MOLECULAR ASPECTS OF ALBINISM IN ANTHER CULTURE DERIVED BARLEY PLANTS.

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by

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ABSTRACT.

MOLECULAR ASPECTS OF ALBINISM IN ANTHER CULTURE DERIVED BARLEY PLANTS.
by Roy P. Dunford.

Haploid cereal plants can be regenerated from single pollen grains via the process of anther culture. Anther culture of cereals is of potential use in crop improvement programmes. One problem associated with anther culture of cereal plants is a high incidence of albino individuals which cannot be used in crop breeding schemes. Albinos derived from barley anther culture (albino pollen plants) are severely pigment deficient and from electron microscopy studies appear to possess plastids that are developmentally arrested at a stage prior to the differentiation of proplastids to mature chloroplasts. The aim of the project has been to investigate some of the molecular aspects of albinism in these individuals.

In vitro propagation experiments were carried out to find the conditions necessary to improve the growth and maintenance of albino pollen plants with the objective of producing a continuous supply of albino tissue for molecular analysis. However, use of various media containing organic and inorganic supplements including a number of plant growth regulators failed to improve the growth of albino plants.

Southern analysis revealed that four out of the five albino plants studied exhibit ptDNA restriction patterns that are different to that expected from the wild type map of the barley plastid genome due to the alteration or deletion of specific ptDNA fragments. One plant appears to contain a major form of ptDNA that has undergone a deletion event removing 75% of all sequences. This confirms that the albino pollen plants examined in this study contain forms of the plastid genome that have undergone structural alteration. I have termed these variant plastid genomes ΔptDNAs. Most of the albino plants studied appear to contain heterogenous populations of ΔptDNAs. One albino barley pollen plant appears to possess an intact plastid genome. For all the albinos studied the overall levels of ptDNA are reduced 5-15 fold compared to the levels found in normal green tissues.

Northern analyses revealed that the transcripts from the ptDNA genes *rbcL* and *psbD-psbC* do not accumulate or are present in albino tissues at $\lesssim$5-10% the level found in seed-derived green shoots. Levels of the plastid encoded 16S and 23S rRNAs are similarly reduced in albino tissues. Further Northern analysis revealed that the abundance of transcripts from the nuclear genes *rbcS* and *cab* are present in most albino plants at $\lesssim$10% the level found in normal green tissues. Southern analysis indicated that the nuclear DNA restriction fragments encompassing the *cab* and *rbcS* genes in two albino plants had not been altered or deleted during the anther culture process.

Analysis of green pollen plants indicated that they contain ptDNA of apparently normal structure and abundance and accumulate transcripts from plastid genes and nuclear genes encoding chloroplast polypeptides to the same levels found in the leaves of light grown seedlings. These results represent the first determination of the levels of photosynthetic gene expression in both albino and green pollen plants.

CONTENTS:

CHAPTER 7: EXPRESSION OF NUCLEAR GENES ENCODING CHLOROPLAST POLYPEPTIDES IN ALBINO POLLEN PLANTS.

CHAPTER 8: DISCUSSION.

COMMONLY USED ABBREVIATIONS.

ATP	Adenosine triphosphate.
bp	Base pair.
BSA	Bovine serum albumin.
C	Celsius.
cm	Centimetre.
CPM	Counts per minute.
CTAB	Cetyltrimethylammonium bromide.
DEPC	Diethyl pyrocarbonate.
dCTP	Deoxycytidine triphosphate
DNA	Deoxyribonucleic acid.
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol.
E	Einstein.
E. coli	*Escherichia coli.*
EDTA	Diaminoethanetetra-acetic acid.
g	Gram(s).
HEPES	N-2-Hydroxyethylpiperizine-N'-2-ethane sulphonic acid.
k	Kilo
kb	Kilobases.
l	Litre.
M	Molar.
m	Milli (10^{-3}).
mm	Millimetre
m^2	Metre squared
MBq	MegaBequerel.
MES	2-(N-morpholino)-ethanesulphonic acid.
min	Minute(s).
MOPS	3-(N-morpholino)-propanesulphonic acid.
n	Nano (10^{-9}).
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form.
OD	Optical density.
PEG	Polyethylene glycol.
psi	Pounds per square inch.
PVP	Polyvinylpyrrolidone.
RNA	Ribonucleic acid.
rpm	Revolutions per minute.
s	Second.
SDS	Sodium dodecyl sulphate.
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol (tris).
μ	Micro (10^{-6}).
UV	Ultraviolet.
V	Volts.
v/v	Volume to volume.
w/v	Weight to volume.

NB. Additional abbreviations are specified where appropriate in the text.

CHAPTER 1: INTRODUCTION.

The work described in this thesis was carried out with the objective of improving our knowledge of the molecular biology of albino barley plants generated from the *in vitro* process of anther culture. In this context the following sections provide an outline of the developmental basis of anther culture and its potential use as a tool in crop improvement programmes. Some of the preliminary studies that have been carried out elsewhere on anther culture derived albinos are described and albinism is further discussed in the context of normal plant development. Emphasis is paid to the molecular events associated with the attainment of photosynthetic competence by plant tissues.

1.1 The Source of Albinos: Anther Culture.

1.1.1 The Development of Pollen.

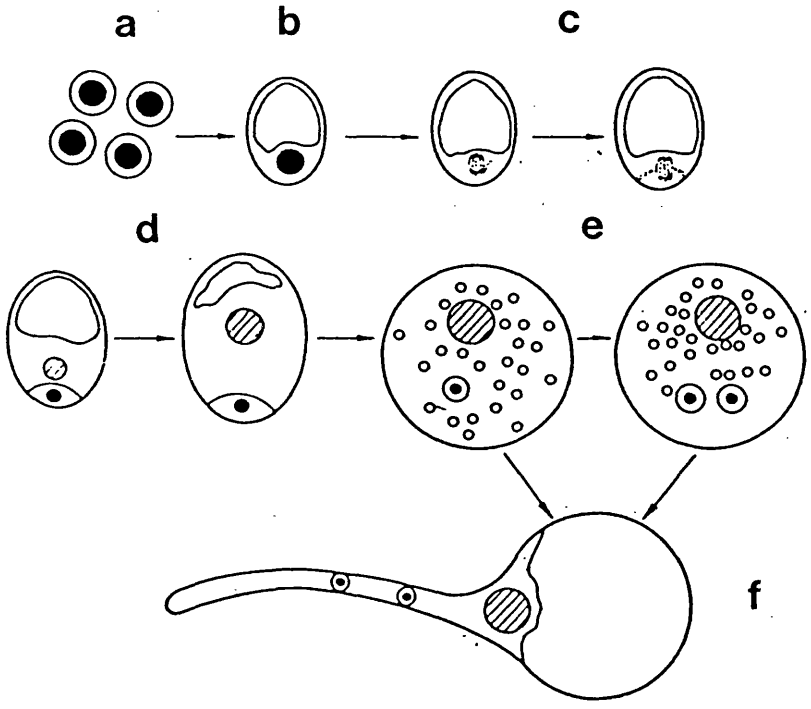
Details of the reproductive biology of angiosperms and in particular the development of the male gametophyte have been reviewed by Frankel and Galun (1977) and only an outline will be given below.

The life cycle of higher plants is divided into two phases, the diploid sporophyte and haploid gametophyte. In angiosperms the sporophyte is represented by somatic tissue comprising the vegetative body of the plant. The male and female gametophytes which develop from, and are maintained by, the sporophytic tissues are relatively small in comparison and are highly specialised for sexual reproduction. The male and female gametophytes in angiosperms are represented by the pollen grain and embryo sac respectively. Pollination and subsequent fertilization results in the fusion of haploid nuclei within these structures resulting in the development of the next diploid sporophyte generation. Details of the development of the male gametophyte or pollen grain are summarised below and in Figure 1.1.

Pollen grains develop within specialised floral structures called

Figure 1.1. Pollen Development.

The developmental sequence of pollen formation in angiosperms. Stage (a) represents a tetrad of four haploid microspores derived from an initial meiotic division of the pollen mother cell. Nuclei are shown by filled circles. Stage (b) shows a uninucleate microspore which has developed a large vacuole. Anthers containing pollen at about this stage are used to regenerate whole plants *in vitro*. Stage (c) represents a uninucleate microspore undergoing mitotic division to generate a bicellular structure, consisting of a large vegetative cell and smaller generative cell as shown in stage (d). The generative and vegetative cell nuclei are represented by filled and hatched circles respectively. Stage (e) represents a microspore that has lost its vacuole and which may undergo a further mitotic division of the generative cell. Stage (f) represents a mature pollen grain that is undergoing germination. The pollen cell wall is not shown. Reproduced from Sunderland (1973).


a
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anthers and arise from the sporophytic tissues via the meiotic division of pollen mother cells (PMCs). By this process the diploid PMCs each generate a tetrad of haploid microspores which subsequently undergo an increase in volume and develop a prominent vacuole. A further highly asymmetric mitotic division results in the production of a large vegetative cell, which represents the majority of the microspore volume, and a smaller generative cell which may undergo division to produce two generative nuclei. The mature pollen grain is composed of the fully developed male gametophyte encased in protective intine (inner) and exine (outer) layers and is released at this stage by the dehiscence of the anther wall.

1.1.2. The Developmental Basis of Anther Culture.

Anther culture is an *in vitro* technique which relies on the suppression of the normal development of the male gametophyte within anthers and the subsequent regeneration of whole plants. Immature anthers are excised aseptically from the donor plants and placed under defined culture conditions which allow optimum regeneration of plants from individual pollen grains. The phenomenon of whole plant regeneration from pollen was first discovered, accidentally, by Guha and Maheshwari (1964). These authors found that excised anthers of *Datura innoxia*, under certain conditions of *in vitro* culture, produced embryo-like structures which were subsequently found to be derived from the pollen within the anthers themselves (Guha and Maheshwari, 1966). Since these initial studies successful demonstrations of the production of pollen derived plants (pollen plants) have been reported for a large number of species including commercially important crops (reviewed by Dunwell, 1984). Most effort has been given to specifying the factors which determine the highest yield of pollen plants from individual anthers. These include the genotype, environment and age of the donor plants, pretreatment of anthers, composition of the anther culture medium and the conditions of light and temperature during culture (Dunwell, 1984). Details of the procedure used to produce the plants described in this study are given in Section 2.17.

One of the most crucial factors involved in anther culture is the stage of pollen development stage at which most efficient regeneration of plants can be achieved. In barley maximum yields of pollen plants are attained by culturing anthers which contain microspores at the mid-uninucleate stage prior to the first mitosis (Huang and Sunderland, 1982; see Figure 1.1.). Subsequent cell division of the microspore then leads to the regeneration of individual plants via a callus stage (Huang and Sunderland, 1982) or by direct embryogenesis (Lyne *et al.*, 1984). In barley a number of different developmental pathways can lead to regeneration from individual microspores and may involve the division of derivatives of the vegetative and/or generative cells (reviewed by Sunderland, 1982).

By consideration of the factors mentioned in the previous paragraph, the efficiency of barley anther culture, in terms of the production of green plants, has improved greatly in recent years. This is commonly expressed as the number of green plants produced per 100 anthers cultured. Typical yields from the work of Huang and Sunderland (1982) were in the region of 2-5 green plants per 100 anthers. However, refinements in anther culture methodology have recently improved yields to over 200 green plants per 100 anthers cultured (Hunter, 1987).

1.1.3. The Application of Anther Culture to Crop Breeding.

Since plants regenerated from pollen are derived from gametophytic tissues they should be haploid. However, in practise, non-haploid plants are also often generated (Dunwell, 1984). In many plant species diploidisation of the haploid plantlets can be achieved by treatment with the mutagen colchicine. Alternatively, in species such as barley spontaneous diploidisation is observed at an early stage in the development of pollen cells that give rise to regenerated plants (Sunderland and Evans, 1980; Lyne *et al.* 1984). When diploidisation occurs the end product of the anther culture process is referred to as a double haploid, homozygous at all genetic loci. Crop breeding programmes often require the establishment of stable homozygous lines from the selected F_2 progeny of hybrid crosses. These stable homozygous lines can then

be used for seed production for use as commercial varieties or in further crosses. The production of a homozygous line from heterozygous plants can be achieved by the time consuming process of self-fertilization and selection over a number of generations. However, the technique of anther culture provides a direct route to the generation of homozygous lines by the production of double haploid individuals. Double haploids can be produced from hybrid F_1 plants and then subjected to field selection for the characteristics of interest. Stable lines can then be established directly by the self fertilization of selected plants and subsequent seed production. The direct selection of double haploid plants offers the potential to produce stable homozygous breeding or varietal lines in a relatively short period of time compared to conventional methods. Improvement of a number of crop species has been reported, including rice, winter wheat, sugarcane, tobacco and pepper (recently reviewed by Morrison and Evans, 1988). In the case of rice, the development of two new varieties took five years as opposed to twelve expected using conventional techniques (Hu and Zeng, 1984).

Aside from any consideration of the efficiency of plant production a major problem associated with cereal anther culture is the high incidence of albino individuals, ie. plants lacking chlorophyll. The occurrence of albinos in the products of anther culture have been reported for the graminaceous species rice (Sun *et al.* 1974), wheat (Bullock *et al.*, 1982) and barley (Clapham, 1973). The number of albinos produced varies and can depend on the species, genotype and physiological status of the donor plant, the pollen stage at which regeneration is induced and the culture conditions (Bullock *et al.*, 1982; Dunwell, 1984). Albino pollen plants are of no use in crop breeding programmes since they remain vegetative and require special culture conditions to survive (see Chapter 4). Albinism therefore represents a major problem in terms of reducing the number of plants that can be used for practical breeding purposes, and as such needs to be overcome before anther culture can be applied more widely to the improvement of cereal crop species. One way to alleviate the problem of albinism could be to improve the overall yield of regenerated plants from anther culture and therefore increase the production of large numbers of useful green plants irrespective of the frequency of albinos. Although

manipulation of anther culture methodology may reduce the practical problems associated with albinism, studies of albino biology may provide a knowledge of the fundamental basis of the phenomenon. Some general studies of albino pollen plants are summarised in the next section.

1.1.4. Previous Studies of Albino Pollen Plants.

Albino pollen plants are severely pigment deficient. However, the general morphology of albino pollen plants is the same as that found for normal green plants (discussed further in Chapter 3). Ultrastructural studies by Sun *et al.* (1974) using transmission electron microscopy techniques have shown that albino pollen plants of rice do not possess mature chloroplasts but instead contain organelles closely resembling proplastids. Proplastids are thought to be progenitor organelles which can differentiate into a range of other plastid types including chloroplasts (Robertson and Laetsch, 1975; Thomson and Whatley, 1980). Similar ultrastructural studies in barley have shown that embryos formed from microspores possess proplastids (Mlodzianowski and Idzikowska, 1980) and that these organelles persist in light-grown albino plantlets (Clapham, 1973). In addition to the presence of proplastids, Clapham (1973) found in some sections of albino tissues 'chloroplasts of almost normal appearance with well developed grana and stromal thylakoids', although no comparison is given between the numbers and occurence of these organelles and proplastids in different albino plants. These observations suggest that, at least in some cases, the development of mature chloroplasts from proplastids may be blocked in albino pollen plants.

Early studies on the molecular biology of albino pollen plants were carried out by Sun *et al.* (1979) following their previous observations on the ultrastructure of rice albinos. These workers described the absence of the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) and also the 23S and 16S ribosomal RNA (rRNA) components of the plastid 70S ribosome in albino pollen plants of rice. The large subunit of Rubisco is known to be encoded by the plastid genome (ptDNA; Coen *et al.*, 1977) as are the 23S and 16S rRNAs (Bedbrook and Bogorad, 1976). Using the rationale that

lack of these plastid components in albino pollen plants could be due to lesions in the plastid genome Day and Ellis (1984, 1985; Ellis and Day, 1986) characterised deleted plastid genomes from wheat and barley pollen plants. This work is summarised in Section 1.4.

From the evidence summarised above it is apparent that any study of the molecular biology of albino pollen plants has to take account of (1) an apparent block to chloroplast development, (2) the absence of specific products of the plastid genome and (3), the possible presence of variant plastid genomes. In this context the following sections summarise aspects of the molecular biology of chloroplasts and their development in normal plants.

1.2. General Features of Chloroplast Development.

The plastids are a diverse group of organelles found in plant cells with different forms arising in a tissue specific manner via a number of alternative but interrelated pathways (Thomson and Whatley, 1980). Examples of the variety of plastid form and function include chromoplasts, which are responsible for the colours found in pigmented tissues such as flower petals and the pericarp of certain fruits, eg. tomato, amyloplasts which contain abundant starch deposits and chloroplasts which provide photosynthetic function in green tissues (Thomson and Whatley, 1980). Because of the biological importance of photosynthesis much effort has been made to characterise the events that define chloroplast maturation in a number of plant species.

In the leaves of cereals such as wheat and barley, cell division is confined to a basal meristem, cell age increasing with the distance from the leaf base. The presence of this developmental gradient in cereal leaves has enabled aspects of cell growth and differentiation, in particular the light induced development of chloroplasts, to be studied as a function of cell age. Cells at the base of developing light grown cereal leaves contain only proplastids, chloroplast development progressing with increasing cell age (Robertson and Laetsch, 1974; Boffey *et al.*, 1979). The progression of early chloroplast differentiation is marked by

increases in plastid size and number due to division and in the DNA and chlorophyll content of individual plastids in addition to an increase in the levels of plastid rRNA (Boffey *et al.*, 1979; Boffey *et al.*, 1980; Barkardottir *et al.*, 1987). The differentiation of chloroplasts from proplastids can also be correlated with increasing photosynthetic capacity in terms of CO_2 fixation and O_2 evolution (Robertson and Laetsch, 1974). The development of photosynthetic acitivity by chloroplasts can be related to the appearance of an extensive internal thylakoid membrane system (Robertson and Laetsch, 1974; Anderson, 1986). Inserted within the thylakoid membranes are several multisubunit protein complexes involved in various aspects of the photosynthetic process. These include photosystems I and II (PSI and PSII) and their associated pigment/protein light harvesting complexes, LHCI and LHCII, the cytochrome *b/f* complex and the thylakoid ATP synthase complex (reviewed by Anderson, 1986). These complexes play a central role in the capture of light energy, electron transport and proton translocation across the thylakoid membrane with the consequent generation of ATP and NADPH (Golbeck *et al.*, 1977). In addition to the thylakoid membrane complexes, chloroplasts contain numerous enzyme activities located in the stroma, including those involved in the photosynthetic carbon reduction cycle which relies on the ATP and reducing potential of NADPH provided by photosynthetic electron transport (Gibbs, 1971). One of these enzymes is ribulose 1,5-bisphosphate-carboxylase-oxygenase (Rubisco) which catalyses the assimilation of carbon from atmospheric CO_2 via the carboxylation of the compound, ribulose 1,5 bisphosphate (Gibbs, 1971). In addition to the development of photosynthetic capacity, mature chloroplasts are also able to perform a number of other biosynthetic functions, including the production of nucleic acids, amino acids, proteins, lipids, and pigments such as chlorophylls, phycobilins and carotenoids (reviewed by Goodwin, 1971).

Ultrastructural and biochemical studies have provided a detailed picture of the events that occur during chloroplast maturation. Additionally, studies at the nucleic acid and protein levels have led to a more detailed understanding of plastid genome structure, the features and regulation of plastid gene expression and the interaction of the plastid and nuclear genetic systems during chloroplast maturation.

1.3. The Chloroplast Genome.

1.3.1. Structure.

Following observations on the non-Mendelian inheritance of mutations affecting photosynthetic functions, Sager and Ishida (1963) characterised a DNA component from *Chlamydomonas* associated with chloroplasts. Further isolation and characterisation of chloroplast associated DNA components were simultaneously carried out with spinach, beet and *Chlorella* (Chun *et al.* 1963) and bean (Kirk, 1963). Since this early work, the chloroplasts of all plant species studied, from algae to angiosperms, have been found to contain a circular genome in multiple copies numbering up to many hundreds and commonly ranging in size from 120kb to over 200kb (Palmer, 1985). In most of the species of land plant studied the general structure of the ptDNA has been conserved and consists of a large inverted repeat (IR) ranging in size from 10-76kb, but being commonly 22-26kb, separating a small single copy region (SSC) from a large single copy region (LSC), commonly in the order of 20 and 80kb respectively (Palmer, 1985). The ptDNA of barley is representative of this structure and is shown in Figure 2.1. Exceptions to this general pattern are characterised by the absence of the inverted repeat and include the legumes pea (Kolodner and Tewari, 1979), broad bean (Koller and Delius, 1980) and the alga *Euglena* (Gray and Hallick, 1978).

The loss of the inverted repeat structure in pea and broad bean is thought to have led to further divergence in the arrangement of their ptDNAs relative to other angiosperm species (Palmer and Thompson, 1982). The gene order in most land plants is highly conserved (Palmer, 1985) and differences, when they do occur, can be accounted for most commonly by simple patterns of inversion (eg. Howe, 1985). However, in pea and broad bean much more complex patterns of multiple inversions have produced a gene order that is very different from that found in other angiosperm species (Palmer *et al.*, 1988). In *Euglena* evidence for substantial modifications in the ptDNA comes from studies on the arrangement of the rRNA (*rrn*) operon. In most other plant species the rRNA operon lies entirely within the inverted repeat, and therefore two copies exist in inverted orientation in each genome. Some strains of *Euglena*

possess three tandemly arranged copies of the *rrn* operon plus an additional copy of the 16S rRNA gene. Evidence for additional variation comes from studies of a strain that possesses five tandem copies of the *rrn* operon plus two additional 16S rRNA genes and another with a single *rrn* operon. This diversity may represent recombinogenic activity within and surrounding the *rrn* operon in *Euglena* (Flamant *et al.*, 1984; Ravel-Chapuis *et al.*, 1984 and references cited therein).

1.3.2. Replication.

Studies of ptDNA replication have been carried out with a number of plant species, eg. pea (Bennett and Radcliffe, 1975) and wheat (Boffey and Leech, 1982). In developing wheat leaves for instance, ptDNA is initially replicated to maintain a constant copy number per plastid as the total number of plastids increases with cell age (Boffey and Leech, 1982). DNA polymerases have been purified from the plastids of pea (McKown and Tewari, 1984) and maize (Carrillo and Bogorad, 1988). Evidence from albino mutants of barley and *Pelargonium*, which fail to synthesise plastid proteins but accumulate ptDNA to normal levels, suggests that these proteins may be encoded by the nuclear genome (Steele-Scott *et al.*, 1982; Hagemannn and Borner, 1978).

Models for the replication of ptDNA molecules have been proposed by Kolodner and Tewari (1975a, 1975b). Using electron microscopy these workers defined regions of pea and corn ptDNA that form displacement loops (D-loops), representing initiation points for replication, approximately 7kb apart on opposite strands of the plastid genome. These D-loops were observed to migrate toward one another and fuse to form a Cairns type replicative intermediate. Once replication of this intermediate had been completed rolling circle replication was seen to initiate from the sites at which Cairns replication had terminated. The authors suggest that the Cairns mechanism may represent the normal pathway for ptDNA replication, while the rolling circle mode may allow rapid increase in ptDNA copy number. Several studies have located regions on the ptDNAs of pea (Kolodner and Tewari, 1975b), *Euglena* (Koller and Delius, 1982) and *Petunia* (de Haas *et al.*, 1986) that may have a

role as replication origins. Mapping of a putative ptDNA replication origin from *Euglena* has been carried out by Koller and Delius (1982). These workers isolated ptDNA from cell-cycle synchronised *Euglena* cultures, cut the ptDNA with restriction enzymes at defined sites on the plastid genome and noted the position of D-loop formation relative to these sites. In addition, cloned regions of *Petunia* ptDNA show sequence homology with a putative replication origin from *Euglena* ptDNA (de Haas *et al.*, 1986). In maize certain regions of the ptDNA are seen to act as preferred templates for the activity of purified maize plastid DNA polymerase raising the possibility that they could be candidates for replication origins *in vivo* (Carillo and Bogorad, 1988). Finally, in wheat and barley, Day and Ellis (1984, 1985) observe that a region of the ptDNA close to one copy of the inverted repeat is always maintained in the deleted ptDNAs found in albino pollen plants (see Section 1.4) which suggests that it could contain a cereal ptDNA origin of replication.

1.3.3. Coding Capacity of the Plastid Genome.

Plastids possess their own transcription and translation systems (Whitfield and Bottomley, 1982; Mullet, 1988) and a large number of genes encoding proteins, rRNAs and tRNAs have been localised on the ptDNAs of a variety of plant species (Whitfield and Bottomley, 1983; Dyer, 1984; Crouse *et al.*, 1985). Nucleotide sequencing of the entire plastid genomes of tobacco and the liverwort, *Marchantia polymorpha* (Shinozaki *et al.*, 1986; Ohyama *et al.*, 1986) has allowed the identity and overall organisation of the majority of plastid encoded genes (approximately 120) to be determined.

The plastid genome encodes all rRNA components of the plastid 70S ribosomes, namely the 5S, 16S and 23S rRNAs. In species with an inverted repeat, the rRNA genes, which are arranged as an operon (*rrn* operon) within this region, are represented twice per genome (see Section 1.3.1 and Figure 5.1). The *rrn* operon from a number of plant species have been well characterised in terms of internal organisation and sequence (see Whitfield and Bottomley, 1983 for review) and it has been demonstrated that the plastid *rrn* operon and its rRNA products bear close resemblance to those of

prokaryotes such as *E. coli*. In higher plants, the rRNA genes (rDNA) are arranged in the order 5'-16S rDNA, spacer, 23S rDNA, spacer, 5S rDNA-3'. The spacer between the 16S and 23S rDNAs has been shown to contain genes encoding tRNAs for isoleucine and alanine and in some species, eg. maize, these genes contain introns (Koch *et al.*, 1981). In addition, the leader sequence 5' to the 16S rDNA in tobacco and maize has been shown to contain a tRNA gene for valine (see Whitfield and Bottomley, 1983). The *rrn* operon is transcribed as a single long precursor which subsequently undergoes processing to generate the individual plastid rRNA species (reviewed by Grierson, 1982).

Sequencing and mapping studies have revealed that ptDNA probably encodes all the tRNA species necessary to permit translation of plastid codons (Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986; Whitfield and Bottomley, 1983). In addition, plastid tRNAs have high sequence homology with prokaryotic tRNAs such as those from *E. coli*. However, in some respects plastid tRNAs do differ from prokaryotic tRNAs in that their genes lack a 3' terminal CAA and many contain intron sequences (Whitfield and Bottomley, 1983).

A large number of protein coding genes have been located on the plastid genome (Crouse *et al.*, 1985; Whitfield and Bottomley, 1983; Shinozaki *et al.*, 1986; Ohyama *et al.*, 1986). These include genes encoding stromal proteins such as the Ribisco large subunit (RbcL) and components of the chloroplast thylakoid membrane protein complexes PSI and PSII, the cytochrome *b/f* complex and ATP synthase complex (see Section 1.2). In addition, sequencing studies have revealed the presence of open reading frames homologous to human mitochondrial NADH-dehydrogenase components, bacterial 4Fe-4S type ferredoxin and components of bacterial nucleotide driven transport systems (Ohyama *et al.*, 1988).

Components of the plastid transcription-translation machinery are also encoded by ptDNA. Genes encoding about a third of the 60 or so ribosomal proteins (Capel and Bourque, 1982) and those encoding translation initiation factors have been located on the plastid genome (Ohyama *et al.*, 1986); Shinozaki *et al.*, 1986; Sijben-Muller *et al.*, 1986). Open reading frames bearing homology to the *E.coli* DNA dependent RNA polymerase α, β and β' subunits have also been

located on ptDNA (Sijben-Muller *et al.*, 1986; Ohme *et al.*, 1986; Cozens and Walker, 1986; Ohyama *et al.*, 1986; Shinozaki *et al.*, 1986).

1.3.4. Features of the Plastid Genetic Machinery.

Isolation of plastid genes has allowed analysis not only of their coding regions but also of the sequences believed to be important in the control of transcription and translation. Sequence analysis and mutagenesis prior to *in vitro* transcription studies in heterologous and homologous sytems have identified regions 5' to a number of chloroplast genes that bear strong structural and functional homology to bacterial promoters (Zech *et al.*, 1981; Gruissem and Zurawski, 1985a, 1985b; Bradley and Gatenby, 1985). The basic features of prokaryotic promoters are also conserved in many plastid promoters, namely in the consensus sequences approximately -10 and -35 in relation to the transcription start site (reviewed by Kung and Lin, 1985). Transcription termination in plastids is also believed to follow a prokaryotic pattern (Whitfield and Bottomley, 1983). Inverted repeat sequences located 3' to a number of plastid genes have been found to have the potential to produce hairpin loop structures that are known to be involved in prokaryotic transcription termination (Rosenberg and Court, 1979) and functional studies have established that plastid RNA polymerase is capable of recognising bacterial termination signals (Chen and Orozco, 1988). However, more detailed examination of the 3' inverted regions in plastid genes has indicated that they may have a limited function in transcription termination but may instead play a role in RNA processing and stabilisation (Stern and Gruissem, 1987).

The patterns of transcription from plastid genes are complex, some genes being transcribed monocistronically, eg. *rbcL* (McIntosh *et al.*, 1980) while others are transcribed in a polycistronic fashion, eg. *psbD-psbC* (Berends *et al.*, 1987) and the *psbB* gene cluster (Westhoff and Herrmann, 1988). Both mono- and polycistronic plastid transcripts may undergo a variety of modification events. These include processing at the 5' end (Mullet *et al.*, 1985, Poulsen, 1985), processing at the 3' end (Stern and Gruissem, 1987),

endonucleolytic cleavage of polycistronic precursor RNAs (Westhoff and Herrmann, 1988), removal of intron sequences by *cis* splicing mechanisms (Westhoff and Herrmann, 1988) and joining of non-adjacent exons by *trans* splicing pathways (Choquet *et al.*, 1987; Koller *et al.*, 1987; Zaita *et al*, 1987).

Many features of the plastid translation apparatus are also prokaryotic in nature. Some, but not all, plastid genes possess sequences located at the 5' region which bear homology with bacterial ribosome binding sites, the so-called Shine-Dalgarno sequence (Whitfield and Bottomley, 1983). Shine-Dalgarno sequences are thought to bind to a specific region at the 3' end of the bacterial 16S rRNA during the initiation of translation (Shine and Delgarno, 1975) and it has been demonstrated that these features are conserved in plastid 16S rRNAs (Whitfield and Bottomley, 1983). As mentioned in Section 1.3.3, sequence analyses have demonstrated the general similarity between the plastid rRNA and tRNAs and those found in *E. coli* (reviewed by Whitfield and Bottomley, 1983). Many of the protein components of the plastid ribosome are also homologous to those found in prokaryotes and the plastid genes encoding some of these proteins are often arranged in a similar manner to the ribosomal protein operons found in *E. coli* (Tanaka *et al.*, 1986).

1.3.5. Regulatory Aspects of Plastid Gene Expression.

Current knowledge of the regulation of plastid gene expression during chloroplast development has been recently reviewed by Mullet (1988) and van Grinsven and Kool (1988).

The mechanisms by which plastid gene expression is regulated during chloroplast development appear to be varied and complex, however, recent studies have revealed a number of possible governing principles. Regulation of plastid gene expression could be achieved by altering the levels of template ptDNA but in developing spinach chloroplasts, overall transcriptional activity of the plastid genes appears to be independent of ptDNA abundance (Deng and Gruissem, 1987). In contrast, changes in the abundance of two plastid transcripts, 16S rRNA and *rbcL* mRNA, have been shown to parallel

changes in the level of ptDNA during the conversion of amyloplasts to chloroplasts in spinach cell suspensions (Aguettaz *et al.*, 1987). This suggests that in some systems changes in the ptDNA template levels may have a role in regulating the expression of plastid genes. In addition to the possible effects of template abundance, a number of *in vitro* studies have shown that the conformation of ptDNA in terms of its superhelical density may have a role in determining the levels of plastid gene expression (Stirdivant *et al.*, 1985; Lam and Chua, 1987; Thompson and Mosig, 1987).

Transcripts from individual plastid genes often accumulate with different kinetics and to different levels during chloroplast development (Rodermel and Bogorad, 1985; Deng and Gruissem, 1987). Functional analyses *in vitro* have shown that the relative strength of plastid promoters can very considerably (Gruissem and Zarawski, 1985b). However, differences in plastid promoter strength may not be the primary mechanism by which differential expression of plastid genes is achieved. The overall transcription of the plastid genome is known to be stimulated by light (Deng and Gruissem, 1987) which can be correlated with an increase in the activity of the plastid RNA polymerase (Apel and Bogorad, 1976). However, under these conditions the relative transcriptional activities of individual plastid genes is the same and furthermore, these relative transcriptional activities do not reflect the different steady state levels of the corresponding RNAs. This evidence suggests that post-transcriptional control of plastid transcript levels, possibly exerted at the level of transcript stability (Mullet and Klein, 1987; Stern and Gruissem, 1987; Deng and Gruissem, 1988; Mullet, 1988), is an important factor in plastid gene expression.

The accumulation of plastid encoded proteins may be regulated by mechanisms that are independent of the abundance of the corresponding mRNAs. In barley, PSI and PSII chlorophyll-binding apoproteins accumulate in a light induced fashion which is not accompanied by an increase in the abundance of the mRNAs that encode them (Mullet, 1988). Furthermore, the mRNAs for these proteins are present at significant levels in the dark whereas their translation products fail to accumulate or do so at low

levels under the same conditions. Finally, illumination of dark grown barley seedlings has been shown to increase the recruitment of specific transcripts into chloroplast polysomes suggesting a light induced stimulation of translation initiation (Klein *et al.*, 1988). Control of plastid encoded protein levels may also be exerted at the level of protein stability and/or turnover. An example of this comes from work with mutants of *Chlamydomonas* which are deficient in components of the PSII complex. These mutants, which are unable to assemble stable PSII complexes, fail to accumulate other PSII subunits possibly due to enhanced degradation of unassembled proteins (Bennoun *et al.*, 1986; Erickson *et al.*, 1986).

1.3.6. Nuclear Gene Expression During Chloroplast Biogenesis.

The plastid genome has insufficient coding capacity to enable production of all the proteins found in mature chloroplasts (Shinozaki *et al.*, 1986). For this reason the light induced development of mature chloroplasts relies on the expression of both plastid and nuclear genes. Many of the protein complexes found in chloroplasts such as those located in the thylakoid membrane, eg. PSI and PSII, are assembled from the products of both nuclear and plastid genomes (Dyer, 1984; Anderson, 1986). This is also true for many stromal polypeptides such as Rubisco whose large (RbcL) and small (RbcS) subunits are coded by the plastid and nuclear genomes, respectively. RbcS and Cab (chlorophyll a/b binding protein of LHCI and II) will be used as examples to describe aspects of expression of chloroplast proteins encoded by the nuclear genome with an emphasis on work carried out with barley, the subject of the present study.

The RbcS and Cab polypeptides are synthesised on cytoplasmic 80S ribosomes as precursor polypeptides, pre-RbcS and pre-Cab. These precursors, which possess N-terminal transit peptides, are targeted to the chloroplast envelope and subsequently undergo ATP-driven, translation independent transport into the organelle (Cashmore *et al.*, 1985). In the case of RbcS, localisation of the precursor to the plastid stroma is accompanied by a two step process that results in the removal of the transit peptide sequences by a

soluble protease generating the mature RbcS polypeptide (Highfield and Ellis, 1978; Smith and Ellis, 1979; Robinson and Ellis, 1984). The mature RbcS then assembles with the plastid synthesised RbcL to produce the functional Rubisco holoenzyme composed of eight small and eight large subunits. Once localised in the plastid stroma, the pre-Cab polypeptide is inserted into the thylakoid membrane, a process which is dependent on the white light induced synthesis of chlorophyll a (Apel and Kloppstech, 1980). Here it undergoes protease mediated cleavage to the mature size by removal of the transit peptide (Schmidt *et al.*, 1981; Chitnis *et al.*, 1988).

The RbcS and Cab polypeptides are known to be encoded by nuclear multigene families in a number of plant species including barley (Barkardottir *et al.*, 1987; see also Chapter 7). Regulation of the expression of these genes has been shown to be operate under the influence of both environmental, ie. light (see review by Tobin and Silverthorne) and developmental factors (Simpson *et al.*, 1986a). In barley the *rbcS* mRNA has been reported to be as abundant in dark grown leaves as it is in those grown in the dark (Batschauer *et al.*, 1986). In contrast, Barkardottir *et al.*, (1987), report a 70% reduction in *rbcS* mRNA abundance in dark grown leaves relative to those grown in the light. Independent cDNA clones were used as hybridisation probes in these experiments and it is possible that they represent different members of the barley *rbcS* gene family. The discrepancy in the patterns of *rbcS* mRNA abundance could therefore be explained if two members of the *rbcS* gene family were regulated differentially, the expression of one being light independent and the other partly light dependent. Evidence for the differential expression of individual *rbcS* genes has been reported for *Petunia* and pea (Dean *et al.*, 1985b; Fluhr *et al.*, 1986). In addition it has been shown that some *rbcS* genes exhibit differential responses to light in developing pea leaves (Fluhr *et al.*, 1986).

In barley, the accumulation of *cab* mRNA is greatly stimulated by white light, the levels of *cab* transcript being very low in the dark (Apel and Kloppstech, 1978; Barkardottir *et al.*, 1987). The phytochrome pigment system has been shown to play a central role in the photoregulation of *cab* gene expression in barley. Etiolated barley seedlings given a pulse of red light stimulate a large

increase in the transcription of *cab* genes and this increase can be reversed by a period of illumination with far red light (Apel, 1979; Gollmer and Apel, 1979; Mosinger *et al.*, 1985).

The expression of genes encoding both *rbcS* and *cab* has been shown to be tissue specific, maximum levels of expression for both genes being seen in leaves, while in non-photosynthetic tissues such as roots the levels of *rbcS* and *cab* expression are very low or undetectable (Simpson *et al.*, 1986a; Barkardottir *et al.*, 1987). From this, it has been concluded that the level of chloroplast development is strongly correlated with the expression of these genes (Simpson *et al.*, 1986a; see Section 1.3.7). In growing barley leaves the abundance of both *rbcS* and *cab* mRNA*s* increase as a function of cell age and advancing chloroplast development (Barkardottir *et al.*, 1986; Viro and Kloppstech, 1980; see also Section 1.2). This increase in transcript abundance is paralled by a similar increase in the level of the RbcS and Cab polypeptides (Viro and Kloppstech, 1980). Detailed functional analyses of DNA sequences 5' to *rbcS* and *cab* genes from a number of plant species have identified regions which specify the light regulated and tissue specific patterns of expression described above (Morelli *et al.*, 1985; Simpson *et al.*, 1985; Simpson *et al.*, 1986b; Nagy *et al.*, 1986; Nagy *et al.*, 1987; Kuhlemeier *et al.*, 1987) and recent studies have identified some of the *trans*-acting factors which may interact with these regions (Green *et al.*, 1987).

1.3.7. The Coordination of Nuclear and Chloroplast Gene Expression.

The assembly of chloroplast multisubunit protein complexes such as PSII involves the expression of genes located on both the nuclear and plastid genomes. The presence of such protein complexes containing fixed stoichiometric amounts of individual subunits suggests that a high degree of co-ordination between the nuclear and plastid genetic systems is necessary during chloroplast biogenesis. An example of this is the tightly coordinated appearance of both the small and large subunits of Rubisco in developing wheat leaves (Dean and Leech, 1982). Another illustration of the requirement for co-ordination comes from the fact that plastid genes are present at a far higher copy number

compared to those encoded by nuclear DNA. For instance, the copy number of the *rbc*L gene in a cereal leaf may reach up to 1000 per plastid in mesophyll cells (Boffey and Leech, 1982) while the number of genes in the barley *rbc*S gene family may be less than ten (Barkardottir *et al.*, 1987; see also Chapter 7). Clearly, mechanisms must exist to compensate for the increased dosage of plastid genes and so ensure that plastid gene products do not accumulate in excess when compared to imported nuclear proteins. The fundamental mechanisms by which such co-ordination is achieved are probably complex and may operate at a number of levels during the transcription, translation and assembly of gene products from both plastid and nuclear genomes.

The transcription of specific plastid (Poulsen, 1983; Tobin and Silverthorne, 1985; Rodermel and Bogorad, 1985) and nuclear genes encoding chloroplast polypeptides (reviewed by Tobin and Silverthorne, 1985) can be enhanced by light. A general stimulation of the transcriptional activity of plastid DNA by light has been reported in spinach (Deng and Gruissem, 1987) and evidence from maize suggests this stimulation of plastid transcription may be correlated with the light induced increase of plastid RNA polymerase activity (Apel and Bogorad, 1976). This increase in activity is not accompanied by any quantitative or qualitative changes in the RNA polymerase itself suggesting the involvement of other factors in the response. However, the identity and origin of these factors, whether they are plastid or nuclear derived, is not known. If the latter were true, then it may serve as a mechanism by which the nucleus could exert a limiting influence on plastid transcription and so allow the coordination of transcription from both compartments. The site of synthesis for the plastid RNA polymerase is thought to be the nucleus (Lerbs *et al.*, 1985). Regulation of plastid transcription could be achieved directly by limiting the availablity of nuclear encoded RNA polymerase subunits. However, it should be noted that genes encoding specific subunits of this enzyme have been located on the plastid genome (Shinozaki *et al.*, 1986). If these genes are active then the plastid may be an alternative site for the synthesis of the RNA polymerase subunits and as such may have a contributory role in the assembly of the active enzyme.

Nuclear gene products may play a part in regulating plastid gene expression at many levels other than transcription. Nuclear mutants of *Chlamydomonas* have been found to be deficient in specific *trans* splicing reactions involved in *psaA* mRNA maturation (Choquet *et al.*, 1988) and the nuclear maize mutant *hcf*38* has been shown to accumulate plastid RNA splicing intermediates (Barkan *et al.*, 1986). This evidence suggests that plastid RNA processing reactions (see Section 1.3.6) could be under the control of proteins encoded by the nucleus. Any regulation of plastid RNA processing could have a direct effect on plastid gene expression. For example, it is known that unspliced plastid mRNAs encoding *petB* and *petD* are not translated into protein (Barkan, 1988). Control at the level of translation could be achieved directly during chloroplast biogenesis by limiting the availability of nuclear encoded ribosomal proteins or by providing factors necessary for the light induced increase in the translation of specific plastid mRNAs (see Mullet, 1988). Nuclear gene products may also exert control over the accumulation of plastid encoded proteins at the post-translational level. Mutants of *Chlamydomonas* deficient in nuclear encoded PSII proteins also fail to accumulate plastid encoded core proteins of PSII (Mayfield *et al.*, 1987). This effect is thought to be mediated by the decreased stability of PSII complexes and the increased turnover of the plastid encoded PSII subunits. In addition, nuclear mutants of *Chlamydomonas* have been shown to have specific effects on the accumulation of the plastid encoded D2 polypeptide of PSII at the level of synthesis and/or degradation (Kuchka *et al.*, 1988).

Clearly, the nucleus may have a major role in achieving coordinated expression of plastid and nuclear genes during chloroplast biogenesis. However, a body of recent evidence suggests that a plastid derived factor (or factors) may also be involved in regulating the expression of nuclear encoded chloroplast polypeptides. Initial work by Bradbeer *et al.*, (1979) using plastid ribosome-deficient rye plants suggested that RNA exported from the plastid into the cytoplasm may regulate nuclear gene expression. However, no passage of RNA across the plastid envelope into the cytoplasm has since been demonstrated. Chloroplast development is thought to play an important part in regulating the expression of nuclear encoded chloroplast proteins in green, light-grown plant

tissues. Evidence for this comes from the observation that genes such as *rbcS* are only expressed in tissues such as leaves, and fail to be expressed in non-photosynthetic tissues such as roots (Simpson *et al.*, 1986a). Further evidence for the control of nuclear gene expression by the plastid compartment has come from the study of plants blocked in chloroplast development due to herbicide treatment or mutation.

