniRef100_Q5VI40 16:501 719:881
UR100:UniRef100_Q2R351 16:501 752:912
UR100:UniRef100_Q9FIJ0 16:489 740:893
UR100:UniRef100_O81212 16:489 740:893
UR100:UniRef100_Q2HXK9 16:501 678:834
UR100:UniRef100_Q1RSM3 16:483 484:639
UR100:UniRef100_A5CBK9 16:498 710:869
UR100:UniRef100_UPI0000196DB3 16:501 665:825
86 . 1 . . . . : . 165

Table 6.6: Homology matches of CDDP9 fragment with their respective E-values, and multiple alignments of deduced amino acid sequences of other related putative proteins.

>CDDP9

AATAACCCTCACTAAATCTGGTGGATGGCGGATGCAAGGCGTGGAAGATCGCGGTGTGATG
ACGGCGTTTATGCAGGCGCTGGGGTTTTAGGGCACAAACAGGGCGGCTGGGAGAAGGTGT
TATCGTCAAATTTGATTTCAAAAGTGATATTGCCTGATGCGCTACGCTTATCAGGCCTACAAT
GTGTGTTGCAATTTACTGATTTCTTTGGATCTTGTAGGCCGGATAAGGCGTTTACGCCGCAT
CCGGCATGAAGCAACGTACTCGATATTAGCAATTTGGCGGCAACCCAAAGTTGCCGATTAAT
GATTAATTCGCATCGGTCGCCGTGCCGTTGGCGTGCCAGTCAAATACGCCGAACTCAAAGC
CTTTCAGATCACCTTTCTCATCCCAGGTCAGCGGTCCCATTACGGGATCCACGGAGTTCGCT
TTCAGGTATTTGGCGATTTCAGCCGGATCGTCAGACTGATTCAGGCCCGCCTGCAAAGAATG
CAGCGCGGCGTAGGTGGTCCAAACGAATGCGCCCATTGGGTCCTGTTTTTTCGCTTTGATC
GCGTCAACAAAGGGTTTGTTCGCCGGAACCTGATCGTAGTTCTTCGGCTTGGTCACCAGCA
GCCCTTCCGCTGATTCGCCCGCAATGTAAGACAGCGAAACGTTAGCCACACCTTCCGGCCC
CATAAACTGAGTTTTCAGCCCTGCCGCGCGTGCCTGACGCAGGATTTGCCCCAATTTCCGG
GTGATAACCGCCGTAGTAAACGAAGTCGATATTCTCTTTTTTCAGACGCGCCGCCCAGCCAT
TTCAGTTGAAAGAGGGGGGGGG

1:	UR100:UniRef100_Q32AS3 Cluster: High-affinity amino acid transpo...	268	3e-78
2:	UR100:UniRef100_Q7UAU9 Cluster: High-affinity amino acid transpo...	268	3e-78
3:	UR100:UniRef100_Q1R5G6 Cluster: Leu/Ile/Val-binding protein; n=2...	268	3e-78
4:	UR100:UniRef100_UPI0000673412 Cluster: COG0683: ABC-type branche...	268	3e-78
5:	UR100:UniRef100_UPI00005F1F2C Cluster: COG0683: ABC-type branche...	268	3e-78
6:	UR100:UniRef100_UPI00005EF2AB Cluster: COG0683: ABC-type branche...	268	3e-78
7:	UR100:UniRef100_P0AD98 Cluster: Leu/Ile/Val-binding protein prec...	268	3e-78
8:	UR100:UniRef100_A2UHR0 Cluster: Extracellular ligand-binding rec...	268	3e-78
9:	UR100:UniRef100_Q83PU1 Cluster: High-affinity amino acid transpo...	266	1e-77
10:	UR100:UniRef100_P25399 Cluster: Leu/Ile/Val-binding protein prec...	263	1e-76
11:	UR100:UniRef100_Q57IR0 Cluster: Leu/Ile/Val/Thr-binding protein;...	259	1e-75
12:	UR100:UniRef100_Q5PJM4 Cluster: ABC superfamily (Bind_prot), bra...	258	5e-75
13:	UR100:UniRef100_P17215 Cluster: Leu/Ile/Val/Thr-binding protein ...	258	5e-75
14:	UR100:UniRef100_A6TF75 Cluster: High-affinity branched-chain ami...	257	8e-75
15:	UR100:UniRef100_A4WFN8 Cluster: Extracellular ligand-binding rec...	250	7e-73
16:	UR100:UniRef100_A0IMZ2 Cluster: Extracellular ligand-binding rec...	196	2e-55
17:	UR100:UniRef100_Q6CZ12 Cluster: Leucine-specific binding protein...	194	9e-55
18:	UR100:UniRef100_Q8X6S7 Cluster: High-affinity leucine-specific t...	188	2e-53
19:	UR100:UniRef100_Q83PU2 Cluster: High-affinity leucine-specific t...	188	2e-53
20:	UR100:UniRef100_P04816 Cluster: Leucine-specific-binding protein...	188	2e-53



The probable gene fragments identified by the BLAST analysis and their sequence similarity to putative genes in other plant species have been summarized based on their putative function in Table 6.7.

Table6.7: Summary of gene fragments and their putative functions with their related accession number for reference.

Candidate gene	Putative functions
CDDP1	Protein of unknown function found in many plant species, Tryptophan repressor/ replication initiator
CDDP3	Aspartic protease from retrotransposons Zinc finger (CCHC-type) evident, RNA-directed DNA polymerase
CDDP5	Water stressed protein widespread in plants; Osmotic stress-activated protein kinase

CDDP8	Respiratory bust oxidase/ NADPH oxidase Drought-induced Erd, insect resistant NOX3, Striga resistant protein, Calcium binding/dependant protein kinase
CDDP9	High affinity amino acid transporter systems, extracellular ligand-binding protein

6.4: Discussion

In order to obtain useful information regarding the molecular responses of cowpea under drought conditions, six cowpea accessions already identified in chapter three as drought tolerant (GH4767, GH2313, GH3679) and drought susceptible (GH3710, GH4778, GH3687), based on physiological and agronomical studies were used in this study. Under the experimental conditions, plant leaves showed no drought induced senescence symptoms during the first 15 days without irrigation. However during the 15-30 days of the drought treatment, there were signs of wilting and when they were rewatered, the drought tolerant accessions were able to recover fully, suggesting the existence of more efficient protection and repair mechanisms in the drought tolerant accessions. Following the PCR differential display method, nine candidate gene fragments were isolated and sequenced. The nucleotide sequences obtained for all the nine CDDP clone fragments were unique and evidently originating from the cowpeas rather than infections or other sources, a common source of variation in differential display experiments. CDDP2 as shown in table 6.1 had six microsatellite repeat of **CATCCT** motif which probably could be exploited for SSR marker design for future drought analysis. When the sequences of the nine fragments were subjected to blastx analysis, CDDP1, CDDP3, CDDP5, CDDP8 and CDDP9 gave high and significant hits (Tables 6.2 to 6.6). The other fragments, CDDP2, CDDP4, CDDP6, and CDDP7 returned no significant hits.

Many changes in gene expression occur in response to water deficit stress. The challenge of the study in this chapter has generally focused on the determination of the changes that support plant adaptation to drought. The five CDDP gene fragments that had significant hits gave very interesting homology matches with sequence similarity to putative genes of other plant species (Table6.7). A system to categorize gene function has been applied to known plant and animal genome. Genes known to be expressed under drought stress has been classified into 13 general categories (http://mips.gsf.de/proj/thal/db/tables/table_func_frame.html. The main concern in this chapter was to determine the mechanisms under which cowpea plant cells respond to drought stress in molecular terms.

The first step in switching on a molecular response in response to environmental signal (drought) is its perception by specific receptors. Upon activation, they initiate a cascade response to transmit the information through a signal-transduction pathway. Urao et al. (1999) were able to show that water deficit, followed by the resulting osmotic stress, triggered the expression of putative osmosensor AtHTK1 in *Arabidopsis*. AtHTK1 was subsequently identified to be the first component 'sensing' changes in osmotic potential inside the cell and triggering the downstream signalling cascade that results in dehydration induced gene expression (Urao et al. 1999). In this study, an osmoregulated ABC super family transporter system was identified in CDDP9 fragment (Table6.7). It is believed that during the development of water stress, the interaction of cationic and anionic amphiphilic substances with plasma-membranes results in changes in the physical state or protein-lipid interactions of membranes that relay osmosensing to cells. Similar occurrence has been reported by Heide and Poolman (2000). Changes in the physical state of membranes may also regulate the activity of major integral membrane proteins, such as aquaporins, which are involved in the control of cell volume or turgor homeostasis (Tyerman et al. 2002).