Mutant maize plants deficient in carotenoid biosynthesis undergo a block in normal chloroplast development when exposed to high light intensities (Bachmann *et al.*, 1969). Carotenoids normally protect chlorophyll from photo-oxidation under these conditions (Anderson and Robertson, 1960). In mutant maize plants lacking carotenoids photo-oxidative damage to the plastids occurs. This block to chloroplast development correlates with the reduced abundance of mRNAs for the nuclear encoded chloroplast proteins RbcS and Cab but not enzymes located in the cytosol such as phosphoenolpyruvate carboxylase (Mayfield and Taylor, 1984; Mayfield and Taylor, 1987). Similar observations are made when carotenoid biosynthesis is blocked by the herbicide Norfluorazon (NF) which, again, leads to photo-oxidative destruction of the chloroplast compartment (Mayfield and Taylor, 1987). Reduced amounts of the translatable mRNAs encoding RbcS and Cab and the corresponding proteins are also found in mustard seedlings treated with NF and grown under high light intensities (Oelmuller and Mohr, 1986). Other studies indicate that the levels of specific mitochondrial and glyoxisomal enzymes are not affected by photooxidative destruction of the chloroplast compartment (Reiss *et al.*, 1983). In barley seedlings *cab* mRNA also accumulates at reduced levels when chloroplast development is blocked by photo-oxidation (Batshauer *et al.*, 1986). Interestingly however, unlike maize and mustard, the levels of barley *rbcS* mRNA are not adversely affected by this treatment suggesting that different factors may influence the expression of *rbcS* in this species. Nuclear run-off transcription assays indicate that the reduced accumulation of *cab* mRNA is due to an inability to enhance the rate of *cab* transcription when barley chloroplast development is blocked by photo-oxidation. Similar experiments have shown that reduced transcription of the *cab* and *rbcS* gene families is responsible for the observed low steady-state levels of their corresponding mRNA in the carotenoid deficient 'ghost' mutant of

tomato (Giulliano and Scolnik, 1988). Taken together, these results suggest the presence of a chloroplast derived factor which can specifically influence the expression of nuclear genes encoding chloroplast polypeptides at the transcriptional level. However, the identity of this putative chloroplast factor remains unknown.

1.4. Molecular Studies of Albino Pollen Plants.

Some of the preliminary studies to characterise albino pollen plants from various species are described in Section 1.1.4. In the following sections the molecular analyses that have been carried out with albino wheat and barley pollen plants are summarised in more detail (Day and Ellis, 1984; Day and Ellis, 1985; Day, 1985; Ellis and Day, 1986).

Investigations of the molecular biology of albino cereal pollen plants has centered on the structure of the plastid genome. The rationale for this came from the observation that albino pollen plants of rice lack major plastid encoded gene products, namely the large subunit of Rubisco and the 23S and 16S rRNAs, one explanation being that this could be due to the presence of defective plastid genomes in these individuals. Southern analysis of digests of total DNA extracted from individual albino wheat and barley pollen plants probed with a series of cloned ptDNA fragments revealed the presence of variant forms of the plastid genome (Day and Ellis, 1984; Day and Ellis, 1985). Although restriction fragments of wild-type size, ie. those expected from the map of intact plastid genome, were detected in albino plants, fragments of altered size were also seen to hybridise to specific ptDNA probes. In addition some probes failed to hybridise to any detectable restriction fragments in digests of DNA from some albino plants. These observations suggested that albino pollen plants contain ptDNAs that have undergone deletion of large tracts of DNA sequence. For simplicity, the variant forms of the plastid genome found in albino pollen plants will be subsequently refered to as ΔptDNAs.

The majority of the albino wheat and barley pollen plants studied appeared to contain heterogeneous populations of ΔptDNAs, as demonstrated by the hybridisation of wild type and non-wild type

restriction fragments to individual ptDNA probes, often at substoichiometric levels. The hybridisation of altered and unaltered ptDNA fragments at high intensity in certain plants suggested that some variant forms of the plastid genome may predominate in these individuals. The assignment of map positions to altered ptDNA fragments in albinos was facilitated by the use of probe DNA fragments with defined positions on the intact barley and wheat plastid genomes (Day and Ellis, 1984; Day and Ellis, 1985). On the basis of these mapping experiments, structures were proposed for circular ΔptDNAs in several albino wheat plants (eg. see Figure 1.2A). In the examples given, the circular molecules appear to be generated by a deletion event fusing two widely separated ptDNA fragments with the removal of all intervening sequences. In one case the deletion events were found to have removed 80% of all wild type ptDNA sequences. Interestingly, some barley pollen plants appeared to contain intact ptDNAs (Day and Ellis, 1985), suggesting that albinism is not necessarily correlated with the presence of ΔptDNAs. Further studies demonstrated that, in addition to circular ptDNA molecules, linear molecules showing homology to ptDNA are also present in albino barley pollen plants (Day and Ellis, 1985). These linear ΔptDNAs were found to range in size from approximately 7-80kb, in some cases forming a series of dimers and higher oligomers of a linear ptDNA monomer. Characterisation of one such monomeric linear ΔptDNA demonstrated that it consisted of an inverted repeat arranged in a hairpin conformation (Ellis and Day, 1986; see Figure 1.2B).

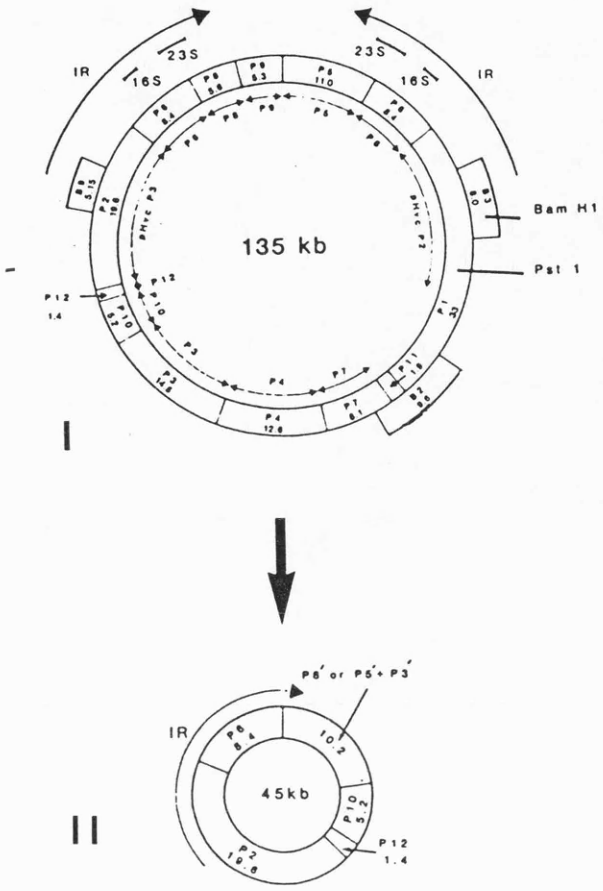
All the pollen plants described in the above studies appeared to retain a specific region of ptDNA close to one inverted repeat. This region is retained in both the circular and linear ptDNAs found in albino pollen plants (Day and Ellis, 1985; Ellis and Day, 1986) and may delimit a DNA sequence necessary for the replication of the plastid genome (see Section 1.3.2). The approximate location of this sequence on the map of barley ptDNA is shown in Figures 5.2 and 5.3.

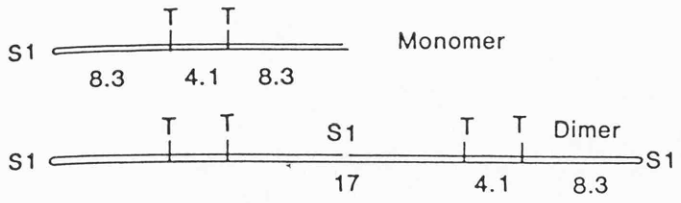
Two possible mechanisms which may be responsible for the generation of circular ΔptDNAs are shown in Figure 1.3. These pathways rely on recombination between short stretches of repeated DNA sequence in direct orientation to each other located in different regions of

Figure 1.2. Examples of Variant Plastid Genomes Found in Albino Pollen Plants.

Panel A: (I) Restriction map of the intact wheat plastid genome (from Day and Ellis, 1984). The position and size, in kb, of specific *Pst* I and *Bam*H I restriction fragments are shown. The inner circles delimit restriction fragments used in the ptDNA Southern analyses of deleted ptDNAs (Day and Ellis, 1984). The long outer arrows indicate the position of large inverted repeat sequences and for reference the locations of the 16S and 23S rRNA genes are shown within this region. (II) Restriction map of a deleted wheat ptDNA (from Day and Ellis, 1984). As in (I), the position and size, in kb, of the remaining *Pst* I derived restriction fragments are shown. Deleted derivatives of *Pst* I restriction fragments are indicated by ' and are numbered according to the origin of sequences present in these fragments. The altered fragment marked (P8' or P5' + P3') is derived from the intact P3 and the intact P8 or P5 fragments shown in (I). The remaining inverted repeat sequences are indicated by a large arrow.

Panel B: Structure of linear hairpin ptDNAs characterised from an albino barley pollen plant (from Ellis and Day, 1986). Restriction sites for *Sst* I are indicated (T) as are sites sensitive to single-strand specific S1-nuclease (S1). The size of restriction fragments are given in kb. The maps of both a monomer and a dimer of the same molecule are shown.

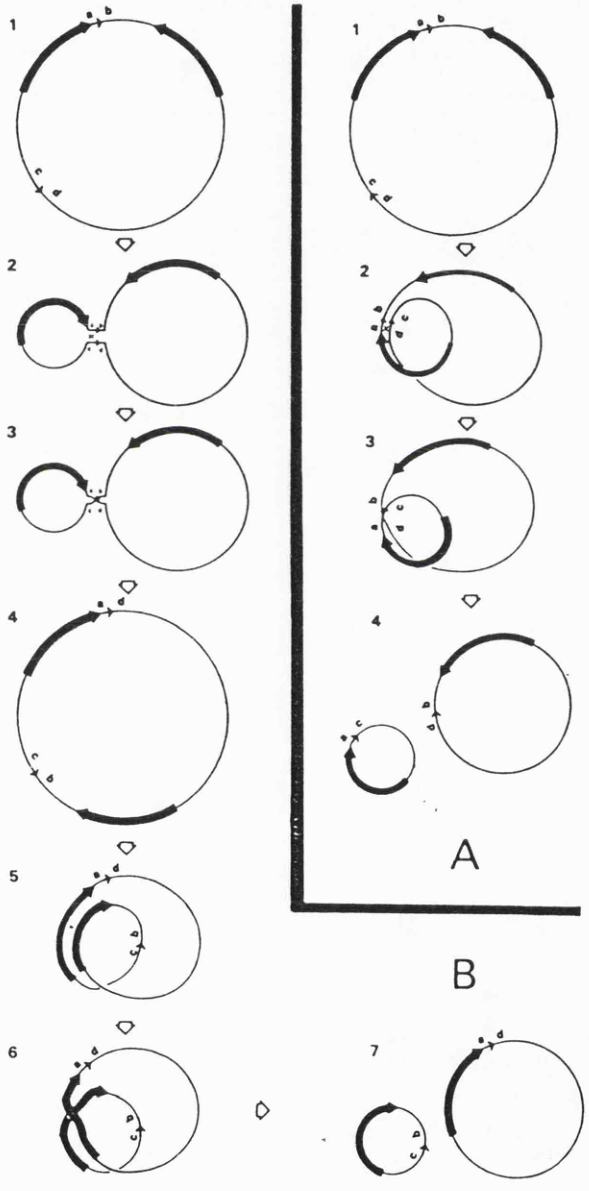

IR
23S
16S
135 kb
Bam H1
Pst 1
I
II
45kb
IR
P12
1.4
A


S1
T
T
8.3
4.1
8.3
Monomer
S1
17
4.1
8.3
Dimer

B

Figure 1.3. Production of Deleted Circular ptDNA Molecules by Recombination.

A single recombination event is shown between two hypothetical small repeats (→) flanked by small single copy sequences (a) and (b), and large copy sequences (c) and (d). In (A) the short repeat sequences are present in direct orientation with respect to each other. In (B) the short repeat sequences are inversely orientated. The location of the large inverted repeat are marked by heavy arrows. In (B) two recombination events, one involving the hypothetical short inverted repeats (steps 1-4) and the other involving the large inverted repeat region of ptDNA (steps 5-7), are required to produce smaller deleted forms of ptDNA (from Day and Ellis, 1984).


1
2
3
4
5
6
7
A
B

ptDNA. Recombination between such direct repeats will lead to the formation of two circular DNA molecules. If one of these molecules lacks sequences necessary for its replication in the plastid then it may be lost. Alternatively, the two circular molecules could be separated due to segregation into different cell lineages. This would generate cell lines with two different circular ΔptDNAs derived from the same recombination event (Day and Ellis, 1984). Although the schemes in Figure 1.3 describe recombination events between sequences in the single copy regions or between the inverted repeats, the proposed structures of some of the circular ΔptDNAs described by Day and Ellis (1984) appear to rely on recombination events between single copy and inverted repeat sequences.

The recombination events outlined above rely on the presence of short repeated sequences and there is evidence that such sequences do exist in ptDNA from a number of plant species (Palmer, 1985). For example, 4-7% of the *Chlamydomonas* plastid genome is composed of dispersed repeats, ranging in size from 100-300bp (Gelvin and Howell, 1979). Hybridisation studies have revealed the presence of small dispersed repeats in the wheat plastid genome (Bowman and Dyer, 1986). One such repeat, 70bp in length and originally present in inverted orientation, has been implicated in the generation of a large sequence inversion known to have occurred during the evolution of the wheat plastid genome (Howe, 1985). Furthermore, another independent inversion has left the three widely spaced copies of this repeat in direct orientation, thus providing the potential for a recombination event leading to deletion of the intervening regions. Homologies between related sequences in the numerous tRNA genes encoded by the plastid genome (Shinozaki *et al.*, 1986) may also provide the potential for recombination-mediated deletion of ptDNA sequences. Such homologies have been implicated as being responsible for specific rearrangements in the plastid genome of *Euglena* (El-Gewley *et al.*, 1984). Studies with *E. coli* have revealed that dispersed DNA sequence homologies of less than 8bp can mediate spontaneous DNA deletion events (Albertini *et al.*, 1982). If sequences like this are present in the ptDNA of cereals such as wheat and barley then they might provide further potential for recombination events that lead to the formation of circular ΔptDNAs.

Although relatively simple events may be responsible for the production of circular ΔptDNAs, the mechanism by which linear ΔptDNAs may be generated in albino pollen plants is unclear, although several possible pathways have been described (Ellis and Day, 1986), one of which is illustrated in Figure 1.4. This process relies on a strand-switching mechanism taking place during replicative migration of a D-loop during the initiation of Cairns-type ptDNA replication (see Section 1.3.2). A linear ΔptDNA with an apparent hairpin conformation similar to that shown in Figure 1.4(vii) has been characterised from an albino barley pollen plant (Ellis and Day, 1986 and Figure 1.2B).

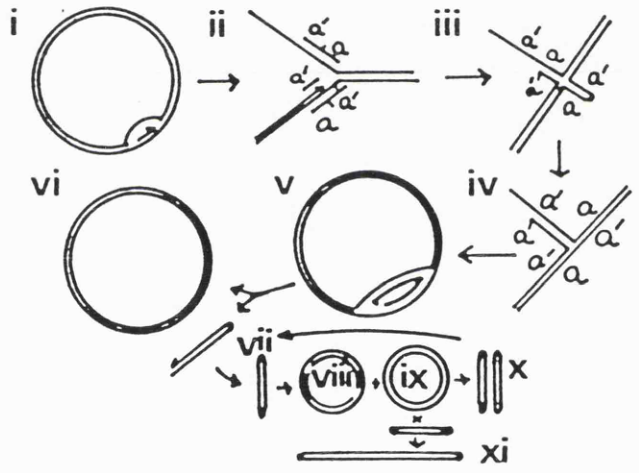
1.5. Other Examples of Deleted Organelle Genomes.

Deleted organelle genomes have been characterised in the chloroplasts of algae and the mitochondrial genomes of fungi and higher plants. Haploid cultures of the unicellular green alga *Chlamydomonas reinhardtii* grown in media containing the thymidine analogue 5-fluorodeoxyuridine (FdUrd) often generate non-photosynthetic, acetate-requiring mutants. The same observation is made when wild type diploid cells are subjected to X-ray treatment in addition to growth on FdUrd (Myers *et al.*, 1982). Mutant strains derived by growth with FdUrd alone have been shown to contain deleted plastid genomes, while in addition to deletions, those generated with a further X-ray treatment have undergone DNA sequence inversions and duplications. Most of the alterations extended symetrically into the inverted repeat regions and often included changes in the structure of restriction fragments encompassing the *rrn* operons. In contrast, one mutant strain was shown to have lost almost all sequences in one copy of the inverted repeat and consequently an entire *rrn* operon. Restriction mapping analyses of bleached, non-photosynthetic mutant strains of *Euglena gracilis* have revealed the presence of deleted ptDNAs (Heizmann *et al.*, 1981). Interestingly, the retained regions of these deleted genomes preferentially included the *rrn* operon, correlating with the presence of low levels of plastid rRNAs.

In addition to reports of altered ptDNAs, deletions have also been found in the mitochondrial DNA of various organisms. Petite strains

Figure 1.4. Possible Mechanism for the Generation of Linear ptDNAs.

(i) Replication is initiated and strand switching of DNA synthesis occurs (ii-iv). This is represented as being due to the looping out of the a-a' hairpin but is not necessarily the mechanism by which strand switching takes place. (v) The whole structure after DNA synthesis has proceeded. (vi) The hairpin molecule is displaced to generate linear and circular structures. (vii-x) The linear molecule can replicate after the open end is sealed. (xi) Recombination between a linear monomer and a circular dimer generates a linear trimer (from Ellis and Day, 1986).


i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
a'
a

of the yeast *Saccharomyces cerevisiae*, characterised by defective respiratory function, have been shown to contain mitochondrial genomes that have undergone deletion events leading to the loss of large tracts of DNA sequence (Locker *et al.*, 1979). The remaining mtDNA is often composed of a particular mtDNA sequence reiterated many times on molecules with circular or linear conformations. Similarly, senescing cultures of the fungus *Podospora anserina* contain a unique form of mtDNA, the SEN DNA, which is composed of a tandemly repeated segment of the normal mitochondrial genome (Jamet-Vierny, 1980). Stopper mutants of *Neurospora crassa*, exhibiting abnormal stop/start growth patterns and loss of respiratory function, possess defective mitochondrial genomes with large deletions, in some cases removing up to 60% of the intact mtDNA (de Vries *et al.*, 1986). Plant mitochondrial DNA has a complex multipartite structure composed of a number of subgenomic circular molecules derived by intramolecular recombination from a larger master chromosome representing the entire mtDNA sequence complexity (Newton, 1988; Lonsdale *et al.*, 1984). Additional intramolecular recombination within the subgenomic circles may provide further structural complexity in plant mtDNA (Lonsdale *et al.*, 1984). Although no net loss of sequence information occurs in this system, the subgenomic circles are essentially deletion derivatives of each of the larger molecules they are formed from, and can thus be compared with the circular ΔptDNAs thought to be present in albino cereal pollen plants (see Section 1.4). Indeed, the mechanisms that have been proposed for the generation of subgenomic mtDNAs in plants and the deleted genomes found in the mitochondria of *S. cerevisiae* and *N. crassa* are very similar to those described in Section 1.4 for circular ΔptDNAs, relying on intramolecular recombination between directly repeated sequences dispersed around the organelle genome (Locker *et al.*, 1979; Lonsdale *et al.*, 1984; de Vries *et al.*, 1986).

1.6. The Aims of This Study.

The general aim of this study was to increase our knowledge of the biology and in particular the molecular aspects of albinism in pollen plants of barley. Specifically:

- To confirm and extend the preliminary ultrastructural studies carried out with anther culture derived albinos of barley (see Section 1.1.4.).

- To improve the general growth and long term maintenance of barley albinos *in vitro* with the objective of providing a continuous supply of material for molecular analysis.

- To confirm the possible presence of ΔptDNA in a number of albino barley pollen plants and determine the extent to which the plastid genomes of these plants may have been altered.

- To determine if, and to what extent the intact regions of ptDNA are expressed in albino pollen plants and in particular to examine what effect, if any, different patterns of ptDNA alterations had on plastid gene expression in general.

- To determine if the expression of nuclear genes encoding chloroplast polypeptides is affected in albino barley pollen plants containing altered chloroplast genomes.

CHAPTER 2: MATERIALS AND METHODS.

2.1. Commonly Used Buffers.

10x MOPS Buffer: 0.4M MOPS (pH 7.0)/ 100mM Sodium acetate/ 10mM EDTA (pH 8.0).

20x SSC Buffer: 3.0M NaCl/ 0.3M Sodium citrate.
pH adjusted to 7.0 with 5M NaOH.

20x SSPE Buffer: 3.0M NaCl/ 0.2M $NaH_2PO_4.2H_2O$/ 0.02M EDTA.
pH adjusted to 7.7 with 5M NaOH.

10x TBE Electrophoresis Buffer: 0.89M Tris base/ 0.89M Boric acid/ 0.02M EDTA (pH 8.0).

TE Buffer: 10mM Tris.HCl (pH 8.0)/ 1mM EDTA (pH 8.0).

2.2. Bacterial Growth Media.

All solid and liquid bacterial media used are described in Miller (1972).

E. coli were grown in Luria Broth (LB) or on Luria Agar (LA) plates at 37°C.

LB media: 10g/l Difco Bactotryptone/ 5g/l Difco Yeast Extract/ 5g/l NaCl-pH adjusted to 7.0-7.4. with 5M NaOH

LB was autoclaved at 15 psi for 15 minutes. Before use 5ml sterile 20% glucose and 1ml filter sterilised 2mg/ml thiamine.HCl were added per litre of LB.

LA for bacterial plates was prepared by adding 12g/l of Difco BactoAgar to LB before autoclaving. If allowed to set LA was melted by heating in a microwave oven.

For selection of *E. coli* during growth the following concentrations of antibiotics were used:

	Liquid culture	Plates.
Tetracycline (Sigma)	12.5μg/ml	12.5μg/ml.
Ampicillin (Sigma)	50μg/ml	50μg/ml.

Tetracycline was prepared as a 12.5mg/ml stock solution in 50% ethanol and stored at -20°C. Ampicillin was added as a powder directly to LB or cooled but molten LA. Antibiotics were added after autoclaving.

2.3. Long-term Storage of *E. coli.* Strains.

This procedure was based on that described by Maniatis *et al.* (1982).

A single colony of an *E. coli* strain was used to inoculate 5ml of LB containing antibiotic to select for maintenance of the plasmid and the culture grown to stationary phase by incubation with shaking at 37°C overnight. Cells were pelleted by centrifugation for 5 mins at 5000 rpm in a Heraeus Christ Minifuge at 4°C and the pellet resuspended in 5ml M9 media.

M9 media was prepared by diluting sterile 10xM9 salts and 100xM9 additive to 1x in sterile Q-water (Q-water is water purified by passage through a Millipore Milli-Q Reagent Grade Water System).

10xM9 salts: 70g/l Na_2HPO_4, 30g/l KH_2PO_4, 5g/l NaCl, 10g/l NH_4Cl.

100xM9 Additive: 24.6g/l $MgSO_4$, 2.2g/l $CaCl_2.6H_2O$.

The cells were pelleted as before, resuspended in 1.5ml M9 media and 0.4ml aliquots mixed with 2.5ml sterile 50% w/v glycerol in 2ml glass screw cap tubes. The mixture was rapidly frozen at -80°C in a dry ice/ethanol bath and stored at -80°C until required. Cells were revived by transferring a small sample from the surface of the frozen aliquot to a plate of appropriate media.

2.4. Transformation of Competent *E. coli* Cells With Plasmid DNA.

The method used was based on that described by Maniatis *et al.* (1982).

Transformations were carried out with the *E. coli* strain HB101, (*F⁻ hsd20 recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 supE44* λ (Maniatis *et al.*, 1982)).

A 1ml overnight stationary phase LB culture of HB101 was used to inoculate 100ml of LB prewarmed to 37°C, which was subsequently shaken vigorously at 37°C until reaching an OD of 0.6 at a wavelength of 600nm. The culture was chilled on ice for 15 mins and the cells were pelleted by spinning at 4000 rpm for 5 mins in a Heraeus Christ minifuge at 4°C. The cells were resuspended in 10ml of ice-cold 0.1M $MgCl_2$ and pelleted again before resuspension in 10ml of ice-cold 0.1M $CaCl_2$. Suspensions were incubated on ice for 20 mins, the cells pelleted again and finally resuspended in 5ml ice-cold 0.1M $CaCl_2$. These cells were competent for transformation. Frozen stocks of competent cells were prepared by adding three volumes of competent cells mixed with one volume of ice-cold 0.1M $CaCl_2$, 50% w/v glycerol. The mixture was dispensed as 200μl aliquots to 1.5ml Eppendorf tubes and quickly frozen using a dry-ice/alcohol bath at -70°C. Frozen aliquots were stored at -70°C.

DNA for cell transformation was mixed with 200μl of competent cells (either freshly prepared or thawed from stock aliquots) and incubated on ice, with occasional agitation, for 30 mins. The mixture was heat-shocked by incubation for 2 mins at 42°C and returned to ice for 20 mins. To each aliquot of transformed cells was added 200μl of LB, and the mixture was incubated (without shaking) at 37°C for 45 mins. Aliquots of the transformation mixture (100μl) or of appropriate dilutions in LB, were plated onto suitable selective media and plates incubated overnight at 37°C.

2.5. Alkaline Lysis Method for the Small-scale Preparation of Plasmid DNA from *E. coli*.

Small-scale preparation of bacterial plasmid DNA was carried out according to the procedure of Birnboim and Doly (1979).

Single bacterial colonies were inoculated with sterile toothpicks into 5ml aliquots of LB under the appropriate antibiotic selection conditions and grown overnight with shaking at 37°C. 1.5ml aliquots of culture were transferred to Eppendorf tubes and the cells harvested by spinning for 2 minutes in a MSE Microcentaur centrifuge at 13000 rpm. After removal of the supernatant the cells were resuspended in 100µl of the following solution:

2mg/ml Lysozyme (Sigma)/50mM Glucose/10mM EDTA/25mM Tris-HCl (pH 8.0)

The samples were incubated on ice for 5 minutes and 200µl of 0.2M NaOH/0.1% SDS was added with gentle mixing until the suspension became clear and viscous. Samples were incubated on ice for a further 5 minutes. 150µl of sodium acetate (pH 4.8) was then added, mixed gently then incubated on ice for 60 minutes. Samples were spun in a MSE Microcentaur centrifuge for 10 minutes at 13000 rpm to yield a clear supernatant which was removed to a new tube. To the supernatant was added 1 ml -20°C ethanol. Samples were mixed well and incubated at -20°C for 30 minutes. The precipitate was collected by centrifugation for 10 minutes and the supernatant discarded. The pellet was dissolved in 100µl of 0.1M sodium acetate/0.05M Tris-HCl (pH 6.0). Nucleic acids were precipitated by adding 1ml -20°C ethanol with incubation at -20°C for 10 minutes. The nucleic acids were collected by a 10 minute spin in a Microcentaur centrifuge at 13000 rpm and the pellet dissolved again in 0.1M sodium acetate/0.05M Tris.HCl (pH 6.0) with precipitation as before. Nucleic acids were collected by a 10 minute spin at 13000 rpm in a Microcentaur centrifuge and dried *in vacuo* for 3-5 minutes. The nucleic acids were redissolved in 50µl sterile Q-water.

5µl aliquots of nucleic acid solution were used in restriction enzyme digests. Contaminating RNA was removed during digestion by

the addition of 1µl 10mg/ml RNAse A (Sigma) which had been boiled for 10 minutes to remove DNAse activity prior to use.

2.6. Large Scale Preparation of Plasmid DNA from *E. coli.*

The method used for large scale preparations of bacterial plasmid DNA was based on that described by Clewell and Helinski (1969).

Cells from single bacterial colonies, frozen glycerol stocks or liquid cultures were inoculated into 5ml LB with the appropriate antibiotic selection and grown overnight, with shaking at 37°C. This culture was then inoculated into 1 litre of LB again with antibiotic selection and incubated with shaking overnight at 37°C. Cells were harvested by centrifugation in a prechilled Sorvall GS3 rotor at 4°C for 5 minutes at 8000 rpm. The supernatant was removed and the cells washed in 50ml TES buffer.

TES buffer: 50mM Sodium chloride/5mM EDTA (pH 8.0)/50mM Tris.HCl (pH 8.0).

The cell suspension was transferred to 30ml screw top Sorvall centrifuge tubes and the cells harvested by centrifugation at 4°C for 10 minutes in a Sorvall SS34 rotor at 8000 rpm. The cell pellet was resuspended in 25ml 4°C 25% sucrose in 50mM Tris.HCl (pH 8.0) and placed on ice. The cell suspension was then incubated for 8 minutes on ice with 3ml fresh 10mg/ml lysozyme (Sigma) in 50mM Tris.HCl (pH 8.0). The mixture was incubated for a further 5 minutes on ice with 3ml 0.25M EDTA (pH 8.0). Cells were lysed by the addition of 30ml lysis cocktail and incubated at room temperature for 15 minutes.

Lysis cocktail: 0.2% Triton X100/50mM Tris.HCl (pH 8.0)/25mM EDTA (pH 8.0).

Lysed cell suspensions were then centrifuged at 17000 rpm for 60 minutes in a Sorvall SS34 rotor at room temperature. The supernatant was removed and the volume measured. For every ml of supernatant 1gm caesium chloride (BRL UltraPure) and 0.1ml 5mg/ml ethidium bromide was added. After the caesium chloride had

dissolved the solution was transferred to 30ml DuPont polyallomer crimp top ultracentrifuge tubes and spun in a DuPont TV850 vertical rotor at room temperature for 16 hours at 40000 rpm. DNA bands were visualised on the CsCl gradient by exposure to short wave UV light. Plasmid bands were removed with a syringe and 1.2mm x 40mm needle. Bands were transferred to 6ml DuPont crimp top ultracentrifuge tubes and respun at room temperature in a DuPont TV865 rotor for 5 hours at 50000 rpm. Plasmid bands were transferred with a syringe and needle to glass Corex centrifuge tubes (DuPont Instruments Ltd.). Ethidium bromide was removed by several extractions with isopropanol and CsCl by dialysis against two changes of sterile Q-water over 2-3 hours. Dialysis was carried out at 4°C. DNA was precipitated by the addition of 2.5 volumes of -20°C ethanol and a 1/10th volume of 3M sodium acetate (pH 5.5) with a 1-2 hour incubation at -20°C. DNA was pelleted by a 10 minute spin in a Sorvall SS34 rotor at 12000 rpm. DNA was dried *in vacuo* for 5 minutes and rehydrated in sterile Q-water.

2.7. Preparation of Total DNA from Plant Tissues.

The method used was based on that described by Murray and Thompson (1980)

Fresh plant tissue was weighed, frozen with liquid nitrogen and homogenised to fine powder with a pestle and mortar. Powdered plant tissue was transferred to a fresh mortar at room temperature and ground further with a volume of CTAB extraction buffer equivalent in mls to twice the mass of plant tissue in grams.

CTAB Extraction buffer: 2% w/v CTAB (Aldrich Chemical Company Ltd.)/100mM Tris.HCl (pH 8.0)/20mM EDTA (pH8.0)/1.4M NaCl/1% w/v PVP (Sigma).

The suspension was transferred to Eppendorf tubes and incubated at 65°C for 10 minutes, with occasional mixing. To the extract was added 1 volume of 24:1 chloroform:octanol. The phases were mixed by shaking and spun in a Microcentaur centrifuge at 13000 rpm for 2 minutes. The aqueous phase was removed to a fresh Eppendorf tube.

The denatured protein interface in the original tube was washed with a 1/5 volume of CTAB extraction buffer, recentrifuged as before and this aqueous phase pooled with the first. A 0.1 volume of 10% CTAB buffer (10% CTAB/0.7M NaCl) was added to the pooled aqueous phase, mixed then incubated at 65°C for 10 minutes prior to reextraction with chloroform/octanol as described. The aqueous phase was transferred to fresh Eppendorf tubes and an equal volume of CTAB precipitation buffer (1% CTAB/50mM Tris.HCl (pH 8.0)/10mM EDTA (pH 8.0)) was added. The mixture was shaken gently and incubated at room temperature for 60 minutes. The precipitated CTAB/DNA complex was pelleted by a 10 minute spin at 13000 rpm in a Microcentaur centrifuge and the supernatant drained off. The pellet was dissolved in 0.5-1ml of 1M NaCl and heated to 65°C to aid dissolution. Nucleic acids were precipitated by the addition of 2 volumes of -20°C ethanol and incubation at -70°C for 1 hour. Nucleic acid was recovered by centrifugation in a Microcentaur centrifuge for 10 minutes at 13000 rpm. The pellet was washed 2-3 times in -20°C 70% ethanol and dried *in vacuo* for 3-5 minutes. Nucleic acids were rehydrated in sterile Q-water.

DNA integrity and the extent of RNA contamination was checked by electrophoresing 2-5μl of nucleic acid solution as described in Section 2.10.

2.8. Estimation of DNA Concentration in DNA Preparations.

DNA concentration and yield were estimated by measuring the OD of DNA solutions at 260nm (OD_{260}) in a Shimadzu UV-240 spectrophotometer and assuming that an OD_{260} of 1.0 is equivalent to a concentration of 50μg double-stranded DNA/ml (Maniatis *et al.*, 1982).

2.9. Restriction Endonuclease Digests of DNA.

2.9.1. Digestion of Plasmid DNA.

Typically, the required quantity of plasmid DNA was digested in a total volume of between 10μl and 100μl. The quantity of enzyme used

and the time of incubation were normally equivalent to a five to ten fold excess digestion. All restriction endonucleases were incubated with DNA at temperatures and in buffering conditions as recommended by the supplier. All restriction endonucleases were purchased from BRL. All restriction enzyme buffers (BRL REact buffers) were supplied by the manufacturers at 10x concentration and stored at 4°C. Table 2.1 specifies the buffering conditions used for each enzyme.

Restriction Endonuclease:	Buffer used
BamH I	3
Bgl I	2
Bgl II	3
EcoR I	3
Hind III	2
Pvu II	2
Pst I	2
Sal I	3
Sph I	2
Sst I	2
Xho I	2

Table 2.1. Buffering Conditions For Restriction Endonucleases.

Numbers refer to the recommended BRL REact restriction endonuclease buffers.
Buffer 1: 10mM $MgCl_2$, 50 mM Tris.HCl pH 8.0
Buffer 2: 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris.HCl pH 8.0
Buffer 3: 10 mM $MgCl_2$, 100 mM NaCl, 50 mM Tris.HCl pH 8.0

All restriction digest reactions were carried out at 37°C.

2.9.2. Digestion of Plant DNA.

Total DNA from plant tissues was digested by an initial incubation with a five to ten fold excess of restriction enzyme for 90 minutes in a volume of 25-50μl as described in Section 2.9.1. After this

time a further aliquot of enzyme was added, again in 5-10 fold excess, in a final volume of 50-100μl and incubated overnight. Digest volumes were adjusted with sterile Q-water. DNA was precipitated from the reaction mix by the addition of a 1/10th volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of -20°C ethanol followed by incubation at -70°C for 1 hour. The DNA was pelleted by a 10-20 minute spin in a microcentrifuge at top speed, dried *in vacuo* for 3-5 minutes and rehydrated in 10-20μl of sterile Q-water.

2.10. Agarose Gel Electrophoresis of DNA.

Agarose gel electrophoresis of DNA was performed essentially as described by Maniatis *et al.* (1982).

Agarose (FMC SeaPlaque or FMC SeaKem) was dissolved in 1xTBE electrophoresis buffer with 0.5μg/ml ethidium bromide by boiling in a microwave oven. The agarose was allowed to cool and then cast into a perspex gel bed with slot former. Gels were submerged in 1x TBE electrophoresis buffer with 5μg/ml ethidium bromide. DNA samples were loaded into gel slots using 6x type I loading buffer (0.25% bromophenol blue/0.25% xylene cyanol/40% w/v sucrose in Q-water) diluted in the sample to 1x. Electrophoresis was carried out at 25V overnight or 100V for 2-3 hours and completed when sufficient resolution was obtained in a given range of molecular weights. DNA was visualised in agarose gels by irradiation with UV light from a Fotodyne transilluminator. Gels were photographed using a Polaroid Land camera with type 57 or 55 film. Estimation of DNA fragment sizes was carried out by comparison with the migration of fragments of known molecular weight derived from lambda bacteriophage DNA digested with the restriction enzyme *Hin*d III.

2.11. Recovery of DNA Fragments from Agarose Gels.

Two methods were used to isolate DNA fragments from agarose gels ready for radiolabelling as described in Section 2.12.2.

Method 1: This method was originally described by Vogelstein and

Gillespie (1979). The procedure and reagents used were supplied commercially by BIO101 as their Geneclean kit. A quantity of plasmid DNA was digested as described in section 2.9.1 to release a known amount of the required restriction fragment. The digest reaction was then loaded onto a 0.8-1.0% low gelling temperature agarose gel (FMC Sea Plaque agarose) and electrophoresed until suitable separation of restriction fragments was achieved. The piece of agarose containing the required fragment was cut out with a scalpel and transferred to a preweighed Eppendorf tube. The tube was weighed again and the mass of the gel slice determined. The gel slice was then macerated with a plastic stirring rod and a volume of saturated NaI (supplied with Geneclean kit) equivalent in μl to 2.5x the mass of the gel slice in mg was added. The agarose was melted by a 10-15 minute incubation at 55°C. 5-10μl of a aqueous suspension of powdered glass (Glassmilk, as supplied with the Geneclean kit) was then added with a 5 minute incubation at room temperature to allow binding of DNA to the glass surface. The Glassmilk was pelleted by a 5 second spin at top speed in a microcentrifuge, the supernatant discarded and the pellet washed three times in 500μl of NEW buffer.

NEW buffer is: 50% (20mM Tris.HCl (pH 7.2)/0.2M NaCl/2mM EDTA) and 50% ethanol.

The washed pellet was then resuspended in 15μl sterile Q-water and incubated at 55°C for 3 minutes to elute DNA from the Glassmilk. The glass was pelleted by a 30 second spin at top speed in a microcentrifuge and the supernatant containing the DNA transferred to a fresh Eppendorf tube. This procedure was repeated with one 15μl aliquot and one 20μl aliquot of Q-water to give a final volume for the eluted DNA solution of 50μl. 5μl of this solution was electrophoresed with known amounts of lamda bacteriophage DNA to estimate the recovery of fragment DNA. The solution of isolated fragment DNA was then diluted to 2.5-10ng/μl. Recovery with this method was commonly in the range 50-100%.

Method 2: This method is described by Dalgleish (1987). A known quantity of the DNA fragment to be isolated was run on a low gelling temperature gel and transferred to an Eppendorf tube as described for Method 1. The gel slice was weighed in the Eppendorf

and a volume of water equivalent in ml to 1.5x the mass of the agarose in g was added. The agarose was melted by boiling for 5 minutes in a 105°C PEG 6000 bath. The DNA concentration was adjusted to 2.5-10ng/µl with sterile Q-water and mixed thoroughly. Isolated fragment preparations were stored at -20°C.

2.12. Southern Analysis.

General details of Southern analysis procedures were based on those described by Dalgleish (1987).

2.12.1. Southern Transfer.

DNA samples were fractionated by electrophoresis on 1 - 0.5% agarose gels (2.10). After electrophoresis the gels were photographed with a ruler alongside for analysis later. Gels were depurinated for 30-45 minutes in 0.25M HCl, denatured for 30 minutes in 0.5M NaOH/1.5M NaCl and neutralised for 30 minutes in 0.5M Tris pH 8.0/1.5M NaCl.

A wick cut from Whatman 3MM paper was soaked in 20x SSC and placed across a glass plate. The glass plate and wick were then placed over a plastic tray containing 20x SSC so that the ends of the wick were submerged. A piece of Whatman 3MM paper was cut to a size 1cm larger than the gel to blotted, soaked in 20x SSC and placed on the flat surface of the wick. Two pieces of Whatman 3MM paper were cut to the same size as the gel, soaked in 20x SSC and placed on the previous sheet of filter paper to act as a pad. The wick and glass plate were covered in SaranWrap and a hole cut with a scalpel around the pad of filter papers to the same size of the gel. The gel was placed onto the pad and air bubbles were removed by rolling a glass pipette along the gel surface. A piece of Hybond-N hybridisation filter (Amersham International) cut to the size of gel to be blotted was wetted with 20x SSC for 30-60 minutes prior to transfer and placed on the gel surface. Any air bubbles between the gel and the Hybond-N were removed and five pieces of dry Whatman 3MM paper cut to the same size as the gel were placed over the filter. A stack of Kimwipe paper towels 6-7cm thick were then placed over the gel and compressed using a suitable weight, eg.

500ml medical flat full of water. Transfers were carried out overnight. After transfer the Hybond-N filter was removed from the gel surface and rinsed in 3x SSC to remove residual agarose. The filter was blotted with Whatman 3MM filter paper and dried further by placing in a 65°C incubator for 10 minutes. DNA was covalently cross linked to Hybond-N by placing the filter on a single layer of SaranWrap over a Fotodyne UV transilluminator and irradiating for 16-20 seconds.

2.12.2. Preparation of Radioactive Probe DNA by Oligolabelling.

Radioactive DNA probes were prepared according to the random hexanucleotide priming method of Feinberg and Vogelstein (1983). DNA fragments used in radiolabelling reactions were prepared as described in Section 2.11.

DNA to be radiolabelled was first denatured by boiling in an Eppendorf tube placed in a 105°C PEG 6000 bath for 10 minutes. The DNA was subsequently placed on ice to prevent renaturation of the denatured DNA.

The radiolabelling reaction was carried out by the addition of the following reagents in the stated order:

25ng DNA in a total volume of 15.5μl,
5μl of OLB C buffer,
1μl BSA (Sigma, 10mg/ml)
1μl DNA polymerase Klenow fragment (BRL),
2.5μl (0.925 MBq) α-^{32}P-dCTP (Amersham International).

Total reaction volume was 25μl. The labelling reaction was carried out at room temperature for 5 hours or overnight.

OLB buffer is prepared as follows:

Solution O: 1.25M Tris-HCl,
0.125M $MgCl_2$,
pH adjusted to 8.0 and stored at 4°C.

Solution A: 1000µl solution O,
18µl β-Mercaptoethanol (Sigma),
5µl each of dATP, dTTP, dGTP.

dNTP's: dATP, dTTP, dGTP. Each 0.1M in T.E.

Solution B: 2M HEPES (pH adjusted to 6.6 with 4M NaOH)

Solution C: Hexadeoxynucleotides. (Pharmacia)
50 OD units dissolved in 550µl TE
to give a concentration of 90 OD/ml. Stored at -20°C.

OLB Buffer: Mix solutions A:B:C: in the ratio of 10:25:15.

The reaction was terminated by the addition of 100µl of stop C buffer (20mM NaCl/20mM Tris-HCl (pH7.5)/2mM EDTA/0.25% SDS/1µM dCTP).

Unincorporated nucleotides were removed by fractionating the stopped reaction mix on a column of coarse or medium grade Sephadex G-50. A glass Pasteur pipette was plugged with polyallomer wool and packed with Sephadex G-50 equilibrated with TE to within 0.5-1cm of the top. The column was washed with 2ml TE (pH 8.0). The reaction mix was applied to the top of the column and the first fraction collected in an Eppendorf tube. A further 13 fractions were collected by applying 100ml aliquots of TE (pH 8.0) to the column. Radiolabelled DNA fragments appear in the first 4-5 fractions containing radioactivity. These were pooled and the number of radioactive counts per minute present in a 1/10th aliquot of the pooled fractions determined by Cerenkov counting in a Beckman LS 6800 liquid scintillation counter. An estimate of the specific activity of the radiolabelled DNA fragment was made by calculating the number of counts per minute per µg of DNA. DNA was routinely labeled to a specific activity of 0.5-1.0 x 10^9 CPM/µg DNA. It was assumed that near 100% recovery of the probe DNA fragment occured in the first four pooled radioactive fractions.

2.12.3. Hybridisation of Southern Blots With Radiolabelled DNA Probes.

Southern blots were sealed into plastic bags (Transatlantic Plastics Ltd) with an electric bag sealer (Calor) ready for prehybridisation and hybridisation to radiolabelled probe DNA. The blots were prehybridised by adding 25ml of the following solution into the plastic bag:

1.5x SSPE/0.5% dried milk (Cadbury's Marvel)/1% SDS/6% PEG 6000.

250µl of 10mg/ml sonicated salmon sperm DNA (Sigma) previously boiled for 10 minutes at 105°C was also added to the prehybridisation buffer. The hybridisation bags were resealed after removal of any air bubbles and prehybridisation was carried out at 65°C with gentle agitation for 2 hours.

The buffer used during hybridisation was the same as that for prehybridisation. Radiolabelled probe DNA was denatured by boiling for 10 minutes in a 105°C PEG 6000 bath. The denatured probe was placed on ice before injection into the hybridisation bag with a 1ml plastic syringe and 1.2mm x 40mm needle. Any air bubbles were removed and the bag was resealed and placed at 65°C with gentle agitation for 16-48 hours.

2.12.4. Washing of Radioactive Southern Blots

After hybridisation excess radiolabelled probe DNA was removed from Southern blots by using either low or moderate stringency washing protocols. All washes were carried out in a shaking air incubator at 65°C.

The low stringency washing protocol included four rinses in 3x SSC/0.1% SDS and four 15-30 minute washes in the same buffer. Moderate stringency washing included four rinses in 3x SSC/0.1% SDS followed by two 15-30 minute washes in 3x SSC/0.1% SDS and finally one or two 15-30 minute washes in 0.5x SSC/0.1% SDS. All washes were carried out in sealable plastic sandwich boxes with gentle agitation.

2.12.5. Autoradiography of Radioactive Southern Blots.

After washing radioactive Southern blots were placed onto a piece of Whatman 3MM paper and covered in SaranWrap. Autoradiography was performed in sealed X-Ray cassettes (Genetic Research Instrumentation Ltd) with tungstate intensifying screens at room temperature or -80°C using Fuji RX or Kodak XAR X-ray film. Autoradiographs were processed in a Agfa-Gevaert Gevamatic-60 film processor.

2.12.6. Analysis of Autoradiographs.

Band sizes on autoradiographs of Southern blots were determined by comparison to the migration of restriction fragments of known size generated by a *Hin*d III digest of lamda bacteriophage DNA.

Band intensities on autoradiographs of Southern blots were determined by laser densitometry using an LKB Ultroscan XL gel scanner.

2.12.7. Preparation of Radioactive Southern Blots for Rehybridisation.

Once a suitable X-ray exposure had been obtained the probe bound to the target DNA and any remaining background was removed by washing the filter in 0.1% SDS heated to boiling and then incubation in the same buffer with gentle agitation at 65°C for 1 hour in a plastic sandwich box. Efficient removal of radiolabelled probe DNA was confirmed by use of a Mini-Monitor or by autoradiography. Rehybridisation with radiolabelled probe DNA was then carried out as described above.

2.13. Preparation of Total RNA from Plant Tissues.

The method used for extraction of total RNA from plant tissues was based on that described by Logemann *et al.* (1987).

Q-water used to prepare RNA extraction solutions was treated with DEPC (Sigma) at 1ml/l for 1 hour. DEPC was then removed by autoclaving at 15 psi for 15 minutes.

Plant tissue was weighed, frozen in liquid nitrogen and homogenised to a fine powder using a pestle and mortar. Powdered plant tissue was transferred to a mortar kept at room temperature and ground further with the addition of a volume of RNA extraction buffer equivalent in ml to twice the mass of the plant tissue in grams.

RNA Extraction buffer: 8M Guanadinium hydrochloride/20mM MES/20mM EDTA/50mM β-Mercaptoethanol

The homogenate was then transferred to sterile Eppendorf tubes. The homogenate was spun for 10 minutes at maximum speed in a microcentrifuge. The supernatant was removed and an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol added. The phases were mixed by shaking and then resolved by centrifugation for 45 minutes at 13000 rpm in a microcentrifuge. The aqueous phase was collected and mixed with 0.7 volumes of -20°C ethanol and 0.2 volumes of 1M acetic acid. Samples were incubated at -70°C for 1 hour before pelleting the precipitated RNA by centrifugation as previously described. The supernatant was removed and the pellet washed twice with 3M sodium acetate (pH 5.2) at room temperature and once with -20°C 70% ethanol. Samples were centrifuged briefly between each wash to prevent the RNA pellet becoming detached form the side of the tube. The RNA was dried *in vacuo* for 5 minutes before rehydration in sterile Q-water.

An estimation of the RNA concentration in a given sample was made by measuring the OD at 260nm (OD_{260}) in a Shimadzu UV-240 spectrophotometer and assuming an OD_{260} of 1.0 is equivalent to 40μg single-stranded RNA/ml (Maniatis *et al.*, 1982).

The integrity of RNA and extent of contamination with DNA was assessed by electrophoresing 3-5μg of nucleic acid on an agarose gel as described in Section 2.14.

2.14. Agarose Gel Electrophoresis of RNA.

Agarose gel electrophoresis of RNA was perfomed essentially as detailed by Maniatis *et al.* (1982).

Agarose gels for the electrophoresis of RNA were prepared by dissolving 1.0-1.5g of FMC SeaKem agarose in 65ml sterile DEPC-treated Q-water by boiling in a microwave oven. The molten agarose was allowed cool to 70°C before addition of 20ml sterile 5x MOPS buffer and 16.7 ml 38% v/v formaldehyde (Fisons AR Grade) in a fume hood. The mixture was swirled gently to mix and cast into a gel bed with slot former.

RNA samples were prepared for electrophoresis by the addition of deionised formamide to a final concentration of 50%, formaldehyde to a final concentration of 8% and 10x MOPS buffer to a final concentration of 1x.

Deionised formamide was prepared as follows: to every 100ml formamide (Fisons AR Grade) was added 10g BioRad AG 501-X8 mixed bed resin and the mixture stirred for 1-2 hours and then filtered three times through Whatman Number 1 filter paper. Deionised formamide was stored at -20°C.

Samples were incubated at 65°C for 10 minutes in order to denature the RNA and subsequently placed on ice to prevent renaturation. 2-3μl sterile loading buffer (50% glycerol/5mM EDTA (pH 8.0)/0.4% bromophenol blue) was added to all samples. Electrophoresis was carried out at 25V overnight in 1x MOPS buffer. RNA was visualised on gels by staining in a solution of 5μg/ml ethidium bromide with subsequent destaining to remove background in several changes of sterile Q-water over 2-3 hours. Where Northern transfer was to be performed RNA markers (BRL) were run in a lane at the edge of the gel which was removed after electrophoresis and stained separately. The part of the gel containing plant RNA samples was left unstained before blotting. As an alternative, a 5mg/ml stock solution of ethidium bromide was added to the agarose before casting the gel at a final concentration of 5μg/ml. This was found to have no effect on subsequent Northern transfer and hybridisation with radiolabelled DNA.

2.15. Northern Analysis.

2.15.1. Northern Transfer.

Transfer of RNA from agarose gels to Hybond-N filter was carried out essentially as described in Section 2.12.1. Gels containing RNA samples were rinsed briefly in sterile Q-water and set up on the blotting apparatus as detailed. Filters were treated after Northern transfer in the same way as Southern blots (2.12.1) except that RNA was cross-linked to the Hybond-N by irradiation with UV light for 50-60 seconds.

2.15.2. Hybridisation of Northern Blots with Radiolabelled DNA.

The conditions used during the hybridisation of Northern blots to radiolabelled DNA are based on those recommended for use with Hybond-N by the manufacturers.

Northern blots were sealed in plastic bags as detailed for Southern blots and prehybridised in 25ml 50% formamide/6% PEG 6000/1.5x SSPE (pH 7.7)/0.1% SDS/5x Denhardt's solution. 250μl of a 10mg/ml solution of sonicated salmon sperm DNA (Sigma) previously boiled for 10 minutes in a 105°C PEG 6000 bath was also added to the prehybridisation mix.

50x Denhardt's solution is: 1% w/v Ficoll/1% w/v PVP/1% w/v BSA.
(All reagents from Sigma)

Blots were incubated at 42°C with gentle agitation for 2-3 hours.

Hybridisation was carried out after removing the prehybridisation buffer and replacing it with 25ml 50% formamide/6% PEG 6000/1.5x SSPE (pH 7.7)/1% SDS/1x Denhardt's solution with the addition of sonicated and denatured salmon sperm DNA as for prehybridisation. Denatured radiolabelled DNA was introduced into the hybridisation bag as detailed for Southern hybridisations. Hybridisations were carried out at 42°C with gentle agitation for 16-48 hours.

2.15.3. Washing, Autoradiography, Analysis and Rehybridisation of Radioactive Northern Blots.

Procedures for the washing, autoradiography, analysis and rehybridisation of Northern blots were carried out essentially as described for Southern blots. Size analysis of bands visualised on autoradiographs of Northern blots hybridised with radiolabelled probe DNA was by comparison with the migration of RNA size markers (BRL).

2.16. Germination and Growth of Barley Seedlings.

To overcome bacterial and fungal infection seeds of the barley variety Igri (Nickersons RPB) were disinfected by the following treatment. Seeds were soaked in 100% ethanol for 20 seconds, rinsed in sterile Q-water and then soaked again in 10% Chloros (ICI) for 1 minute. The seeds were rinsed and blotted dry on sterile Kimwipe paper towels in a lamina flow hood and then placed in sterile Kilner jars on a pad of moist sterile Kimwipe tissue at a density of 50-100 seeds per jar. The sealed jars were then placed in a Fitotron constant environment cabinet (Fisons) until harvesting. To obtain tissue germinated under conditions of darkness Kilner jars were wrapped in a double layer of aluminium foil.

Light and dark grown shoot and root tissue was harvested 8-10 days after germination. Harvesting of dark-germinated barley plants was carried out under a green biological safelight. All tissue was rinsed in sterile Q-water, blotted dry and then plunged into a liquid nitrogen bath before storage in sealable plastic bags at -70°C.

2.17. Generation of Green and Albino Plants Via Anther Culture.

Regeneration of plants from the pollen of barley (*Hordeum vulgare.* variety Igri) was carried out as described by Hunter (1987).

Barley plants were grown in Conviron S10H controlled environment cabinets at 12°C under a mixture of fluorescent and tungsten

lighting ($350\mu E/m^2/s^1$) on a 16 hour light/8 hour dark cycle at 60-80% relative humidity. Tillers were harvested and the spikes, containing anthers, dissected out under aseptic conditions after surface sterilisation with ethanol. The stage of anther development was determined on anthers taken from a central floret. The anthers were squashed gently in acetocarmine (Sigma) and examined microscopically. Only those spikes with anthers containing microspores at the mid-uninucleate stage (Huang and Sunderland, 1982) were used subsequently. Microspore viability was determined by counting the number of pollen grains with a nucleus or nuclei of normal appearance. Only spikes with anthers containing 40% viable pollen were selected. Each selected spike was placed in one compartment of a two-compartment Petri dish with 1ml sterile water in the remaining compartment to maintain humidity. The spikes were given a cold pretreatment by placing the Petri dishes at 4°C for 28 days (Huang and Sunderland, 1982). After this time anthers were again examined by acetocarmine staining to determine microspore viability. As previously, only anthers containing 40% viable pollen were selected. Anthers were cultured in 5cm Petri dishes containing 10ml callus induction medium (Foroughi-Wehr *et al.*, 1976: see Section 2.18) with the substitution of maltose in place of sucrose. The media was solidified with 0.8% FMC Sea Plaque agarose. Cultures were incubated in the dark at 25°C for 28 days then transferred to continuous white light. Green and albino plantlets were then harvested at intervals and transferred under aseptic conditions to a variety of growth media as described in 2.18.

2.18. Media for the Growth and Maintenance of Plants Regenerated from Anther Culture.

2.18.1. Basal media:

All media were made up in either Q-water or double distilled water (DDW). Unless specified all media were autoclaved at 10 psi for 15 minutes before the addition of non-autoclavable supplements. Difco Bacto-Agar was used as a solidifying agent at 6g/l and was added prior to autoclaving. In the the majority of cases media were poured into washed and sterile 500ml Powder Round Jars (FSA Lab

Supplies).

Basal media were made up a follows. For the specification of media constituents see Table 2.2.

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962):

4.71g/l MS Redimix powder (Flow Laboratories Ltd), 20g/l Sucrose (BRL Ultrapure).

pH adjusted to 5.6 with 0.1M KOH.

Half strength MS medium MS/2:

As above but 2.35g/l MS Redimix powder.

Foroughi-Wehr callus induction (FH) medium (Foroughi-Wehr *et al.*, 1976):

3.22g/l modified Murashige's minimal organics medium (supplied as a ready mixed powder and modified to specification by Imperial Laboratories (Europe) Ltd), 20g/l Sucrose.

pH adjusted to 5.6 with 0.1M KOH.

Gamborg's B5 medium (Gamborg, 1970):

3.88g/l B5 Redimix powder (Flow Laboratories Ltd)/ 20g/l Sucrose.

Redimix powder was occasionally replaced by use of stock solutions as specified below. It should be noted that there are slight differences in the final concentrations of some constituents between B5 prepared from Redimix powder and stock solutions (see Table 2.2.).

100ml B5 micronutrient stock was prepared by adding 1ml each of the following stock solutions to 90ml Q-water: 7.5mg/ml KI, 2.5mg/ml $NaMoO_4.2H_2O$, 0.25mg/ml $CuSO_4.5H_2O$ and 0.25mg/ml $CoCl_2.6H_2O$. Other compounds were added dry as follows: 0.03g H_3BO_3, 0.1g $MnSO_4.H_2O$,

Table 2.2. Final Concentrations (mg/l) of Various Tissue Culture Media Constituents.

	Medium.			
	B5.	B5 Redimix.	MS.	FH.
Macronutrients.				
KNO_3	2500	3000	1900	1900
KH_2PO_4	–	–	170	170
$CaCl_2.2H_2O$	150	150	440	439.8
$MgSO_4.7H_2O$	250	250	370	370.6
NH_4NO_3	–	–	1650	165
$(NH_4)_2SO_4$	134	134	–	–
$NaH_2PO_4.H_2O$	150	169	–	–
Micronutrients.				
KI	0.75	0.75	0.83	0.83
H_3BO_3	3.0	3.0	6.2	6.2
$MnSO_4.H_2O$	10.0	13.2	22.3	22.3
$ZnSO_4.7H_2O$	2.0	2.0	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025
Organics.				
Inositol	100	100	100	100
Nicotinic Acid	1.0	1.0	0.5	–
Pyridoxine.HCl	1.0	1.0	0.5	–
Thiamine.HCl	10	10	0.1	0.4
Glycine	–	–	2.0	–
FeNaEDTA	28	40	36.7	36.7

0.02g $ZnSO_4.7H_2O$. Volume was made up to 100ml with Q-water.

100ml B5 organic stock prepared by adding 100mg nicotinic acid, 100mg pyridoxine.HCl and 1g thiamine.HCl to Q-water.

100ml Fe:Na_2EDTA stock prepared by dissolving 0.28g in Q-water.

All stock solutions were filter sterilised by passage through a Nalgene disposable filter unit and stored at 4°C.

B5 was prepared by adding 10ml/l micronutrient stock, 10ml/l FeNaEDTA stock, 1ml/l organic stock to Q-water, 20g/l sucrose with inositol and major elements added dry to final concentrations specified in Table 2.2.

2.18.2. Media Containing Nitrogenous Supplements.

All stock solutions of nitrogenous supplements were filter sterilised by passage through a 115ml disposable Nalgene filter unit. All stock solutions were stored at 4°C.

A 50mM glutamine (Sigma) stock was prepared by dissolving 1.461g glutamine in 200ml DDW. MS with supplementary glutamine was made by the addition of stock glutamine at 100ml/l to the autoclaved and cooled medium to give a final concentration of 5mM.

Casein hydrolysate (CH, Sigma) was added to MS before autoclaving at 1g/l or by the addition at 50ml/l of a filter sterilised 5mg/ml stock solution to autoclaved and cooled B5 giving a final concentration of 250mg/l. In this case B5 was prepared from stock solutions as described above but without the addition of KNO_3 or $(NH_4)_2SO_4$ but with KCl added at 2.95g/l to make up available K^+ ions.

200ml 20x amino acid mix (20x AA) prepared by dissolving 3.504g glutamine, 1.064g aspartic acid, 0.696g arginine and 0.03g glycine in Q-water. Media containing 1x AA and 2x AA mix were prepared by adding 20x AA mix at 50ml/l and 100ml/l respectively to autoclaved and cooled B5 prepared without inorganic nitrogen sources as

described above.

B5 was also prepared from stock solutions with twice the amount of KNO_3 added (5.0g/l).

2.18.3. Media Containing Supplementary Plant Growth Regulators.

All plant growth regulators were purchased from Sigma. Stock solutions of plant growth regulators were filter sterilised by passage through a 115ml disposable Nalgene filter unit and stored at 4°C.

Cytokinin:

6-Benzylaminopurine (BAP) was prepared as a 1mg/ml stock by dissolving 100mg BAP in a minimum volume of 0.1M KOH, with dilution to 100ml in DDW. MS was supplemented with BAP to concentrations of 0.1, 0.5 and 1.0mg/l by the addition of 100, 500 and 1000µl respectively of the stock solution to 1 litre autoclaved and cooled basal medium. MS/2 was prepared with a final concentration of BAP of 1mg/l.

Auxins:

Indole-3-acetic acid (IAA) and naphthalene acetic acid (NAA) were prepared as 1mg/ml stocks and added at 1ml/l to autoclaved and cooled B5 giving a final concentration of 1mg/l.

2,4-Dichlorophenoxyacetic acid (2,4-D) was prepared as a 2mg/ml stock and added at 1ml/l to autoclaved and cooled B5 giving a final concentration of 2mg/l.

Gibberellic Acid:

Gibberellic acid (GA_3) was prepared as a 173ng/ml stock solution and added at 1, 2 and 4ml/l to autoclaved and cooled B5 to give final concentrations of 173ng/l, 346ng/l and 692ng/l repectively.

2.18.4. Media Containing Supplements of Undefined Composition:

Malt extract:

Malt extract (Oxoid) was added at 4g/l to B5 before autoclaving to give a final concentration of 0.4% w/v.

Barley Seedling Extract:

Extracts of barley seedlings were prepared as follows: 100g of fresh shoots from 8-10 day-old barley seedlings were harvested and homogenised to a fine powder with a pestle and mortar in liquid nitrogen. The homogenate was transferred to a mortar at room temperature and ground further with the addition of approximately 200ml Q-water. The slurry was filtered through two layers of muslin and several times through Whatman Number 1 filter paper. The pH of the filtered extract was adjusted to 5.6 and stored at -20°C. MS containing 10% barley seedling extract was prepared by adding extract at 100ml/l before autoclaving.

Potato Extract:

Extracts of potato tubers were a kind gift from Keith Fuell (Sittingbourne Research Centre) and were prepared according to the protocol of Chuang *et al.* (1978). Briefly, 100-200g of fresh potato tubers (var. Desiree) were cleaned and the sprouts, if any, removed. The tubers were cut into small pieces, boiled for 25-30 minutes in a given volume of DDW and filtered through several layers of muslin. The residual potato pieces were re-extracted in the same manner and the two liquors combined, adjusted to pH 5.6-5.8 and stored at -20°C until use. 1 litre batches of MS/2 containing 5% and 20% potato extract were prepared by adding the extract at 50 or 200ml/l before autoclaving.

2.19. Growth Conditions.

Plants were grown in Fisons Fitotron 600H or Vindon S13F constant environment incubators under a mixture of fluorescent and tungsten lamps with a 16 hour light/8 hour dark cycle at 25°C.

2.20. Electron Microscopy.

The ultrastructural studies described in Chapter 3 were carried out according to methods used by the Analytical Chemistry Division, Shell Research Centre, Sittingbourne (Glauert, 1975). A brief summary of the sample preparation procedure is given below.

1 x 3mm pieces of green or albino leaf tissue from plants regenerated via anther culture were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) for 3 hours. After washing in the same buffer the samples were post-fixed in 2% osmium tetraoxide in the same phosphate buffer for 2 hours. The samples were then dehydrated by soaking in a graded series of alcohols of increasing concentration and embedded in resin.

Ultrathin sections of tissue were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, then viewed in a JEOL 100CX or Phillips 300 electron microscope at 60kV.

RESULTS.

CHAPTER 3: GENERAL FEATURES AND ULTRASTRUCTURE OF ALBINO POLLEN PLANTS.

3.1. General Features of Albino Pollen Plants.

Chlorophyll-deficient plants generated from barley anther culture were first described in detail by Clapham (1973). These plants ranged in colour from white through to yellow and pale green and often included chimaeric forms, eg. pigmented and white longitudinal leaf sectors, pigmented leaf tips and albino leaf bases. The majority of the plants generated from anther culture during this study were green or white with the occasional occurrence of variegated leaves with green/white longitudinal stripes. One individual changed from white to pale yellow over the course of four weeks under a 16 hour light/ 8 hour dark cycle.

The 1-3 month old albino plants used in this study were 5-10cm tall with leaves ranging in width from 2-5mm and in length from 5-10cm. Albinos exhibited a range of tillering frequencies, ie. the number of tillers per plant, ranging from none to five or more. In general no rooting was observed for any of the albinos (but see Section 4.4). In contrast green pollen plants were commonly at least 2-3 times larger in size than albinos, often filling a 500ml culture vessel with many tillers (≥10) and in most cases displayed vigorous rooting into the culture media.

3.2. Ultrastructure of Albino Pollen Plants.

The ultrastructure of plastids from albino barley and rice pollen plants has been described by Clapham (1973) and Sun *et al.* (1974) respectively. These workers reported the absence of mature chloroplasts in albino plants but the presence of structures closely resembling proplastids which are thought to be the precursors of chloroplasts (Robertson and Laetsch, 1974). To confirm and extend these observations the ultrastructure of albino and green pollen plants was examined using transmission electron microscopy as described in Section 2.20. General features of plant cell ultrastructure have been described by Robards (1970) and more specific details of plastid ultrastructure by Muhlethaler (1971).

These were used as references in interpreting the electron photomicrographs presented in Figures 3.1-3.5.

The mesophyll cells from a green pollen plant are compared with those from an albino in Figure 3.1. Mesophyll cells from the green pollen plant are characterised by the presence of several chloroplasts at the periphery of the cell. Mesophyll cells from albinos often contained a few (1-3) structures which closely resembled proplastids (see Figures 3.2-3.4) although in some cells these structures were absent altogether. In some sections of green leaf tissue (data not shown) the presence of other organelles such as mitochondria and the nucleus can be seen, again in a thin strip of cytoplasm at the cell periphery. Mitochondria of normal appearance can also be seen at the periphery of mesophyll cells from albino leaf tissue (Figure 3.4). The most striking feature of a large proportion of mesophyll cells from albino leaves is the presence of a granular material that wholly or partially fills the vacuole. One explanation could be that this is an artefact of the sample preparation procedure used before observation under the electron microscope. Deposition of some substance (or substances) in the vacuole could also be the result of some unique but uncharacterised feature of albino physiology. Although the identity of this substance is unknown, histochemical tests might aid in establishing its chemical nature, eg. protein, lipid.

The structure of plastids from the leaf mesophyll cells of green and albino pollen plants are compared in Figure 3.2. Further details of the plastids found in the mesophyll cells of albino leaves are shown in Figures 3.3 and 3.4.

Plastids seen in sections of green tissue have internal features characteristic of mature chloroplasts whereas those in albino plants strongly resemble relatively undifferentiated proplastids with little internal structure. A highly organised thylakoid membrane system consisting of grana stacks and stromal lamellae is clearly visible in the mature chloroplast. However, these structures are not visible in proplastids from albino tissues. One proplastid in Figure 3.2 contains a disorganised mass of vesicular membranes which is reminiscent of the prolamellar body of etioplasts from dark grown leaves. These membrane structures can

also be seen in the proplastids shown in Figures 3.3 and 3.4. Lipid storage deposits in the form of plastoglobuli are also present in both chloroplasts and proplastids. Proplastids also appear to contain localised deposits of phytoferritin (Muhlethaler, 1971) which are not visible in the chloroplasts of green tissues. Phytoferritin may act as an iron source during the light-induced development of the photosynthetic apparatus and disappears during chloroplast maturation (Muhlethaler, 1971). A double membrane envelope can clearly be seen delimiting the chloroplasts of green mesophyll cells and the proplastids of albino tissues. In general the proplastid-like structures from albino plants appear to be slightly smaller and less regular in shape than mature chloroplasts found in green tissues (note scale bar, Figure 3.2). Most of the proplastids appear to contain structures resembling vacuoles. This was also observed in plastids in the albino barley pollen plants studied by Clapham (1973). The identity of these structures is unknown but they could be the result of invaginations of the plastid envelope. This feature was not seen in mature chloroplasts.

A chloroplast and plastid-like structure from the green and albino sectors of a variegated leaf are shown in Figure 3.5. Both structures appear to be surrounded by cytoplasm at the periphery of the cell. The chloroplast seen in the green sector contains an extensive thylakoid membrane system and numerous plastoglobuli. In contrast the plastid-like structure in the albino sector contains no organised internal features although a delimiting double membrane does appear to be present. A few plastoglobuli can be seen but other features such as internal membranes and phytoferritin deposits are absent. Granular inclusions can also be seen in the interior of the plastid-like structure which are not present in the normal chloroplast or when looking at the plastids from albino pollen plants that are shown in Figures 3.2-3.4. These inclusions can also be seen in the cytoplasm surrounding the plastid but their identity is unknown.

Since the entire length of the variegated leaf was sectored longitudinally it suggests that the distinction between pigmented and albino tissues may originate in the basal meristem. The meristem would presumably be divided longitudinally into cells giving rise to albino and green cell lines. Chimaeric meristems of

this type have been described by Tilney-Basset (1986). Although no tissue was available to study this phenomenon in more detail, it might be possible using light-microscopy to trace green and white cell lines to their origins and so characterise the structure of a variegated meristem in more detail.

From these studies it appears that mature chloroplasts are absent from albino pollen plants and the albino sectors of variegated leaves. Instead these tissues appear to contain structures that strongly resemble proplastids. Proplastids represent a relatively undifferentiated form of plastid which can develop into photosynthetically competent chloroplasts in illuminated leaves (Robertson and Laetsch, 1974). Albino pollen plants were grown under the same conditions of illumination as green individuals (16 hours of light per day) which suggests that there may be some block operating to prevent the light-induced development of mature chloroplasts in albino leaves.

3.3 Summary.

Albino barley pollen plants are severely pigment deficient, are smaller in size and have a lower tillering frequency than green pollen plants. Major differences can also be seen at the ultrastructural level. Firstly, the vacuoles of many mesophyll cells from albino leaves contain an electron-dense material of an unknown nature. Secondly, in contrast to the leaves of green pollen plants, albinos do not contain mature chloroplasts. Instead, the mesophyll cells of these individuals contain organelles that closely resemble proplastids which are thought to develop into chloroplasts in normal illuminated leaves (Robertson and Laetsch, 1974). This suggests that there may be some block operating to prevent the light-induced differentiation of proplastids into chloroplasts in albino pollen plants.

Figure 3.1. Mesophyll Cells of Albino and Green Pollen Plants.

Panel A: Mesophyll cells from albino leaf tissue. The cell on the right hand side has electron-dense material completely occluding the large central vacuole (VG).

Panel B: Mesophyll cells from a green leaf tissue. Note the presence of several chloroplasts per cell located at the cell peripheries (c) and the apparent absence of chloroplasts in the albino.

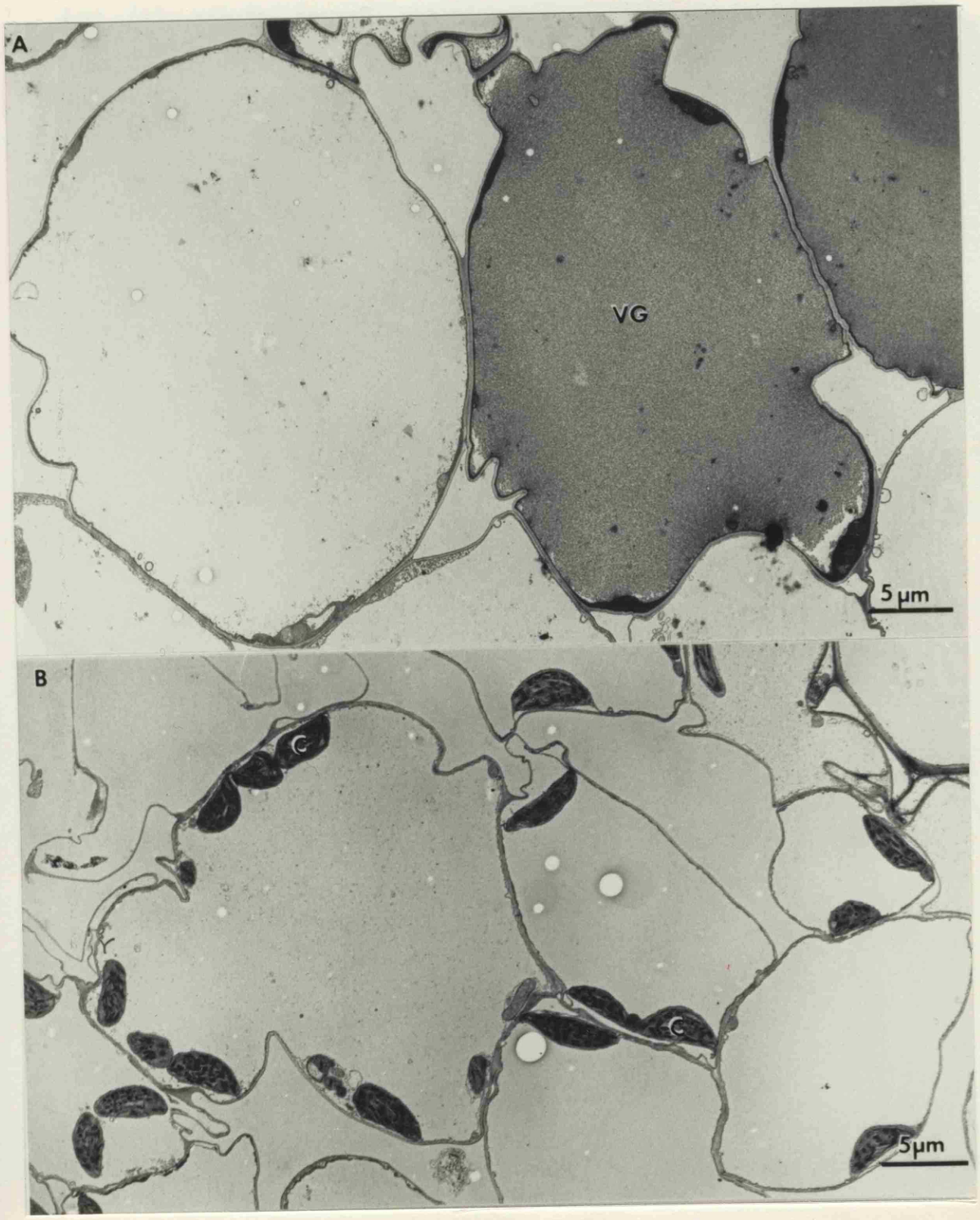

A
VG
5 µm
B
C
C
5µm

Figure 3.2. Comparison of Plastids from Mesophyll Cells of Green and Albino Pollen Plants.

Panel A: Plastid from green plant showing structural characteristics of mature chloroplasts. Granal stacks (g) and stromal lamellae (l) of the thylakoid membrane system are clearly visible. Lipid deposits may also be present (p). Other features include the cell wall (cw) and the plastid envelope (pe).

Panel B: Proplastid-like structures from an albino mesophyll cell. Note that these structures are smaller in number than the chloroplasts found in green mesophyll cells. Deposits of lipid (p) and phytoferritin (f) may be present. A disorganised mass of vesicular membranes (v) is present in one plastid as indicated. Proplastids also appear to contain structures resembling vacuoles (c). Other features include the cell wall (cw) and the plastid envelope (pe).

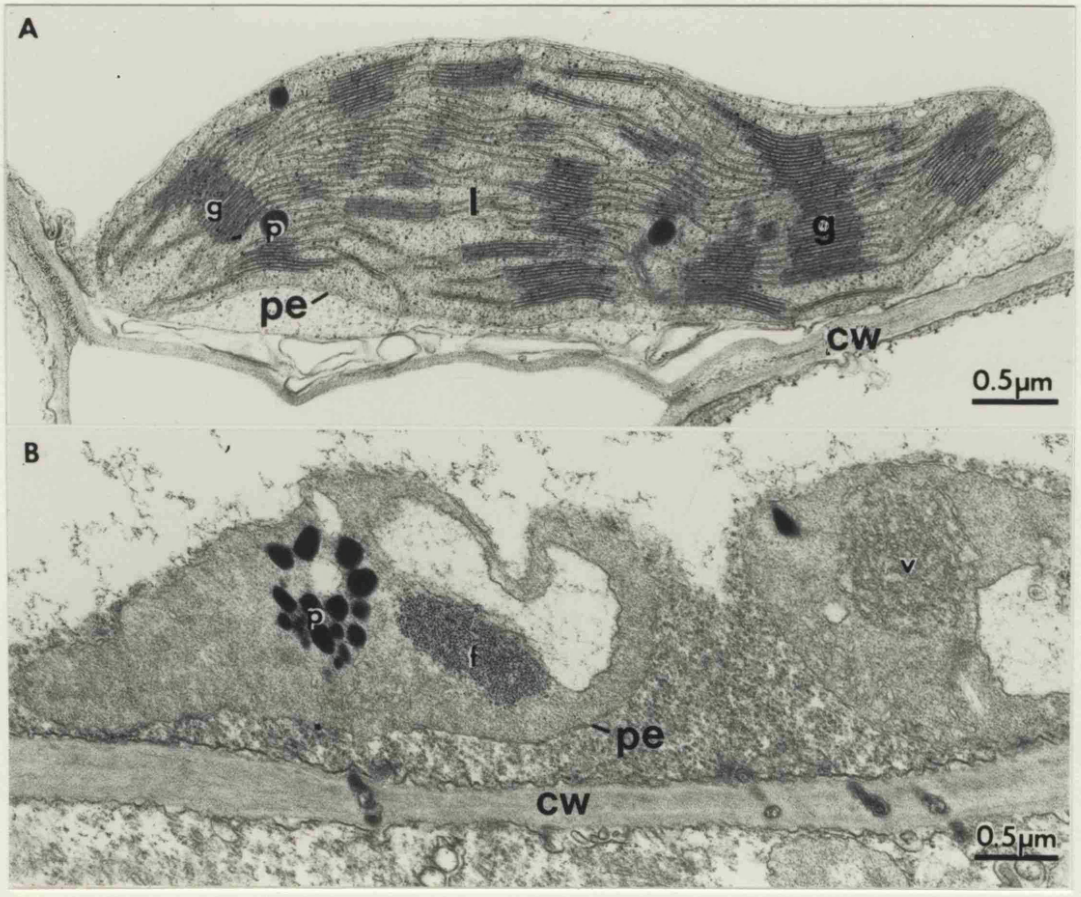

A
g
p
l
g
pe
cw
0.5µm
B
v
p
f
pe
cw
0.5µm

Figure 3.3. Structure of Mesophyll Cell and Plastid from Albino Barley Pollen Plant.

Panel A: Mesophyll cell with large central vacuole (o) and peripheral cytoplasm containing proplastid-like structure (p) and structures closely resembling mitochondria (m).

Panel B: Higher magnification of structures seen in panel A. The proplastid appears to contain lipid deposits (l), phytoferritin (f) and a disorganised membrane system (Vm). Structures closely resembling mitochondria (m) can be seen surrounding the proplastid. Note the presence of a vacuole-like structure in the proplastid.

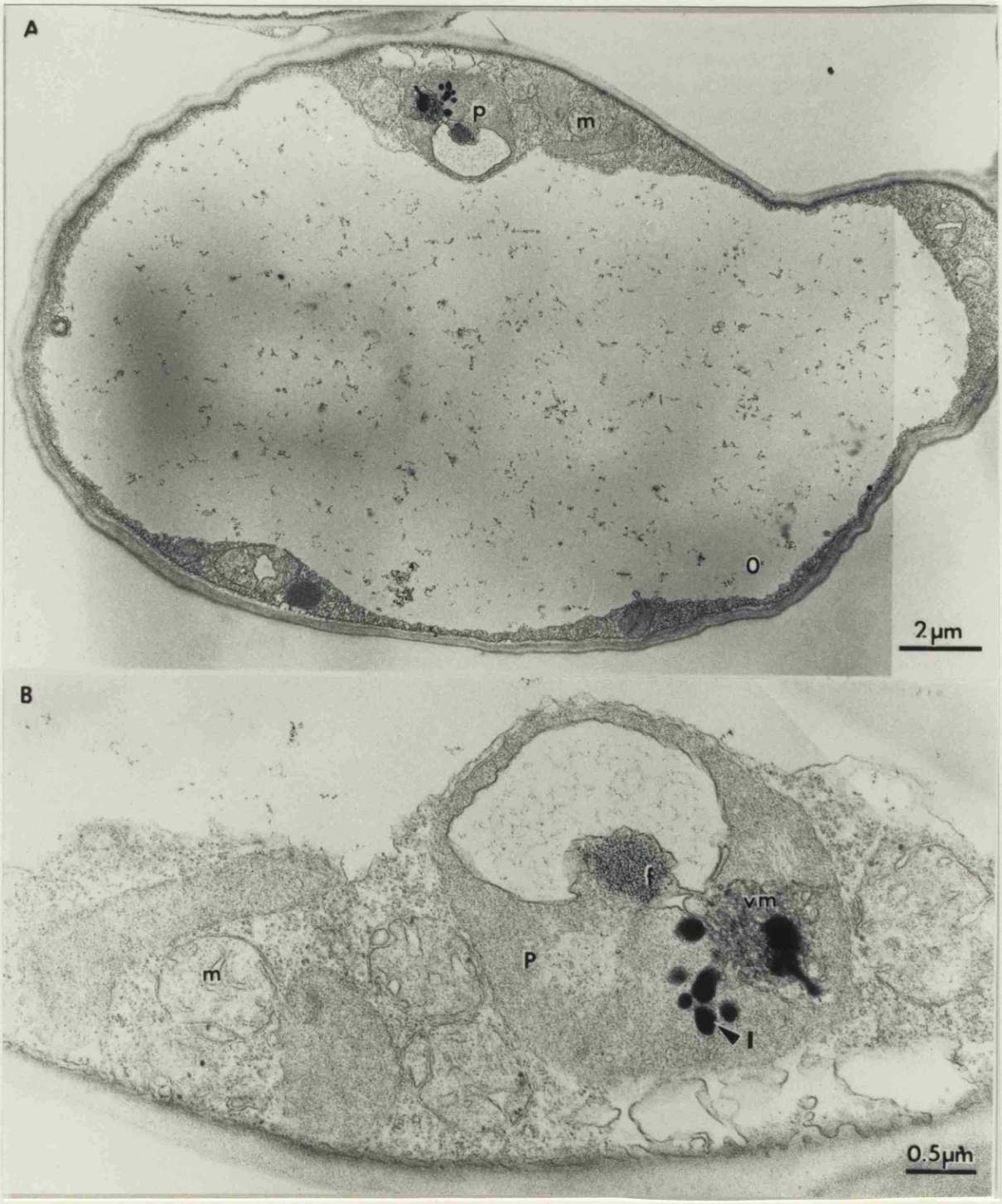

A
P
m
O
2 µm
B
f
vm
P
m
I
0.5µm

Figure 3.4. Details of the Structure of Proplastid-Like Structures from Mesophyll Cells of Albino Barley Pollen Plants.

Panel A: Several proplastids in a mesophyll cell (p). CW=cell wall.

Panel B: Magnification of proplastid with lipid (l) and phytoferritin (f) deposits and disorganised mass of vesicular membranes (vm). Note presence of double membrane plastid envelope (pe).

Panel C: Magnification of vesicular membrane.

Panel D: Magnification of phytoferritin deposits.