After the first perception of osmotic changes during water stress, the signal transduction cascade is known to involve protein phosphorylation and dephosphorylation mediated by several protein kinases and phosphatases whose genes have been shown to be up-regulated by dehydration stress (Bray, 2002a). Changes in cytoplasmic Ca^{2+} concentration are likely to mediate the integration of different signalling pathway. In the study certain protein kinases were observed to have been up-regulated as CDPK was recorded in gene fragment CDDP1. Osmotic stress activated-protein kinase was also identified in CDDP5. It has been observed that most of the abundant regulatory protein kinases involved in abiotic stress signalling are Ca^{2+} dependant (CDPK) (Knight and Knight, 2000) and mitogen activated (MAPK) (Kizis et al., 2001). Several MAPKs and CDPKs have been identified in plants subjected to water stress and shown to be involved in transducing the dehydration signals sensed at the plasma membrane to the nucleus (Ramanjulu and Bartels, 2002).

After the first stress-recognition events, cell-to-organ drought mediated responses are believed to diverge in different pathways according to the involvement or not of ABA. In the ABA-dependent pathway, the accumulation of ABA activates various stress-associated genes, some of then being recognized as stress adaptive. These gene products are either functional (such as aquaporins or the enzymes of osmoprotectants biosynthesis) or regulatory (such as protein kinases). The ABA-independent pathway is still poorly understood, but is known to be rapidly induced by water stress (Kizis et al., 2001; Shinozaki et al, 2003). In the study, the identification of components like Cytochrome b-245, NOX3, NADPH oxidase, respiratory burst oxidase, putative uncharacterized proteins and many others in all the gene fragments seem to suggest that they were involved in all these mechanisms.

There are other genes that are completely unknown in that an mRNA (or EST) has not been previously identified and there is no amino acid sequence homology with other proteins of known function. Bray, (2002b) had considered

these categories to include the one called 'cell rescue, defence, and cell death and ageing'. Many of the genes known to be induced by water-deficit are placed in this category even though an exact function is not understood. In table 6.7 genes induced by water-deficit stress have been summarized and from the discussion thus far it has been recognized that to follow the mechanisms of how plant cells respond to water stress deficit involves the initial perception of the stress, signalling events, ABA-mediated responses, non-ABA-mediated responses, various biochemical mechanisms which include osmotic adjustments and activities of LEAs as well as cell protection mechanisms. These have been categorized according to the gene fragments identified in this study as follows;.

Metabolism

It has been known for some time that cellular metabolism is altered by water-deficit stress (Blum et al., 2001), however, detailed analyses on the effect of stress on the majority of the enzymes in individual metabolic pathways is lacking. Of changes that are known, it remains unclear which are brought about as an adaptive response and which and which represent lesions in metabolic pathways. Most work has concentrated in the areas of photosynthesis (carbon and energy metabolism; Chaves, 2002), carbon and nitrogen utilization (Foyer et al., 1998), and the synthesis of small molecules that potentially play a role in osmotic adjustment (Shoemaker et al., 2002). The gene fragments identified in this study are largely involved in amino acid, phenylpropanoid and fatty acid metabolism. Nitrate reductase activity has been shown to be decreased by water-deficit stress in a number of species including cowpea and Arabidopsis (Foyer et al., 1998).

Transcription

Transcription factors are required to regulate changes in gene expression in response to water-deficit stress. The *Arabidopsis thaliana* genome contains numerous transcription factors, many of which are specific to plants (Riechmann et al., 2000).Several different classes of transcription factors were induced by

water stress in this study (Table6.7), including ethylene response element binding protein 4, zinc finger, Peptidase aspartic and DREB2A. The figure 6.2 shows a putative zinc finger fragment identified in the study aligned to a component from *Medicago trancatula;*

```
Query: 155 VVVLYDSEATHSFLSNECLRKLGLMMRELGCELIVMTSASGEVSTSI 15
          + V + D+ ATH F++ +C+  LGL + ++  E++V T   G V+TS+
Sbjct: 236 LVAIIDTSATHCFIAFDCVSALGLDLSDMNGEMVVETPVKGSVTTSL 282
```

Figure 6.2: Zinc finger, CCHC-type; query is a portion of the CDDP3 fragment; sbjct is *Medicago trancatula*; zinc finger tips are shown in red and underlined.

These are all likely to be involved in the up-regulation of genes, many of which are signalled through ABA. A stress induced histone was also identified which are similar to a stress-induced histone H1, H1-S, identified in tomato and its relatives (Scippa et al., 2000). This histone may play a role in maintaining chromatin structure during water deficit.

Cellular communication/ Signal transduction

In this study few potential signalling molecules were identified. However, many genes have been identified that play a potential role in the regulation of cellular processes in response to water-deficit (Table 6.7). The Shinozaki/Yamaguchi-Shinozaki group (2003) in Japan has identified many of these genes in stress libraries. These implicate CDPK and MAPK cascades as well as phosphatidylinositide signalling pathways.

Cell rescue, defence, cell death and ageing

A number of genes that may be classified in the cell rescue or defence category are known to be up-regulated in response to water-deficit stress. The two largest groups identified so far are in the amelioration of oxidative stress and/or in defence against pathogens. In this study, glutathione-S-transferase, L-ascorbate peroxidase, and putative lectin, were observed to be involved in cell defence, which seems to be correlated by work done by Borsani et al. (2001).

Uncharacterized/ unclassified/ Novel genes

Notably, the hydrophilic proteins that that have been called LEA proteins remain a very interesting group. These proteins have been classified into several groups based on amino acid sequence (Bray et al., 2002b). The different characteristics predicted from protein structure indicate that the proteins may play distinct roles in cells subjected to water-deficit stress, although the molecular function remains unclear. The results of this study also indicated that a substantial amount of the genes identified had been placed under the unclassified category which included most of the LEA proteins like group II LEA, and uncharacterized putative proteins.

Abiotic stress related genes

It is interesting to note that a number of the gene fragments identified in the study were placed in this category. Studies carried out on effect of abiotic stress on plants have demonstrated that a high complexity of interactions occur between different abiotic stress factors. It is therefore likely that one single stress factor triggers the activation of several signalling pathways (divergence). In contrast, different stimuli might provoke a similar cellular effect, thus certain shared signalling mechanisms, and the subsequent cell responses can be active under different stress conditions (convergence). Within the general frame work of abiotic stress one can encounter conditions like low temperature or cold stress, dehydration or drought stress, salinity or salt stress and many others. It is therefore not surprising that the abiotic stress related genes observed in this study seems to confirm this general principle. It appears that drought stress triggers the production of ABA, which in turn induces various genes, since most genes that respond to abiotic stress (drought, cold, salt stress) are induced by ABA as indicated by Shinozaki et al (2003). It was observed in this study that the genes found in this category had functions that were related to all the categories proposed. For example, Chlorophyll a/b binding protein and water stressed proteins identified in gene fragment CDDP5, could be placed under metabolism

and energy category, Replication protein A1 could be considered under protein synthesis and cell division, whilst, Osmotic stress-activated protein kinase could be under metabolism and cell defence.

Protein destination

Many genes that are induced are involved in the fate of proteins that have been synthesized. Water deficit may cause proteins to become aggregated or malformed which may either require protein degradation or chaperone activity. Accordingly, some mRNAs of genes that encode proteases and chaperones are induced. In this study the induced genes were mostly ubiquitin, cysteine protease, metallopeptidase, and Cysteine protease inhibitor.

The multi-sequence alignments of the CDDP gene fragments with other homologous stress responsive proteins (Table6.2 to Table6.6) showed that some of the similarities amongst the sequences were high. Due to the high homology observed, certain primers could be designed from the region for conserved orthologue synteny (COS) marker analysis in future.