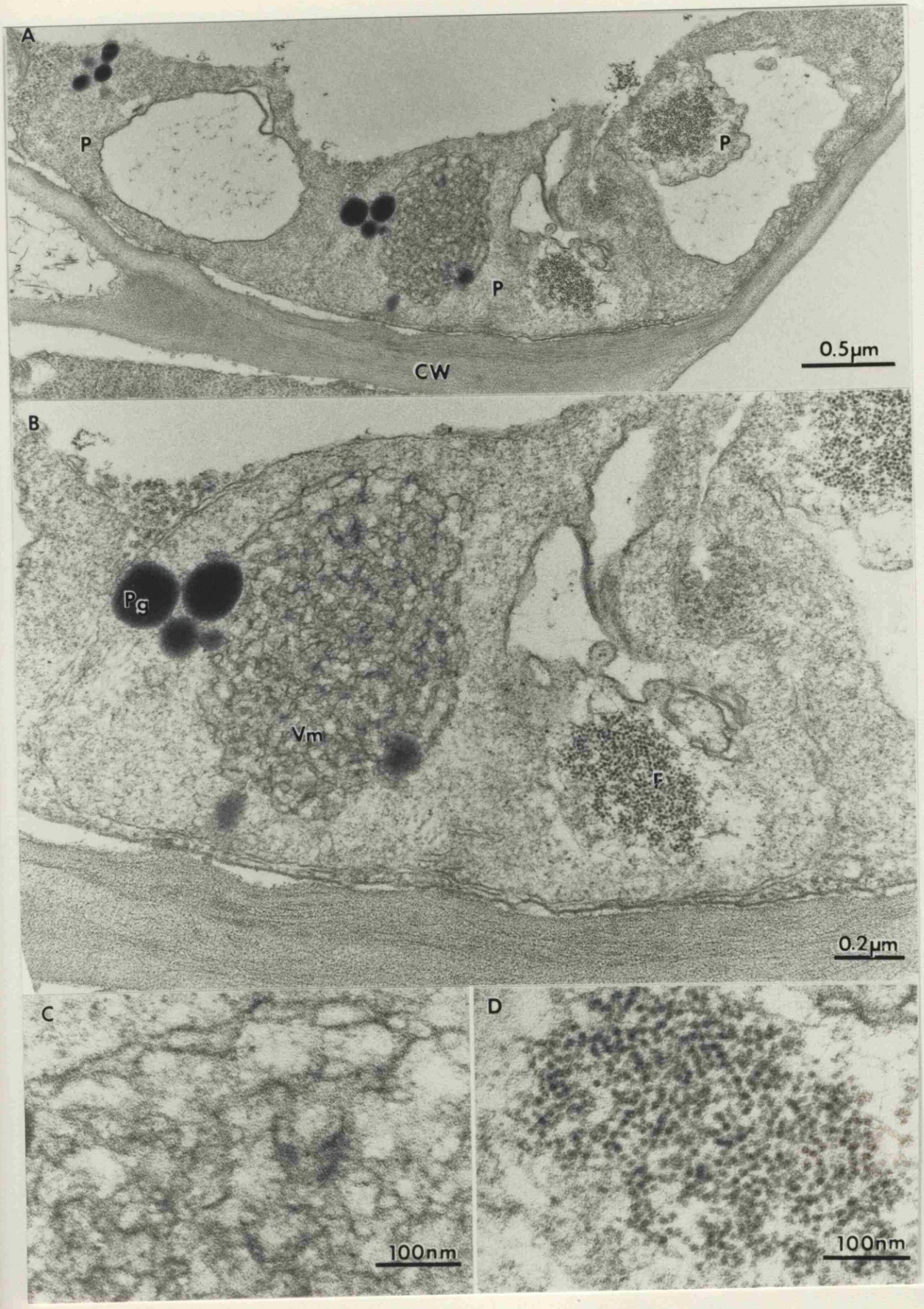
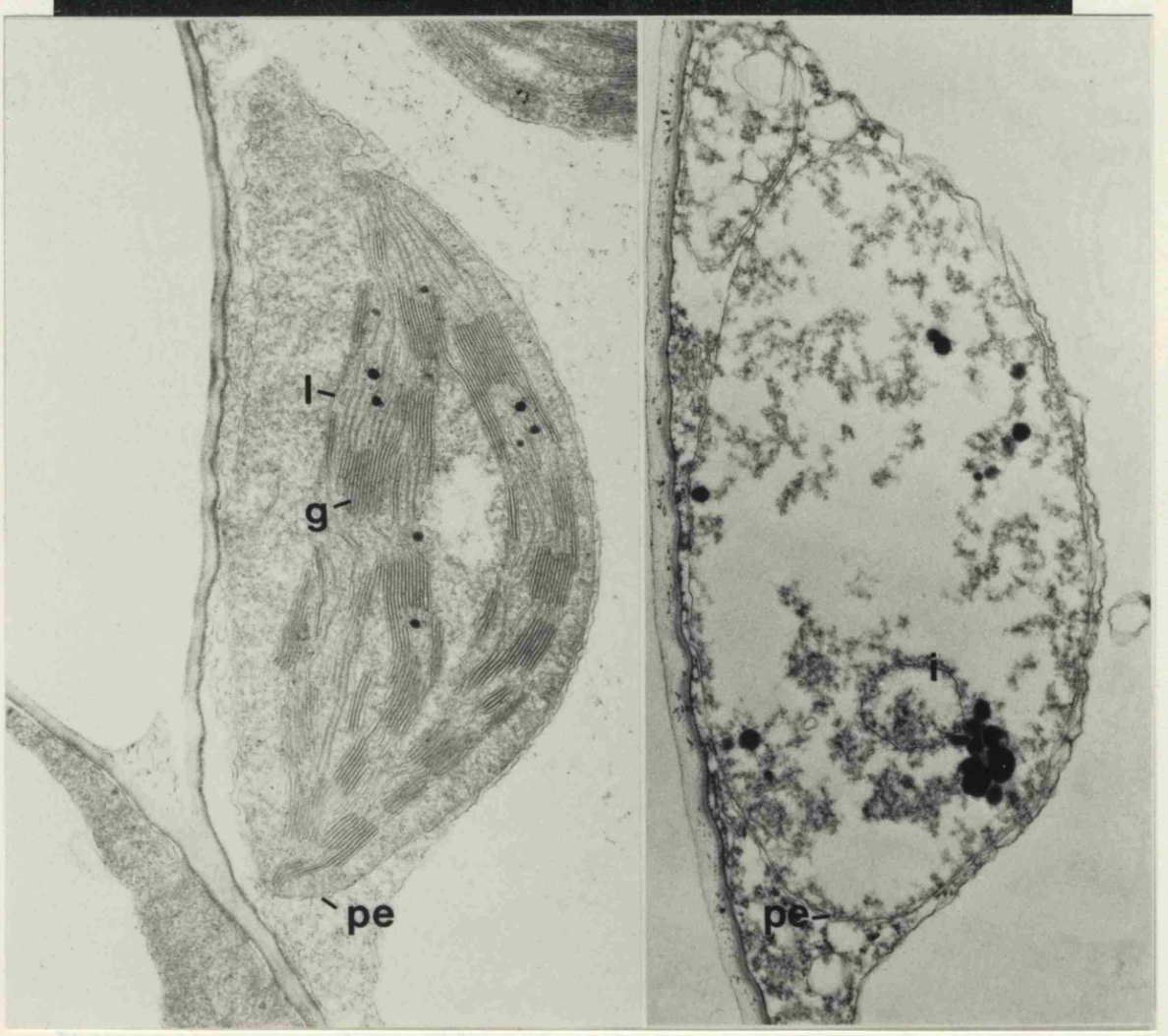

A
P
P
P
CW
0.5µm
B
Pg
Vm
F
0.2µm
C
100nm
D
100nm

Figure 3.5. Ultrastructural Features of Green and White Sectors of A Variegated Barley Pollen Plant.

Panel A: Section of variegated leaf showing green and white sectors.

Panel B: Chloroplast from green sector of variegated leaf. Features include granal (g) and stromal lamellae (s) of thylakoid the membrane system and the plastid envelope (pe).

Panel C: Plastid-like structure from white sector of variegated leaf. Note lack of almost all internal structure apart from granular inclusions as indicated (i). Plastid envelope (pe) is visible as indicated.


l
g
pe
l
pe

CHAPTER 4. *IN VITRO* PROPAGATION OF ALBINO POLLEN PLANTS.

4.1. General Approach.

Albino barley plants regenerated from anther culture generally remain very small, limiting the yields of tissue which can be obtained from such individuals. In order to carry out extensive studies of the molecular aspects of albinism it would be ideal to have available large quantities of plant material, thus allowing extractions of nucleic acids and proteins to be carried out from a number of individuals. Furthermore, it would also be an advantage for those sources of tissue to be maintained over a period of time, thus allowing any specific aspects of albino molecular biology to be investigated in more detail.

Several features of the growth pattern of barley could be exploited to achieve the long-term *in vitro* propagation of albino plants. Barley, in common with many grasses, produces several tillers which are stems carrying the foliage leaves and floral parts generated from a axillary meristem at the base of the plant. Tillers have the potential to be separated from the rest of the plant and could be cultured individually *in vitro*. It might be expected that the individual tillers might in turn produce tillers of their own and so allow further subdivision and propagation. Since a line of tillers cultured individually will have originated from the same plant they will presumably be genetically identical. In addition, since the foliage of barley plants originates from a basal meristem it could be possible to harvest albino leaf tissue by periodically cutting above the meristem under aseptic conditions each time allowing regrowth of the foliage. This could be used as an alternative or a supplement to the clonal propagation of tillers. The principal aim, therefore, was to devise a reliable culture medium which would allow vigorous tillering and good growth of foliage.

It was a common observation that during the *in vitro* culture of regenerants derived from anther culture, green individuals exhibited much more vigorous tillering and growth of foliage on both supplemented and unsupplemented media than albinos. Plants

grown *in vitro* were transferred to fresh media every 4-5 weeks. Despite this, albino plants commonly only survived for about three months before showing signs of senescence such as browning and curling of the leaf tip. Green plants derived from anther culture usually survived for much longer periods. It is not at present possible to define the nature of a deficiency that could reduce the growth of albino plants compared to green plants. However, a number of media were formulated to attempt an improvement of albino tillering and leaf growth. These included media with supplementary inorganic and organic nutrients, plant growth regulators and various undefined additions such as plant extracts. A summary of all the media used is presented in Table 4.1.

In the following experiments albino plants (in groups of 5-20) were compared visually with controls to assess the ability of different culture media to improve overall growth as defined by increased tillering frequency, leaf growth and rooting, etc. (see above). No specific measurements were made since visual comparison was thought to be sufficient to detect major differences in the ability of the various media to improve albino growth.

4.2. Comparison of Albino Plant Growth on Different Basal Media.

A variety of basal media were compared to see if any preferentially supported albino growth. MS and MS/2 when compared showed no detectable difference in their support of albino growth. These media contain much higher levels (10- and 5-fold more, respectively) of ammonium ions compared to FH, the medium used in the initial regeneration of plants from anthers. It was possible that initial culture on a medium with low levels of NH_4 followed by transfer to one containing higher levels of this ion might have a deleterious effect on albino growth. However, when plants regenerated on FH were transferred to FH or B5 (another medium with relatively low levels of NH_4) for 4-5 weeks no improvement in growth was seen when compared to that on MS or MS/2. In the following experiments the basal medium used is specified as appropriate.

Table 4.1. Summary of Media Used in Attempts to Improve Albino Growth.

Abbreviated names are shown on the right. Details of the preparation and constituents of all media are given in Section 2.18.

Basal Media:	Abbreviation:
Murashige and Skoog Medium	MS
Half-Strength MS	MS/2
Foroughi-Wehr Callus Induction Medium	FH
Gamborg's B5 Medium	B5
Basal Media with Nitrogenous Supplements:	
MS with 5mM Glutamine	MS+Glu
MS/2 with 5mM Glutamine	MS/2+Glu
MS with Casein Hydrolysate	MS+CH
B5 with Casein Hydrolysate	B5+CH
B5 with Amino Acids (Glutamine, Aspartic Acid, Arginine and Glycine)	B5+AA B5+2AA

Media with casein hydrolysate and amino acids mix were prepared without inorganic sources of nitrogen (see Section 2.18.2).

B5 with Additional KNO_3	B5+2N
Basal Media with Supplementary Plant Growth Regulators:	
MS with 0.1, 0.5 and 1.0mg/ml Benzylamino Purine	MS+0.1BAP MS+0.5BAP MS+1.0BAP
MS/2 with 1.0mg/ml Benzylamino Purine	MS/2+BAP
(MS/2 with 1.0mg/ml Benzylamino Purine and 5mM Glutamine	MS/2+BAP+Glu)
B5 with 1mg/l Indoleacetic Acid	B5+IAA
B5 with 1mg/l Naphthaleneacetic Acid	B5+NAA
B5 with 2mg/l 2,4-Dichlorophenoxyacetic Acid	B5+2,4D
B5 with 0.5, 1 and 2mM Gibberellic Acid	B5+0.5GA_3 B5+1.0GA_3 B5+2.0GA_3
Media with Supplements of Undefined Composition:	
B5 with 0.4% Malt Extract	B5+ME
MS with 10% Barley Seedling Extract	B5+BE
MS/2 with 5 and 20% Potato Extract	MS/2+5PE MS/2+20PE

4.3. Growth of Albino Plants Using Media Containing Nitrogenous Supplements.

Albino barley plants derived from anther culture lack mature chloroplasts (see Chapter 3) and probably do not perform photosynthesis. All *in vitro* culture media contained sucrose at 20g/l thus allowing heterotrophic growth of albino plants. However, chloroplasts perform a wide range of other metabolic functions including nitrogen assimilation and amino acid biosynthesis. It is possible that the block on chloroplast maturation seen in albino plants may have a profound effect on these aspects of metabolism and so contribute to a deficiency in albino growth. Furthermore, the addition of nitrogenous supplements to media used to maintain albino barley plants may compensate for any such deficiency and could lead to improved growth.

One of the first media tested in this context was B5 supplemented with twice the concentration of KNO_3 hence increasing the amount of available nitrate ions in the media for uptake and assimilation by the albino plants. In this experiment, increasing the amount of available nitrate had no detectable effect on albino growth when compared to untreated plants. As albino plants do not produce roots except under certain hormone treatments, this might lead to an inability to take up nitrate ions present in the growth medium. Plants treated with 1mg/l IAA produced many adventitious roots at their base after several weeks incubation (see Section 4.4). However, when additional KNO_3 was included in this treatment no detectable effect was seen on albino growth.

Since albino plants may be deficient in nitrogen assimilation it was decided to supplement basal media with a variety of amino acids to investigate their effect on albino growth. MS and MS/2 were supplemented with 5mM glutamine and Casein hydrolysate (CH), which contains a range of amino acids from the hydrolytic degradation of casein (MS+CH, B5+CH). In the case of B5+CH the basal medium was prepared without inorganic sources of nitrogen. B5 was also prepared with the addition of the amino acids aspartic acid, glycine, glutamine and arginine, again without inorganic sources of nitrogen (Toriyama and Hinata, 1985). In all these cases no enhancement of albino growth was observed in comparison to

untreated control plants after 4-5 weeks incubation.

4.4. Media with Supplementary Plant Growth Regulators.

Exogenous plant growth regulators (PGRs) are widely used to modify the growth of plant tissues *in vitro*. The experiments described below were designed to investigate if the application of a number of PGRs with a variety of effects could be used to improve tillering and the growth of foliage in albino plants.

Labes and Byshka (1979) have demonstrated that the cytokinin benzylamino-purine (BAP) promotes tillering of green barley plants *in vitro*. Maximum tillering was stimulated using concentrations of BAP between 5 and 10mg/l. Experiments were designed to compare the growth and tillering of albino plants cultured on MS and MS/2 media supplemented with 0, 1, 5 and 10mg/l concentrations of BAP. After incubation for one month no detectable difference was found between groups of albino plants treated with different concentrations of BAP. Although BAP did not radically improve leaf growth or tillering at concentrations up to 10mg/l after 4 weeks of culture it was possible that it might have an effect at a lower concentration after a extended period. To test this albino plants were cultured on MS and MS/2 supplemented with BAP at a concentration of 1mg/l for several months with transfer to fresh media every month. However, after several months culture on media containing 1mg/l BAP no increase in tillering frequency was observed.

Since albino plants do not produce roots (see Section 3.1) this may impair their ability to assimilate nutrients from the culture media *in vitro* with a correspondingly detrimental effect on growth. One of the effects of the group of PGRs known as auxins is to promote adventitious root formation (reviewed in Jacobsen, 1983). A number of auxins were tested to determine if they could induce root formation in albinos and if this had any effect on albino growth. Albino plants were grown on unsupplemented B5 media and B5 supplemented with indoleacetic acid (IAA) and naphthaleneacetic acid (NAA) both at a concentration of 1mg/l and the synthetic auxin 2,4-dichlorophenoxy-acetic acid (2,4D) at a concentration of 2mg/l.

Plants grown with auxins showed no improvement in growth compared to untreated plants after incubation for one month. However, IAA and NAA at a concentrations of 1mg/l did induce adventitious root formation in 90% of treated individuals.

Gibberellins are considered to have a stimulatory effect on plant growth (Jones and MacMillan, 1984). Experiments were designed to compare the growth of albino plants grown on B5 supplemented with gibberellic acid (GA_3) at concentrations of 0, 0.5, 1.0 and 2.0nM. After incubation for one month plants treated with GA_3 were taller (10-12cm) with thinner foliage when compared to untreated individuals. However, it was estimated that no overall increase in the amount of available tissue had occured.

4.5. Media with Supplements of Undefined Composition.

The use of complex media such as those based on vegetable extracts might improve albino growth by supplying a wide range of nutrients such as carbohydrates, inorganic ions, vitamins and amino acids (Thorpe, 1982). However, in such experiments it would be difficult to determine the exact factors in the complex media responsible for any improvement in albino growth. A number of media with supplements of undefined composition were tested for their ability to improve the growth of albinos. Experiments were carried out to compare the growth of albino plants grown on unsupplemented MS/2 media and that containing 5% and 10% potato extract, B5 with 10% barley seedling extract and B5 with 0.4% malt extract. The composition of media containing potato extracts were based on those described by Chuang *et al.* (1978). Barley seedling extract was added at the level suggested for the addition of potato extract. The level of malt extract added was arrived at empirically and was based on the concentrations of other complex additives, eg. yeast extract which are used in bacterial media such as Luria Broth (see Chapter 2). Ideally, a range of concentrations of each extract should have been tested for their effect on albino growth. However, this was not possible due to a limiting number of plants available for study. After several weeks incubation no improvement in growth was seen with plants grown on any of the supplemented media compared to plants grown on basal media alone.

4.6. Strategies for Harvesting and Propagating Albino Tissue.

Tillers were separated from a small number of plants and transferred onto a variety of supplemented and unsupplemented media. As described above, vigorous growth of the individual tillers could not be achieved. Also, it was hoped that the separate tillers from an individual would produce tillers of their own, but in no case was this observed. It is therefore doubtful whether individual albinos can be maintained for extended periods of time using the propagation of tillers as outlined in Section 4.1. In a number of individuals foliage was harvested in such a way as to make sure that tissue was removed from 1cm above the leaf base, ie. above the meristematic region. The plants were then allowed to grow back after a period of several weeks. Often what remained of the cut leaves showed signs of senescence with no new growth. Any re-growth was most often in the form of entirely new leaves. However, as a result of these experiments, no net increase in the quantity of albino tissue was achieved.

4.7. Summary.

The general aim of the *in vitro* culture experiments described was to formulate a reliable medium that would promote vigorous leaf growth and tillering in albino plants. This could then be used to generate large amounts of tissue from individual albino barley plants and allow the maintenance of these plants over an extended period of time. As a result individuals could be examined at the molecular level and then specific aspects arising from this could be analysed in more detail at a later stage. None of the culture conditions described in the preceding sections led to the enhancement of albino growth or their tillering frequency. As a result the strategies devised in Section 4.1 for the maintenance and propagation of albino plants met with limited success.

The underlying cause of poor growth in albinos is possibly very complex making it difficult to define an *in vitro* culture medium to compensate for this. Furthermore, it is possible that several factors are acting together to limit the growth of these plants requiring that more extensive experiments be carried out than was

feasible in the scope of this study. Material for subsequent analysis was grown on unsupplemented B5 medium which yielded small but sufficient quantities of tissue (0.5-1.0g) for an examination of at least some of the molecular aspects of the albino condition.

CHAPTER 5. THE STRUCTURE OF THE PLASTID GENOME IN ALBINO BARLEY POLLEN PLANTS.

5.1 Introduction.

Albino pollen plants of wheat and barley have been shown to contain deleted forms of ptDNA (Day and Ellis, 1984; Day and Ellis, 1985; see also Section 1.4). Therefore, as part of an extended investigation of albinism it was necessary to confirm this observation in my own population of albino pollen plants. Furthermore, this might allow correlations to be drawn between specific patterns of ptDNA deletion and any other features associated with albinism at the molecular level. The following sections describe restriction mapping experiments to elucidate the structure of ptDNA in normal seed-derived plants, albinos and green plants regenerated from anther culture.

5.2 Mapping The Plastid Genome of the Barley Variety Igri.

The restriction map of barley ptDNA has been determined previously (Poulsen, 1983; Day, 1985). However, there is evidence to suggest that intraspecific variation can exist in the ptDNA restriction maps of species belonging to the genus *Hordeum* including *H. vulgare* as well as certain conifer species (Holwerda *et al.*, 1986; Wagner *et al.*, 1987). The available restriction maps of barley ptDNA, corresponding to that of the varieties Bonus and Sabarlis, are very similar to each other. Since the variety used in this study was Igri, the possibility that there are differences between the ptDNA restriction patterns of this variety and others, eg. Sabarlis, due to intraspecific variation cannot be excluded. Therefore, restriction mapping experiments were performed to test the structural similarity between the ptDNA of Sabarlis and Igri. This exercise was also useful to test the procedures to be used later in the restriction mapping of ptDNA from albino plants.

A plasmid library of 10 *Pst* I restriction fragments covering 100% of Sabarlis ptDNA designated pHvCP1-10 (Day and Ellis, 1985; see Figure 5.1) was transformed into *E. coli*. Plasmid DNA was prepared

from bacterial transformants carrying each of the plasmids and insert DNA excised by digestion with *Pst* I. Each insert fragment (P1-10) was isolated and purified in preparation for radiolabelling for use in hybridisation experiments. 5μg aliquots of total DNA from Igri seedlings were digested with the restriction endonucleases *Pst* I, *Sal* I, *Xho* I and with combinations of *Pst* I with *Sal* I (*Pst* I + *Sal* I) and *Pst* I with *Xho* I (*Pst* I + *Xho* I). The restriction digests were fractionated by agarose gel electrophoresis, transferred to nylon hybridisation filters and hybridised with the ptDNA fragments P1-10. After autoradiography of the Southern blots, fragments hybridising with each of the ptDNA probes were compared in size to those determined from the *Pst* I, *Sal* I and *Xho* I restriction maps of Sabarlis ptDNA. For each of the enzymes used, singly and in combination, no major differences were seen in the size of the Igri ptDNA fragments and those expected from the ptDNA map of Sabarlis (Day, 1985). It was therefore concluded, at this level of analysis (±200bp), that Igri has a similar ptDNA restriction fragment pattern to that of Sabarlis for each of the enzymes used. The ptDNA restriction fragment map of *H. vulgare* var. Igri is presented in Figure 5.1.

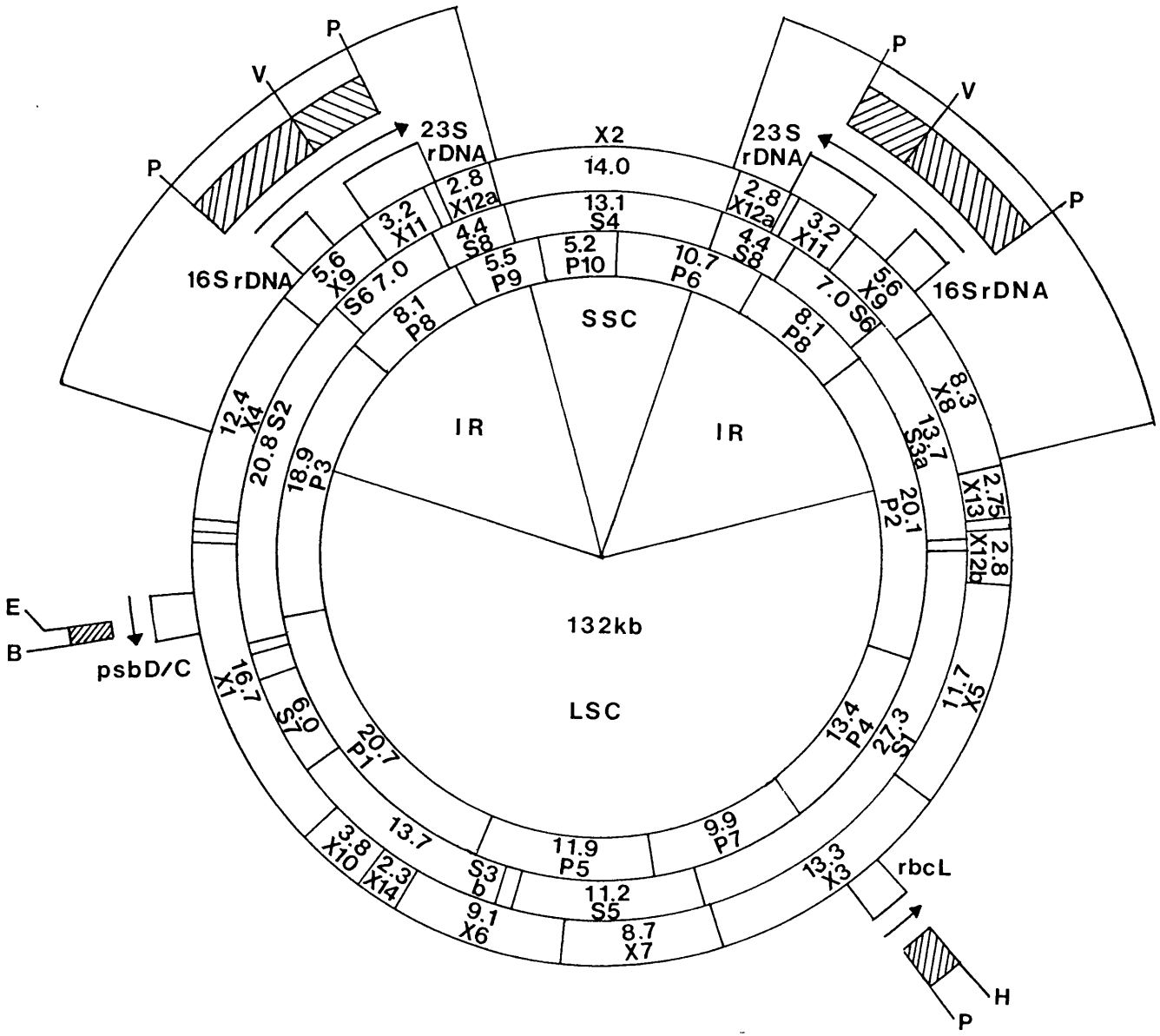
5.3. Mapping of the Plastid Genome in Albino Plants.

5.3.1. Approach.

Five albino pollen plants weighing approximately 1.0g were chosen for analysis. The plants were divided lengthways into two portions weighing approximately 0.5g. One portion from each individual was used for extraction of total DNA and the other for extraction of total RNA (see Chapter 6). Yields were in the range of 15-100μg of total DNA per sample. Two duplicate 5.0μg aliquots of total DNA from each albino were digested with the restriction enzymes *Pst* I and *Pst* I + *Xho* I, fractionated by electrophoresis on agarose gels and transferred to separate nylon hybridisation filters. To detect ptDNA restriction fragments in digests of total albino DNA Southern blots were probed sequentially with the *Pst* I derived ptDNA probes P1-P10. Successive hybridisations with different radiolabelled ptDNA fragments were carried out by an initial hybridisation, followed by autoradiography and stripping of hybridised probe from

Figure 5.1. Structure of the Barley Plastid Genome.

Inner circles show cleavage sites for the enzymes *Pst* I (P), *Sal* I (S) and *Xho* I (X). The sizes of all restriction fragments are given in kb. Restriction fragments are designated by the letter representing the enzyme used followed by a number (redrawn from Ellis and Day, 1985). The positions of the large single copy region (LSC), large inverted repeat (IR) and small single copy regions (SSC) are marked. Open segments on the outside of the restriction map mark the positions of the genes *rbcL*, *psbD-psbC* and also 16S and 23S rDNA sequences (Oliver, 1984; Berends *et al.*, 1987). Arrows indicate the direction of transcription. Hatched segments represent the restriction fragments used as hybridisation probes in the Northern blot analyses described in Chapter 6, the letters indicating the restriction enzymes used to generate them (Oliver, 1984; Berends *et al.*, 1987: B=*Bam*H I, E=*Eco*R I, H=*Hind* III, P=*Pst* I, V=*Pvu* II).


P
V
P
23S rDNA
16S rDNA
X2
14.0
2.8 X12a
3.2 X11
4.4 S8
5.6 X9
7.0 S6
13.1 S4
5.2 P10
5.5 P9
8.1 P8
SSC
IR
IR
12.4 X4
20.8 S2
18.9 P3
132kb
LSC
E
B
psbD/C
16.7 X1
6.0 S7
20.7 P1
13.7
3.8 X10
2.3 X14
S3
b
9.1 X6
11.9 P5
11.2 S5
8.7 X7
9.9 P7
13.4 P4
27.3 S1
13.3 X3
rbcL
H
P
10.7 P6
13.7 S3a
20.1 P2
2.75 X13
2.8 X12b
11.7 X5
8.3 X8
7.0 S6
5.6 X9
3.2 X11
4.4 S8
2.8 X12a
16S rDNA
23S rDNA
P
V
P

the filter as detailed in Section 2.12.7. After autoradiography of the hybridisation filters, the restriction fragment patterns obtained were compared to those expected from the map of barley ptDNA presented in Figure 5.1. A summary of the restriction fragments detected in these experiments is presented in Tables 5.1 and 5.2 and representative autoradiographs of each successive hybridisation with ptDNA probes P1-P10 are shown in Appendix 5.1. The data presented in Tables 5.1 and 5.2 is shown graphically in Figures 5.2 and 5.3.

Although efforts were made to check the efficiency of the stripping process carried out between successive hybridisations with different ptDNA probes by monitoring or autoradiography, the probe fragment was sometimes not entirely removed prior to subsequent rehybridisation. Because of this, alignment of autoradiographs obtained from the same Southern blot probed successively with different ptDNA fragments sometimes revealed bands that could be attributed to 'print-through', ie. where radiolabelled probe from a previous hybridisation produced a signal on an exposure of the same filter rehybridised with a second DNA fragment. The bands seen in these cases were not included in Tables 5.1 and 5.2 due to the difficulty in distinguishing whether they were really due to 'print-through' or the presence of authentic fragments hybridising to the second ptDNA probe.

In addition to band sizes, Tables 5.1 and 5.2 indicate the relative levels of the different ptDNA restriction fragments seen in each of the albino plants examined. Underloading of the lanes carrying control digests of seedling DNA resulted in the bands corresponding to ptDNA fragments being very faint compared to those found in albinos. Because of this it was more convenient to use A320 as an internal standard by which to estimate the relative intensity of ptDNA fragments in other plants (see Table 5.1 and Appendix 5.1). Visual comparison showed that the ratio of band intensities between digests of A320 and seedling DNA were similar for each ptDNA fragment used as a probe, indicating that the ptDNA fragments seen in A320 are present at the same stoichiometry as those in the intact plastid genome (see also Section 5.3.6). In contrast, the ratio of intensities between ptDNA fragments in other albinos and those seen in the control differed over a wide range. Where

Table 5.1. Summary of Restriction Fragments Found In Southern Analyses of ptDNA from Albino Plants.

The size of restriction fragments, in kb, hybridising to ptDNA probes P1-P10 are shown for *Pst* I (P) and *Pst* I + *Xho* I (PX) digests of DNA extracted from albino plants A301 and A302 (see Appendix 5.1). Fragments derived from digests of green leaf DNA (Wild Type) are included for comparison. Probe fragments are indicated on the far left. The intensity of albino ptDNA fragments relative to those found for A320 (see Section 5.3.1) is given in brackets: w = weak hybridisation, m = medium, no mark = strong hybridisation comparable to A320. An asterix (*) denotes an unusually high level of hybridisation relative to other ptDNA restriction fragments.

	Wild Type.		A301.		A307.	
	P	PX	P	PX	P	PX
P1	20.7	12.0	20.7	12.0	20.7(*)	18.6
		3.8		8.1(w)	11.0	12.0
		2.6		3.8		3.8
		2.3		2.6		2.6
				2.3		2.3
P2	20.1	12.4	20.1	13.8(w)	16.6(w)	12.4(w)
		8.3	16.6(w)	12.4(w)		4.6(w)
		4.6	14.8(w)	11.0(w)		2.8(w)
		2.8	13.2(w)	9.1(w)		
		0.6	11.0(w)	8.3(m)		
			9.8(w)	6.6(m)		
			5.8(w)	5.4(w)		
				4.6		
				4.3(w)		
				2.8		
				0.6(w)		
P3	18.9	12.4	18.9	12.4(m)	12.4(m)	12.4(m)
		8.3	13.5(m)	10.9(w)	9.3(m)	9.3(m)
		4.7		9.6(w)	7.6(w)	8.7(w)
		0.6		8.3	6.2(*)	7.1(w)
				6.8(m)	2.7(w)	5.6(w)
				5.6(w)	2.6(w)	4.7(*)
				4.7(m)	1.3(w)	2.8(w)
				4.3(w)		2.6(w)
				0.6(w)		2.2(*)
						0.5
P4	13.4	6.7	13.4	20.9(w)	13.4(w)	6.7(w)
			6.7(m)	13.0(m)		
			4.3(w)	12.0(w)		
				10.5(w)		
				9.6(w)		
				6.7		
				6.3(w)		
				4.3(w)		

Table 5.1 Continued.

	Wild Type.		A301.		A307.	
	P	PX	P	PX	P	PX
P5	11.9	6.5 5.4	11.9	6.5 5.4	-	-
P6	10.7 5.5	7.1 2.8 1.7 0.8	10.7 5.5	7.1 2.8 1.7 0.8	10.7(m) 5.5(m)	7.1(m) 2.8(m) 1.7(m) 0.8(m)
P7	9.9	6.6 3.3	9.9	6.6 3.3	19.5(w) 9.9(w) 8.3(w)	6.6(w) 6.0(w) 3.3(w) 2.9(w) 2.0(w)
P8	8.1	5.0 3.1	8.1	5.0 3.1	8.1(m)	5.0(m) 3.1(m)
P9	10.7 5.5	7.1 2.8 1.7 0.8	10.7 5.5	7.1 2.8 1.7 0.8	10.7(m) 5.5(m)	7.1(m) 2.8(m) 1.7(m) 0.8(m)
P10	5.2	5.2	5.2	5.2	5.2(m)	5.2(m)

Table 5.2. Summary of Restriction Fragments Found in Southern Analyses of ptDNA from Albino Plants.

The size of restriction fragments, in kb, hybridising to ptDNA probes P1-P10 are shown for *Pst* I (P) and *Pst* I + *Xho* I (PX) digests of DNA extracted from albino plants A308, A309 and A320 (see Appendix 5.1). Fragments derived from digests of green leaf DNA (Wild Type) are included for comparison. Probe fragments are indicated on the far left. The intensity of albino ptDNA fragments relative to those found for A320 (see Section 5.3.1) is given in brackets: w = weak intensity, m = medium intensity, no mark = strong hybridisation, comparable to A320. An asterix (*) denotes an unusually high level of hybridisation relative to other ptDNA restriction fragments.

	Wild Type.		A308.		A309.		A320.	
	P	PX	P	PX	P	PX	P	PX
P1	20.7	12.0	9.3	9.3	20.7(m)	12.0(m)	20.7	12.0
		3.8				3.8(m)	15.8(w)	3.8
		2.6				2.6(m)		2.6
		2.3				2.3(m)		2.3
P2	20.1	12.4	20.1	12.4	24.0(w)	12.4(m)	20.1	12.4
		8.3	8.3(m)	7.4(m)	20.1(w)	11.8(w)	17.8(w)	8.3
		4.6		0.6(w)	17.8(m)	8.3(w)		7.3(w)
		2.8			13.5(w)	6.5(w)		4.6
		0.6(w)			10.7(w)	4.6(w)		2.8
					7.8(w)	4.1(w)		0.6(w)
						2.8(m)		
						0.6(w)		
P3	18.9	12.4	18.9(*)	12.4(*)	18.9(w)	12.4(m)	18.9	12.4
		8.3	8.3(m)	7.4(m)	16.6(m)	8.3(w)		8.3
		4.7		4.7(*)		4.7(m)		4.7
		0.6(w)				4.2(w)		0.6(w)
						0.6(w)		
P4	13.4	6.7	22.4(w)	12.6(w)	13.4(w)	6.7(w)	13.4	6.7
P5	11.9	6.5	-	-	11.9(m)	6.5(m)	11.9	6.5
		5.4				5.4(m)		5.4
P6	10.7	7.1	4.3(w)	15.1(w)	10.7	7.1	10.7	7.1
	5.5	2.8	2.0(w)	2.4(w)	5.5	2.8	5.5	2.8
		1.7		1.3(w)		1.7		1.7
		0.8		0.8(w)		0.8		0.8
P7	9.9	6.6	-	-	9.9(m)	6.6(m)	9.9	6.6
		3.3				3.3(m)		3.3
P8	8.1	5.0	14.5(w)	5.0(m)	14.1(w)	7.1(m)	8.1	5.0
		3.1	8.1(w)	3.6	8.1	5.0		3.1
			7.6(w)	3.1(w)		3.1		
			3.6					

Table 5.2 Continued.

	Wild Type.		A308.		A309.		A320.	
	P	PX	P	PX	P	PX	P	PX
P9	10.7	7.1	4.3(w)	15.1(w)	10.7	7.1	10.7	7.1
	5.5	2.8	2.0(w)	2.4(w)	5.5	2.8	5.5	2.8
		1.7		1.3(w)		1.7		1.7
		0.8		0.8(w)		0.8		0.8
P10	5.2	5.2	-	-	5.2	5.2	5.2	5.2

Figure 5.2. Summary of *Pst* I Derived ptDNA Fragments Found in Albino Plants A301 and A307.

The lower bar represents the linear map of intact barley ptDNA showing the position of *Pst* I derived fragments (P1-10; see Figure 5.1). The position of inverted repeat sequences are represented by horizontal lines below the map. The small bar at the junction of P1 and P3 indicates the position of a *Sst* I fragment shown to be retained in the ptDNA of albino barley pollen plants studied by Day and Ellis (1985). The positions of the genes *rbcL*, *psbC* and *psbD* and also the 16S and 23S rDNA sequences are indicated by open boxes below the intact restriction map. The scale is in kb, as indicated.

The positions of *Pst* I derived fragments found in albino plants A301 and A307 are shown above the map of intact barley ptDNA. This represents data summarised from Table 5.1 and the autoradiographs shown in Appendix 5.1. Further details of mapping are given in Sections 5.3.2-5.3.6. Vertical dotted lines show the limits of intact *Pst* I fragments. Filled bars represent fragments with an intensity similar to that found for A320 (see Figure 5.3 and Section 5.3.1). Hatched and open bars represent fragments with intermediate and low intensity, respectively, in the same comparison. Filled bars marked with an asterix (*) represent fragments that are unusually intense compared to others (see section 5.3.4). Bars flanked by horizontal dotted lines represent restriction fragments where a map location on the intact plastid genome was unable to be assigned. Bars with cross hatched ends indicate fragments that are larger than the hybridising ptDNA probe. The numbers within these fragments indicate their size in kb.

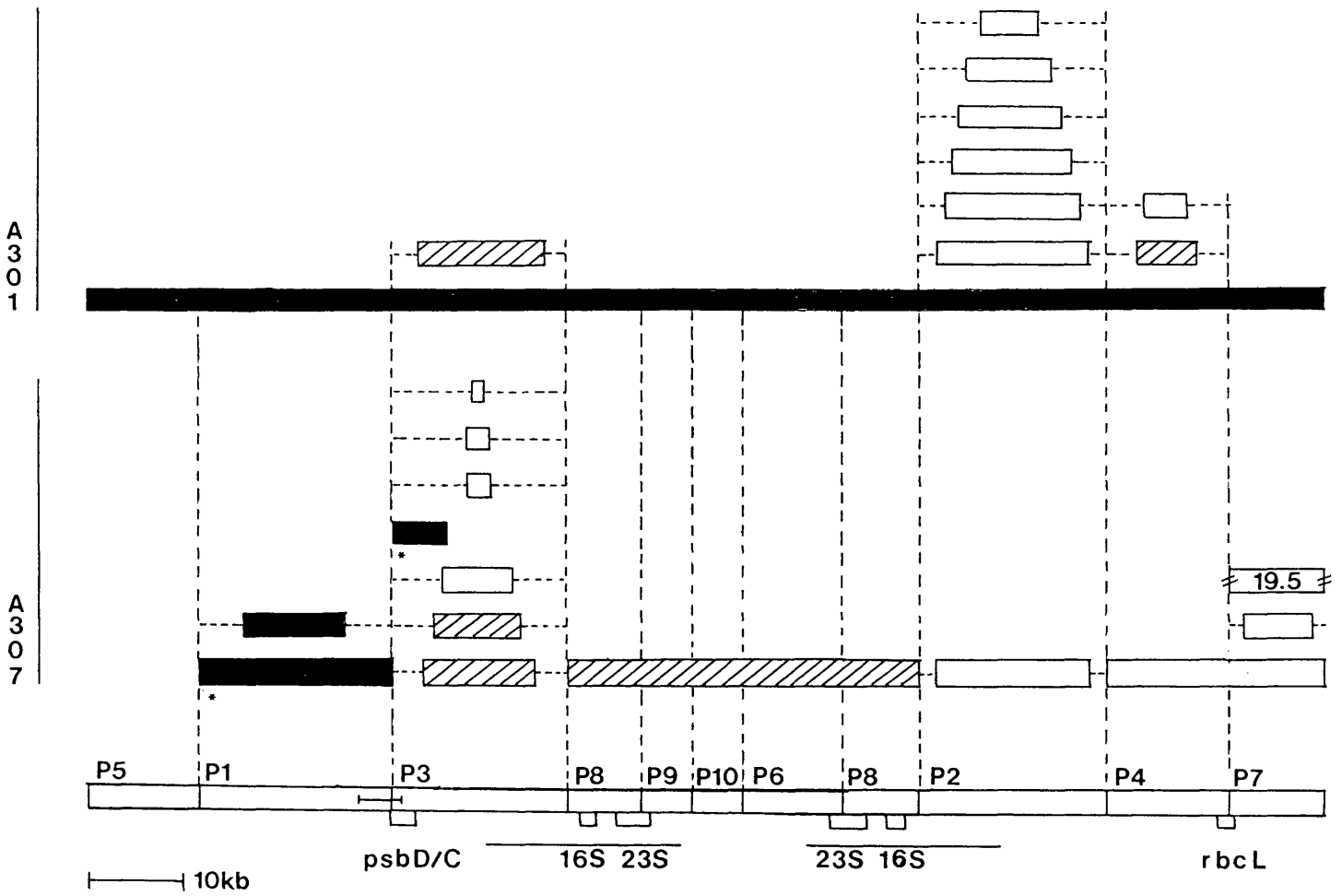
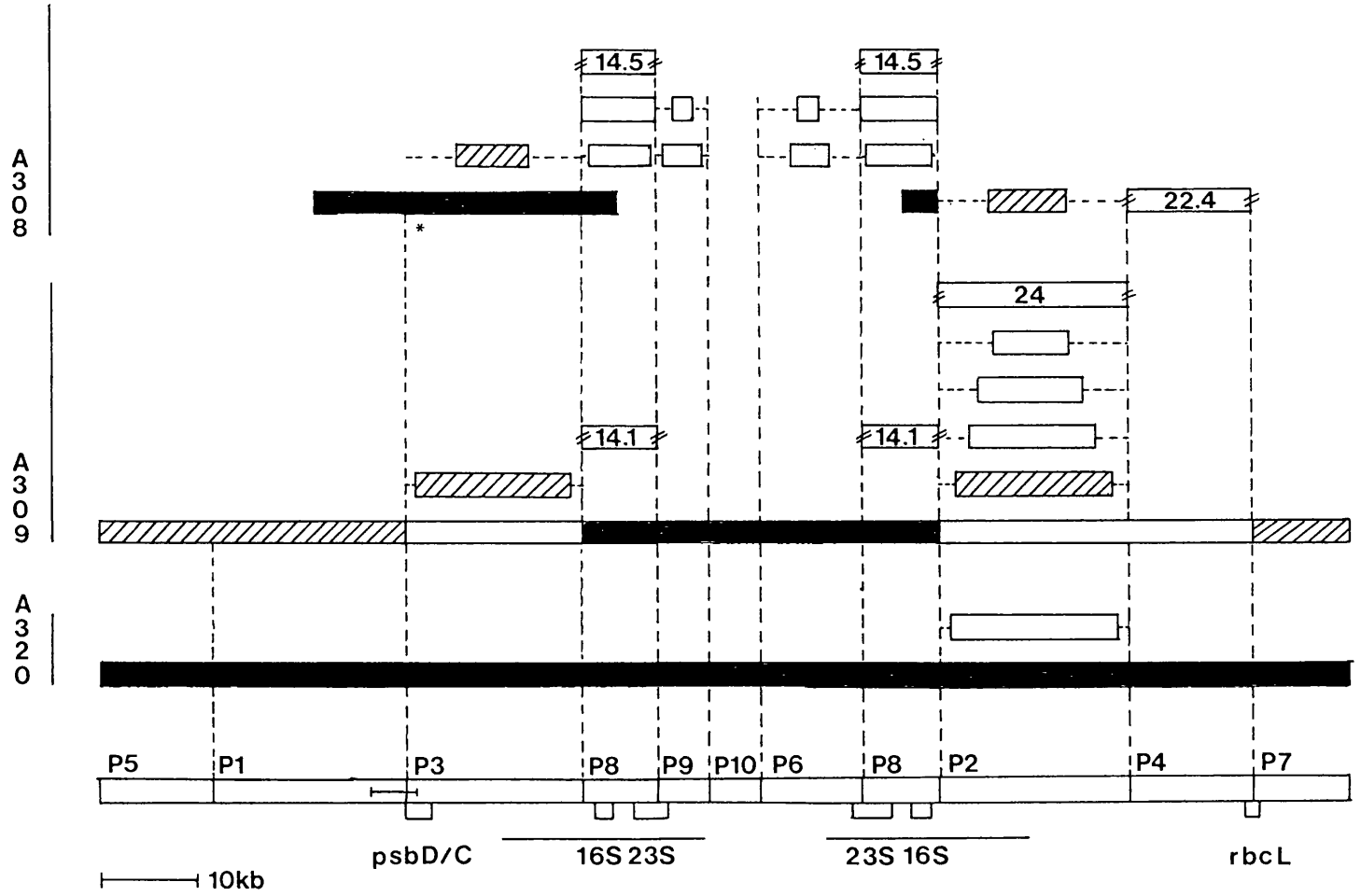

A301
A307
19.5
P5
P1
P3
P8
P9
P10
P6
P8
P2
P4
P7
psbD/C
16S 23S
23S 16S
rbcL
10kb

Figure 5.3. Summary of *Pst* I Restriction Fragments Found in Albino Plants A308, A309 and A320.