6.5. Conclusions

- Plants exposed to different water regimes induced different responses to gene expression. The study also indicated that differential display PCR technique may be an effective method for drought gene expression analysis of stressed and unstressed plants for the identification of drought genes of interest.
- The study was able to identify nine candidate gene fragments induced by drought stress. The identified potential candidate gene fragments were successfully cloned, sequenced and characterized.
- The identified differentially expressed genes from the cowpea accessions considered to be drought tolerant could generally be linked to the

physiological and morphological studies in chapter three and the other chapters

- It is now important to carry out the experiment on a much larger scale, perhaps with a target of 100 differentially displayed genes from each category of tolerant and of susceptible accessions. The comparison of this number of genes should then be analysed for 1) different responses in the genes that are turned on; and 2) different alleles where similar genes are found in both plant groups.
- It is important that the identified potential gene fragments are further tested and then verified whether they would show any inducible differences in expression, using Northern blotting or real-time PCR.

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CHAPTER SEVEN

7.0. Drought genes and tolerance in Ghanaian cowpea accessions- General implications of experiments.

7.1. Introduction

During a typical life cycle, plants are exposed to a wide range of environmental changes that may disturb the normal growth and development they accomplish in optimal growth conditions. Evolution has led to the development of mechanisms aimed to increase their tolerance to these negative factors, including both physical adaptations and several complex mechanisms of interactive cellular and molecular changes triggered after the onset of various stresses. In general terms, the first step for this process consists of the perception of the adverse situation. Then, through a release or activation of second messengers, different signalling cascades are set in motion in order to relay the information. Transcription effectors can be induced and activated in that way, bringing about the expression of specific stress-responsive genes, which encode proteins involved in the protection of the plant cell against the effects of the damaging situation and/or the repair of the injuries already caused. The current approaches to elucidate the molecular mechanisms modulating the stress signalling networks are based on the control of the expression of specific stress-related genes. In order to easily interpret the analysis of stress signalling pathways in the laboratory, plants need to be isolated from other stresses. However, the studies carried out to date have demonstrated a high complexity of interactions occurring between different abiotic, and even biotic, stress factors. It is therefore likely that one single stress factor triggers the activation of several signalling pathways (divergence). In contrast, different stimuli might provoke a similar cellular effect, thus certain shared signalling mechanisms, and the subsequent cell responses can be active under different stress conditions (convergence). The completion of the *Arabidopsis thaliana* genome sequence in 2000 (Arabidopsis Genome Initiative,

2000), followed by the nearly completed genome sequence for rice in 2002 (Goff et al., 2002; Yu et al., 2002), has caused much excitement in the field of plant research. Rapidly following these landmark efforts are advances in characterization of genomes of other crops including maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and legumes such as soybean [Glycine max (L.) Merr.] and barril medic (*Medicago truncatula* Gaertner; Ware et al., 2002; Lunde et al., 2003; Shoemaker et al., 2002; Young et al., 2003). The accumulating information allows plant researchers to explore new paradigms to address fundamental and practical questions in a multidisciplinary manner.

Although new research fields such as metabolomics have emerged as post-genomic era technologies (Phelps et al., 2002), challenges still lie ahead in answering how genomics will aid in crop improvement from a practical standpoint (Osterlund and Paterson, 2002). Under field conditions, crop plants often experience abiotic stresses including water deficits, high temperatures, salinity, and aluminum (Al), low soil fertility, and mechanical wounding. Such stresses can reduce yields to 20% or less of potential yields (Wang et al., 2003). Reviews in plant water deficit stress responses have been discussed in chapter one (Chaves et al., 2002; Griffiths and Parry, 2002; Munns, 2002; Zhu and Xiong, 2002; Rao and Cramer, 2003). Drought provides one of the major limitations to food production worldwide. In some parts of the world, particularly in the tropics and other locations where most of the world's poor people reside, drought is endemic. Moreover, many parts of the Earth's surface are not arable primarily because of severe water limitations, and the amount of land with these problems grows every year. Hence, improved tolerance to drought has been a goal of crop improvement programmes since the dawn of agriculture. As already stated in previous chapters, drought tolerance appears to be a complex problem, with many contributing loci that show efficacy only in a subset of circumstances (Lebreton et al. 1995; Ribaut et al., 1997; Tuinstra et al. 1996; 1997; Nguyen and Blum, 2004). Thus, progress in understanding the basic physiological and

molecular nature of drought tolerance has been slow (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 1997).

In trying to achieve my general objective of the study of Ghanaian cowpeas for drought tolerance, I adopted a top-down approach (i.e. phenotype to gene) by beginning with physiological analyses of cowpea plants for drought tolerance. This involved analyzing cowpea for stress physiology and agronomic traits (e.g., survivability, grain quality), in chapter three. Crop phenotyping is a key preparative step proceeding trait gene discovery by functional genomics approaches (Nguyen and Blum, 2004). Phenotyping allows establishing screening environments and procedures, including identifying appropriate stress levels for differentiating genotypes and developmental stages most responsive to a given stress. Genetic resources in the crop are selected based on phenotyping, which reveals phenotypic and physiological traits required for a particular stress environment (e.g., stress-tolerant versus stress sensitive germplasm). Although only one measure of drought tolerance was used here, it is often important to try to partition the components of complex and continuously variable (quantitative) traits to assist understanding their genetic control.

Diversity can provide one means of sustaining and improving the livelihoods of farmers where or when environmental conditions are unfavourable in production systems in terms of reliability and sustainability of yield. Much of the world's agricultural biodiversity is found in environments marginal for agricultural production. Loss of such diversity or genetic choices diminishes farmers' capacities to cope with extreme temperatures, drought and salinity. Farmers in stress-prone areas have to take particular care not to introduce non-adapted material which often fails and can lead to erosion of the desired characteristics in local varieties through unwanted crossing. Thus, in the face of constant vulnerability to loss of key stress-resistant types, the management of appropriate diversity of a specific crop constitutes a significant component of livelihood strategies of farmers in highly stressed production environments. Estimation of

genetic diversity in cultivated crops is essential for breeding programmes and for the conservation of genetic resources (Soleimani et al., 2002). All genetic resource conservation activities require characterization of the diversity present in both the gene pools and the gene banks (Karp et al., 1997). Assessment of the genetic diversity in *V. unguiculata* is, therefore, of crucial importance for developing conservation strategies for this economically important crop species. In the first instance, this usually involves description of variation for morphological and physiological traits. However, morphological variation is often found to be restricted and genotype expression is generally influenced by environmental conditions, thereby, constraining the analysis of variation. These limitations have resulted in the deployment of molecular techniques that analyze polymorphism at the DNA level directly.

Genomics-based technologies could then be applied to dissect the genetic variability of the selected genetic resources. In order to study genetic diversity in the cowpea germplasm collected from Ghana and elsewhere, three multi-locus PCR techniques were used in chapter four for the molecular analysis. Technologies from functional genomics (e.g., differential display PCR) are being used extensively for novel genes identification and characterization because they can analyze large numbers of genes in parallel, thus providing an overall picture of how genes are regulated under stress conditions. This differs from a structural genomics approach (i.e., mapping) in which one or a few genes are studied to determine their location on individual chromosomes. With a functional genomics approach, thousands of genes are screened using mRNA expression profiling and sequence information coupled with bioinformatics. This allows for the selection of a group of candidate genes that are putatively involved in expression of phenotypic and physiological traits. Chapters five and six were based on these techniques for candidate gene analysis and gene expression profiles and analysis from drought stressed and unstressed cowpea plants. In the future these genes identified could be examined for "proof of concept" by genetic transformation (over-expression and/or knock-out of the genes) followed by trait

analysis (Meagher, 2002; Wang et al., 2003) and for creating molecular markers to be tested in further physiological studies and through field breeding programs.

For genomic strategies to successfully support crop improvement, key components are required. Availability of diverse genetic resources allows for the identification of superior alleles for genes controlling traits of interest, and an active breeding program is essential to ensure that these genes can be recombined and selected in combinations that result in agronomically useful populations or lines (Hoisington et al., 1999). Access to physiological expertise and an understanding of phenotypic and physiological aspects of traits of interest are also essential (Miflin, 2000; Bruce et al., 2002). Water-deficit stress-responsive genes and non-stress responsive genes are applicable in breeding for desirable traits. Sources of these genes will come from both in-house research and publicly available information from model plants such as Arabidopsis (Bressan et al., 2001; Hall et al., 2003).

The objective of the study in this chapter is to identify if the drought tolerance phenotype identified in Chapter three within the germplasm under analysis can be linked with any of the markers found in Chapters four and five, or possibly even with any genes identified in the smaller number of accessions analyzed for gene expression in Chapter six.

7.2. Materials and methods

Chapter seven is a compilation of all the experimental work from chapters three, four, five, and six and therefore all the materials and methods used in these respective chapters (sections 3.2, 4.2, 5.2, and 6.2) have been adopted for this comparative study.

7.3. Results of morphological and physiological screening, genetic diversity, candidate gene and gene expression analysis.

7.3.1. Screening of Ghanaian Cowpeas for Drought Tolerance

In all 106 cowpea accessions collected from across the length and breadth of Ghana were used for this study. Twelve of the sample used did not germinate initially and were not included in the analysis. Based on the criteria of the study, about 70% of the accessions used were considered to be drought tolerant and 30% as sensitive or susceptible to drought stress. Table 7.1 below shows the source of collection against the number of cowpea accessions.

Table7.1: Performance of cowpea under drought stress and their source of collection.

	Drought Tolerant	Drought Susceptible
Area of collection	**No. of accessions**	**No. of accessions**
Middle belt (MB)	**22**	**5**
North-east (NE)	**13**	**2**
North-west (NW)	**16**	**3**
South-east (SE)	**13**	**5**
South-west (SW)	**5**	**10**
Total	**69**	**25**

The dendrogram constructed on the basis of the data generated from the 10 qualitative and quantitative traits on 106 accessions (Table 3.3) divided them into four major clusters with numerous sub-clusters (Fig.7.1). The statistical analysis on the morphological quantitative traits, plant height (cm) and seed weight (g), of the 106 accessions indicated a negative correlation between the two traits with a correlation coefficient value (r) of 0.0605. The Covariance between the parameters was 0.7705 and a standard deviation value of 4.9436.

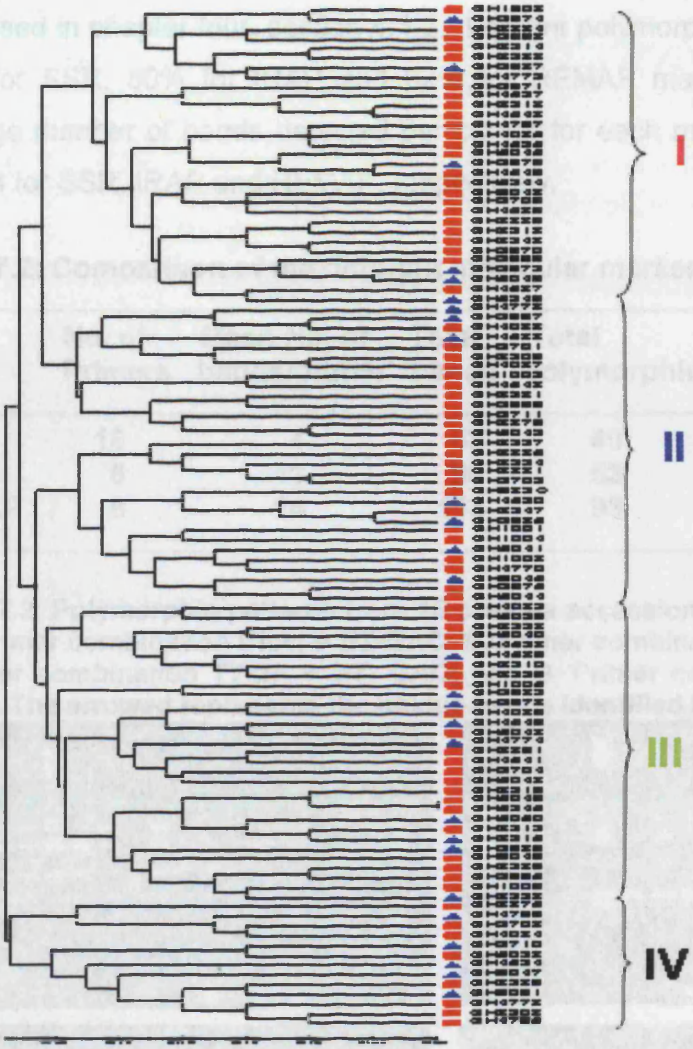


Figure7.1: UPGMA Dendrogram of 106 cowpea accessions using 10 qualitative and quantitative morphological traits. Four major clusters were observed. ■ = Drought tolerant; ▲ = Drought susceptible

7.3.2. Genetic diversity analysis using SSR, IRAP and REMAP techniques

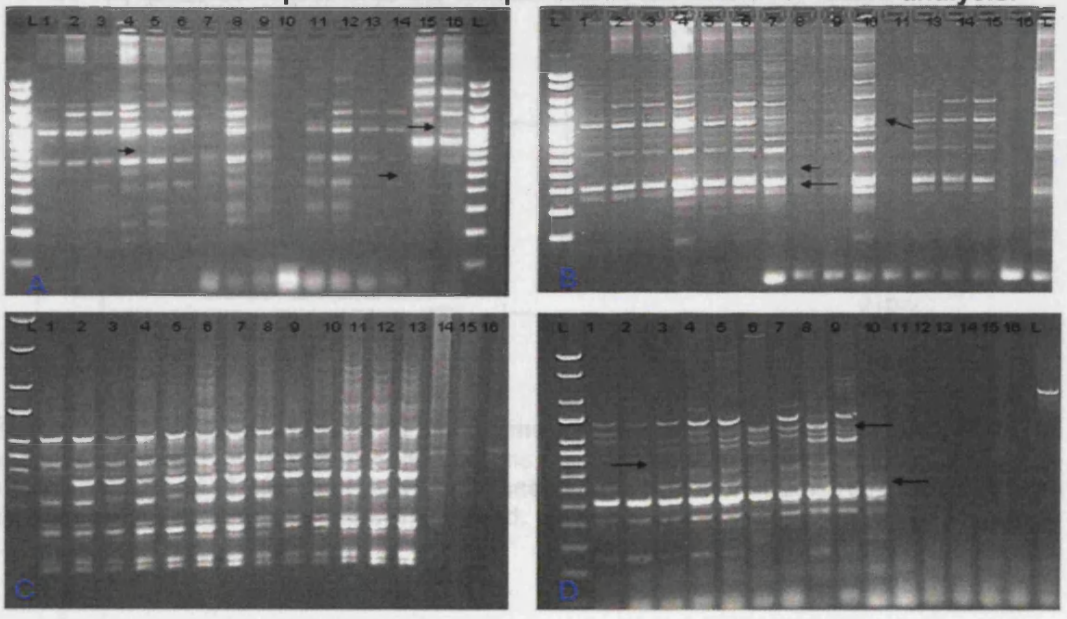
Polymerase chain reaction (PCR) based DNA multi-locus markers, SSR, IRAP, and REMAP techniques were used to investigate the extent of genetic variability within and among the 106 cowpea accessions from Ghana, ten varieties from IITA, Nigeria, and five accessions from supermarket shops in United Kingdom. The selections of these marker types were based on their relative technical simplicity, level of polymorphism they detect and cost effectiveness as already

discussed in chapter four, section 4.1.2. Percent polymorphic bands varied from 75% for SSR, 80% for IRAP and 82% for REMAP markers (Table7.2). The average number of bands detected per primer for each marker type was 4, 11, and 14 for SSR, IRAP and REMAP, respectively.

Table7.2: Comparison of the different molecular markers used in the study.

	No. of Primers	Mean No. of bands/primer	Total bands	Total polymorphic bands	%Polymorphic bands
SSR	**16**	**4**	**53**	**40**	**75%**
IRAP	**6**	**11**	**66**	**53**	**80%**
REMAP	**8**	**14**	**114**	**93**	**82%**

Fig. 7.2: Polymorphic patterns from 16 cowpea accessions generated by REMAP. A. Primer combination Cicer + BT-GAC; B. Primer combination Cicer + BT-CTG; C. Primer combination TY-2R + BT- GAC; and D. Primer combination TY-2R + BT-CTG. The arrowed represents the unique bands identified for future analysis.