For key refer to legend in Figure 5.2.


A308
A309
A320
14.5
14.5
22.4
24
14.1
14.1
*
P5
P1
P3
P8
P9
P10
P6
P8
P2
P4
P7
psbD/C
16S 23S
23S 16S
rbcL
10kb

possible, laser densitometry was used to obtain a more accurate estimate of relative band intensity (see Section 5.3.6).

The following sections include a summary of the data presented in Tables 5.1 and 5.2, and the structure of the ptDNA in some albino plants is discussed in more detail. For simplicity, bands that correspond in size to those expected from the restriction map of barley ptDNA are termed wild type, and fragments that are of altered size, non-wild type.

5.3.2. Albino Plant A308.

The ptDNA restriction fragment pattern of A308 has been extensively altered from that of the wild type. The only major bands detected in restriction digests of total DNA from A308 hybridise to probe fragments P1, P3 and P8. It does appear however that that probe P2 also hybridises to bands of 12.4kb (*Pst* I + *Xho* I) and 20.7kb (*Pst* I) at high intensity. These are probably derived from P3 since they correspond in size to fragments that would be detected due to cross homology between the regions of P2 and P3 encompassing sequences in the inverted repeat region (IR: see Figure 5.1). The absence of restriction fragments corresponding specifically to P2 (see Table 5.2) suggest that this region of the plastid genome has been lost in plant A308. The presence of 22.4kb *Pst* I and 12.6kb *Pst* I+*Xho* I derived bands hybridising to probe P4 could be due to cross-homology between P4 and P3 (see Section 5.7). The ptDNA probes P5, P6, P7, P9 and P10 hybridised weakly to bands of non-wild type size or showed no detectable hybridisation. This indicates that the major form of ptDNA in A308 has lost the small single copy region (SSC) and most of the large single copy region (LSC) possibly during extensive single or multiple deletion events.

Wild-type size *Pst* I and *Pst* I + *Xho* I derived restriction fragments hybridise to ptDNA probe P3 in digests of total DNA from A308 (see Figure 5.4A). In addition, weakly hybridising bands of 8.3kb and 7.4kb derived from *Pst* I and *Pst* I + *Xho* I digests respectively are also detected with this probe. It is interesting to note that these bands are also present at the same level when P2 is used as a probe. The significance of this is unclear but may

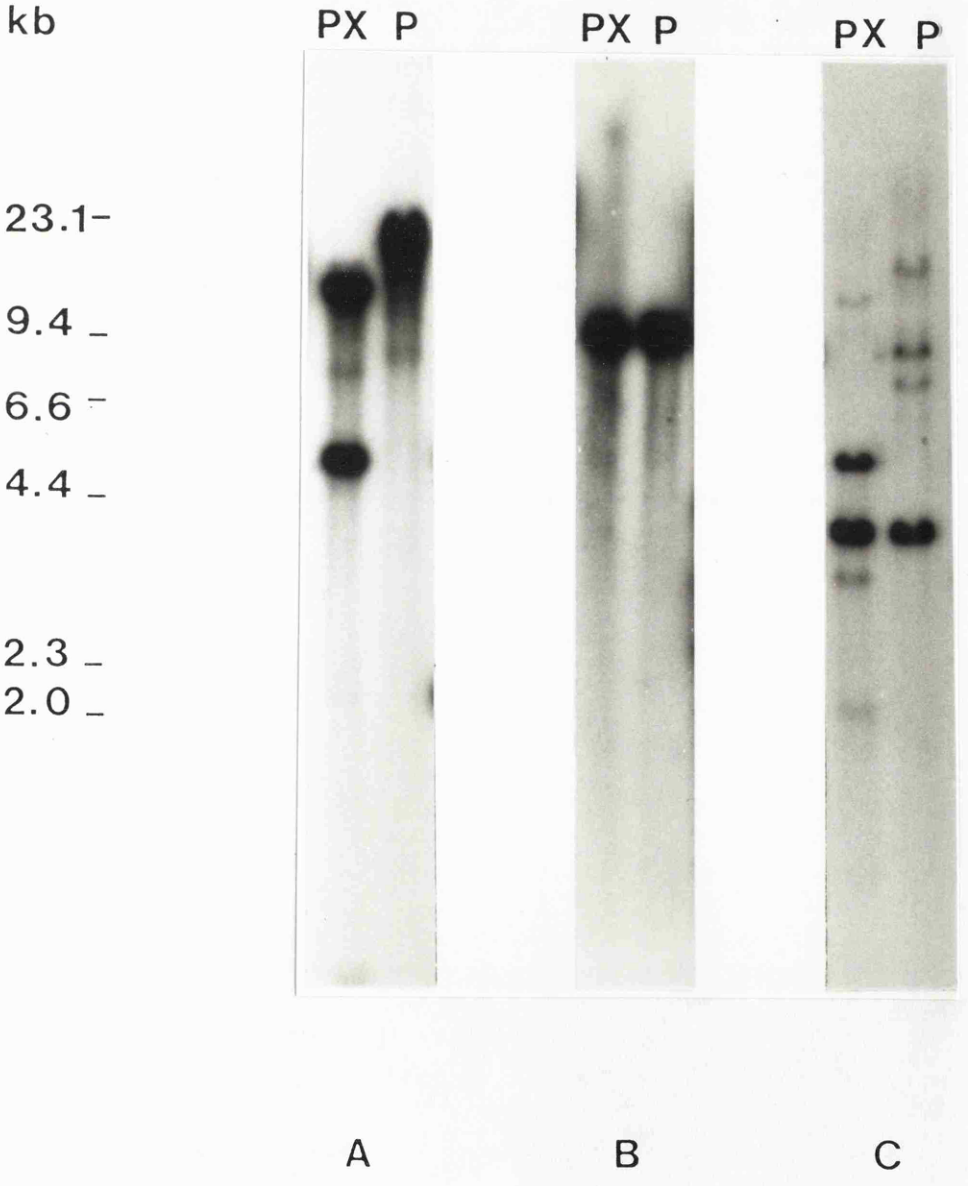
mean that these fragments are included in the region of homology between P2 and P3. As indicated before, P3 also hybridises strongly to bands of wild type size and also of non-wild type size at lower intensity. The intensities of the wild type bands are unusually high relative to those detected elsewhere in the ptDNA of A308 and also in other plants. Laser densitometry scans of these bands indicate that they are present at an intensity twice that of those seen in A320. This is puzzling especially since the flanking fragments P1 and P8, although altered, are comparable in intensity to those seen in A320.

A major band of 9.3kb can be seen in both *Pst* I and *Pst* I + *Xho* I digests when ptDNA fragment P1 is used as a hybridisation probe (see Figure 5.4B). Similarly, the major band seen in both digests when P8 is used as a probe is 3.6kb (see Figure 5.4C). These bands represent truncated forms of the wild type 20.7kb P1 (P1') and 8.1kb (P8') fragments. It should be noted that in both cases the same size fragment is generated with the enzymes *Pst* I and *Pst* I + *Xho* I. This suggests that the variant fragments P1' and P8' may be derived from a *Pst* I digest only. Further restriction mapping data (data not shown) indicates that the 3.6kb P8' fragment is a truncated form of a 5.2kb *Pvu* II + *Pst* I derived sub-fragment of P8 (illustrated in Figure 5.1; see also Section 6.3.4). Bands corresponding in size to wild type fragments are also present but at lower intensity than the 3.6kb bands as are weakly hybridising bands of non-wild type size. The presence of wild type and non-wild type size bands at different stoichiometries suggests that A308 may contain multiple forms of the plastid genome.

Linear and circular structures have been proposed for a number of ΔptDNAs found in albino wheat and barley plants derived from anther culture (Day and Ellis, 1984; Day and Ellis 1985). If the major restriction fragments described above are located on the same molecular species then the most abundant form of ptDNA in A308 appears to include *Pst* I derived fragments of 18.9kb (P3), 9.3kb (P1') and 3.6kb (P8'). However, it should be noted that P3 appears to be present at a higher abundance than either P1' or P8' indicating that its copy number is higher than the other major ptDNA fragments in A308. One explanation is that A308 possesses a major ΔptDNA with one copy each of P3, P1' and P8', the higher

Figure 5.4. Major ptDNA Restriction Fragments Seen in Albino A308.

Autoradiographs of lanes containing fractionated total DNA from A308 digested with *Pst* I (P) or *Pst* I + *Xho* I (PX) and probed with ptDNA fragments P3 (Panel A), P1 (Panel B) and P8 (Panel C). Size markers indicated in kb on the left. The hybridisation filters were washed as described in Appendix 5.1. Autoradiographs represent 16-96 hour exposures to X-ray film at -80°C.


kb
PX P
PX P
PX P
23.1
9.4
6.6
4.4
2.3
2.0
A
B
C

levels of P3 being taken account of by the presence of separate molecular species containing P3. It should be noted in this context that the presence of minor wild type and non-wild type bands hybridising to probe P8 suggests the presence of multiple forms of ΔptDNA in A308. Alternatively, the major form of ptDNA in A308 may contain more copies of P3 than the fragments P1' or P8'. Lastly, P1' and P8' may exist on a separate ΔptDNA to P3, the P3 fragment respresenting an autonomous molecular species (for further discussion see Section 8.2.2). Although a number of alternatives are possible, in the following discussion it will be assumed for simplicity that the first model is correct.

Placing P3, P1' and P8' on the wild type restriction map suggests two structures, one circular and one linear which are consistent with the restriction fragment data (see also Figure 5.2). Both molecules are 31.8kb in size, which represents the deletion of 75% of all ptDNA sequences, including the SSC and portions of the LSC and IR regions. However, both types of molecule rely on different mechanisms for their generation. First, it should be noted that P1, P3 and P8 lie adjacent to each other on the circular map of wild type barley ptDNA (see Figure 5.1) but that there are two copies of the fragment P8 located in each IR region. The linear molecule could be generated by a deletion event that ends in P1 and the copy of P8 adjacent to P3 leaving 9.3kb and 3.6kb derivatives of these fragments flanking the intact P3. The free ends of the altered fragments P1' and P8' in this structure (see Figure 5.5) would explain why the same size fragment is generated by *Pst* I and *Pst* I+*Xho* I digests since no *Xho* I sites exist between the free ends and the *Pst* I sites flanking P3.

A simple scheme linking the two ends of P1' and P8' in a circular molecule would involve their fusion as a result of recombination between sequences in both fragments (see Section 1.4). This would mean that the same size *Pst* I derived fragment, representing the fusion fragment P1'-P8', would be detected using both P1 and P8 as hybridisation probes. This is not the case in A308. Two hypothetical two step pathways to generate circular ΔptDNA consistent with the restriction fragment data from A308 are outlined in Figure 5.6. These deletion events include recombination between LSC and SSC regions, SSC and IR regions and between

Figure 5.5. Possible Structure of a Linear ΔptDNA in Albino A308.

The inner circle represents a *Pst* I derived map of the intact plastid genome. Numbers refer to *Pst* I derived fragments P1-10. The outer segment represents the possible map position of the major restriction fragments described in Section 5.3.2. P1' and P8' represent altered versions of the intact fragments P1 and P8. Deltas (Δ) show the possible end-points of unspecified deletion events occurring in P1 and P8. The sizes of intact and altered ptDNA molecules found in normal plants and A308, repectively, are indicated in kb.

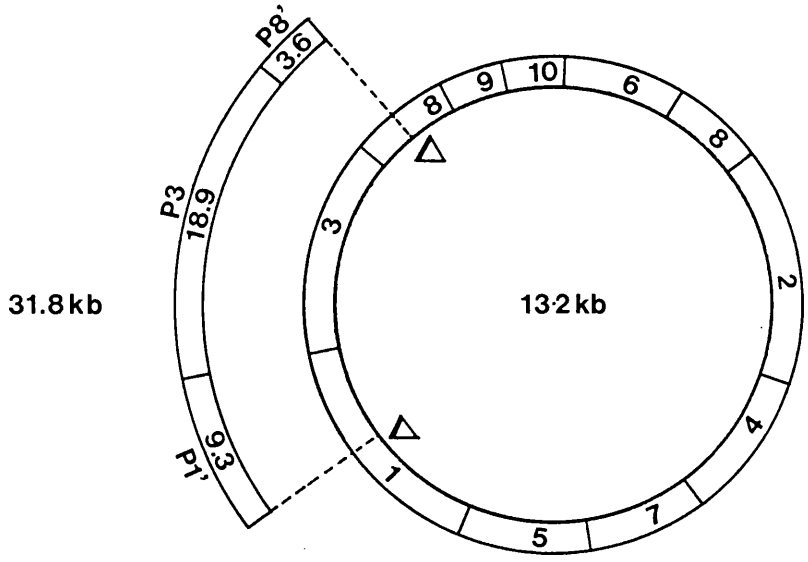

P8'
3.6
P3
18.9
P1'
9.3
31.8 kb
8
9
10
6
8
2
4
7
5
1
3
13·2 kb

Figure 5.6. Possible Recombination Schemes for the Generation of Circular ΔptDNA Molecules in A308.

Two alternative schemes are shown in A and B, steps I-III. Step I shows the intact barley plastid genome. The map positions of *Pst* I derived restriction fragments P1-10 are indicated. The filled lines on the inside of the intact map indicate the position of inverted repeat sequences. Radial dotted lines specify endpoint positions for the initial deletion event, Δ^1. Concentric dotted lines represent sequences removed during this deletion event. Step II shows the remaining ptDNA after event Δ^1 and the positions of the endpoints for the second deletion event, Δ^2. The position of deletion endpoints in P8, pathway A, are unclear (see Section 5.3.2) but possible locations are delimited by twin radial dotted lines. Step III indicates the remaining ptDNA after event Δ^2. The fusion of fragments P1 and P2, and P1 and P6 are indicated as 1+2 and 1+6, respectively. Fragment 8' refers to the altered version of P8 generated in pathway A.

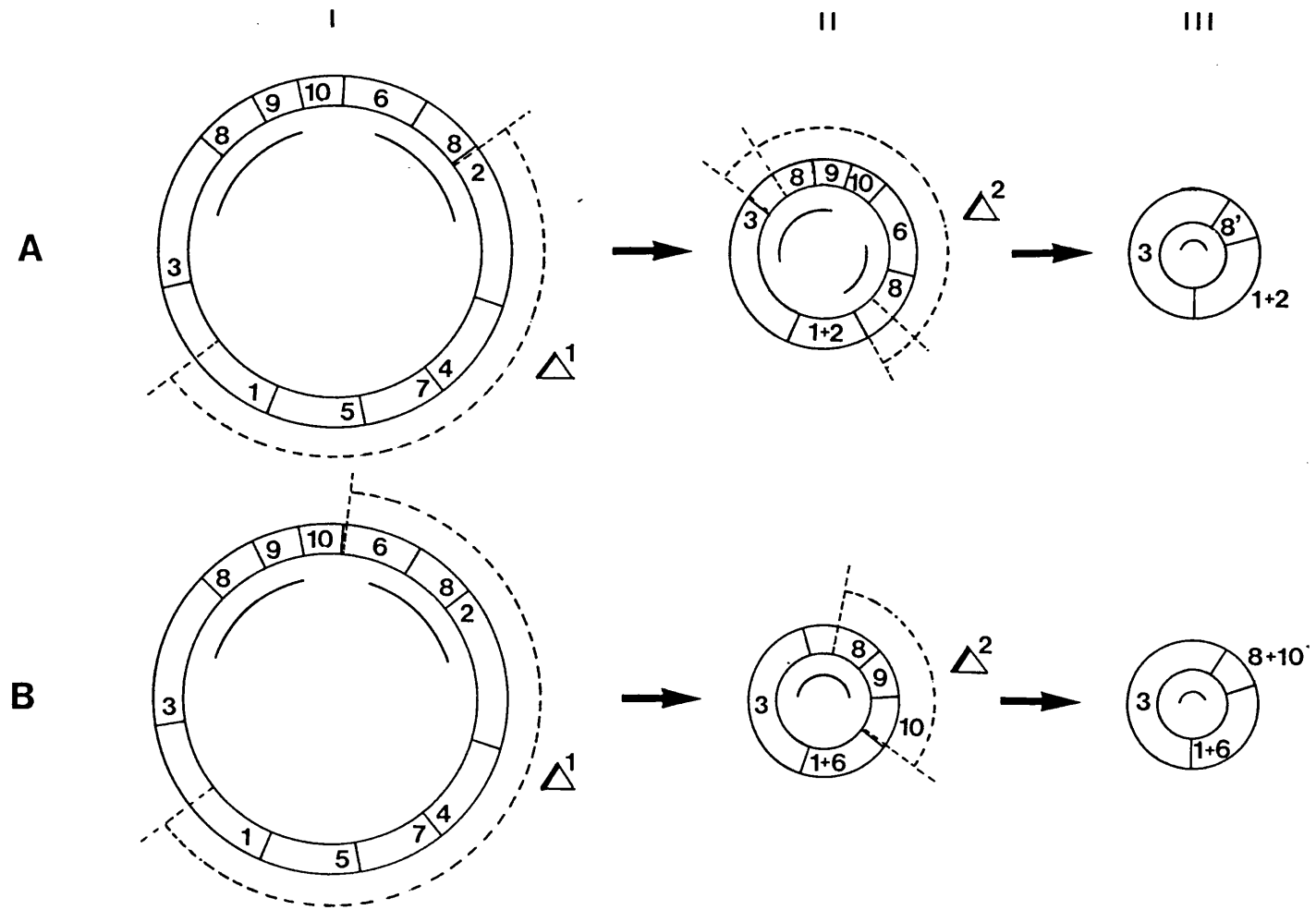
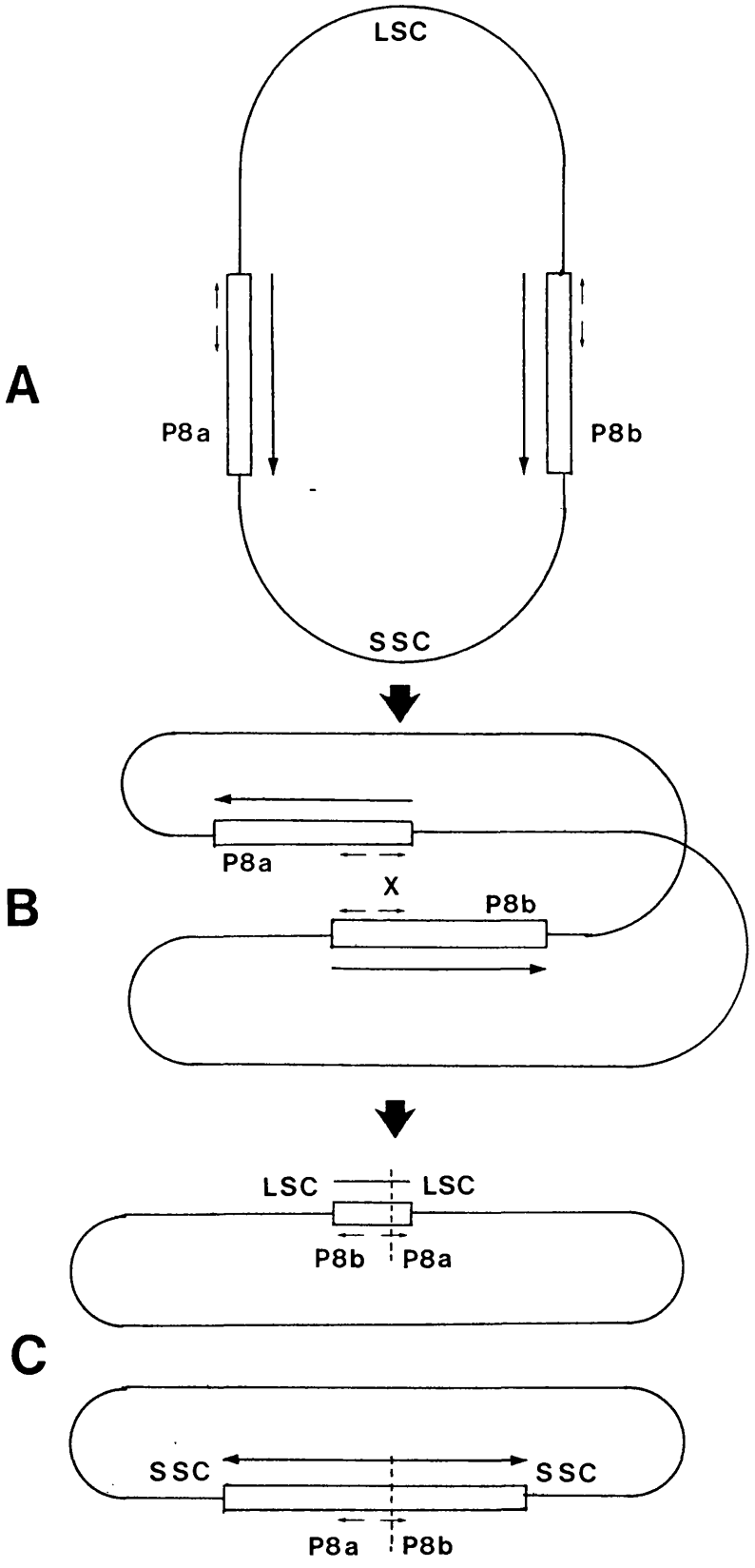

I
II
III
A
B
10
9
8
6
8
2
3
1
5
7
4
Δ1
Δ2
1+2
8'
1+6
8+10

Figure 5.7. Scheme for A Deletion Event Mediated by Recombination Between the Inverted Repeats of ptDNA.

Panel A: Diagram of the barley plastid genome (not to scale). Positions of large (LSC) and small (SSC) single copy regions are indicated. Open bars represent the two copies of fragment P8 (P8a and P8b) located in the inverted repeat region. For simplicity, these are the only sequences shown in the inverted repeat. The inverted orientation of fragments P8a and P8b is indicated by long arrows. Small inverted repeats within P8a and P8b are indicated by short arrows.

Panel B: Recombination between small inverted repeats in direct orientation located within the end of each copy of P8.

Panel C: The products of recombination described in Panel B. These molecules are deletion derivatives of each other. Open bars represent altered versions of P8a and P8b generated by fusion between the two intact fragments. Dotted vertical lines represent the position of the recombination event. Regions representing P8a and P8b in the altered fragments are indicated and their proximity to either LSC or SSC regions is indicated.


A
LSC
P8a
P8b
SSC
B
P8a
X
P8b
C
LSC
LSC
P8b
P8a
SSC
SSC
P8a
P8b

sequences in the two IRs. Although two pathways are outlined in Figure 5.2, the scheme does not take into account the presence of two isomers of ptDNA differing in the relative orientation of the two single copy regions (Palmer, 1983). However, a similar series of events can be envisaged with either ptDNA conformation. It is important to note some assumptions that must be made about the events outlined in Figure 5.2. Fusion of P1 with each of the fragments detailed in pathways A and B must lead to their almost complete deletion except for the *Pst* I site distal to the junction of P1 and P3. If larger tracts of these fragments were left after fusions with P1 the 9.3kb derivative would be detected when they were used as probes in hybridisation experiments. The same rationale applies to the first fusion of P8 with P6 and the second between P10 and the remaining copy of P8 as outlined in pathway B.

It should be noted that step II of pathway A relies on recombination between small repeats in direct orientation located in each of the copies of P8, which in turn lies within the large inverted repeat region. To enable this, identical, small inverted repeats (IRs) have to be present in each copy of the larger IR region. Sequences in each of the smaller IRs will always be in direct orientation to one another. Recombination between these sequences will lead to a deletion event. This is shown schematically in Figure 5.7. The 3.6kb P8' fragment would in this case represent a fusion between the two ends of the intact copies of P8 proximal to the LSC (see Figure 5.7C). Small inverted repeat sequences do occur in this region of the barley plastid genome, flanking or within the 16S rDNA (see Figure 5.1). Such sequences provide the potential for secondary structure formation, eg. stem-loops, in the 16S rRNA and its precursor (Tohdoh and Sugiura, 1982, Whitfield and Bottomley, 1983).

5.3.3. Albino Plant A301.

Pst I and *Pst* I + *Xho* I restriction digests of total DNA from albino plant A301 probed in Southern hybridisation experiments with probes P1-P10 contain all the fragments expected from the map of wild type ptDNA at a high intensity (see Figure 5.2). However, for a number of probes restriction fragments of non-wild type size are

also observed, eg. when using P2 and P3 which lie at the LSC and IR border and also P4 which is located in the LSC. The non-wild type bands cover a wide size range and are often present at different stoichiometries to each other as well as to bands of wild type size (see Figure 5.2). This suggests that several independent events might have occurred to generate the range of fragments observed and that A301 may therefore contain multiple forms of the plastid genome. However, because of the complexity of the observed restriction pattern it was not possible to assign particular bands to individual molecular species in albino plant A301.

5.3.4. Albino Plant A307.

Hybridisation experiments to investigate the ptDNA structure of A307 reveal bands of wild type and non-wild type hybridising to probes P1-P10 (see Table 5.1 and Figure 5.2). A large number of aberrantly sized restriction fragments with different stoichiometries occur when P3 is used as a probe. If these are the products of independent events that have generated ΔptDNA molecules in A307 then it implies, as for A301, that a number of different types of these molecules exist in this individual.

An interesting feature of A307 is that the ptDNA fragments P1 and P3 hybridise to bands at an unusually high intensity compared to other probes. P1 and P3 are adjacent in the plastid genome and P3 covers the border of the LSC with one IR (see Figure 5.1). Strongly hybridising fragments of wild type size are detected with the probe P1 although non-wild type size fragments are also present (see Table 5.1). P3 hybridises strongly to a *Pst* I derived fragment of 6.2kb and *Pst* I + *Xho* I derived fragments of 4.7 and 2.2kb. Only the 4.7kb fragment is of wild type size. These fragments are not detected when P2 is used as a probe. Since P2 shares an extensive region of homology with P3 in the form of IR sequences it suggests that the unusually intense bands are derived from sequences in the LSC (see Figure 5.2). Laser densitometry of these bands indicate that the 6.2kb and 4.7kb bands are at a level twice that of P3 derived bands in A320. It is interesting, however, to observe that the 2.2kb fragment appears to be at an even higher intensity: six times that of P3 derived bands detected in digests of A320 DNA (see

Section 5.4).

5.3.5. Albino Plant A309.

Pst I and *Pst* I + *Xho* I digests of total DNA from A309 probed with ptDNA fragments P1-P10 reveal all the restriction fragments expected from the wild type map of the barley plastid genome (see Table 5.1 and Figure 5.2). But, again, fragments of non-wild type size were present often at intensities different from the wild type fragments. This suggests that in A309 multiple forms of the ptDNA may exist including ΔptDNAs. Interestingly, fragments hybridising to probes covering the LSC region (P1-P4) are present at a much lower intensity compared to those located in the IR and SSC regions. Under-representation of these fragments suggests that some forms of ΔptDNA in A309 have lost most or all of LSC region.

5.3.6. Albino Plant A320.

Probe fragments P1-10 detect bands of wild type size and uniformly high-intensity in *Pst* I and *Pst* I + *Xho* I digests of total DNA from A320 (see Table 5.1 and Figure 5.2). If these fragments lie on the same molecular species then it is possible that the major form of the plastid genome in albino plant A320 is intact as far as can be determined by restriction analysis with the enzymes *Pst* I and *Xho* I. These enzymes have relatively few recognition sites (11 and 23 respectively) in the barley plastid genome and generate fragments often exceeding 10kb in size. Small differences in the size of large fragments such as these are difficult to detect since resolution in this size range is limited using standard agarose gel electrophoretic techniques (Maniatis *et al.*, 1982). In contrast to *Pst* I and *Xho* I, digestion of the plasmids pHvCP1-10 with *Eco*R I and *Bam*H I indicates that barley ptDNA contains approximately 30 and 50 recognition sites for these enzymes, respectively (data not shown). As a result, digestion with these enzymes cleaves most of the larger *Pst* I and *Xho* I ptDNA fragments into a size range between 10kb and 500bp where maximum resolution can be achieved during agarose gel electrophoresis of DNA (Maniatis *et al.*, 1982). Hence, this would provide a means of resolving whether small,

previously undetectable deletions or rearrangements were present in the ptDNA of A320.

To test this, 5 and 10μg aliquots of DNA from A320 were digested with *EcoR* I and *EcoR* I + *BamH* I, respectively, in parallel with control digests of equal amounts of DNA from the leaves of green seedlings. Digests were fractionated by electrophoresis on 30cm long 1% agarose gels at 20V for 16 hours, conditions that were chosen to allow good separation of all restriction fragments. The fractionated digests were transferred and fixed to hybridisation filters and probed sequentially with the ptDNA fragments P1-10. Autoradiographs of these Southern hybridisation experiments show that the restriction fragment patterns of DNA from A320 are identical to those found in wild type controls (data not shown). This provides further evidence to support the hypothesis that the albino pollen plant A320 may contain an intact plastid genome. The resolution in these experiments was estimated to be sufficient to distinguish restriction fragments differing in size by 25-50bp.

The observation that A320 appears to contain a plastid genome that is indistinguishable from the wild-type at the level of analysis used means that albinism in pollen derived plants cannot necessarily be correlated with major alterations in the ptDNA. It should also be noted in this context that albino pollen plant A301 also contains all the restriction fragments expected from the wild type map of barley ptDNA, although no further restriction fragment analysis was carried out with this individual.

In addition to the major bands described above, the presence of non-wild type fragments at lower intensity (see Figure 5.3) indicate that ΔptDNA molecules may also exist in this individual. A320 was used as an internal standard when comparing the relative intensity of different restriction fragments from other plants. The rationale for this is described in Section 5.3.1.

5.4. The Abundance of ptDNA in Albino Pollen Plants.

It has been demonstrated that total DNA from albino cereal pollen plants contains a reduced level of ptDNA compared to that found in

normal green tissues (Day and Ellis, 1984). Therefore, in addition to structural alterations, changes in the abundance of ptDNA can also occur in albino pollen plants. Experiments were performed to test whether this was also true for the albino plants used in the plastid genome mapping experiments described above.

The crude measure of relative band intensity outlined in Tables 5.1 and 5.2 was made, using A320 as an internal standard, according to the rationale detailed in Section 5.3.1. A more accurate measurement of the relative levels of ptDNA in albino plants and normal green tissues was made by carrying out the following experiment. Equal amounts of total DNA from albino plant A320 and green seedling leaves were digested with *Eco*R I or *Eco*R I + *Bam*H I to generate a range of restriction fragments (see Section 5.3.6), fractionated by agarose gel electrophoresis, transferred and fixed to a nylon filter and hybridised sequentially with the ptDNA probes P1-P10. Autoradiographs of the hybridisation filters were scanned using a laser densitometer and from this the relative intensity of bands representing ptDNA in digests of albino and seedling DNA were estimated. For each of the probes used, bands representing ptDNA fragments in digests of total DNA from A320 were estimated to be approximately 1/5th the intensity of the corresponding bands from the control. Therefore, the overall level of ptDNA in albino pollen plant A320 was estimated to be about 20% of that found in the leaves of green seedlings. Since the same reduction in band intensities was seen for fragments encompassed by both the IR and single-copy regions it suggests that these regions of the plastid genome of A320 are present at the same copy number as found in the ptDNA of normal plants. Under-representation of IR fragments in the plastid genome of A320 might have indicated that a previously undetectable deletion event had removed one copy of this region of the plastid genome. This appears not to be the case and supports the notion that A320 may contain an intact plastid genome.

The estimation of albino ptDNA levels described above assumes that the amounts of nuclear DNA and mtDNA are the same per cell for both green and albino plants. If this were not the case then it could lead to a incorrect determination of ptDNA levels in albinos. For instance if albinos contained greatly increased amounts of mtDNA compared to green plants then the level of ptDNA found in A320

would be an underestimate using the method described above.

The intensity of bands representing ptDNA fragments in other albino plants were determined relative to the internal standard A320 by laser densitometer scanning of the autoradiographs shown in Appendix 5.1. From this it was therefore possible to estimate the abundance of specific ptDNA sequences relative to those found in green tissues. PtDNA fragments P8 and P10 are representative of probes hybridising to the most intense ptDNA fragments in digests of albino DNA (see Table 5.1, except note that some P1/P3 derived fragments seen in A307 and A308 are of unusually high intensity: see later). These probably represent sequences that are part of the major forms of ptDNA in the albino plants studied. The intensity of these major albino ptDNA fragments detected with probes P8 and P10 relative to those found in green tissues are shown in Table 5.3. This data indicates that these sequences are present in albino plants at levels approximately 6-20% of those found in normal green tissues. This reduction in the levels of ptDNA fragments in albino plants is in general agreement with the data of Day and Ellis (1984).

Laser densitometer scanning of digests of DNA from the albino pollen plants A307 and A308 probed with P1 and P3 indicate that some ptDNA fragments hybridising to these probes are present at levels exceeding those found in digests of total DNA from A320. In A308 fragments of wild type size hybridising to probe P3 are present at approximately twice the level found in A320 and therefore approach 40% of that in normal green leaves. Similar levels are seen for the 6.2kb *Pst* I and 4.7kb *Pst* I+*Xho* I derived fragments hybridising to P3 and wild type P1 derived fragments in albino A307 (see Section 5.3.4). In addition, a 2.2kb *Pst* I+*Xho* I derived fragment also hybridses to P3 in A307, however this fragment is present at six times the level of P3 derived fragments in A320 and therefore 120% of that in green leaves. The reason why these fragments are of unusually high abundance in albino plants A307 and A308 is discussed further in Chapter 8.

Table 5.3. Estimation of the Relative intensity of major *Pst* I restriction fragments hybridising to P8 and P10 in albino pollen plants compared to that in green leaves.

Band intensities were determined by laser densitometer scanning of the autoradiographs IIX and X, shown in Appendix 5.1. The values represent band intensities calculated for each plant relative to that found for the internal standard A320 and from this the intensity relative to that found for green leaves (see Section 5.4). In the case of A308, the major band hybridising to P8 is 3.6kb in size rather than the expected 8.1kb. The intensity of this band was compared to similar sized fragments in a *Pst* I+*Xho* I digest of DNA from A320. Bands hybridising to P10 are absent in plant A308 and therefore no determination was made in this case.

	P8.		P10.	
Albino Plant	Relative to A320	Relative to green.	Relative to A320	Relative to green.
A301	0.69	0.14	0.64	0.13
A307	0.35	0.07	0.32	0.06
A308	0.6	0.12	-	-
A309	0.92	0.18	0.83	0.17
A320	1.0	0.2	1.0	0.2

5.5. The Structure and Abundance of the Plastid Genome in Other Tissues.

To test for the presence of ΔptDNAs in tissues other than those from albino pollen plants and green seedlings total DNA was prepared from the leaves of etiolated seedlings, roots, five green pollen plants and pollen plant V1. V1 was originally selected as an albino regenerant which after four weeks growth under standard conditions of illumination (see Section 2.19) turned pale yellow in colour. DNA extracted from etiolated seedlings, roots, green pollen plants and V1 was digested with the restriction enzymes *Pst* I and *Xho* I singly or in combination. The digests were fractionated by agarose gel electrophoresis, transferred and fixed to nylon hybridisation filters and probed with the ptDNA fragments P1-10. For digests of DNA extracted from etiolated leaves, green pollen plants and V1 only fragments of wild-type size were seen to hybridise to the probes P1-P10 at a uniformly high intensity. Similarly, only wild-type fragments were seen in digests of root DNA but these were present at levels approximately 5% of that found in green tissues. This data suggests that all regions of the plastid genome were present in the tissues examined. Minor bands, not expected from the map of wild type barley ptDNA, were also present in some of these digests and are discussed in Section 5.6. No other fragments of non-wild type size could be detected in these experiments. This evidence suggests that the plastid genome in these tissues is intact and that the existence of major forms of ΔptDNA is confined to albino plants only. However, the presence of ΔptDNAs at very low abundance cannot be discounted at this level of analysis (see Section 8.2).

Experiments were also carried out to determine the abundance of the plastid genome in green pollen plants and V1. In the ptDNA mapping experiments described above, although equal amounts of DNA were loaded for each green pollen plant, lanes carrying digests of DNA from seed-derived green leaves were underloaded (data not shown). As a result, no conclusions could be drawn about the relative abundance of ptDNA in green pollen plants and seed derived leaves. To resolve this, equal amounts DNA from one of the green pollen plant previously examined and seed derived green leaves were digested with *Pst* I + *Pvu* II, fractionated by agarose gel

electrophoresis, transferred and fixed to a nylon filter and hybridised to the ptDNA fragment P8. Bands corresponding to those expected from the map of barley ptDNA were seen to hybridise to P8 at similar intensities in both digests (data not shown), suggesting that ptDNA was present in the green pollen plant at the same level as that found in seed derived green leaves. During the structural analyses of ptDNA in green pollen plants all the ptDNA probes, including P8, hybridised to wild type fragments at a similarly high intensity in each individual. This suggests that ptDNA is present at normal levels in all the green pollen plants examined. The abundance of ptDNA in plant V1 was compared directly to that in seed derived green leaves by hybridisation of the ptDNA probes P1-10 to equal amounts of digested DNA from each tissue (see mapping experiments above). Each of the probes hybridised to ptDNA fragments at a similar intensity in both cases suggesting that V1 contains normal levels of ptDNA.

5.6. The Uniform Presence of Non-Wild Type Restriction Fragments Hybridising to Barley ptDNA Probes.

During the Southern blot analyses described in Sections 5.3 and 5.5 certain ptDNA probes were seen to hybridise to non wild-type fragments irrespective of the plant tissue used. For instance these fragments were seen in digests of total DNA from albino pollen plants, green pollen plants and the roots and leaves of seed grown individuals. Two of the most striking examples are seen in hybridisation experiments using ptDNA fragments P8 and P5 as probes. Two fragments of 8.3kb and 4.7kb hybridise to probe P5 and a single band of 11.7kb hybridises to P8 in *Pst* I + *Xho* I or *Xho* I digests of total DNA from albino and green pollen plants, root and green leaf tissue (Figures 5.8 and 5.9). This pattern of digestion suggests that the fragments hybridising to P5 and P8 are derived from a *Xho* I digest alone. These fragments are clearly visible in digests of total DNA even when the proportion of ptDNA in the sample is greatly reduced, for example in albino leaves and seed-derived roots where the level of ptDNA was estimated by laser densitometry to be five and twenty-fold less than that in green leaves respectively. This suggests that the presence of these fragments is independent of the abundance of ptDNA in the tissues

Figure 5.8. Non-Wild Type Restriction Fragments Hybridising to P5 in a Number of Different Barley Tissues.

Autoradiographs of Southern blots probed with ptDNA fragment P5. Lanes A and B carry DNA from albino pollen plants A320 and A308, lanes C and D carry DNA from the roots and leaves of light grown seedlings and lane E carries DNA from the green pollen plant G14. 5μg aliquots of digested DNA were loaded per lane. All DNA samples were digested with the enzymes *Pst* I + *Xho* I, except in lanes C and D where *Xho* I only was used. Lanes A and B, C and D, and E alone represent the results of separate Southern analyses. The position of the 8.3kb and 4.7kb non-wild type fragments (see text) are indicated by arrows. The more intense bands in lanes A, C and E represent wild type ptDNA restriction fragments (see Table 5.1). The positions of size markers, in kb, for lanes A and B only are indicated on the left. Size markers for other lanes are omitted for simplicity. Filters were washed under the condition of moderate stringency detailed in Section 2.12. Autoradiographs represent 16-96 hour exposures to X-ray film at -80°C.