The band patterns obtained though polymorphic also showed that they were conserved. A number of unique bands were identified as shown by arrows in Fig. 7.2. These bands will be analyzed later for further clues.

To study the distribution of genetic variation in the cowpea accessions for the SSR and IRAP marker systems, UPGMA clustering method was used. The results indicated interesting variations among the accessions used for the study (Fig.7.3: A and B).

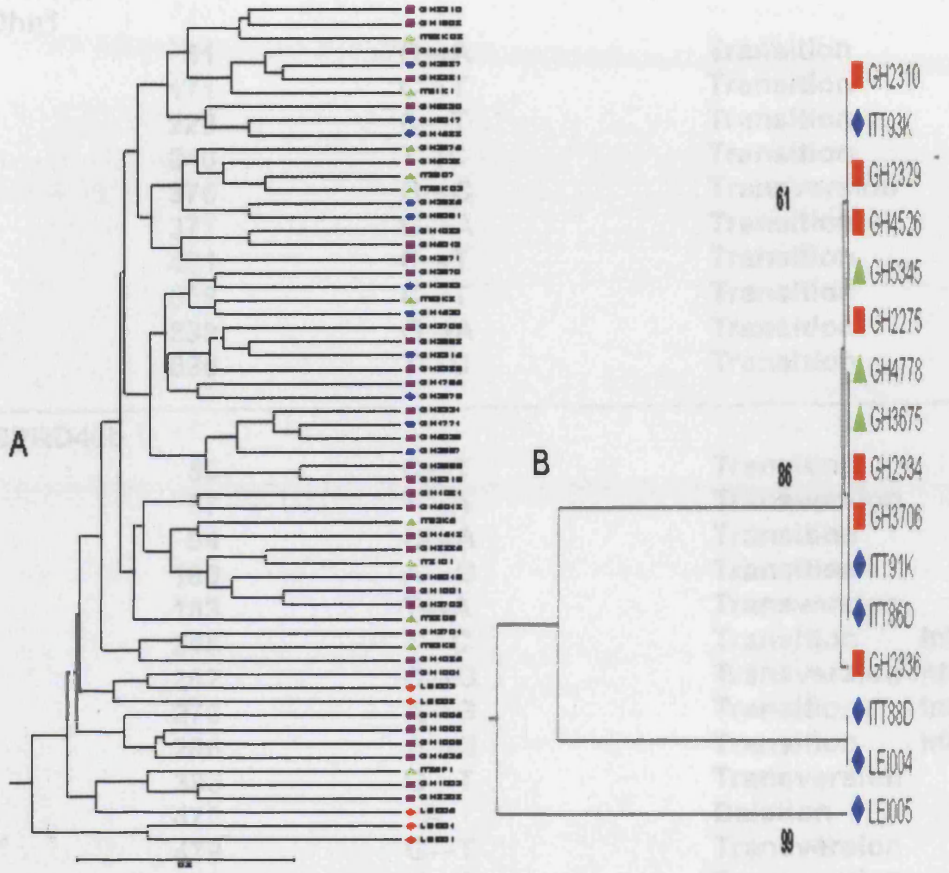


Figure7.3: Phylogenetic models of the two marker systems for diversity studies. A. Model of SSR data based on 16 primer combinations for 61 cowpea accessions using UPGMA clustering method. B. Model of IRAP data based on six primer combinations for 16 cowpea accessions using UPGMA clustering method.

7.3.3. Candidate gene cloning and analysis

In order to gain a better insight of the molecular mechanisms of drought stress responses in certain Ghanaian cowpea accessions, known and characterized drought inducible genes were used as a source for cloning, sequencing and comparative analysis of the genomic DNA of the lines used for the study.

Table7.4: Details of SNPs, deletions, insertions, identified in Dhn1 and CPRD (8, 14, 22, 46b, and 86). Positions of polymorphism are from the start of fragment amplification.

Fragment	Position	Sequence change	Nature	Region
Dhn1				
	81	G→A	Transition	
	171	C→T	Transition	
	228	C→T	Transition	
	240	T→C	Transition	
	376	G→C	Transversion	
	377	G→A	Transition	
	481	C→T	Transition	
	508	C→T	Transition	
	599	G→A	Transition	
	636	T→C	Transition	
CPRD46b				
	56	C→T	Transition	
	67	T→A	Transversion	
	94	G→A	Transition	
	180	A→G	Transition	
	183	T→A	Transversion	
	245	T→C	Transition	Intron
	262	C→G	Transversion	Intron
	276	A→G	Transition	Intron
	296	A→G	Transition	Intron
	385	G→T	Transversion	
	472	A	Deletion	
	479	G→T	Transversion	
	482	G→C	Transversion	
	485	A→G	Transition	
	545	C→A	Transition	
	712	A→G	Transition	
	738	A→G	Transition	
	759	G→A	Transition	
	876	G→A	Transition	
	1031	T→C	Transition	
CPRD86				
	176	A→T	Transversion	
	279	A→T	Transversion	
	407	T→C	Transition	Intron
	504	T→C	Transition	

CPRD8			
70	**T→C**	**Transition**	
118	**T**	**Deletion**	**Intron1**
154	**T**	**Deletion**	**Intron1**
189	**T→C**	**Transition**	**Intron1**
360	**A→G**	**Transition**	
439	**T→C**	**Transition**	**Intron2**
537	**C→T**	**Transition**	
925	**T→C**	**Transition**	
976	**A→G**	**Transition**	
CPRD14			
91	**T→C**	**Transition**	
364	**C→T**	**Transition**	**Intron1**
396	**G→A**	**Transition**	**Intron1**
401	**G→T**	**Transversion**	**Intron1**
402	**T→C**	**Transition**	**Intron1**
540	**T→G**	**Transversion**	
580	**T→G**	**Transversion**	
610	**A**	**Insertion**	
627	**T→C**	**Transition**	
628	**G**	**Insertion**	
652	**C/A**	**Insertion**	
675	**T**	**Insertion**	
728	**T→G**	**Transversion**	**Intron2**
1055	**G→A**	**Transition**	
1118	**T→C**	**Transition**	
CPRD22			
115	**T→C**	**Transition**	
157	**G→T**	**Transversion**	
172	**T→C**	**Transition**	
177	**G→A**	**Transition**	
235	**C→T**	**Transition**	
241	**G→T**	**Transversion**	
248	**G→A**	**Transition**	
424	**T→C**	**Transition**	
541	**A→G**	**Transition**	
712	**A→G**	**Transition**	
830	**G→A**	**Transition**	
900	**T→A**	**Transversion**	
902	**T→A**	**Transversion**	
918	**T→A**	**Transversion**	

On the basis of a search for homology of the CPRD gene fragments sequenced as well as the CDDP gene fragments identified using the BLAST programme, various putative genes and proteins were found to have sequence identity with the identified fragments were subsequently put into two groups as shown in Table7.5 below.

Table7.5 Characterization of drought inducible genes identified in candidate genes and gene expression studies

Group 1; **Consist of functional proteins that function in stress tolerance {LEA proteins, water stress-inducible proteins, dehydration-inducible proteins, osmo-protectant biosynthesis-related proteins, etc}**
Group2; **Consist of regulatory proteins, protein factors involved in further regulation of signal transduction and gene expression. {Transcription factors like zinc finger, CADK, MAPK, NADPH, protein kinases, protein phosphatases}**

7.4. Discussion

The present study showed that the 106 Ghanaian accessions of cowpeas collected for the study had considerable genetic variation in morphological and agronomic traits. The smallholder farmers traditionally collect seeds from those plants with better growth forms under their environment. Each year the selection of seeds is limited to a few chosen individuals and seeds are bulked and kept for the next planting. Thus the local varieties grown in the various regions of Ghana have adapted to local conditions and farmers' practices, and represent unique sources of diversity. Many useful traits have developed in these areas following natural and farmer's selection over the years. The results from Table7.1 indicates that if the landscape of Ghana is divided into three blocks of northern part, southern part, and the middle belt as a transition zone, where the climatic conditions experienced in the other two blocks abounds, then out of the 69 accessions found to be generally drought tolerant from the physiological and morphological analysis, 51 (29 from north; 22 from middle belt) of them were

from the northern zone: In the north, less than 15% of the accessions were classed as susceptible; in contrast, 66% of the accessions from the southwest were susceptible to drought. As reported by FAO (http://www.fao.org/ag/agL/swlwpnr/reports/y_sf/z_gh/gh.htm), rainfall in Ghana generally decreases from south to north and the wettest area is the extreme southwest. The trend of drought tolerance follows this observation, and suggests that farmers by all intents and purposes tend to keep seeds of the products that did well within the framework of the environmental challenges faced during the planting season for the next cropping season. These accessions could offer new alleles for abiotic stress tolerance in cowpea.

The clustering pattern based on morphological and physiological traits indicated that adaptive selection may have caused the accessions to maintain their distinct identities. The fact that most accessions clustered separately from the Leicester outgroup (Figure 7.1) indicates that cowpea farmers in Ghana have been selecting for different morpho- agronomic traits or alternatively, the consequences of a genetic bottleneck where the full range of alleles were never available to farmers in Ghana could be in action. Thus within the accessions there exists a vast amount of variation. Even though some degree of selection is practiced by farmers, there is no strict isolation of such cowpea fields from the neighbouring farms so gene flow between cowpea plants within the same farm as well as between different farms is likely to occur. Stoddard (1986) has reported that bees can enhance pollination of *Vicia* up to 194m from the hive, although Ibarra-Perez (1996) noted that less than 3% of ovules were cross-pollinated from plants 0.76 m apart.

From the four clusters identified from this study (fig.7.1), the upper 25% of the accessions could be used to represent those with the highest average diversity and therefore crosses for improvement of the accessions to drought could be considered. Despite their importance, the genetic characterization of cowpea accessions has been largely ignored until very recently. Genetic characterization

in cowpea accessions has been primarily pursued by using morphological traits (Singh et al. 2002, Hall et al. 2003). The pot evaluation screening method was successful in identifying probable drought tolerant accessions from susceptible ones, though further field evaluation should be considered for comparative analysis. It also showed that morphological traits could be valuable tools for cowpea characterization with the limitations of the influences of the environmental condition on the phenotypic characters. Ideally for the results to be considered appropriate the sample size should be about 50 to100 accessions as useful scale for testing, but needs to be on a bigger scale than carried out here with respect to number of markers and the scoring of more phenotypic characters.

The next step in the study was the characterization of the 121 cowpea accession for genetic diversity at the molecular level using DNA markers in order to evaluate their potential in cowpea breeding programmes. The SSR, IRAP and REMAP markers were thus used with the aim of identifying the cultivars having unique alleles at a given loci and further characterize for markers associated with abiotic stress tolerance, as these are the populations most likely to contain new alleles in general and potentially for stress-related loci. The results obtained indicated that all the three multi-locus PCR based molecular markers were highly polymorphic (Table7.2). The range of pairwise differences obtained for the three marker systems used indicated that most of the accessions may be closely related as the differences were very minimal. The large differences were observed between the out-group and the Ghanaian accessions which may probably be due to the fact that they are cultivars developed from genetically distant parents. This result suggests that these markers can be used to predict similarities among the accessions. This result is consistent with that of Li and colleagues (2001). The cluster analysis of the three marker systems showed similar patterns with the out-group being clearly separated from the other accessions, though the accessions could not be separated into distinct groups based on their already determined drought status (Fig.7.3). However, several

factors may affect the genetic relationship among cultivars, such as number of markers used, distribution of markers in the genome of working samples, and the nature of evolutionary mechanisms underlying the variation measured (Brown et al., 1996). The genetic characterization data obtained could provide useful information for utilizing these populations in cowpea breeding programmes to create abiotic stress tolerant cowpea.

It must be pointed out that the experimental design of this study was not such that analysis would enable a QTL (quantitative trait locus) identification and characterization. A set of unrelated (or unknown relationship) plants was used in the study, and drought tolerance, measured as in Chapter three, was scored as a binary variable. However, the consequences of water stress are continuous variables and certainly involve interactions with numerous gene loci. In the future it would be appropriate to carry out a QTL analysis using one or more appropriate mapping populations from the cowpea lines analyzed here and a range of mapped markers based on published cowpea data. The polymorphisms identified between lines here mean that appropriate parents could be picked based on drought responses in Chapter 3, building an F_2 mapping population of 100 or more individuals to score for drought response characteristics, and both SSR and other classes of markers could be used for the QTL analysis. This type of QTL population has the problem that drought scoring cannot be replicated at different sites or different years; therefore a recombinant inbred population would be better. Although this will take several years to generate, it would have multiple mapping applications so would be a good target to start its production in the near future.

Preliminary analysis of the nature of polymorphism in the CPRD gene fragments indicates a range of SNPs and a few indels with respect to reference sequences of the genes isolated by others in cowpea and other legumes (Table 7.4). SNPs have been found to occur with a frequency from 1 in every 202 bp in the mouse genome and 1 in 1000 bp in the human genome (Lindblad-Toh *et al.*, 2000;

Wang *et al.,* 2003). There are various methods for SNP detection and scoring. More commonly used are gel-based sequencing and high-density variation-detection DNA chips (Wang *et al.,* 2003). SNP has many advantages and great potential for many applications. Since SNPs exist over the whole genome of organisms with a relatively high frequency, they could facilitate the development of highly dense genetic function maps that would be highly valuable for genome analysis. Moreover, as the sequence context of the SNPs is already known, it has the potential for automation and can facilitate the genetic study of associating sequence variations with heritable phenotypes on a large scale. Because of this, there has recently been considerable interest in SNP discovery and detection for genome analysis of plant (Chen *et al.,* 2002). In this study, within all the coding sequences, a SNP frequency of between 1 in 100 and 1 in 70 bases was observed in the six genes analyzed. Overall, there were 44 transitional (A/G or C/T) changes and 18 transversions (A/T, A/C, G/C, G/T), The excess of transitional changes is considered as typical in plant genes, and indicate mutation of methylated cytosine to thymine, although Keller et al. (2005) point out that the data for this conclusion is limited and the result may not be universal.

The results of the homology matches obtained from the sequences of the candidate gene fragments as well as the gene expression analysis of the stressed and unstressed drought tolerant and susceptible cowpeas as summarised in Table 7.4 indicate the range and complexity of gene array involved in responses to drought tolerance. Probing breeding populations that show improved traits during environmental stress with genomic tools is expected to uncover important gene networks involved in drought tolerance. Sun et al. 2001, applied cDNA microarray analysis to discover maize genes expressed in the ear that alter their expression due to water stress during flowering. The cDNA for selections of maize genes corresponding to cell cycle, known stress responses, carbohydrate metabolism, and ABA responsive genes were included on the microarrays. Of the cell cycle genes, they noted three general patterns of

gene expression modulation: (1) an increase in mRNA levels during the period of stress; (2) a decrease in mRNA during the stress followed by an increase during subsequent recovery; (3) an increase in mRNA only during recovery period. These results suggest distinct function for members of the cell cycle regulation gene family during dehydration and recovery. Similar patterns of expression in response to water stress were also noted in this study as shown in Table 7.4 of the groups of functional and regulatory genes and proteins identified when the cowpea plants were subjected to drought and allowed to recover. The results of the study therefore provide compelling leads for further analysis.

7.5: Conclusions

- The screening and characterization study has shown that the Ghanaian cowpea accessions used had considerable genetic variation in morphological and agronomic traits. The mode of selection of seeds for the planting season also has had influence on the variability encountered with most of the drought tolerant accessions identified coming from the northern block of the country.
- The results of the molecular diversity analysis with the three multi-locus PCR based molecular markers showed that these markers were highly polymorphic and have high discriminatory power. The results also suggest that these markers could be used to predict similarities among accessions and for planning of crosses of parental lines for population and QTL studies.
- The candidate gene analysis of the CPRD gene fragments using genomic DNA of the cowpea accessions showed the high polymorphic nature of the gene fragments, the structure of the gene with their intronic regions, the identification of significant SNPs within both the coding and the non-coding regions and the characterization of these gene fragments as having high homologies with certain putative genes involved in abiotic stress.