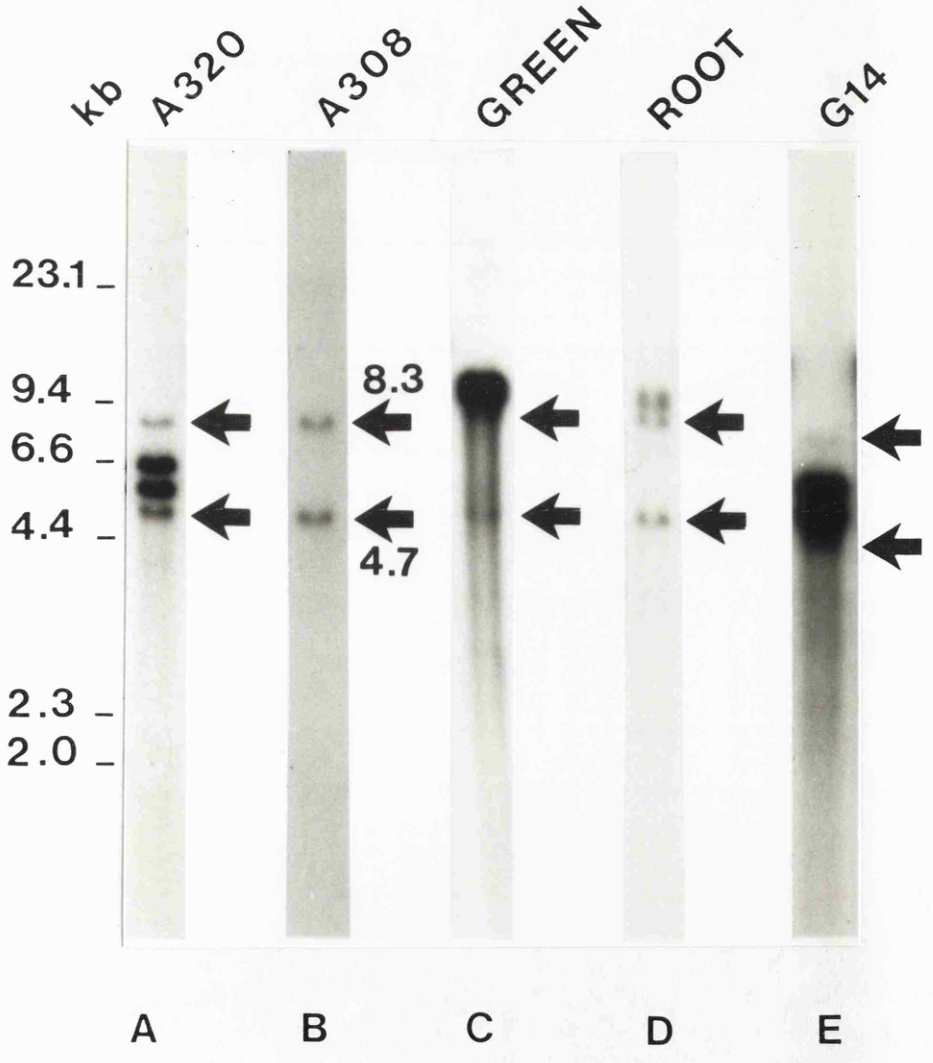
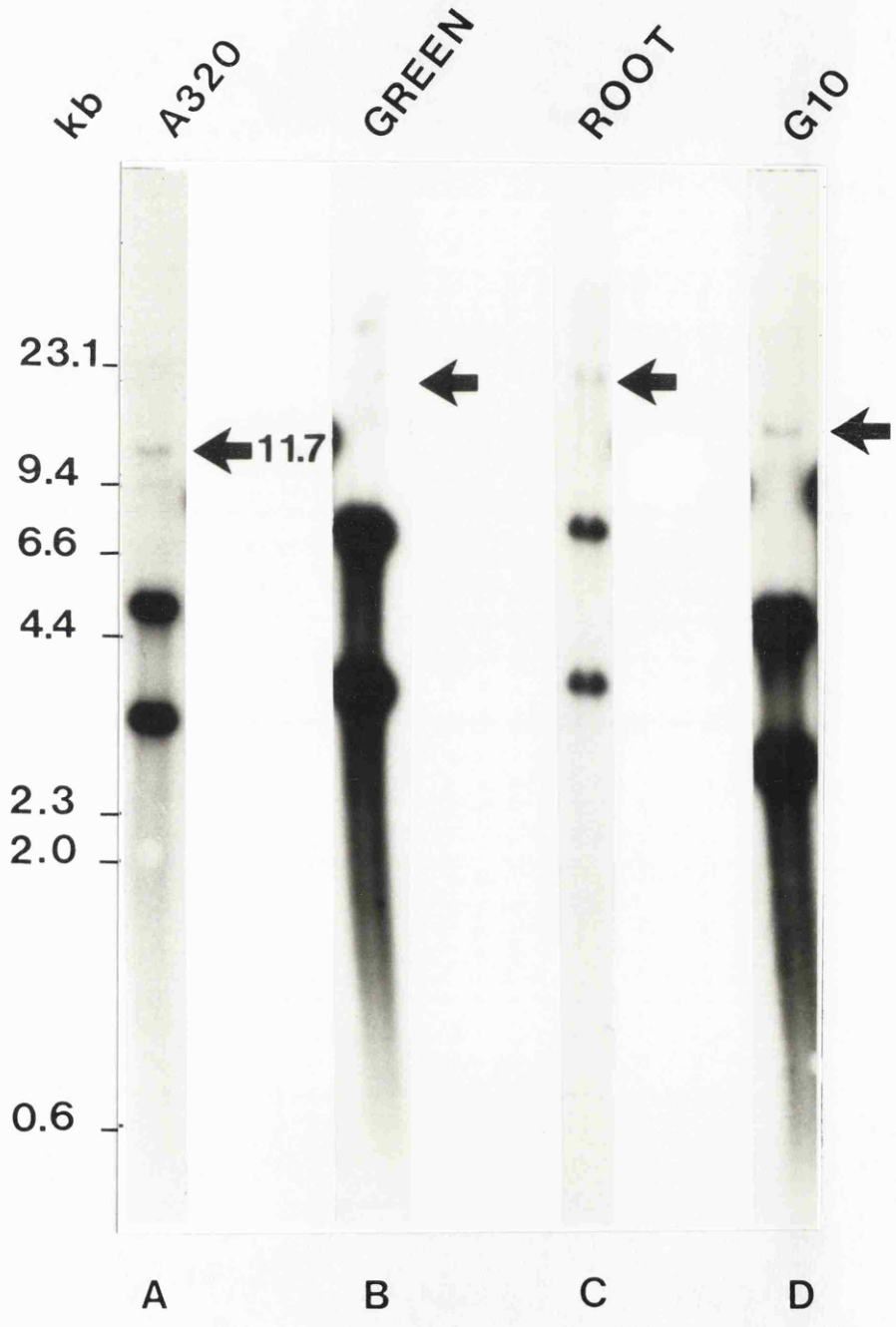

kb
A320
A308
GREEN
ROOT
G14
23.1
9.4
6.6
4.4
2.3
2.0
8.3
4.7
A
B
C
D
E

Figure 5.9. Non-Wild Type Restriction Fragments Hybridising to P8 in a Number of Barley Tissues.

Autoradiographs of Southern blots probed with the ptDNA fragment P8. Lane A carries DNA from albino plant A320, lanes B and C carry DNA from the leaves and roots of light grown seedlings and lane D carries DNA from the green pollen plant G10. 5μg aliquots of digested DNA were loaded per lane. All DNA samples were digested with *Pst* I + *Xho* I except in lanes B and C where *Xho* I alone was used. Lanes A alone, B and C, and D alone represent the results of different Southern analyses. The position of the 11.7kb non-wild type fragment is indicated by an arrow. More intense bands represent wild type ptDNA restriction fragments (see Table 5.1). The positions of size markers, in kb, for lane A only, are indicated on the left. Size markers for other lanes are omitted for simplicity. The washing conditions and exposure times for the hybridisation filters are as detailed in Figure 5.8.


kb
A320
GREEN
ROOT
G10
23.1
9.4
6.6
4.4
2.3
2.0
0.6
11.7
A
B
C
D

examined. The fragments hybridising to P5 are also seen in an albino plant that has undergone deletion of the wild-type P5 fragment, suggesting that their presence may be independent of the overall ptDNA structure. Taken together, these observations suggest that the fragments seen to hybridise to P5 and P8 may be located on a DNA molecule other than the plastid genome. Although the identity of these fragments is unknown, there is evidence to suggest that ptDNA sequences are present in other cellular compartments such as the nucleus and mitochondrion (Lonsdale, 1985; Timmis and Scott, 1985; Schuster and Brennicke, 1988). Therefore, further characterisation of the sequences described above might include the hybridisation of P5 and P8 to DNA specifically isolated from plastids, mitochondria and nuclei. Since the origin of these bands remains unknown they are not included in the data summarised in Tables 5.1 and 5.2.

5.7. The Detection of Cross-Homology Between Different Regions of Barley ptDNA.

During the characterisation of the ptDNA of wheat and barley pollen plants Day (1985) observed that certain regions of the plastid genome exhibit distant cross-homology. In particular, weak cross-hybridisation was seen between fragments corresponding to P2 and P4 on the map of barley ptDNA (see Figure 5.1). Bowman and Dyer (1986) have performed Southern hybridisation experiments designed to detect and map small repeats in the wheat plastid genome by the detection of cross-homology between different ptDNA restriction fragments. Such short repeat regions have been implicated in the mechanisms which may be responsible for the generation of circular ΔptDNA in albino pollen plants (see Section 1.4). On this basis an attempt was made to detect cross-homologies between different *Pst* I restriction fragments derived from the barley plastid genome (see Figure 5.1). Detection of cross-homologies such as these could be used as a preliminary study to define the presence and location of short repeat regions and their possible involvement in the generation of ΔptDNA in albino pollen plants.

Plasmids pHvCP1-10 were digested with the restriction enzyme *Pst* I to release the insert ptDNA and fractionated by agarose gel

electrophoresis, loading 250ng DNA per lane. The gels were run for a period sufficient to separate the insert DNA well away from that of the vector. This procedure was duplicated ten times and each gel was blotted onto hybridisation filters. Each of the ptDNA fragments P1-10 was radiolabelled and presented to one of the filters in Southern hybridisation experiments. Every ptDNA fragment was therefore hybridised with every other sequence in the barley plastid genome. A low stringency post-hybridisation washing strategy (see Section 2.12) was chosen to allow stable hybrid formation between short or dispersed regions of DNA homology. The radioactive Southern blots were autoradiographed and reciprocal cross hybridisation between different ptDNA fragments noted. These results are summarised in Table 5.4. As examples two representative autoradiographs are shown in Figure 5.10.

In the experiments described above, a band corresponding in size to the vector plasmid DNA (pAT153) was seen to hybridise to all the ptDNA probes at variable levels. This could be explained by cross-homology between the ptDNA probe and filter-bound vector DNA. However, in a separate experiment, only barely detectable hybridisation could be seen between a purified pAT153 probe and filter-bound ptDNA fragments (data not shown). It was considered unlikely that these extremely weak cross-homologies could account for the sometimes high level of hybridisation seen between certain ptDNA probes and filter-bound vector DNA. Instead, the most likely explanation was that vector DNA was present as a contaminant in preparations of the various ptDNA probe fragments. Furthermore, any cross-hybridisation between contaminant vector DNA and filter bound ptDNA fragments was considered too weak to confuse the assessment of authentic ptDNA/ptDNA hybrids.

Most of the ptDNA/ptDNA hybrids detected in these experiments are very weak. The most intense hybrids are formed between P2/3 and P4. A similar pattern of cross-hybridisation in the barley plastid genome was also reported by Day and Ellis (1984) and can be seen in the ptDNA mapping experiments described in the previous sections when filters probed with P4, P2 or P3 are exposed for extended periods of time (eg. the 22.4 *Pst* I derived band seen to hybridise to P4 in A308 is similar in size to P3 and could be due to cross homology between these two fragments). Other, weaker

Table 5.4. Barley ptDNA fragments showing cross-hybridisation.

Hybridisation between identical fragments and fragments that share large regions of homology, eg. within the inverted repeat: P2 and P3; P6 and P9, is denoted by an asterix (*). Weak hybridisation between non-homologous fragments is denoted by a tick (✓).

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10
P1	*		✓		✓					
P2		*	*	✓						
P3	✓	*	*	✓			✓			
P4		✓	✓	*						
P5	✓				*	✓				
P6					✓	*			*	
P7			✓				*		✓	✓
P8								*	✓	✓
P9						*	✓	✓	*	
P10							✓	✓		*

Figure 5.10. Cross-Homologies Between Different ptDNA Restriction Fragments.

Panel A: Autoradiograph of Southern blot probed with ptDNA fragment P1. Lanes P1-P10 carry 250ng of the plasmids pHvCP1-P10, respectively, digested with the enzyme *Pst* I. Weak cross homologies between ptDNA fragments are indicated with an asterix (*). More intense signals correspond to hybridisation between the probe, P1 and digests of pHvCP1 on the filter. Arrow indicates position of the vector, pAT153.

Panel B: Autoradiograph of Southern blot probed with ptDNA fragment P4. Cross homologies are indicated by asterixes. Other details are as Panel A.

Hybridisation filters were washed as detailed in the text. Autoradiographs represent 2 hour exposures to X-ray film at room temperature.

P10
P9
P8
P7
P6
P5
P4
P3
P2
P1
A
*
*

P10
P9
P8
P7
P6
P5
P4
P3
P2
P1
B
*
*

cross-homologies between ptDNA fragments are unlikely to be detected in these experiments.

As outlined in Section 1.4 short repeated DNA sequences could be involved in the production of circular ΔptDNAs in albino pollen plants. Sequences of this kind have been localised on the plastid genome of wheat (Bowman and Dyer, 1986). The experiments described above represent a preliminary study to localise similar sequences on the plastid genome of barley. Although cross-homologies have been detected between different barley ptDNA fragments (see Table 5.4), the nature of the sequences responsible for the formation of weak hybrids is unknown. However, the low level of cross-hybridisation between these fragments indicates that the regions of homology may be extremely short or that they are dispersed within tracts of non-homologous sequence. Localisation of the cross-hybridising sequences to smaller restriction fragments might enable their position on the plastid genome to be defined more closely. Sequencing of these fragments may then provide details of the structure of the cross-hybridising regions. The involvement of repeated sequences has been implicated in the generation of defined alterations during evolution of the wheat plastid genome (Howe, 1986). However, the involvement of similar sequences in the generation of specific ΔptDNAs in albino plants has not been demonstrated. If the observed cross-homologies in barley DNA are due to the presence of short repeated sequences then their involvement in the generation of ΔptDNAs could be investigated by comparing their map position on the plastid genome with the location of defined deletion end-points in these molecules.

5.8. Summary and Conclusions.

Four out of the five albino plants studied (A301, A307, A308 and A309) appear to contain major altered forms of the plastid genome. In contrast, green pollen plants, albino A320 and plant V1 appear to contain intact ptDNA. In some cases these alterations have involved the deletion of large tracts of the plastid genome, eg. A308, A307. The albino A308 appears to have lost approximately 75% of all ptDNA sequences. Because A320 contains an abundant form of

ptDNA which appears to be intact albinism in pollen plants cannot necessarily be correlated with major changes in the plastid genome. However, in this case, and for the other plants examined, the presence of small insertions, deletions and single base pair changes cannot be discounted at the level of analysis used. The extent of the deletion events generating ΔptDNAs appears to include in most cases large portions of the LSC region since sequences in this region are most often underrepresented in albino plants (see Figures 5.2 and 5.3). Many plants have altered restriction fragments which are derived from ptDNA fragments P2 and P3 (see Table 5.1) which suggests that these regions, which include the borders between the IR and LSC regions, could be acting as preferential targets for the mechanism(s) responsible for the generation of ΔptDNA (see Section 1.4). None of the barley ΔptDNAs characterised by Day and Ellis (1985) appear to contain sequences homologous to the IR regions. In contrast, albino plants A301, A307, A309 and A320 contain major forms of ptDNAs which include fragments representing sequences in the IR and SSC regions. This suggests that the events operating to produce ΔptDNA molecules in these albino pollen plants have left different regions of the plastid genome intact than previously reported. The observation that some ptDNA probes hybridise to multiple bands at different stoichiometries in albino plants suggests that all the albino pollen plants studied may contain mixed populations of ptDNA molecules (see also Chapter 8).

It has been noted that the ΔptDNAs from albino pollen plants always retain a region covering the junction of fragments P1 and P3 and which may contain sequences sufficient for the replication and/or maintenance of the plastid genome (Day and Ellis, 1984; Day and Ellis, 1985; Ellis and Day, 1986). All the albino plants used in this study have also retained this region of ptDNA (see Table 5.1 and Figure 5.1). However, since in most cases mixed populations of ptDNA molecules appear to exist in albino plants it has not been possible to determine if all molecular species contain these sequences. Indeed, the complexity of restriction patterns seen in most cases has meant that assignment of particular restriction fragments to individual forms of ptDNA has been not been possible. However, an attempt has been made to suggest possible structures for the major form of ptDNA in albino plant A308, an individual

showing a much less complex restriction pattern (see Figure 5.2). If the major ΔptDNA of A308 is circular then the available restriction fragment data suggest that multiple deletion events may have occurred to generate this structure, rather than single deletion events as proposed by Day and Ellis (see Section 1.4). Pathways of different levels of complexity could therefore be operating to generate circular ΔptDNA in albino pollen plants.

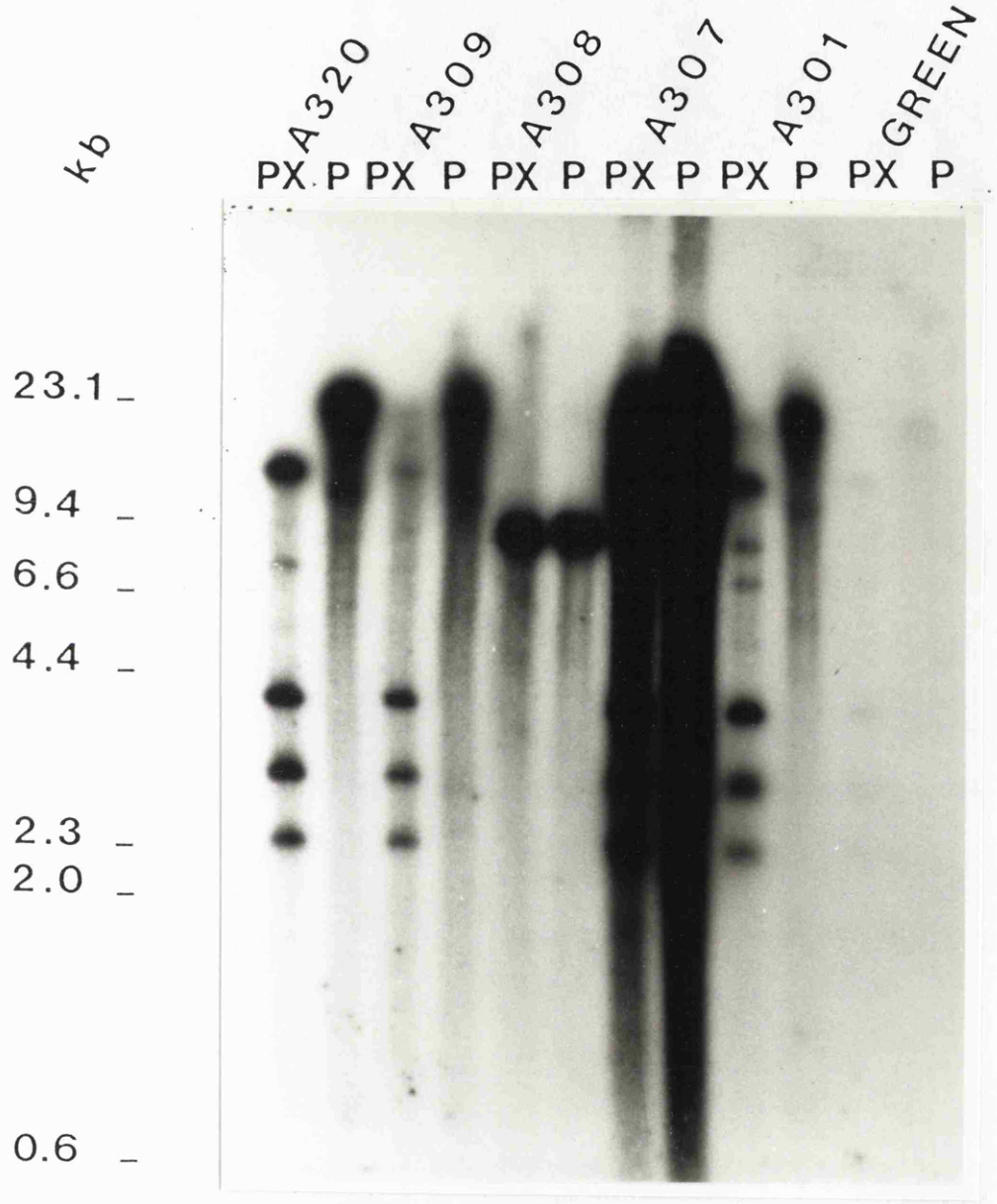
Short repeated sequences could be involved in the generation of ΔptDNAs in albino pollen plants (see Section 1.4). The experiments described in Section 5.6 represent a preliminary study to try to detect the presence of such short repeated sequences on the barley plastid genome. A number of barley ptDNA restriction fragments have been shown to cross-hybridise (see Table 5.3) but the exact location and structure of the sequences responsible for this cross-hybridisation were not determined.

In addition to changes in the structure of the ptDNA of albino pollen plants a reduction in the abundance of ptDNA restriction fragments is also seen. Restriction fragments representing the major forms of ptDNA in albino plants are present at levels 5-15 fold less than those found in the leaves of seed-derived green tissues. However, certain ptDNA restriction fragments found in albino plants A308 and A307, specifically in the region of P1 and P3 are present at amplified levels compared to other areas of the plastid genome. In contrast to albino plants, green pollen plants and plant V1 appear to accumulate ptDNA at the levels found in the leaves of green seedlings.

APPENDIX 5.1.

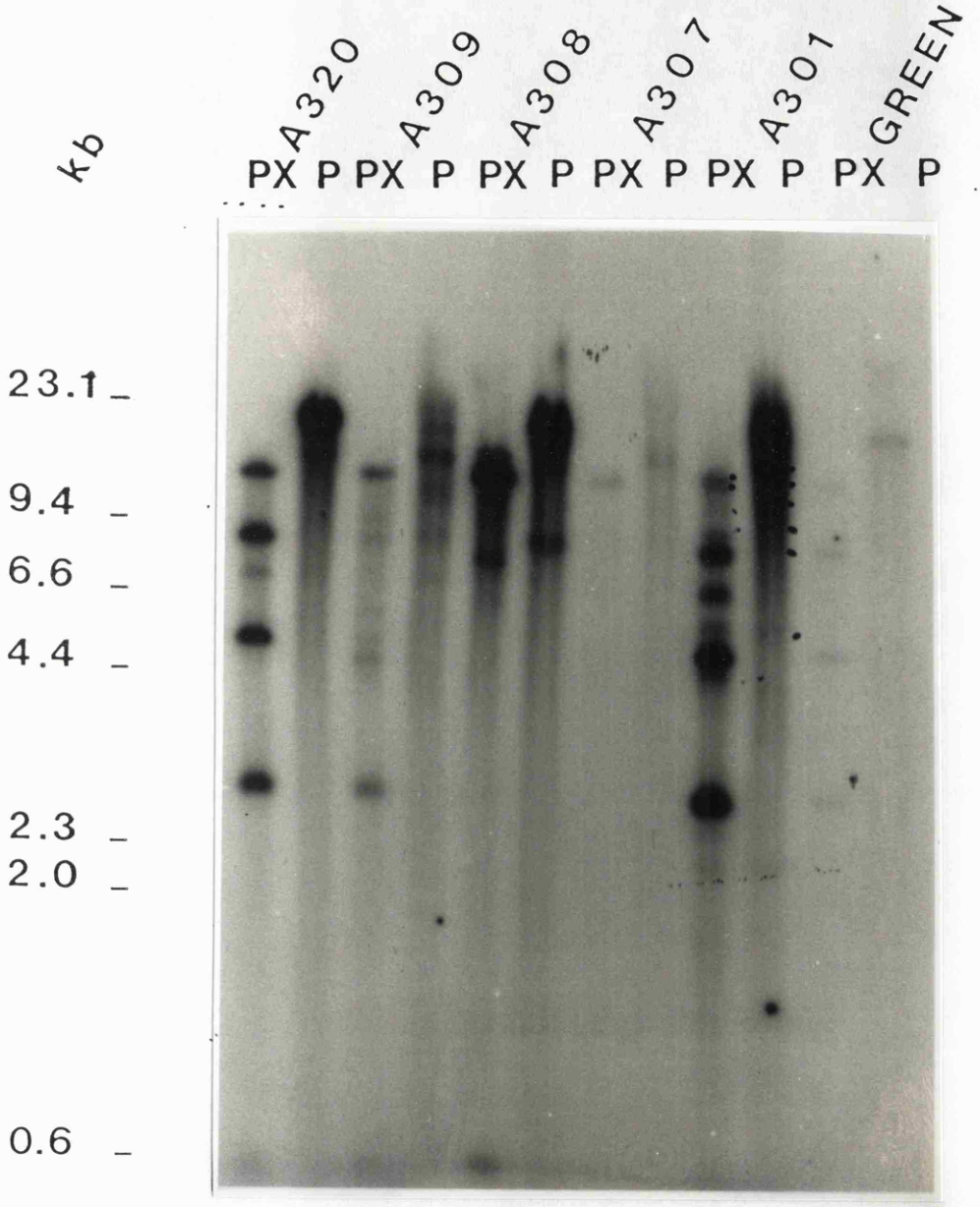
APPENDIX 5.1. Southern Analysis of ptDNA in Albino Pollen Plants.

Figures I-X show representative autoradiographs of the Southern hybridisations described in Section 5.3. In each case the figure number is indicated in the bottom right corner. Lanes carrying DNA from albino plants A301, A307, A308, A309 and A320 and seed derived green leaves (GREEN) are marked accordingly. 5μg aliquots of digested albino DNA were loaded per lane. Lanes carrying DNA from seed derived green leaves were accidentally underloaded. Lanes carrying DNA digested with *Pst* I + *Xho* I or *Pst* I alone are indicated as is the ptDNA probe used in each case. The position of size markers, in kb, is indicated on the left of each autoradiograph. Hybridisation filters were washed under the conditions of moderate stringency detailed in Section 2.12. Autoradiographs represent 16-96 hour exposures to X-ray film at -80°C.