- The study identified many abiotic or drought inducible genes in cowpea from the DD-PCR and the candidate gene fragment analysis. The results indicated cross-talk between the signalling and functional regulatory mechanisms within the stress mitigation systems. The BLAST analysis also indicated that some of the identified inducible genes in cowpea were common with stress genes in many other plant species like *Arabidopsis* and rice.
- The design of the study, the top-down approach (i.e. phenotype to gene) has been successful as a lot of useful links have been identified that can broaden the general approach the identification of genes related to abiotic stress in general and drought tolerance in general.

CHAPTER EIGHT

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CHAPTER EIGHT

8.0: Diversity and abiotic stress in cowpea: general discussion, conclusions and further research.

8.1. Introduction

Food shortages in Africa are strongly associated with environmental calamities. The major abiotic stress factors affecting food production in sub- Saharan Africa are low soil fertility, drought, salinity, soil acidity and heat stress. They are expressed in crops through a series of morphological, physiological, biochemical and molecular changes that affect plant growth and productivity (Wang et al., 2003). Resistance to abiotic stress factors is multigenic, as well as quantitative in nature. Attempts with conventional breeding methods to develop crop varieties tolerant to multiple abiotic stresses have been only partly successful. Efficient identification, isolation and use of favourable genes for breeding stress-tolerant genotypes may require other efficient tools, including molecular markers, functional genomics, and in the long term, transgenic technology.

In parts of the world where people live, drought is perhaps the most limiting factor to crop production, and the situation is expected to deteriorate in Africa. The current trends in land degradation, desertification and climatic variability have been predicted to intensify because of global warming. The erratic supply of rainfall across seasons, poor soil-water-holding capacity and poor management of water resources has led to drought occurring, on average, once every 3 years in eastern and western Africa for the last 30 years, causing human and environmental disasters. For instance, drought has affected common bean production in eastern, central and southern Africa to cause losses of more than 395,000 t each year (Amede et al., 1990). Although challenging, drought tolerance can be improved through conventional breeding, using existing genetic

diversity. Newer methods, involving molecular markers and comprehensive gene expression profiling, provide opportunities for directing the continued breeding of genotypes that provide stable grain yield under widely varied environmental conditions (Bruce et al., 2002).

Cowpea has many drought-tolerance characteristics which makes it a model for drought tolerance studies as stated earlier in chapter one section 1.6. These characteristics also make cowpea one of the most important feed and food crop in the arid and semiarid regions of the world (Singh et al. 2002). Cowpea is an important food legume and an essential component of cropping systems in the drier regions and marginal areas of the tropics and subtropics covering parts of Asia and Oceania, the Middle East, southern Europe, Africa, southern USA, and Central and South America. It is particularly important in West Africa with over 9.3 million hectares and 2.9 million tonnes annual production. With about 25% protein in its grains, cowpea is an important source of quality nourishment to the urban and rural poor who cannot afford meat and milk products (Poor man's meat). Cowpea haulms contain over 15% protein and constitute a valuable source of fodder. However, cowpea production in much of the tropics is still limited by drought (Nguyen and Blum, 2004). Thus, developing genotypes that have an advantage under water-limited conditions is a major challenge for the improvement of cowpea. Results from breeding programmes (Singh et al., 2002 ; Hall et al. 2003) indicate that advances in crop improvement are more likely if drought tolerance traits are selected in addition to yield *per se.* In this study a top-down approach (i.e. phenotype to gene) by beginning with physiological and morphological analyses of cowpea plants for drought tolerance, through to gene expression of stressed and unstressed cowpea plants for the identification of genes linked to drought tolerance and its phenotypic traits for possible QTL exploitation eventually, have been pursued and the results discussed.

8.2: Genetic diversity and identifying sources of tolerance

8.2.1: Morphological screening of germplasm for tolerance

Analyses of the extent and distribution of genetic variation in a crop are essential in understanding the evolutionary relationships between accessions and to sample genetic resources in a more systematic fashion for breeding and conservation purposes. Traditionally, genetic resources in cowpeas are classified based on morphological markers. However, these morphological traits used in classification of cowpea to different accessions are conditioned by a relatively small number of genes. On the other hand, important traits which are related to habitat adaptation and exhibit enormous variability among cowpea germplasm are complex and quantitatively inherited. Hence, classifying germplasm based solely on a few discrete morphological characters may not provide an accurate indication of the genetic divergence among the cultivated genotypes of cowpea. Some characters that may be used for classification may be variable depending on growth conditions of the plant, and not genotype. The results for the screening experiment in chapter three exhibited some interesting trends. It was realized that the local farmer had an in depth knowledge about the climatic constraints and the selection method to be adopted to ensure some amount of yield every growing season. Thus the results showed that the drought tolerant accessions identified mostly were from the northern block of Ghana where the rainfall distribution is erratic and highly unreliable.

For the current upsurge in research into conservation agriculture (CA) to be successful, it is crucial to acknowledge that application will largely depend on the value that agricultural and molecular biology researchers place upon certain assets controlled by the poor and hitherto neglected farmers, specifically germplasm and related local knowledge. It should be noted that these regions inhabited by such farmers serve as reservoirs of genetic diversity for the development of genomics-based tools for increasing the ability of agricultural research to make use of such genetic diversity in developing improved crop varieties. In particular, the study noted that molecular markers can help

researchers both to search germplasm collections for potentially valuable breeding materials and to make use of them by introgressing desirable traits into elite cultivars. It follows, then, that an agricultural research institute (or a network of such institutes) that has access to these tools will be in a position to make use of unconventional breeding materials such as farmers' traditional varieties. This means that traditional varieties, together with farmer knowledge about the characteristics of such varieties, will take on enhanced value to researchers as genomics-based technologies become available to them, provided that the researchers are interested in making use of the genetic resources known to farmers. However, there are some scientists, who regard these farmer assets as being irrelevant to their work. Such scientists make use of the genetic material that is already held in germplasm collections, augmenting this by collecting wild relatives of the crop in question, and modern technologies are enabling them to screen accessions for desirable traits by examining their genomes, rather than their phenotypes. This approach is forcefully advocated by Tanksley and McCouch (1997), who assert that 'there is tremendous genetic potential locked up in seed banks that can be released only by shifting the paradigm from searching for phenotypes to searching for superior genes with the aid of molecular linkage maps'. This then suggests two alternative approaches to broadening the genetic base of plant breeding. The first as advocated by Hall et al (2003), rely on the insights of the rural people to find plants whose phenotypes expressed desirable traits for further analysis as against the sidelining of the rural farmer in the second approach.

The wooden box screening method (Singh et al. 1999b) adopted for the study was successful in partitioning the germplasm to drought tolerant and susceptible lines. The results of the cluster analysis though informative, could not place these accessions into distinct groupings. However, analysis of the sources of collection against their respective drought status could give a fair idea of the origins and therefore further characterization of the germplasm and molecular analysis was proposed. More screening methods for drought, specifically field tests, replicates

between years and sites should be conducted since the field analysis give the actual character that breeders want. However, there is the need also to conduct laboratory-based experiments which are much less labour-intensive, potentially more reliable, more reproducible and allowing larger screening projects. Laboratory tests can also enable screening for stresses not yet encountered in the field, resistance to new pathogens for example. Therefore, it would be ideal if the field tests are carried out and developed in parallel with lab-based approaches such as the box test used here, or new approaches such as growth in different osmolarities, or even tissue-culture based tests, bearing in mind that the box method only addresses just an aspect of field drought tolerance.

8.2.2: Molecular diversity analysis using molecular markers

The material sampled here demonstrated that there is a substantial pool of diversity in Ghanaian cowpea germplasm. The results obtained when three multi locus PCR based molecular markers were used to characterize the 121 cowpea accessions were very informative. The three markers were highly polymorphic and could identify all the accessions used. The use of REMAP and IRAP markers in this study is the first of its kind in the analysis of Ghanaian cowpeas. There is therefore the need to design more primers and increase the germplasm base so that the potential in these two molecular techniques could be fully tapped. The results indicated that genome fingerprinting has a major role to play in the characterization of population structure and in the analysis of variability in cowpea. As yet, there is no evidence that the germplasm diversity of cowpea has been fully sampled and further and deeper sampling across Africa is still required. The strategies and potential of initiatives such as that of the Kirkhouse Trust (http://www.kirkhousetrust.org/) in establishing and equipping mobile DNA and genotyping laboratories in Ghana are particularly important in this regard. The accessions analyzed here have a largely different profile of diversity from those previously sampled. The Ghanaian cowpeas were different from the Leicester outgroup, but was somehow closely related to the IITA, Nigerian samples. Even in the important IITA study (Singh et al. 2002), a small number of

accessions (seven of the 100) were very different from each other and the bulk of the collection analyzed. Molecular markers are increasingly being used to assess the diversity of germplasm collections, including collections of landraces and their wild relatives (Langridge et al. 2001).

8.3: Candidate gene fragment and gene expression analysis

Plants exposed to different water regimes induced different responses to gene expression. The study confirmed that differential display PCR technique is an effective method for drought gene expression analysis for the identification of novel genes. Nine candidate gene fragments induced by drought stress were identified in chapter six from the DD-PCR analysis. The identified candidate gene fragments were successfully cloned, sequenced and characterized. There are two general approaches to identify and isolate genes involved in drought tolerance (Mullet et al. 2001). First, genes are targeted that show relatively rapid changes in expression at the RNA level in response to water limitation. Second, genes involved in drought tolerance are identified and isolated using map-based gene discovery technique. In this study the approach adopted was to design primers from fragments of candidate drought inducible genes, and the primers used to amplify corresponding fragment lengths from genomic DNA of cowpeas. Then the successful products were cloned and analyzed. Genes induced under water-deficit stress can be divided into two groups. The first code for proteins directly involved in protection, the second for proteins involved in regulation of signal transduction and gene expression (Thomashow, 1999; Shimamoto and Kyozuka, 2002; Zhu and Xiong, 2002; Shinozaki et al. 2003). These two major groups were also identified in the study. Following cellular perception of water loss, a signalling mechanism must be activated to induce specific genes (Bray, 2002b). Different conditions induce different stress-induced genes and thus there must be several different signalling mechanisms. Some may lead to similar responses, but other relate to differences in onset of the drought whether regularly at a particular point in the season, or with gradual or sudden onset.

Differences were identified in the expression analysis. Gene identification will be useful to conventional breeders using diversity in the species and for transgenic approaches with genes from the same and different species. Several different experimental systems, including lower and higher plants and microbes, have been analyzed for plant abiotic stress responses (Grover et al., 2003). Stress response has been analyzed at the molecular level to discover stress proteins, stress genes, stress promoters, trans-acting factors that bind to stress promoters and signal transduction components involved in mediating stress responses (Chaves et al. 2002). The functional relevance of stress-associated genes is being tested in different trans-systems, including yeast and higher plants. To overcome the scarcity of abiotic-stress-specific phenotypes for conventional genetic screenings, molecular genetic analysis, using a stress-responsive promoter-driven reporter, is a potential alternative to genetically dissecting abiotic-stress-signalling networks in plants (Xiong et al., 2001).

8.4: Problems encountered and their solutions

The cowpea seeds used for the study were mostly acquired from the open market and from local farmers and so could not obtain adequate passport data of the lines from their source but rather from the Plant Genetic Resource Centre at Bunso, in Ghana.

The design of the experiment for screening of plants for drought tolerance was such that six plants were made to grow in the same pot for the preliminary study which resulted in overcrowding of the plants and atypical root environments. Subsequently replicates of three plants per pot in two sets of the same accession were adopted.

In the preliminary experiment, the seedlings were exposed to a night temperature of 15°C increasing to a maximum of 35°C during the day with a 6-hour supplementary light to stimulate the temperature regimes. The plants did not grow well, but when the supplementary light was increased to 10 hours the plants became well established and were adopted.

8.5: Conclusions

- The study was aimed to attempt to identify DNA polymorphisms linked to drought stress that could be used in cowpea breeding programmes for drought tolerance. The approach was therefore to use the top-bottom experimentation system starting with the screening and identification of the phenotypes and subsequently to the identification of candidate gene fragments. All the physiological and molecular techniques used for the study were observed to be relevant and adequate for the task set.

- The pot screening method which was adopted for the screening of the cowpea lines into drought tolerant and susceptible varieties was successful. The results obtained indicated that there were more drought tolerant accessions in the germplasm used than the susceptible ones. This is significant in the sense that cowpea plant is generally considered to be fairly tolerant to drought stress. It was also realised that the morphological characters were inadequate to effectively serve as a means of determining the variability of the accessions used. Therefore it was proposed that genomic approaches be adopted for the diversity and genomic characterisation.

- The diversity analysis was performed using three different molecular markers, SSR, IRAP and REMAP techniques. The results indicated that these markers were highly polymorphic, they could all uniquely identify the accessions and they had very high correlation coefficient values amongst them. From the results of the cluster analysis crosses of the lines could be planned for further analysis.

- The results from the fragment candidate gene cloning and sequencing analysis indicated that there was enormous diversity amongst the accessions used. SNPs were identified in both the exonic and the intronic regions, as well as some deletions and insertions were also identified. The

SNPs were observed to be largely transition in character with less than 20% transversions. Analysis of the clones with other plant species indicated that they were putatively involved in combating drought stress or serving as transport systems for signalling purposes. In the future, approaches to this part of the study will change as DNA sequencing ("the $1000 human genome") technologies become faster and cheaper.

- The study also identified potential abiotic or drought inducible gene fragments in cowpea from the DD-PCR and the candidate gene fragment analysis, like water stressed proteins, osmotic stress-activated protein kinase, respiratory bust oxidase and calcium binding/NADPH oxidase. The results indicated cross-talk between the signalling and functional regulatory mechanisms within the stress mitigation systems. The BLAST analysis also indicated that some of the identified inducible genes in cowpea were common with stress genes in many other plant species like *Arabidopsis* and rice. The design of the study, that is, the top-down approach (i.e. phenotype to gene) has been successful as a lot of useful links have been identified that could broaden the general approach in the identification of genes related to abiotic stress in general and drought tolerance in general. Genes induced under water-deficit stress were divided into two groups. The first code for proteins directly involved in protection, the second for proteins involved in regulation of signal transduction and gene expression. Following cellular perception of water loss, a signalling mechanism must be activated to induce specific genes as indicated by Bray (2002b). Different conditions induce different stress-induced genes and thus there must be several different signalling mechanisms that are useful for future identification and analysis. Technologies such as microarrays for gene expression analysis, as well as real time, quantitative PCR, have considerable potential as alternative routes to identify and confirm responses of plant genes to abiotic-stress.

Further Research

Molecular markers closely linked to genes of agronomic importance have been demonstrated to be useful tools for indirect selection in a barley breeding programmes (Jefferies *et al.* 2004). Marker-assisted selection is time-efficient, non-destructive and depending on linkage relationships, characterized by low selection error. Marker assisted selection should be done on a case by case basis. Further investigation for drought tolerance will be required to establish the importance of the identified genomic regions in other backgrounds. Also, field evaluation is required to establish the effectiveness of the drought screening system in modelling water responses and in evaluating the stability of QTLs across environments.

The QTLs and molecular markers for drought tolerance provide further evidence for the inheritance of the stem reserve mobilization. Compared with conventional methods, QTLs and molecular markers provide breeders new alternatives for selection. Marker assisted selection can accelerate breeding by reducing the time to develop new cultivars (Bruce et al. 2002; Mayes et al 2005). Further research is needed on molecular markers and QTL mapping to screen potential parents for drought tolerance in cowpea.

As there is the possibility of the whole genome of cowpea being sequenced in the not very near future by the Timko group (2006), microarray analysis will be used to identify candidate genes. Genes that are induced by stress are ideal for comparative microarray analysis. A typical experiment will be to challenge an array of ESTs with RNA extracted from stressed and unstressed tissues. A comparison of the two will identify genes that are up-regulated in the stressed tissues and those which are down-regulated or switched off.

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