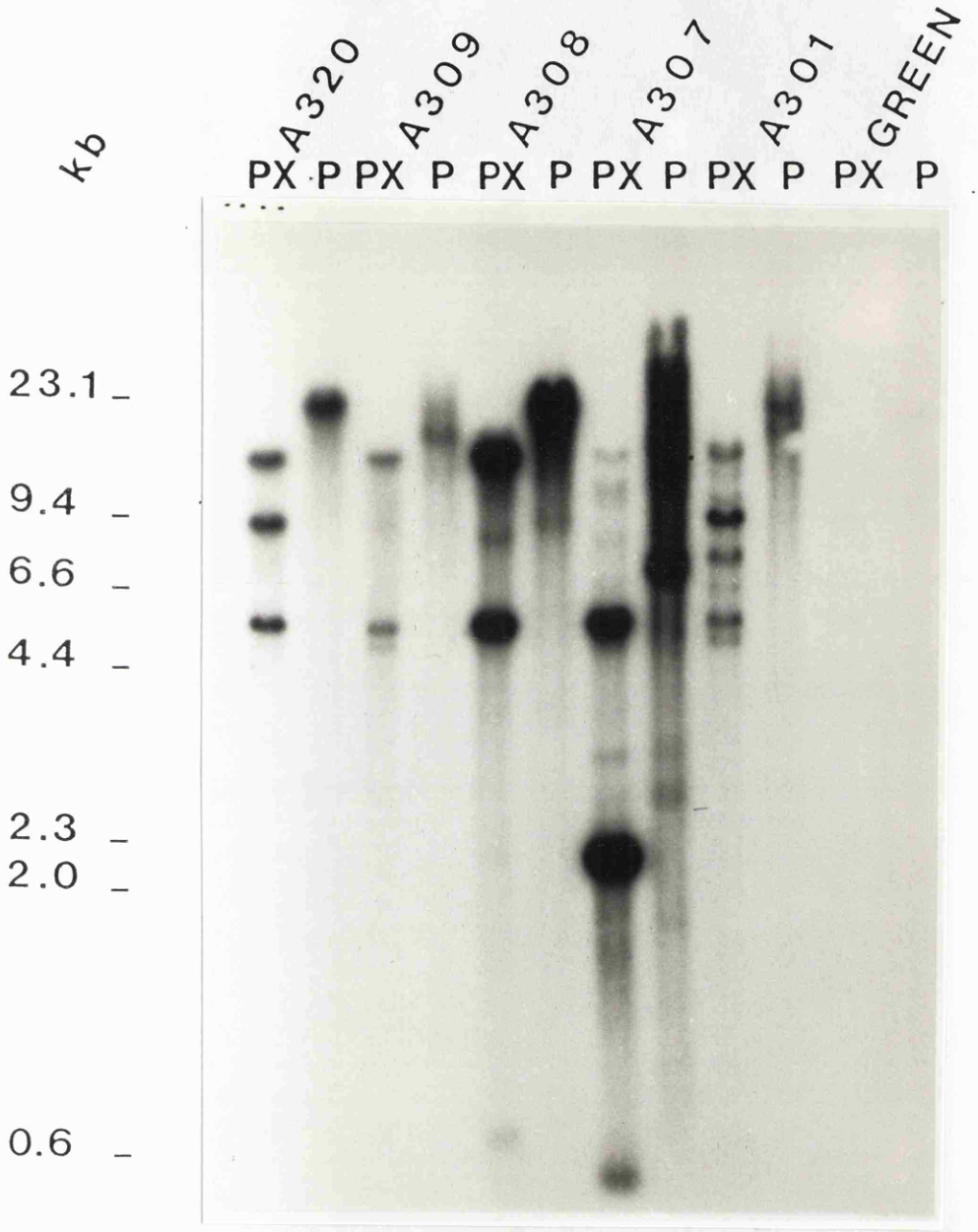
P=Pst1 PX=Pst1+Xho1

Probe=P1



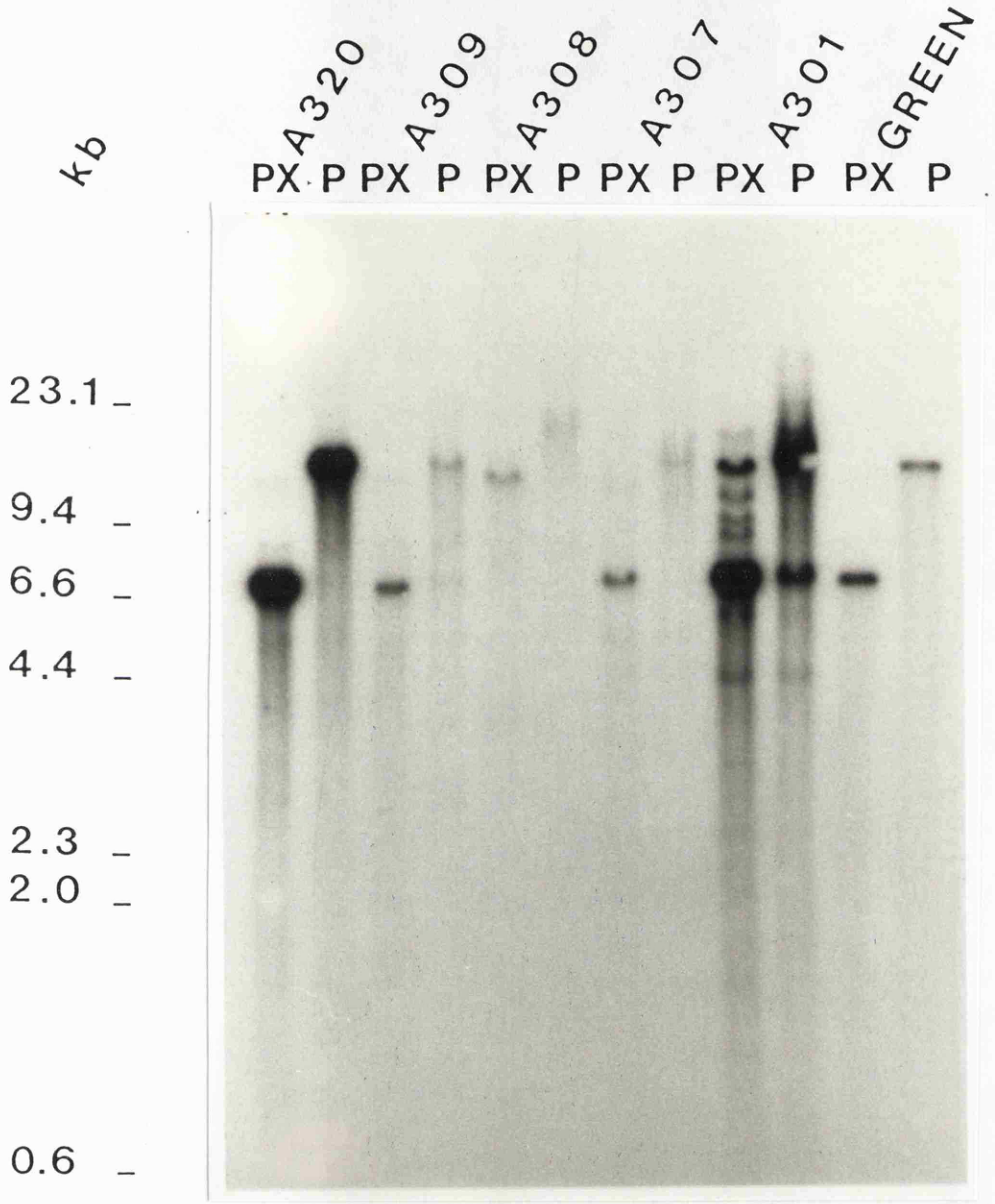
P=Pst1 PX=Pst1+Xho1

Probe=**P2**



P=Pst1 PX=Pst1+Xho1

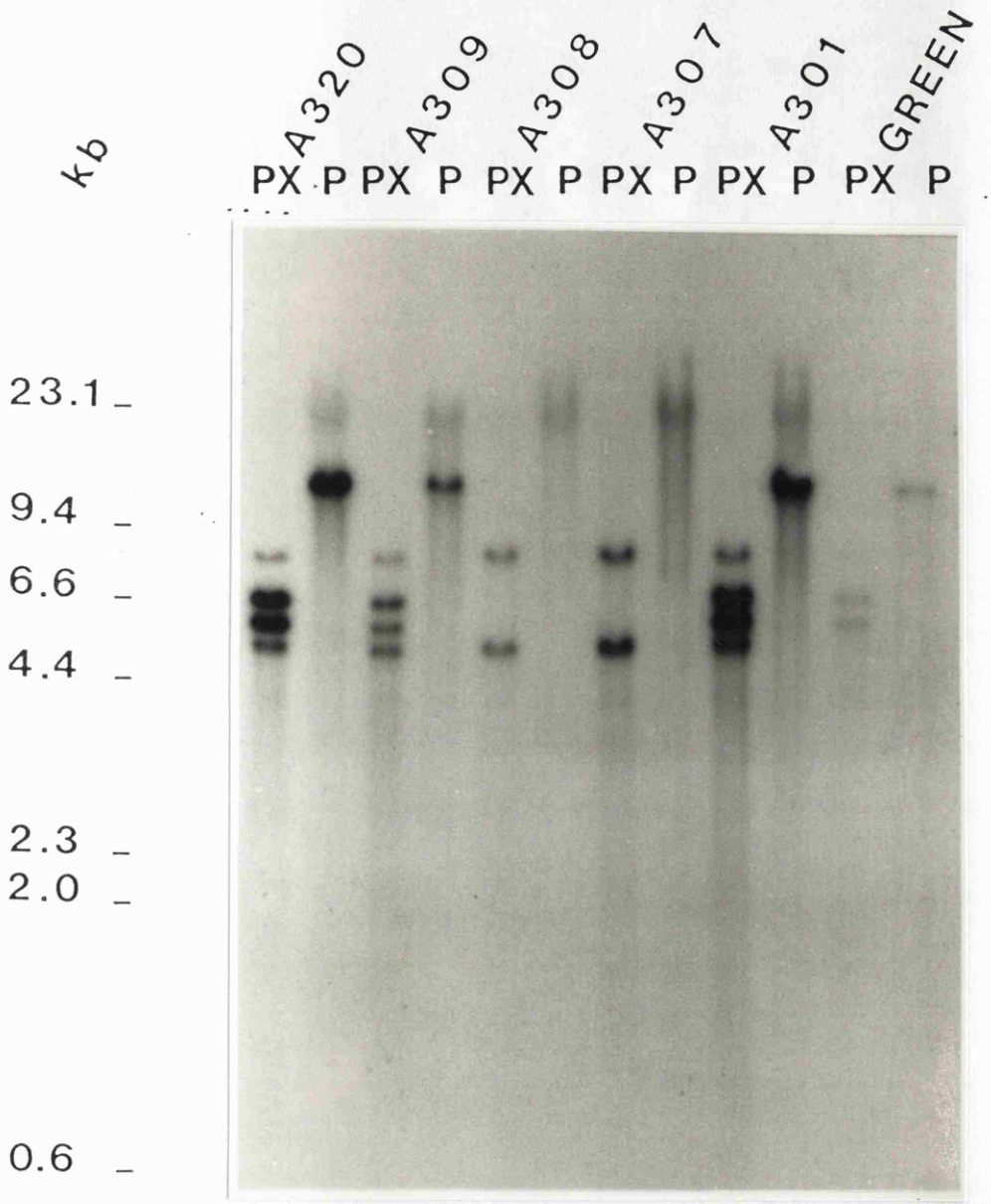
Probe=**P3**



P = Pst1 PX = Pst1+Xho1

Probe = **P4**

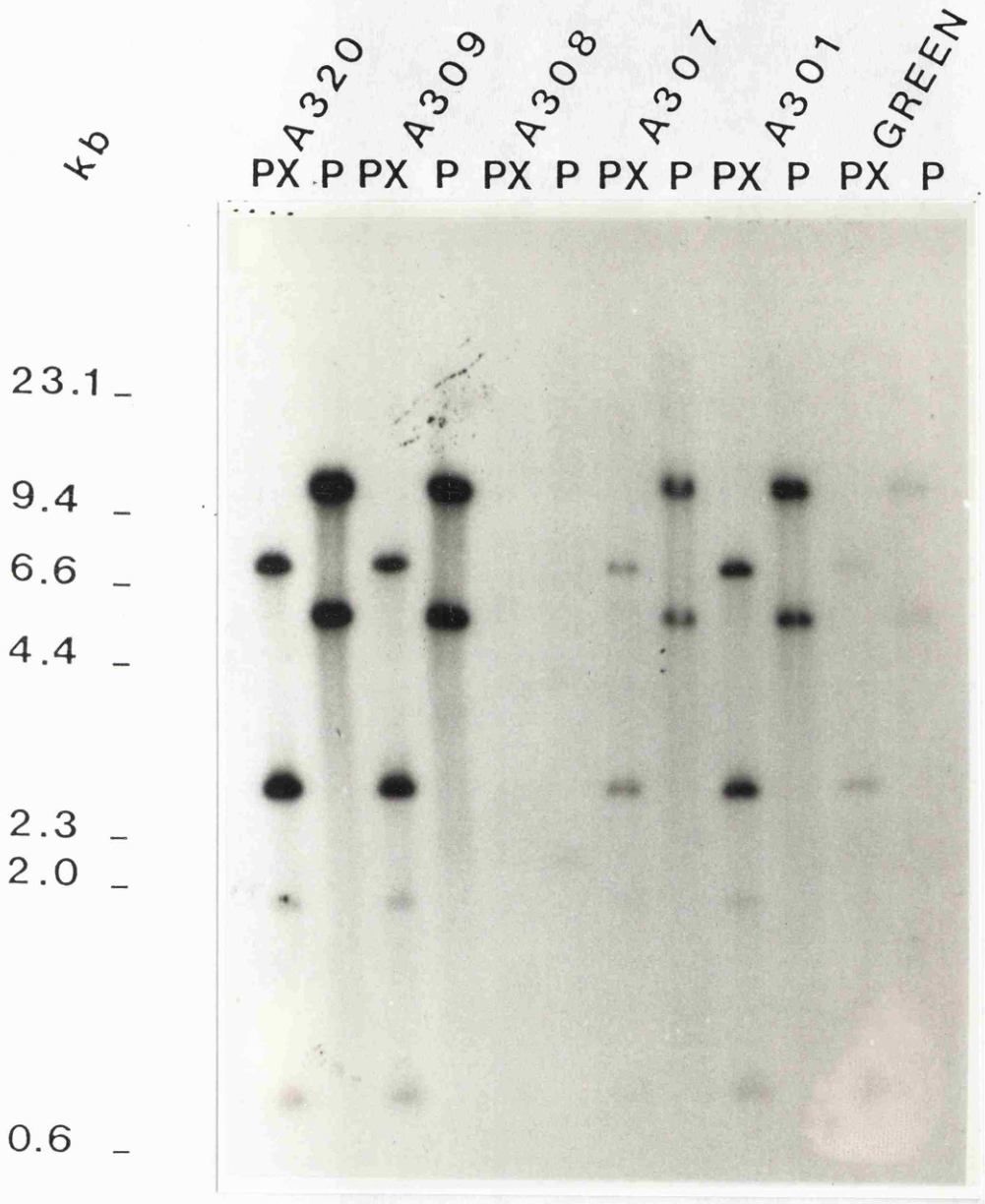
IV



P=Pst1 PX=Pst1+Xho1

Probe=**P5**

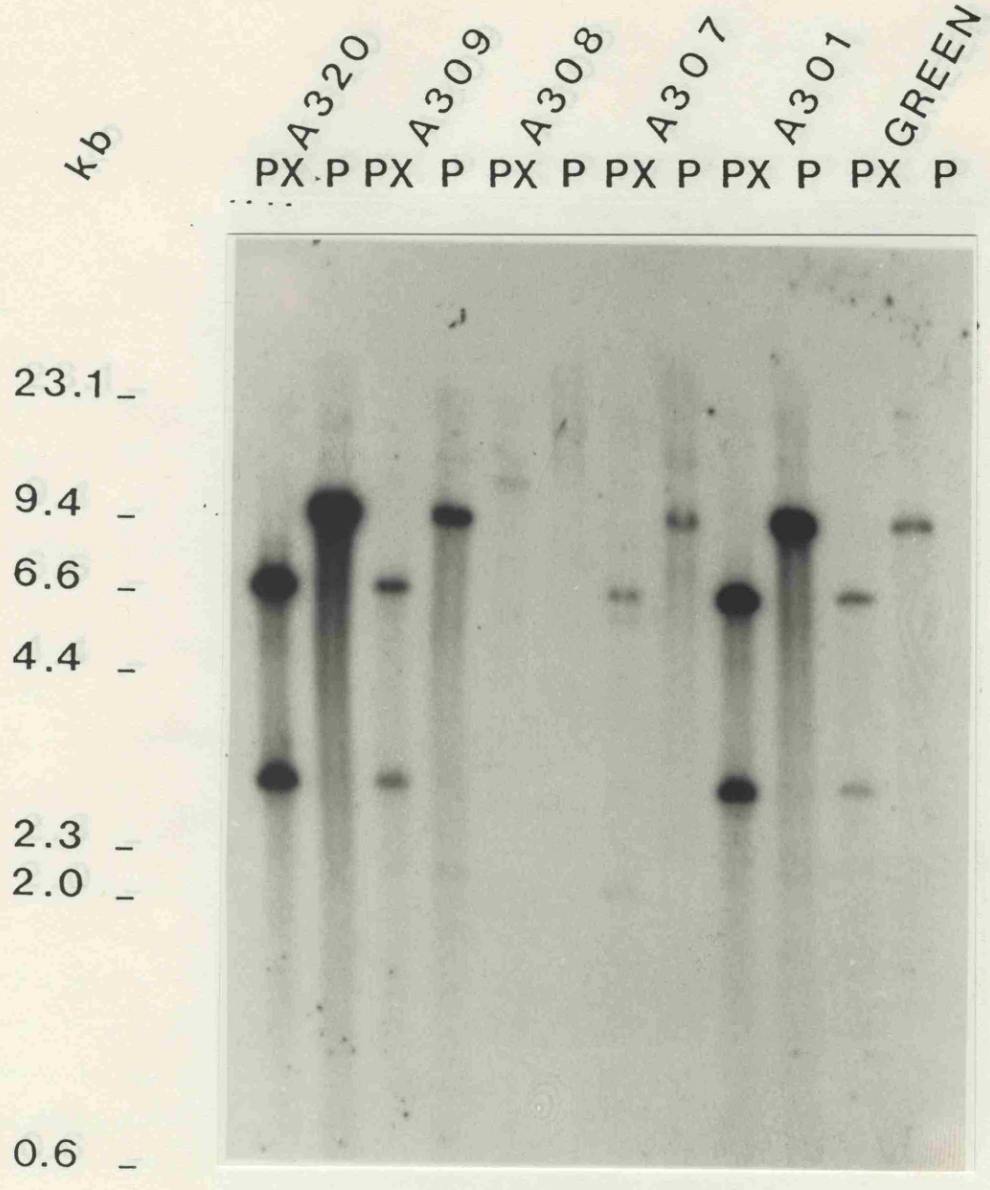
V



P=Pst1 PX=Pst1+Xho1

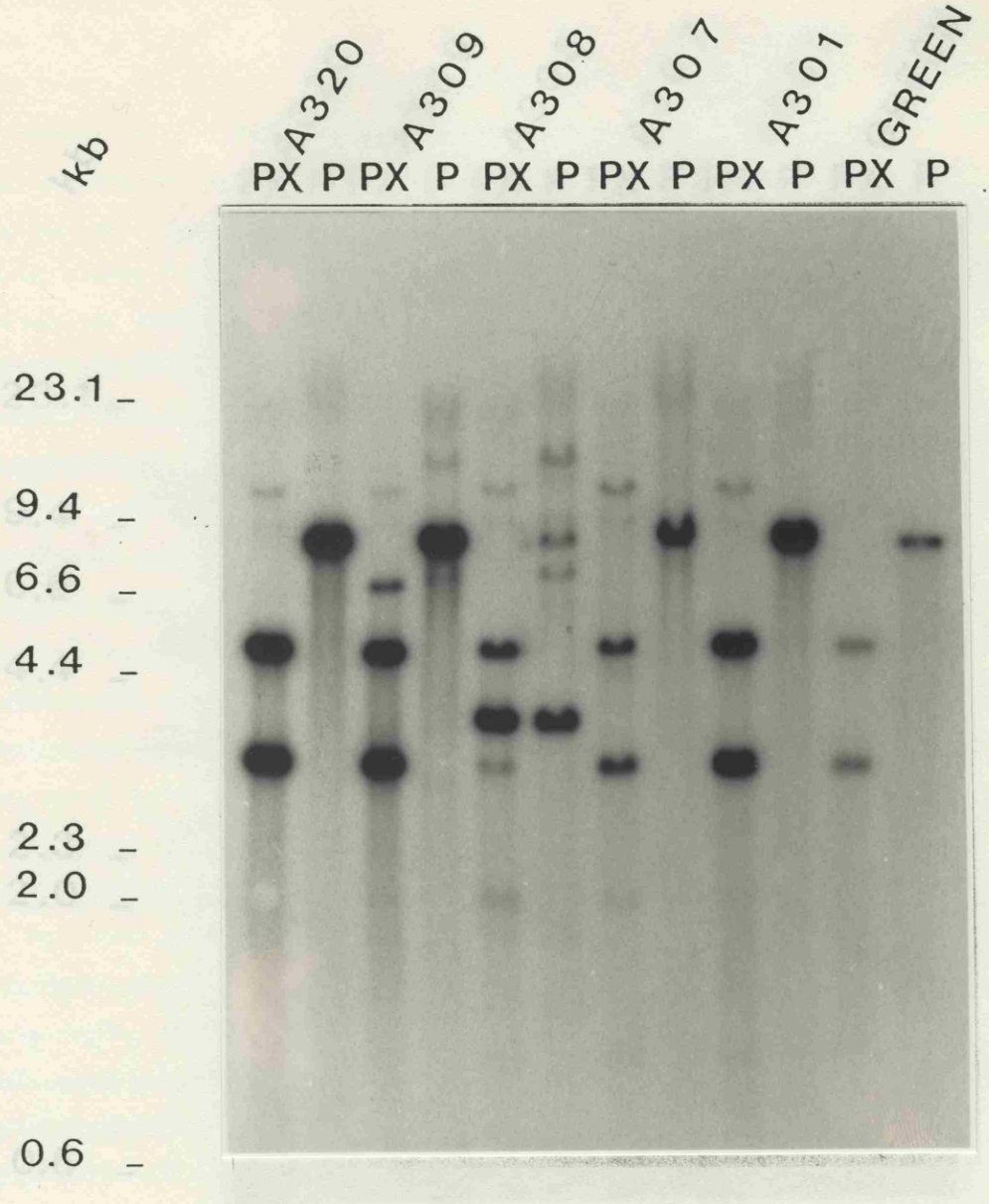
Probe=P6

VI



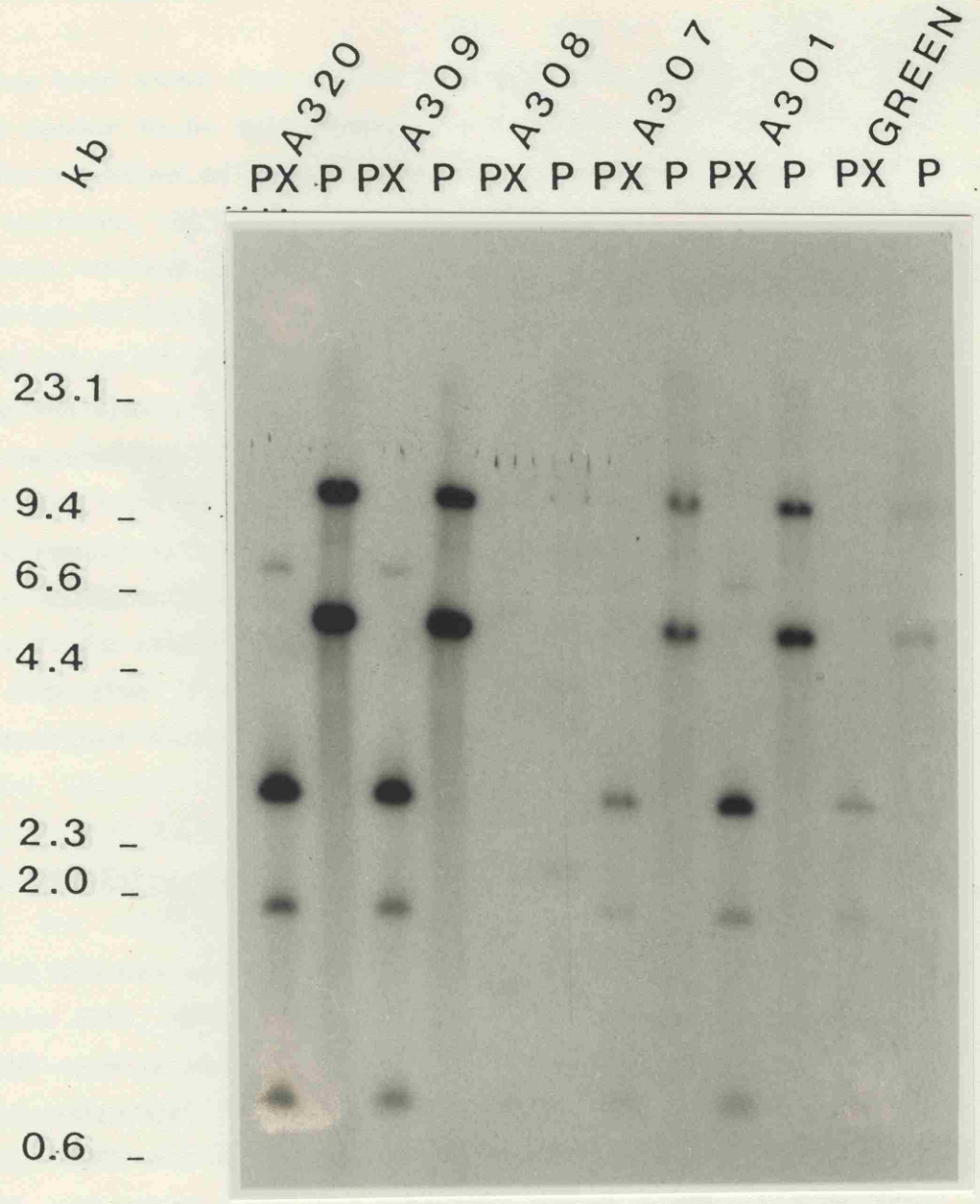
P=Pst1 PX=Pst1+Xho1

Probe=P7



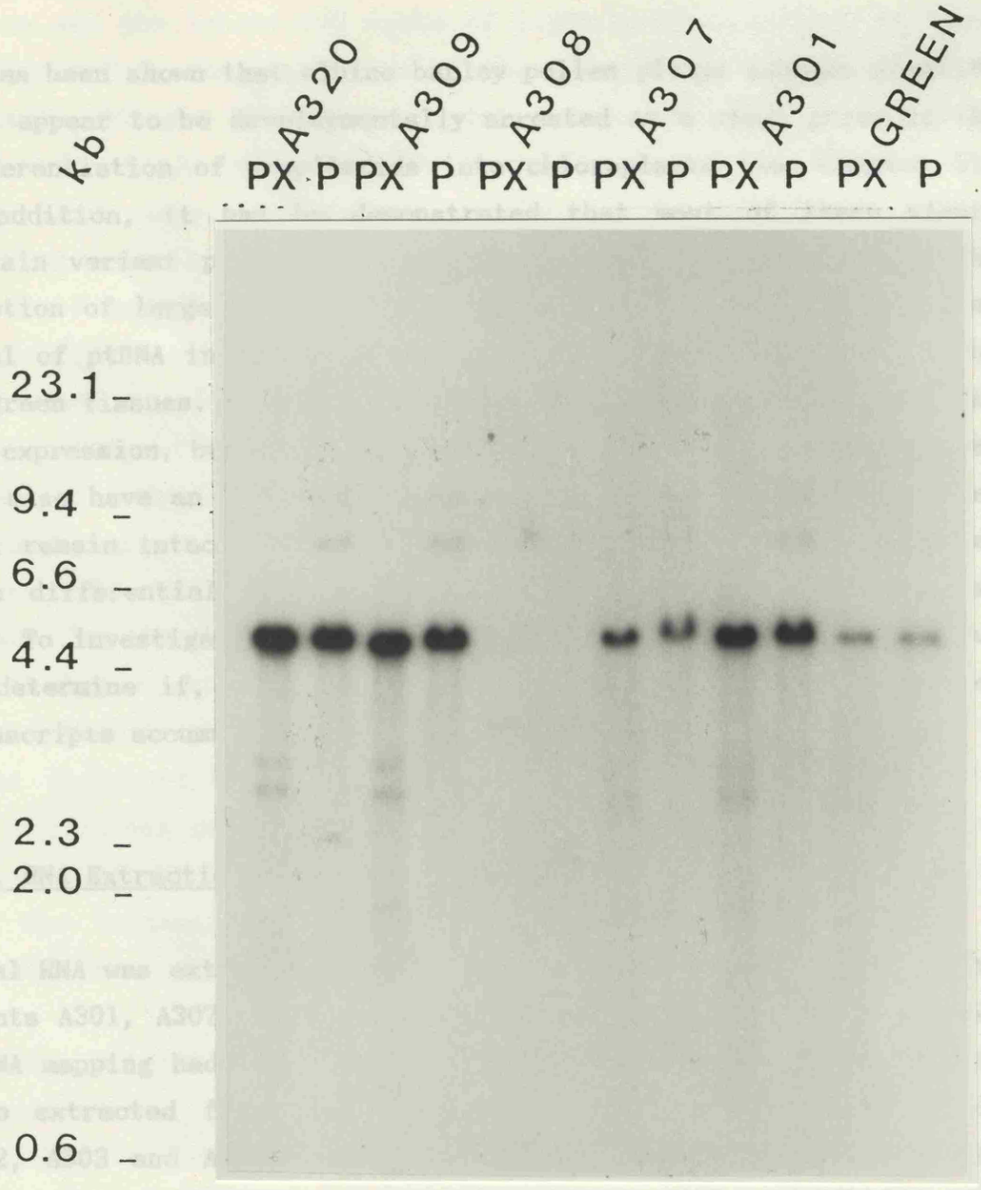
P=Pst1 PX=Pst1+Xho1

Probe=P8



P=Pst1 PX=Pst1+Xho1

Probe=P9



P=Pst1 PX=Pst1+Xho1

Probe=P10

X

CHAPTER 6: EXPRESSION OF THE PLASTID GENOME IN ALBINO POLLEN PLANTS.

6.1. Introduction.

It has been shown that albino barley pollen plants possess plastids that appear to be developmentally arrested at a stage prior to the differentiation of proplastids into chloroplasts (see Chapter 3). In addition, it has be demonstrated that most of these plants contain variant plastid genomes that appear to have suffered the deletion of large tracts of DNA sequence. Furthermore, the general level of ptDNA in albino plants is reduced relative to that found in green tissues. Deletion of a plastid gene will obviously abolish its expression, but the presence of deletions in the plastid genome may also have an effect on the transcription of the DNA sequences that remain intact. Moreover, different patterns of deletions may have differential effects on the transcription of the remaining DNA. To investigate albinism further, experiments were carried out to determine if, and to what extent a number of plastid encoded transcripts accumulate in albino barley pollen plants.

6.2. RNA Extractions from Plant Tissues.

Total RNA was extracted from tissue remaining from the five albino plants A301, A307, A308, A309 and A320 and the plant V1 for which ptDNA mapping had been carried out (see Chapter 5). Total RNA was also extracted from four additional albino pollen plants A501, A502, A503 and A504, two anther derived callus samples C470 and C505 and green leaf and root tissue from barley seedlings. The protocol used for total RNA extractions was based on that of Logemann *et al.* (1987) since this method was found to give superior yields of total RNA than other methods and also to be easily adapted for extractions from relatively small quantities of tissue. These considerations made the method ideal for RNA extractions from limited amounts of albino tissue where maximum yields of RNA were essential.

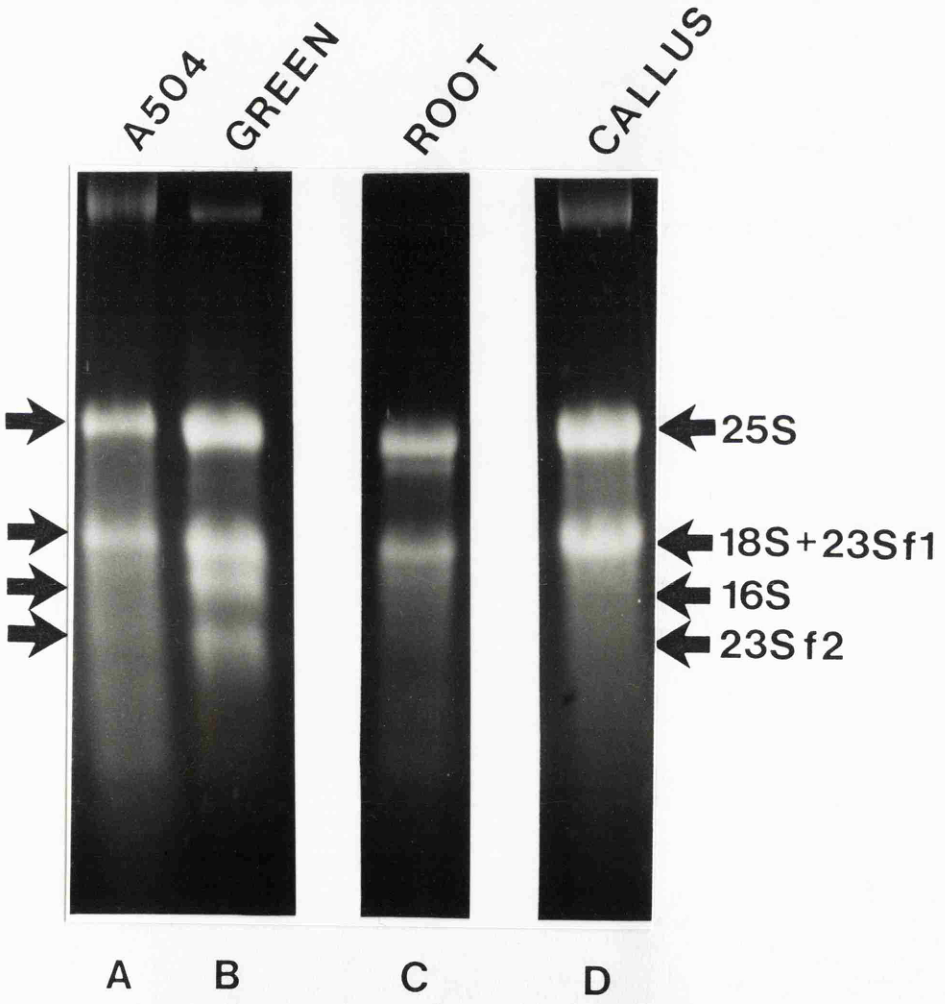
Yields of total RNA from samples of albino tissue weighing ≤0.5g

were estimated by spectrophotometry to range from 15-70μg. To test the integrity and size of total RNA extracted from the various samples, aliquots from each were fractionated by electrophoresis on agarose gels under denaturing conditions (see Chapter 2). The typical appearance of RNAs extracted from an albino, pollen derived callus and the leaves and roots of light grown seedlings is shown in Figure 6.1. The appearance of the major bands representing the cytosolic 25S and 18S rRNAs were taken as a measure of the level of integrity of the extracted RNA (see Figure 6.1.). In all cases these bands were discrete and clearly visible with little of the smearing associated with degraded RNA (Selden, 1987). It can be seen in Figure 6.1 that a high molecular weight band is present in samples of total RNA from albino and green tissues. This probably represents low levels of contaminant DNA isolated from the plant tissue along with RNA (Logemann *et al.*, 1987; Poulsen, 1983). The levels of contaminating DNA were judged from electrophoresed RNA to be roughly equivalent for each of the different tissues. The small amount of contaminating DNA could have been removed from the samples by the action of RNAase-free DNAase. However, blotting of an ethidium bromide stained gel containing fractionated RNA samples onto Hybond-N and subsequent examination of the filter under UV light indicated that little or no DNA had been transferred under the conditions used (data not shown). This observation eliminated the possibility that hybridisation between radiolabelled probe DNA and the contaminant DNA could interfere with the detection of plastid transcripts in total RNA samples.

Samples of total RNA from different plant tissues were prepared at concentrations ranging from 0.3-1.6μg/μl. In the experiments described in following sections RNA samples from different plants were divided into two groups: those with relatively high (≥0.6μg/μl) and those with lower (≤0.6μg/μl) concentrations of total RNA. Separate Northern hybridisation experiments were carried out for 'high' and 'low' concentration RNA samples. To compare transcript levels in different plants it was necessary to use comparable amounts of total RNA from each tissue. Aliquots of total RNA preparations containing approximately 7μg and 3.5μg of nucleic acid were taken from the high and low concentration RNA samples respectively. To check further whether these aliquots contained comparable amounts of RNA, samples were fractionated by agarose gel

Figure 6.1. The Appearance of Total RNA from Albino, Callus, Root and Leaf Tissue.

Samples of total RNA from albino A504 (lane A), the green leaves (lane B) and roots (lane C) of light grown seedlings and pollen derived callus C505 (lane D) were electrophoresed and visualised by staining with ethidium bromide and subsequent UV illumination as described in Section 2.14. Lanes B and D contain 5μg RNA and lanes A and C contain 3μg RNA. The position of the plastid and cytosolic rRNA bands are indicated with arrows: 25S = 25S rRNA, 23Sf1 and 23Sf2 = breakdown products of plastid 23S rRNA, 18S = 18S rRNA, 16S = plastid 16S rRNA.


A504
GREEN
ROOT
CALLUS
25S
18S+23Sf1
16S
23Sf2
A
B
C
D

electrophoresis, blotted and fixed onto nylon filters and hybridised with a radiolabelled probe representing a portion of the wheat 25S rDNA which encodes the 25S rRNA component of cytosolic 80S ribosomes. This was prepared as a *Sal* I subfragment of the plasmid pTA71 (Gerlach and Bedbrook, 1979) which contains the entire rDNA repeat unit of wheat. Figure 6.2A shows the restriction map of pTA71 and the location of the 25S rDNA probe fragment. The result of hybridising the 25S rDNA probe with the 'high' concentration group of RNA samples along with equal amounts of total RNA extracted from seed-derived leaf and root tissue is shown in Figure 6.2B. 25S rRNA is one of the most abundant species of RNA in all plant tissues (see Figure 6.1) therefore the level of hybridisation to the rDNA probe can be used as an estimate of total RNA content per sample. The levels of hybridisation in each lane indicates that the volumes of RNA solution applied to the gel contained equivalent amounts of RNA and that similar quantities of RNA were transferred from each lane to the filter during Northern blotting. A similar result was obtained using the 'low' concentration RNA samples.

6.3. Levels of Plastid rRNAs in Albino Tissues.

6.3.1. General Approach.

In barley, the plastid rRNAs are encoded in a single transcription unit located in the inverted repeat region of the plastid genome. The position of the 16S and 23S coding regions on the map of barley ptDNA is shown in Figure 5.1. In higher plants, this transcription unit gives rise to a polycistronic RNA which includes the 23S and 16S rRNAs (see Section 1.3.3). This undergoes processing to generate the individual plastid rRNA species (reviewed by Grierson, 1982).

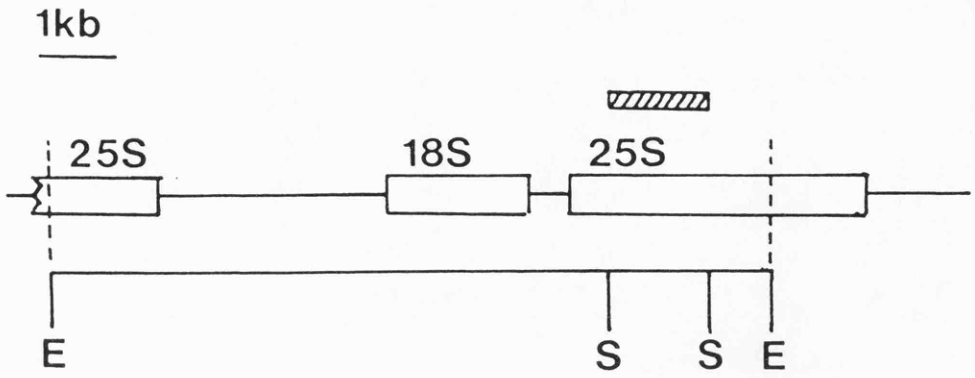
A striking feature of total RNA isolated from albino tissues, as visualised by ethidium bromide staining of agarose gels, is the absence of bands corresponding to chloroplast 23S and 16S rRNAs (see Figure 6.1). This feature has also been reported for albino pollen plants regenerated from rice anthers (Sun *et al.*, 1979). However, it is possible that the plastid rRNAs are present in

Figure 6.2. Hybridisation of 25S rDNA Probe to Total RNA Samples.

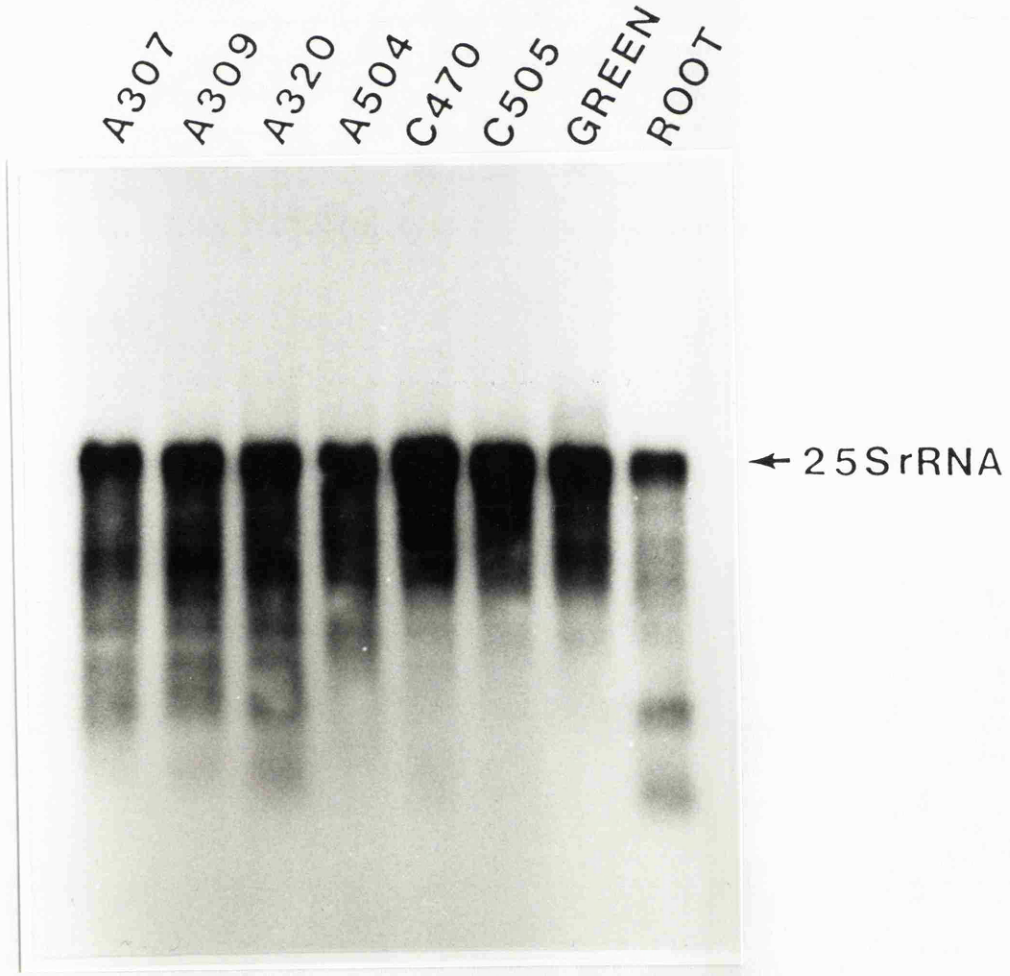
Panel A: Simplified restriction map of wheat rDNA repeat unit (redrawn from Gerlach and Bedbrook, 1979). Restriction enzyme sites are indicated: S = *Sal* I, E = *EcoR* I. Vertical dotted lines denote the limits of the insert of plasmid pTA71 (Gerlach and Bedbrook, 1979). The position of 18S and 25S rRNA genes are represented by open boxes. The position of the 25S rDNA specific probe fragment is indicated by a hatched bar.

Panel B: Autoradiograph of a Northern blot probed with the 25S rDNA probe detailed above. The source of the RNA is indicated at the top of each lane: A307, A309, A320, A504 = RNA from albino pollen plants, C505 and C470 = RNA from pollen derived callus samples, GREEN = RNA from the leaves of light grown barley seedlings, ROOT = RNA from the roots of barley seedlings. 3.5μg of total RNA was loaded per lane. The position of the 25S rRNA band is indicated with an arrow. The hybridisation filter was washed under conditions of moderate stringency as specified in Section 2.15. The autoradiograph represents a 4 hour exposure to X-ray film at room temperature.

A


1kb
25S
18S
25S
E
S
S
E

B


A307
A309
A320
A504
C470
C505
GREEN
ROOT
25SrRNA

samples of total RNA from albino tissues but at levels beyond the limit of detection using ethidium bromide staining of agarose gels.

6.3.2. Detection of 23S rDNA Transcripts.

To test whether plastid rDNA transcripts are present in albino pollen plants, equal amounts (see Section 6.2) of total RNA from a number of individuals were fractionated by agarose gel electrophoresis, blotted and fixed onto hybridisation filters and hybridised with probes encompassing the whole 16S rRNA gene and most of the 23S rRNA gene. These probes are derived from two *Pst* I + *Pvu* II derived subfragments of the pHvCP8 ptDNA insert which includes most of the plastid *rrn* operon (see Figures 5.1 and 6.8). The result of hybridising total RNA from various albino and control tissues with a probe encompassing the 5' end of the barley 23S rDNA (see Figure 5.1) is shown in Figures 6.3 and 6.4. These autoradiographs were obtained after exposing the hybridisation filters to X-ray film for approximately 3-5 hours. The major bands seen in the positive control lane, carrying total RNA from the leaves of green seedlings, represent the two specific breakdown products of the native 23S rRNA with sizes of 1.9kb and 1.0kb (Poulsen, 1983). Also visible in these lanes is a faint band of 2.9kb which may represent intact 23S rRNA. The specific 23S rRNA breakdown products are absent in samples of total RNA from root and callus tissue even when the bands representing 23S rRNA in the positive control lanes are over exposed. As can be seen from Figures 6.3 and 6.4 bands hybridising to 23S rRNA are absent in most albino pollen plants again, even when the positive control is relatively overexposed. However, in two cases a very faint band can be seen to hybridise to the 23S rDNA probe in lanes carrying total RNA from albino plants A320 and A501. In plant A320, this band is 2.9kb in size and therefore suggests that very low levels of the intact 23S rRNA may be present in this individual. The 1.0kb and 1.9kb 23S rRNA breakdown products are also very faintly visible on the original autoradiograph but at lower intensities than the 2.9kb band. The relatively low intensities of these bands compared to the 2.9kb band indicates that the 23S rRNA is predominantly in the intact form and has not undergone breakdown to the same extent as seen in the positive control. The band seen in RNA from plant A501

Figure 6.3. Detection of 23S rRNA in Albino Plants.

Autoradiograph of Northern blot hybridised with a barley 23S rDNA probe. The source of RNA is indicated above each lane: A307, A309, A320, A504 = RNA from albino pollen plants, C470 and C505 = RNA from pollen derived callus samples, GREEN = RNA from the leaves of light grown barley seedlings, ROOT = RNA from the roots of barley seedlings. 3.5μg of total RNA was loaded per lane. The top arrow on the right indicates the position of the intact 23S rRNA band and lower arrows indicate the position of the more abundant 23S rRNA 1.9kb and 1.0kb breakdown products. The position of a 2.9kb band in A320 is also indicated. The position and size, in kb, of the RNA molecular weight markers is shown on the left. The hybridisation filter was washed under conditions of moderate stringency as detailed in Section 2.15. The autoradiograph represents a 3-5 hour exposure to X-ray film at room temperature.

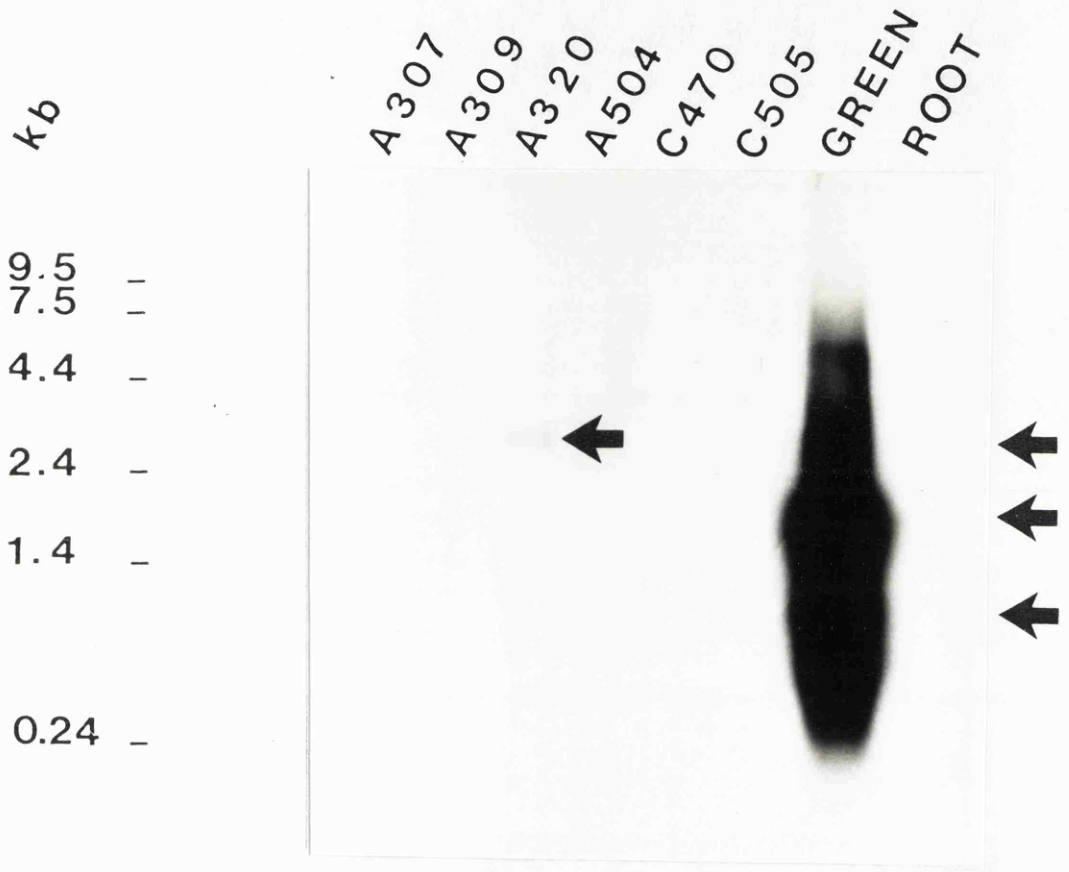
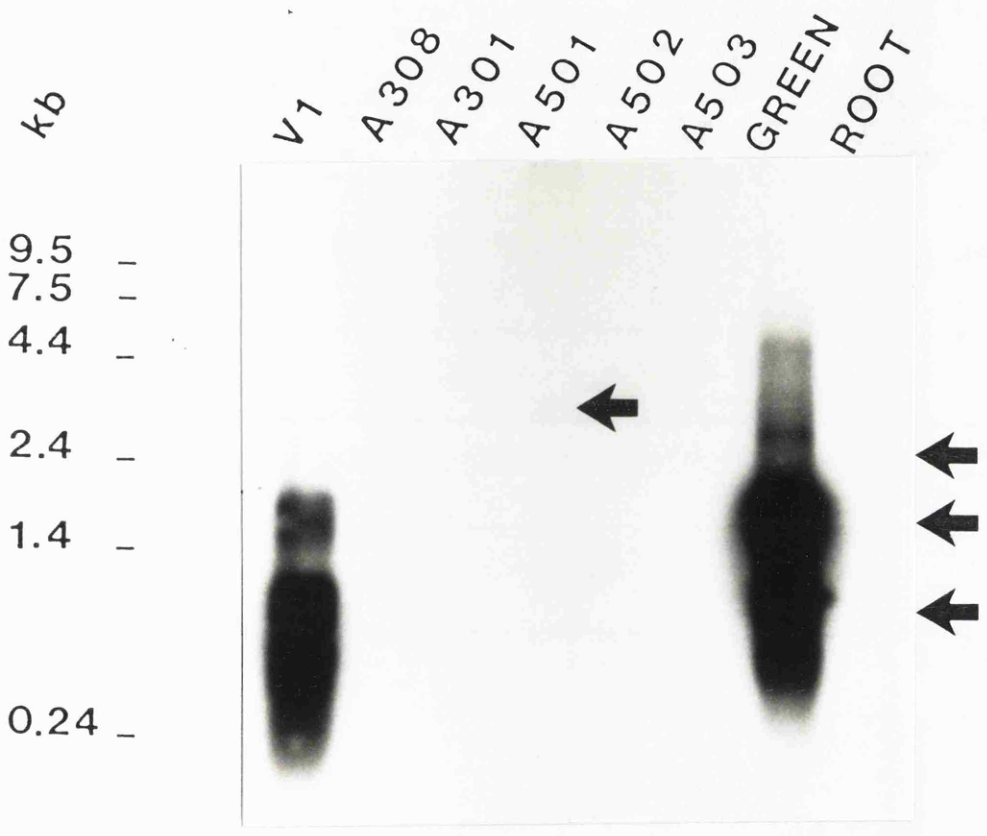

kb
9.5
7.5
4.4
2.4
1.4
0.24
A307
A309
A320
A504
C470
C505
GREEN
ROOT

Figure 6.4. Detection of 23S rRNA in Albino Pollen Plants.

Autoradiograph of Northern blot hybridised with a barley 23S rDNA probe. The source of RNA is indicated above each lane: V1 = RNA from pollen plant V1, A308, A301, A501, A502 and A503 = RNA from albino pollen plants, GREEN = RNA from the leaves of light grown barley seedlings, ROOT = RNA from the roots of barley seedlings. 3.5μg of total RNA was loaded per lane. The upper arrow on the right indicates the position of the possibly intact 23S rRNA band and the lower arrows indicate the position of the more abundant 23S rRNA 1.9kb and 1.0kb breakdown products. The position of a 3.4kb band in A501 is also indicated. The position and size, in kb, of the RNA molecular weight markers are shown on the left. The hybridisation filter was washed under conditions of moderate stringency as detailed in Section 2.15. The autoradiograph represents a 3-5 hour exposure to X-ray film at room temperature.


kb
V1
A308
A301
A501
A502
A503
GREEN
ROOT
9.5
7.5
4.4
2.4
1.4
0.24

is again extremely faint but its position is marked on Figure 6.4. The size of this band is 3.4kb which is slightly larger than the intact 2.9kb 23S rRNA. Although the identity of this band remains unclear, it is interesting to note that its size is similar to that estimated for the precursor of the mature 23S rRNA in the spinach rRNA processing pathway (Grierson, 1982). If this band represents low levels of such a precursor molecule then it implies the presence of some block to the final 23S rRNA processing event in plant A501 (see Chapter 8). Comparison of the intensities of bands seen hybridising to the 23S rDNA probe in plants A320 and A501 and those in the positive control indicate that their levels are ≤1% of those found in normal green leaves.

Total RNA from plant V1 contains relatively intense bands that are similar in size to those seen in the positive control. However, in this sample the stoichiometry of the two 23S breakdown products is different to that found in RNA from green tissues. Much less of the 1.9kb fragment appears to be present relative to the smaller 1.0kb fragment and this difference in stoichimetry is also accompanied by a substantial increase in the smearing of the 23S rRNA bands and particularly of the smaller breakdown product, which suggests that the RNA in this sample may be degraded to a greater extent than in other cases. Estimation of the abundance of these bands indicates that taken together, they are present at 10-50% the level found in green leaves.

6.3.3. Detection of 16S rDNA Transcripts.

Total RNA samples from a variety of albino tissues, the leaves and roots of light grown seedlings and anther derived callus samples were hybridised with a probe encompassing the whole barley plastid 16S rDNA region (see Figure 5.1). Representative autoradiographs from this experiment are shown in Figure 6.5, 6.6 and 6.7. The positive control sample was total RNA extracted from the leaves of green seedlings. A number of bands can be seen in the positive control lane, the most prominent of these being 1.6kb in size. This probably represents 16S rRNA since its size is close to that expected for the plastid 16S rRNA from other plant species (Schwarz and Kossel, 1980). However, a number of other less intense bands

Figure 6.5. Detection of 16S rRNA in Albino Plants.

Autoradiograph of Northern blot hybridised with a 16S rDNA probe. The source of the RNA is indicated above each lane: ROOT = RNA from the roots of barley seedlings, GREEN = RNA extracted from the leaves of light grown barley seedlings, V1 = RNA from pollen plant V1, A307, A308, A309, A320, A501, A502, A503 and A504 = RNA from albino pollen plants, C470 = RNA from the pollen derived callus sample, C470. 1μg of total RNA was loaded per lane. The arrow at the right indicates the position of the 1.6kb 16S rRNA band. The position and size, in kb, of the RNA molecular weight markers are given on the left. The hybridisation filter was washed under the conditions of moderate stringency specified in Section 2.15. The autoradiograph represents a 3 hour exposure to X-ray film at room temperature.

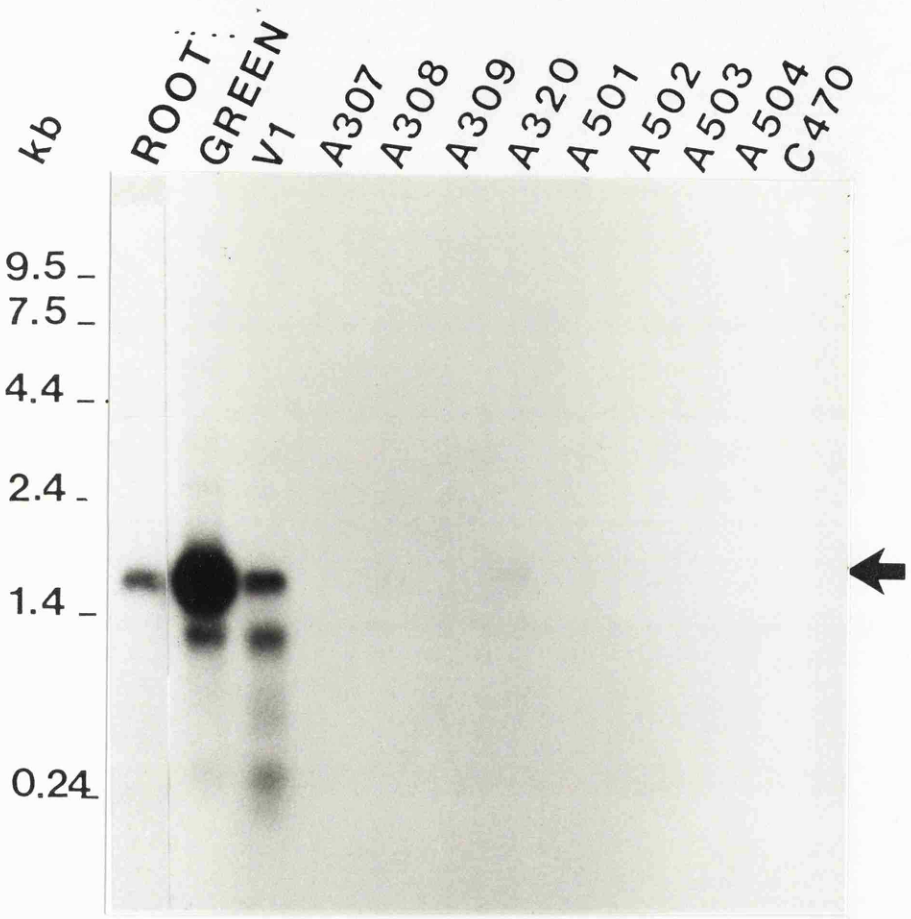

kb
ROOT
GREEN
V1
A307
A308
A309
A320
A501
A502
A503
A504
C470
9.5
7.5
4.4
2.4
1.4
0.24

Figure 6.6. Detection of 16S rRNA in Albino Plants.

Autoradiograph represents a 16 hour (overnight) exposure of the hybridisation filter in Figure 6.5. The arrow at the left indicates the position of the 1.6kb 16S rRNA band. The lane carrying root RNA has been omitted for simplicity.

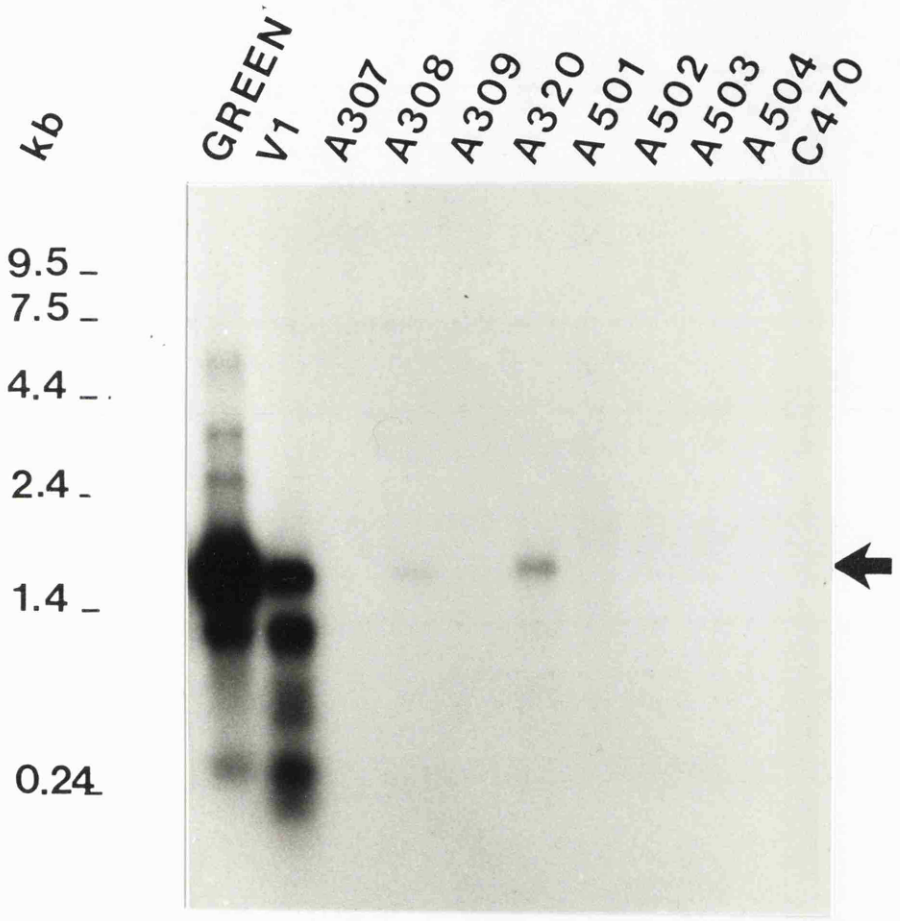
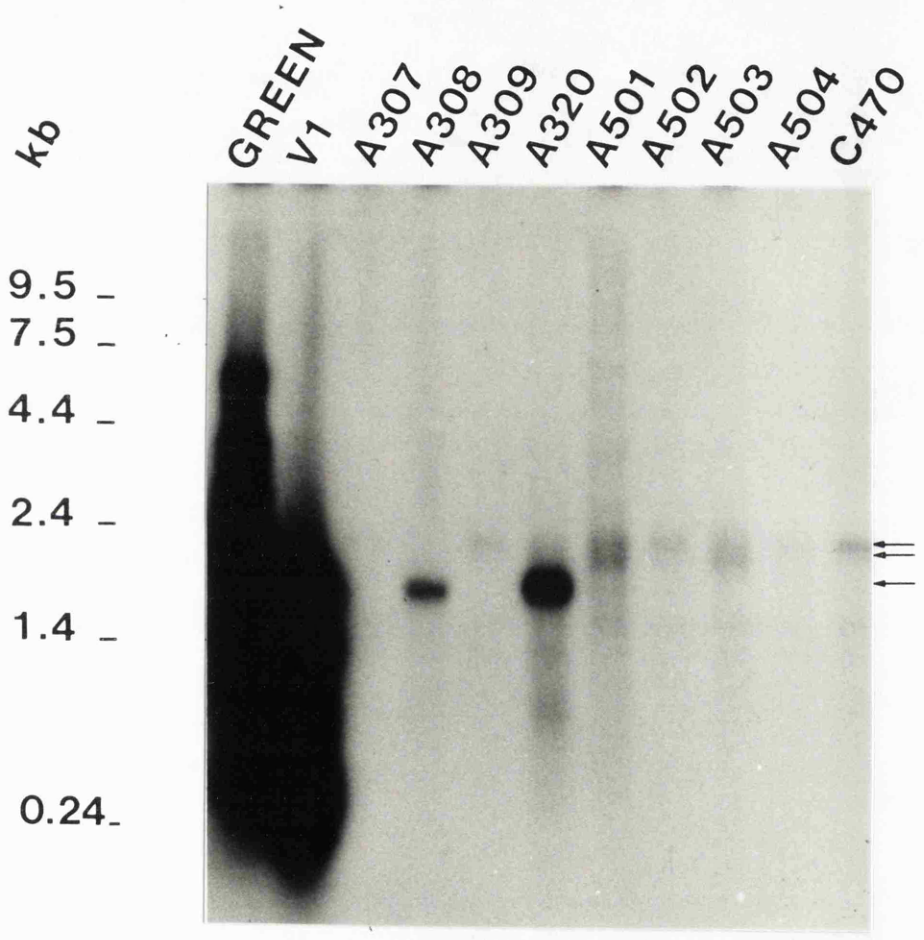

kb
GREEN V1
A307
A308
A309
A320
A501
A502
A503
A504
C470
9.5
7.5
4.4
2.4
1.4
0.24

Figure 6.7. Detection of 16S rRNA in Albino Plants.

The autoradiograph represents a 3 day exposure of the hybridisation filter shown in Figure 6.5. The small arrows at the right indicate the position of the 2.0kb and 1.8kb RNAs described in the text. The position of the 1.6kb 16S rRNA band is also indicated.


kb
GREEN
V1
A307
A308
A309
A320
A501
A502
A503
A504
C470
9.5
7.5
4.4
2.4
1.4
0.24

are present and it is unclear which molecular species they represent. As observed for the 23S rRNA, some of the smaller bands, the most prominent being 1.3kb and 0.35kb in size, might represent specific breakdown products of the native 16S rRNA. Transcripts larger than the 16S rRNA band, of 2.8kb, 3.5kb and 5.5kb are also present. The detection of transcripts of similar size, originating from this region of the barley plastid genome has also been reported by Poulsen (1983). It is possible that these bands represent intermediates in the post-transcriptional processing pathway that generates the individual rRNA species from the initial polycistronic plastid rDNA transcript (reviewed by Grierson, 1982). However, although details of this processing pathway are unknown for barley, none of the bands seen in the Northern hybridisation experiments described above are similar in size to the putative intermediates in the 16S rRNA processing pathway of spinach (Grierson, 1982). It should also be noted that the probe fragment used in this hybridisation experiment includes sequences 5' to the *rrn* operon. This raises the possibilty that some of the bands present in RNA from normal green leaves could correspond to transcripts originating from regions 5' to the rDNA operon and which overlap into the ptDNA region encompassed by the 16S rDNA probe fragment (see Figures 5.1 and 6.8). As yet, no transcription units have been mapped to this area of the barley plastid genome. However, data from the sequencing of the entire plastid genome of tobacco (Shinozaki *et al.*, 1986) indicate that several open reading frames are present in this region. More detailed transcript mapping in this area of the plastid genome would be necessary to elucidate the origin of these additional bands further.

Interestingly, a band corresponding in size to the 16S rRNA can also be seen in the lane carrying total root RNA. The presence of the 16S rRNA in root tissues has also been reported in spinach (Gruissem and Deng, 1988). This is in contrast to the situation seen for the 23S rRNA which fails to accumulate in the roots of barley seedlings (see Section 6.3.2). Since the two rRNAs are generated from the same primary transcript (Grierson, 1982), the differential accumulation of the 16S rRNA may reflect it's greater stability over that of the 23S rRNA in roots.

Bands hybridising strongly to the 16S rDNA probe are present in the

lane carrying total RNA from pollen plant V1. A prominent band of 1.6kb corrsponding in size to 16S rRNA is present at an abundance approximately 10% of that found in green leaves. No bands of a molecular weight higher than that of 16S rRNA are present in this sample. However, other bands of lower molecular weight are also present in this lane. A prominent band of 1.3kb is shared between these two samples as is a fainter band of 0.35kb, however these are of a higher intensity in V1. Over-exposure of the lane carrying total RNA from V1 revealed another shared band of approximately 0.7kb. The identity of these prominent bands in plant V1 is unknown.

Faint bands corresponding in size to 16S rRNA can be seen in lanes carrying RNA from albino plants A308 and A320 after a 3 hour exposure of the hybridisation filter. These can be seen more clearly when the filter is exposed overnight (see Figure 6.6). Comparison of the intensity of these bands in the different samples indicates that 16S rRNA is more abundant in A320 than in A308 and that overall this transcript is present in albinos at levels $\leq$1% of that found in green leaves. Exposing the hybridisation filter for 3 days (Figure 6.7) revealed bands of 2.0 and 1.8kb in samples A501 and A503. In samples A309, A320, A502, A504 and C470 only the 2.0kb band is visible. However, the presence of the 1.8kb transcipt in A320 may be difficult to determine since, if present, it would be obscured by overexposure of the 16S rRNA band. The 1.8 and 2.0kb transcripts were not detected in plants A307 or A308. The presence of these bands in the positive control was unable to be investigated due to over-exposure of the band corresponding to the 16S rRNA and their identity remains unclear. However, the size of these bands is very similar to that of the mitochondrial 18S rRNA. Furthermore, weak homology has been found between the plastid 16S rRNA and mitochondrial 18S rDNA of maize (Stern and Lonsdale, 1982). It is therefore possible that either the 1.8kb or 2.0kb bands represent the mitochondrial 18S rRNA detected by hybridisation to the 16S rDNA probe used in these experiments. However, the validity of this is uncertain since A307, A308 and V1 lack these bands entirely which would imply a reduction in the levels of 18S rRNA in these individuals, a fact that remains to be established.

Interestingly, the size of the 1.8kb and 2.0kb bands is similar to proposed intermediates in the processing pathway that generates mature 16S rRNA from the initial polycistronic rRNA transcript (see Grierson, 1982). This raises the possibility that some albino plants may be defective in efficient rRNA maturation, thus leading to the accumulation of processing intermediates. However, a more thorough characterisation of the identity of the RNA species described above would be needed to explore this possibility further.

6.3.4. Correlations Between rDNA Expression and ptDNA Structure.

The data presented above indicates that most albino barley pollen plants do not accumulate plastid rRNA transcripts and those that do do so to a limited extent. Furthermore, the presence of variant plastid genomes in albino plants raises the possibility that the reduced abundance of plastid rDNA transcripts is due to major structural changes in the ptDNA of these individuals.

Mapping of the ptDNA from albino plants and plant V1 indicates that in all individuals except A308, the restriction fragments encompassing the 16S and 23S rDNAs are intact (see Figures 5.2 and 5.3). A308 possesses an altered ptDNA fragment which may include only an intact 16S rDNA (see below). These fragments represent some of the most abundant ptDNA sequences present in albino pollen plants. In plant V1 these restriction fragments are present at the level found in green leaves. The Northern blot experiments described in the previous sections have shown that only plants A320, A308 and V1 accumulate plastid rRNAs. A320 and plant V1 contain both 23S and 16S rRNA while A308 contains only the 16S rRNA. The abundance of these transcripts in the albinos and plant V1 was estimated to be ≤1% and 10-50% of that found in green leaves, respectively. The reduction in the abundance of plastid rRNAs appears to be much greater in proportion than the observed decrease in the abundance of the ptDNA fragments encompassing them in albino plants. This suggests that the low levels of plastid rRNAs may be due primarily to a decrease in the transcription of the rRNA operon rather than a reduction in DNA template availability. The reduced abundance of plastid rRNAs seen in plant

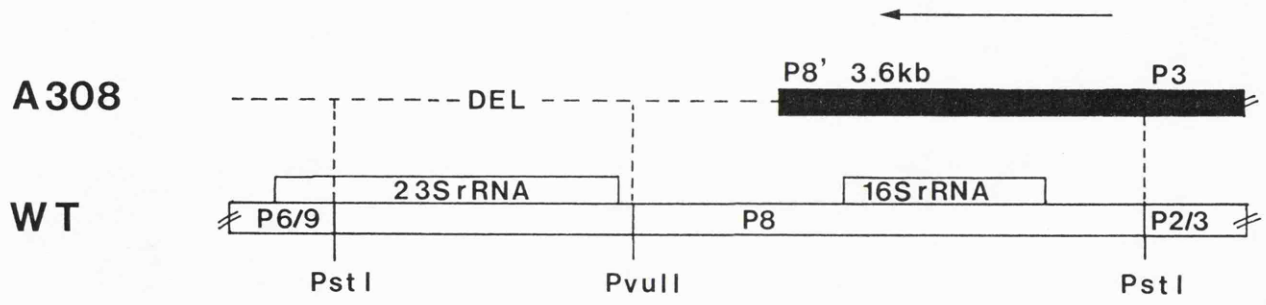
V1 which has normal levels of rDNA, provides support for this. However, a decrease in stability or an increase in transcript turnover cannot be discounted.

As detailed in Section 5.3.2, the ptDNA restriction fragment P8, which encompasses the plastid rRNA operon, has undergone major structural alteration in pollen plant A308. The major band hybridising to probe P8 is 3.6kb in size and is derived from a *Pvu* II derived subfragment which encompasses the 16S rDNA. Restriction fragments of wild-type size are also present in digests of DNA from A308 but are present at a lower intensity than the 3.6kb band. Proposals for the possible structure of a ΔptDNA in albino A308 are presented in Figures 5.5 and 5.6. One possible structure of the remaining portion of ptDNA fragment P8, shown in relation to the position of the 16S and 23S rDNAs, is presented in Figure 6.8. The deletion events that might have generated the restriction pattern seen in A308 appear to have removed the portion of P8 encompassing the 23S rDNA but have left the 16S rDNA intact. This correlates well with the observation that A308 accumulates 16S rRNA but not 23S rRNA. This evidence indicates that even though A308 may contain a truncated rDNA operon, transcription can occur from the remaining portion and presumably any necessary processing of this transcript can also occur. Alternatively, the remaining portion of the *rrn* operon in A308 could represent a fusion between the two copies of P8 present in the inverted repeat region (see Figure 5.7 and Section 5.3.2). An event like this could lead to the fusion of the 5' ends of the 16S rDNAs (see Figure 5.1 and 5.7). Moreover, if this were the case, it still allows the production of a transcript with the same size as the authentic 16S rRNA in A308.

However, it is possible that the 16S rRNA transcript is derived from the fragments of wild-type size seen hybridising to probe P8 in A308. In this case and others, where multiple forms of the plastid genome exist in albino plants, it would be difficult to assign a particular transcript as being derived from a specific ptDNA fragment. Albino plant A501 appears to contain a low abundance RNA species homologous to 23S rRNA but no 16S rRNA. However, in contrast to A308, it is not known whether this correlates to specific structural alterations in the rDNA of this plant.

Figure 6.8. Possible Structure of *rrn* Operon in Albino A308.

Lower bar indicates the structure of the intact barley plastid *rrn* operon (WT). Restriction sites for *Pst* I and *Pvu* II are indicated by vertical filled and dotted lines. The identity of the *Pst* I derived restriction fragments are given (see also Figure 5.1). The open bars above the restriction map respresent the position of the 16S and 23S rRNA genes. The filled bars indicate the possible structure of the remaining rDNA sequences in the most abundant form of ptDNA in A308 based on the Southern analyses described in Section 5.3.2. DEL = ptDNA sequences that have been deleted in A308. P8' denotes a truncated version of the intact fragment, P8. The arrow indicates the direction of transcription for the *rrn* operon.


A308
WT
DEL
P8' 3.6kb
P3
23SrRNA
16SrRNA
P6/9
P8
P2/3
PstI
PvuII
PstI

Since in some albinos there appears to be no accumulation of plastid rRNAs despite the presence of intact restriction fragments encompassing the rDNA operon (A301, A307 and A309), it is possible that the overall structure of the ptDNA in these plants may be having an effect on gene expression. The accumulation of both 16S and 23S rRNAs can be correlated in plant A320 with the presence of a restriction fragment pattern that is indistinguishable from the wild type (see Figure 5.3 and Section 5.6). In contrast, plant A301 also possesses all wild type ptDNA restriction fragments at a relatively high abundance but does not accumulate plastid 23S rRNA. Consideration of the ptDNA integrity of the only other albino plant to accumulate 16S rRNA, A308 (see Figure 5.3), reveals that major changes have taken place, including the apparent deletion of large tracts of DNA. Clearly, using these examples, no correlation can be made between the overall level of integrity of the plastid genome and the extent to which the plastid rRNAs accumulate in albino plants.

6.4. Levels of *rbc*L mRNA in Albino Plants.

The location of the gene encoding the large subunit of Rubisco, *rbc*L, on the barley plastid genome is shown in Figure 5.1. The position of a *Pst* I + *Hin*d III derived subfragment encompassing most of the *rbc*L coding region is also shown. This restriction fragment was used as a hybridisation probe to detect *rbc*L mRNA in total RNA preparations from albino pollen plants. The result of these Northern blot experiments are shown in Figure 6.9.

A major transcript of 1.7kb is detected in positive control lanes carrying total RNA from seed-derived green leaves. This corresponds closely in size to the *rbc*L mRNA reported by Poulsen (1983) and also to *rbc*L mRNAs found in spinach (Zurawski *et al.*, 1981) and maize (McIntosh *et al.*, 1980). A smaller transcript of 1.4kb was also seen to hybridise to the *rbc*L probe in addition to the main 1.7kb mRNA. This band, although difficult to discern in Figure 6.9, can be seen on the original autoradiograph but is clearly visible in Figure 6.12. The presence of two *rbc*L transcripts of similar size to those described above has been previously reported for barley (Poulsen, 1984) and similar transcript patterns have been

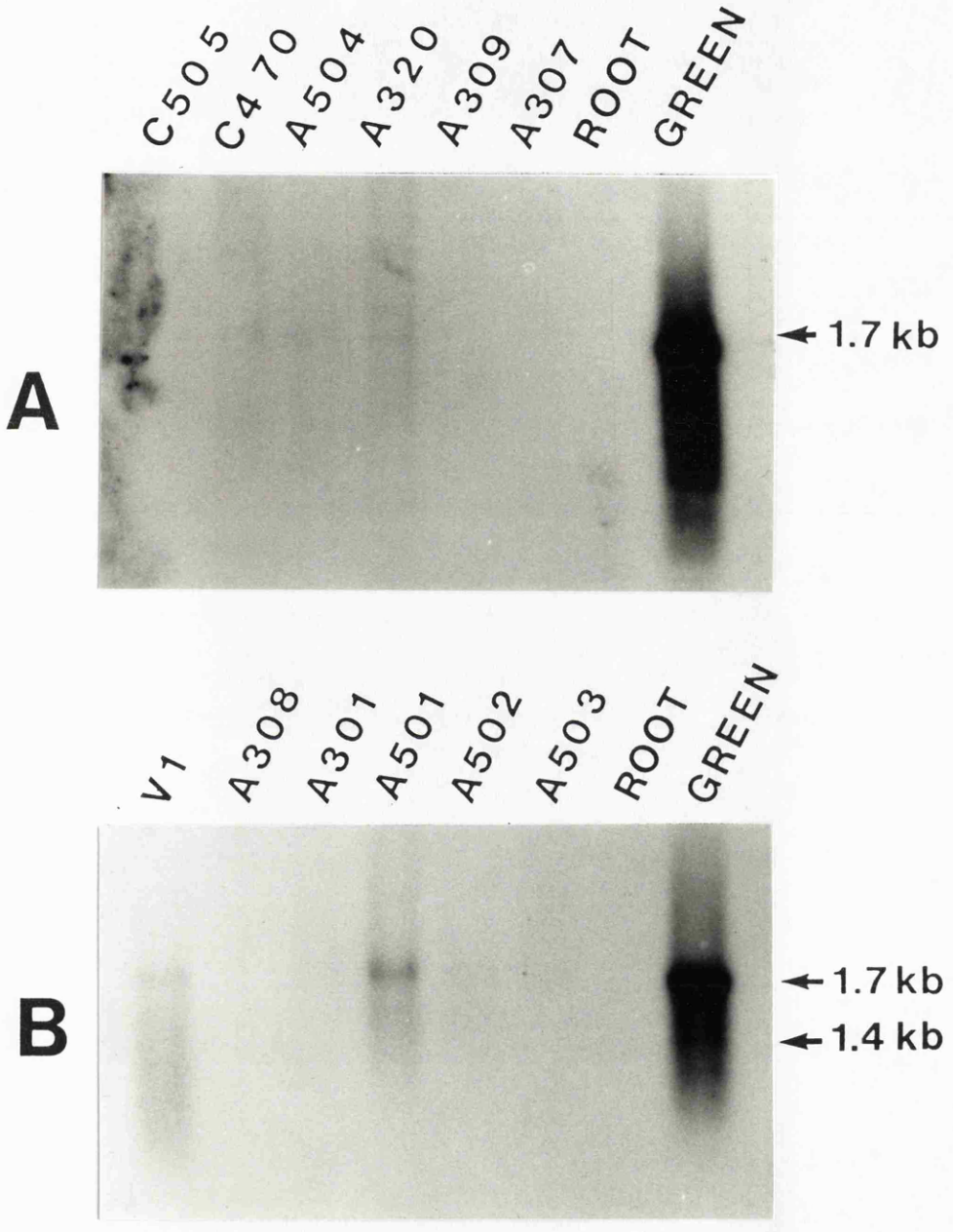
Figure 6.9. Detection of *rbcL* Transcripts in Albino Plants.

Autoradiographs of Northern blots hybridised with a *rbcL* probe. The source of RNA is indicated above each lane.

Panel A: C505, C470 = RNA from pollen derived callus samples, A504, A320, A309 and A307 = RNA from albino pollen plants, ROOT = RNA from the roots of barley seedlings, GREEN = RNA from the leaves of light grown barley seedlings. 7.0μg of total RNA was loaded per lane. The position of the 1.7kb *rbcL* mRNA is indicated by an arrow on the right.

Panel B: V1 = RNA from pollen plant V1, A308, A301, A501, A502 and A503 = RNA from albino plants, ROOT and GREEN as panel A. 3.5μg of total RNA was loaded per lane. The positions of the 1.7kb and 1.4kb *rbcL* transcripts are indicated on the right.

Both autoradiographs represent 16 hour exposures to X-ray film at -80°C. The hybridisation filters were washed under the conditions of moderate stringency specified in Section 2.15.


C505
C470
A504
A320
A309
A307
ROOT
GREEN
A
1.7 kb
V1
A308
A301
A501
A502
A503
ROOT
GREEN
B
1.7 kb
1.4 kb

described for *rbcL* in pea, maize and spinach (Mullet *et al.*, 1985; Woodbury *et al.*, 1988). It has been suggested that the shorter transcript may be derived by a processing event occurring at the 5' end of the longer one. The 1.7kb transcript is undetectable in lanes carrying root total RNA illustrating the strong tissue specific expression of this gene in barley seedlings. Similarly, *rbcL* transcripts are undetectable in RNA extracted from pollen derived callus samples.

In all the albinos tested, the major 1.7kb *rbcL* mRNA is present at reduced levels and in some cases is undetectable. Plant V1 shows a slightly higher level of the 1.7kb transcript than other individuals as does albino plant A501. The smearing of the band in V1 indicates that RNA extracted from this individual may be degraded to a greater extent than that from other plants (see Section 6.3.2). The level of *rbcL* transcript in albino plant A501 was found by laser densitometry to be approximately 10% the abundance of that found in normal green tissues and represents the highest level of *rbcL* mRNA in all the albino plants studied. The low intensity of bands representing *rbcL* mRNA in lanes carrying RNA from other albino tissues and V1 made it difficult to determine the levels of this transcript by laser densitometry. However, it was estimated that the *rbcL* mRNA was present in these plants at a level ≤5% of that found in normal green leaves.

As for the reduced abundance of plastid rRNAs, the low levels of *rbcL* transcripts in albino plants A301, A307, A308, A309 and A320 could be due to structural changes in the ptDNA fragments encompassing and surrounding the *rbcL* gene in these individuals.

Three of the albino plants for which ptDNA mapping has been carried out, A307, A308 and A309, have no detectable *rbcL* transcript while the remaining two, A301 and A320, show levels ≤5% of that found in normal green tissues. The presence of low levels of *rbcL* mRNA in A301 and A320 can be correlated with the presence of intact ptDNA restriction fragments encompassing the *rbcL* gene (see Figures 5.2 and 5.3) and all other wild type restriction fragments at high abundance. Similarly, the abundance of *rbcL* mRNA is also very low in pollen plant V1 despite the presence of an apparently intact plastid genome at the same abundance as that found in the leaves of

light grown seedlings (see Section 5.5). As for the plastid rRNAs, the reduction in the abundance of *rbcL* mRNA in these plants is far greater than the estimated reduction in the ptDNA levels encompassing the *rbcL* gene, suggesting that the observed low levels of *rbcL* mRNA may be due to a reduction in the transcription of the *rbcL* gene. Although, as pointed out previously, a reduction in the stability of the mRNA cannot be discounted (see Chapter 8).

In similar comparisons to those described above, the failure of *rbcL* mRNA to accumulate in A307, A308 and A309 can be correlated with the reduced abundance (A307, A309) or absence (A308) of the wild type ptDNA fragments encompassing the *rbcL* gene (see Figures 5.2 and 5.3). Under-representation of the restriction fragments encompassing the *rbcL* gene in albino plants may have an effect on its transcription by limiting the amount of available template for the plastid RNA polymerase (see Chapter 8). However, it is possible, from the observations with A301 and A320, that this could also be superimposed on an overall reduction in *rbcL* transcription. In addition, it is possible, in the absence of more accurate mapping data, that some of the non-wild type restriction fragments seen, for instance, in A307 and A308, have undergone loss or disruption of the regions encompassing *rbcL*, hence leading to a further reduction of the abundance of transcripts from this gene.

6.5. Levels of *psbD-psbC* Transcripts in Albino Pollen Plants.

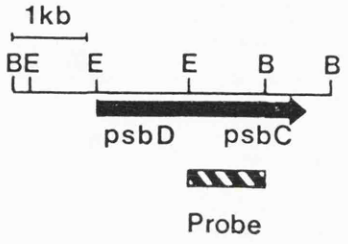
The genes *psbC* and *psbD*, which encode components of the chloroplast photosystem II complex, lie adjacent to each other in the LSC region of the barley plastid genome (see Figure 5.1). The *psbD-psbC* gene cluster has been cloned and the complex patterns of transcription from this region of the plastid genome have been characterised in etiolated and illuminated barley seedlings (Berends *et al.*, 1987; Gamble *et al.*, 1988). Several RNA species have been shown to originate from this area of the plastid genome (see below) including transcripts that encompass both *psbD* and *psbC* and others which encompass *psbC* only. A simplified restriction map of the region of barley ptDNA (Berends *et al.*, 1987) covering the *psbD-psbC* transcription unit is shown in Figure 6.10A. An *EcoR* I + *BamH* I subfragment covering a portion of the *psbC* gene was

Figure 6.10. The Pattern of Transcription from *psbD-psbC* in Barley Seedlings.

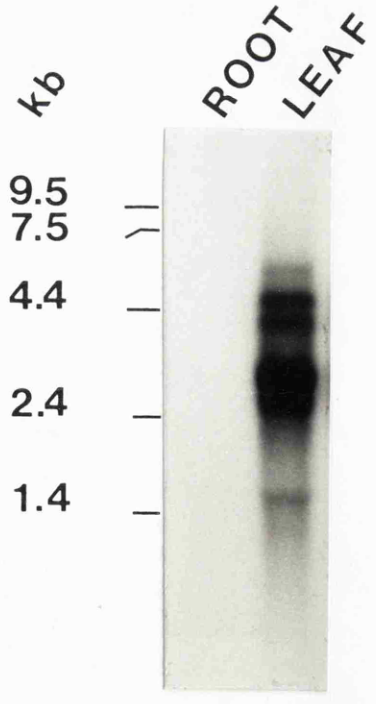
Panel A: Simplified restriction map of the *psbD-psbC* region of barley ptDNA (redrawn from Berends *et al.*, 1987). The positions of restriction sites are indicated. B = *Bam*H I, E = *Eco*R I. The filled arrow indicates the position and direction of transcription of the *psbC* and *psbD* coding sequences. The hatched bar below the restriction map shows the probe fragment used in hybridisation experiments.

Panel B. Autoradiograph of Northern blot of RNA from the roots (ROOT) and leaves (LEAF) of light grown barley seedlings. 10μg of total RNA was loaded per lane. The position and size, in kb, of the RNA molecular weight markers is given on the left. The autoradiograph represents a 16 hour exposure to X-ray film at -80°C. The hybridisation filter was washed under the conditions of moderate stringency detailed in Section 2.15.

A



B



used to probe RNA extracted from the leaves and roots of light grown seedlings and the result of this hybridisation is shown in Figure 6.10B. The major transcript observed is 3.2kb in size with fainter bands of 5.6kb, 4.8kb, 4.0kb, 2.6kb and 1.7kb. This transcript pattern is similar to that reported by Gamble *et al.*, (1988) in Northern analyses using RNA extracted from the plastids of illuminated barley seedlings hybridised with the DNA fragment shown in Figure 6.10A. No *psbD-psbC* derived transcripts are detected in total RNA extracted from root tissue and this was therefore used as a negative control.

To test for the presence of *psbD-psbC* transcripts in albino plants equivalent amounts of total RNA extracted from a number of individuals were hybridised to the probe shown in Figure 6.10A. The results of these experiments are presented in Figure 6.11, A and B. The controls were, again, total RNA extracted from the leaves and roots of light grown seedlings. The control lane carrying total RNA from green tissues in Figure 6.11B shows a number of transcripts of various sizes hybridising to the *psbC* probe. The major band seen in the control lane is 3.2kb. Bands of approximately 4.8kb, 4.0kb and 1.7kb are also faintly visible in the control lane but at a lower abundance than the 3.2kb transcript. This pattern is similar to that seen in Figure 6.10B. Only the 3.2kb and 1.7kb bands can be seen in the positive control lane in Figure 6.11A possibly due to the high levels of background hybridisation.

From the results shown in Figure 6.11, the levels of transcripts originating from the *psbD-psbC* region are greatly reduced in albino pollen plants, anther derived callus samples and plant V1 compared to that found in green leaves. In most cases, only the 3.2kb and 1.7kb transcripts are visible, eg. A504, Figure 6.11A; A308, Figure 6.11B, and even then are only barely detectable on the original autoradiograph. The high backgrounds seen in lanes carrying total RNA samples made accurate laser densitometry of individual bands difficult, however, the general level of *psbD-psbC* transcripts was estimated to be ≤5% of the level found in normal green leaves. Albino pollen plants A501, A502 and A504 appear to have slightly higher levels of *psbD-psbC* transcripts which were estimated to be approximately 10-20% of that found in green leaves. Interestingly, albino pollen plants A501 and A502 have transcript patterns which

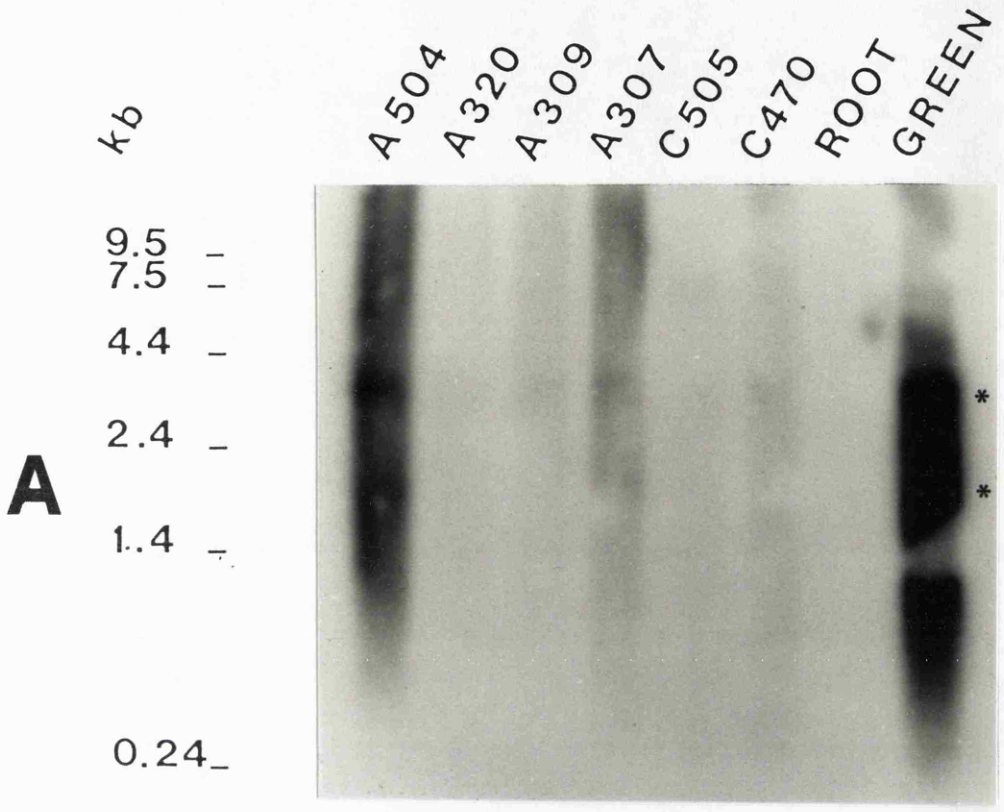
Figure 6.11. Detection of *psbD-psbC* Transcripts in Albino Plants.

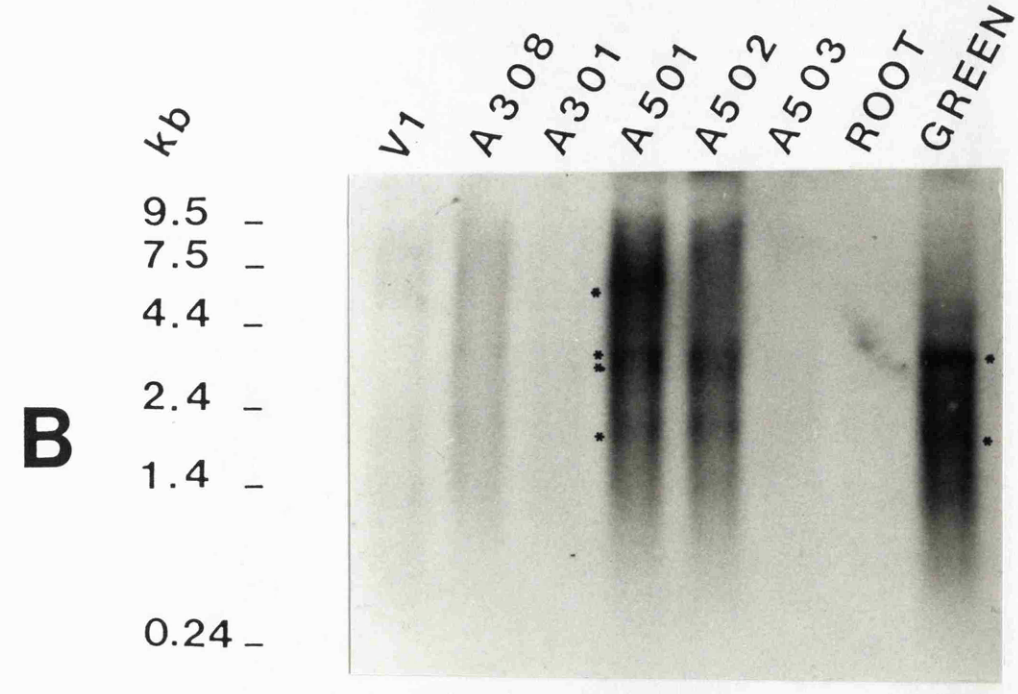
Autoradiographs of Northern blots hybridised with the *psbD-psbC* probe shown in Figure 6.10A. The source of the RNA is indicated above each lane.

Panel A: A504, A320, A309 and A307 = RNA from albino plants, C505 and C470 = RNA from pollen derived callus samples, ROOT = RNA from the roots of barley seedlings, GREEN = RNA from the leaves of light grown barley seedlings. 7.0μg of total RNA was loaded per lane. The positions of the 3.2kb and 1.7kb *psbD-psbC* transcripts are indicated with asterixes (*).

Panel B: V1 = RNA from pollen plant V1, A308, A301, A501, A502 and A503 = RNA from albino pollen plants, ROOT and GREEN as panel A. 3.5μg of total RNA was loaded per lane. The positions of 3.2kb and 1.7kb *psbD-psbC* transcripts are indicated with asterixes. Additional 5.5kb and 2.9kb bands found only in A501 or A502 are similarly marked.

Both autoradiographs represent 16 hour exposures to X-ray film at -80°C. The hybridisation filters were washed under the conditions of moderate stringency detailed in Section 2.15.


A
kb
9.5
7.5
4.4
2.4
1.4
0.24
A504
A320
A309
A307
C505
C470
ROOT
GREEN
*
*


B
kb
9.5
7.5
4.4
2.4
1.4
0.24
V1
A308
A301
A501
A502
A503
ROOT
GREEN
*
**
*
*
*

are different to that seen in the control. In addition to the 3.2kb and 1.7kb transcripts, which are present at equal intensity, both plants appear have a unique band of 2.9kb. A501 has an additional unique transcript of about 5.5kb. The 2.9kb and 5.5kb bands are similar in size to the 2.6kb and 5.6kb transcripts seen in samples of green leaf RNA (Figure 6.10B) and it is possible that this slight discrepancy in size could be due to differences in the migration of the RNA molecular weight markers between the two gels. However, since it is not known whether these transcripts represent the same molecular species in A501, A502 and green leaves, it is possible they represent novel RNAs generated due to alterations in the ptDNA of plants A501 and A502. This possibility could not be explored due to a lack of ptDNA mapping data from these individuals.

As in Sections 6.3-6.5, a comparison was made between the levels of *psbD-psbC* transcripts and the structure of the ptDNA found in albino pollen plants A301, A307, A308, A309, A320 and V1. The *psbD-psbC* transcription unit lies at the junction of P3 and P1 (see Figure 5.1). This area is encompassed by a 4.7kb *Pst* I + *Xho* I subfragment of P3 and a 12.0kb *Pst* I + *Xho* I subfragment of P1. All the albino pollen plants for which ptDNA mapping experiments have been carried out, including the pale yellow plant V1, possess intact versions of these fragments. However, in albino A308 fragment P1 has been truncated to 9.3kb but may still include the region encompassing the 3' end of the *psbD-psbC* transcription unit (see Section 5.3.2). In albino plants A301 and A320 the 4.7kb and 12.0kb P3 and P1 derived fragments are present at a relatively high abundance (see Table 5.1 and Figures 5.2 and 5.3). In contrast, the P1 and P3 derived fragments are under-represented in albino A309 (see Figure 5.3). The 4.7kb and 12.0kb P3 and P1 derived fragments are present at high levels compared to other areas of the plastid genome in albino plant A307. Similarly, the abundance of the 4.7kb P3 derived fragment also appears to be amplified in plant A308, however in this case there is no concomitant increase in the abundance of the flanking 9.3kb P1 derived fragment. Plant V1 possesses levels of the P3 and P1 subfragments encompassing the *psbD-psbC* transcription unit at similar levels to those found in normal green tissues.

These observations show that, irrespective of the abundance of the restriction fragments encompassing the *psbD-psbC* genes or the integrity of the remaining plastid genome, transcripts from this region of ptDNA are present at levels ≤5% of those found in green tissues. Again, the reduction in the level of *psbD-psbC* transcripts compared to that in normal green leaves are, in general, lower than the reduction in the abundance of the restriction fragments encoding them. This suggests that transcription of these genes or the stability of the corresponding mRNAs may be reduced in albino pollen plants and plant V1.

6.6. Levels of Plastid Transcripts in Green Pollen Plants.

As an additional control, studies on the transcription of the ptDNA found in green pollen plants were carried out. Total RNA was extracted from two green pollen plants and equal amounts fractionated by agarose gel electrophoresis. The RNA was transferred and fixed onto nylon hybridisation filters and hybridised with radiolabelled DNA probes for *rbcL* and the *psbD-psbC* transcription unit as described in the previous sections. The levels of plastid rRNAs were investigated by comparing the intensity of ethidium bromide stained rRNA bands in equivalent amounts of fractionated total RNA from green pollen plants and seed-derived green tissues. The results of these experiments are shown in Figure 6.12. In all cases the pattern and abundance of transcripts from each of the transcription units investigated appear to be the same as that found in seed-derived green tissues.

6.7. Summary.

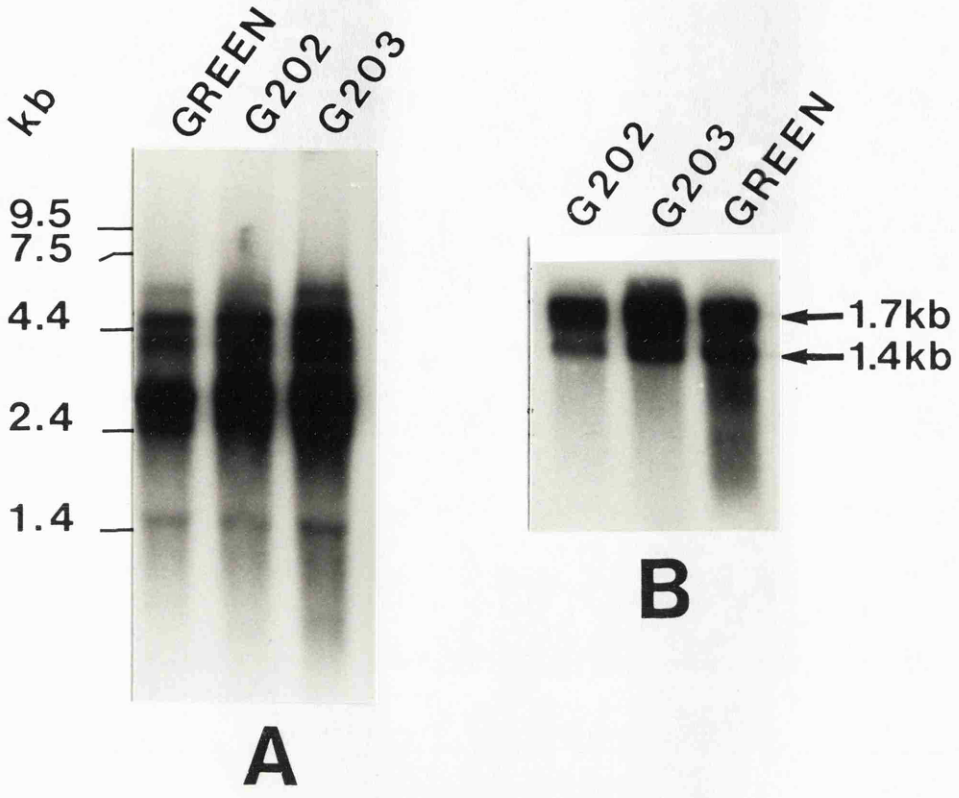
The studies described above were carried out to determine if, and to what extent a number of plastid encoded genes were expressed in albino barley plants. The levels to which transcripts from the plastid *rrn* operon, the *rbcL* gene and the *psbD-psbC* gene cluster accumulate in a number of albino plants is summarised in Table 6.1. In general, plastid encoded transcripts either fail to accumulate or do so to levels that are much lower than those found in the leaves of light grown barley seedlings.

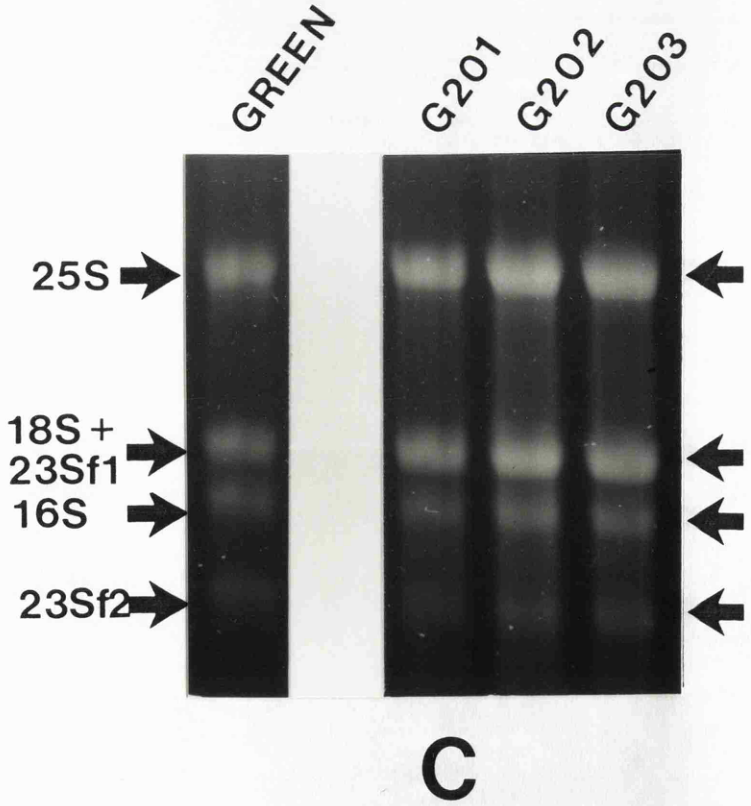
Figure 6.12. The Levels of Plastid Transcripts in Green Pollen Plants.

Panel A: Autoradiograph of Northern blot hybridised with *psbD-psbC* probe detailed in Figure 6.10A. Source of RNA is shown above each lane: GREEN = RNA from the leaves of light grown barley seedlings, G202 and G302 = RNA from green pollen plants. 10μg of total RNA was loaded per lane. The position and size, in kb, of the RNA molecular weight markers is shown on the left. The filter was washed under the conditions of moderate stringency detailed in Section 2.15. Autoradiograph represents a 16 hour exposure to X-ray film at -80°C.

Panel B: Autoradiograph of Northern blot hybridised with *rbcL* probe. Sources of RNA are indicated above each lane and are the same as for panel A. 10μg of total RNA was loaded per lane. The positions of the 1.7kb and 1.4kb *rbcL* transcripts are marked by arrows. Filter washing and exposure to X-ray film was carried out as for panel A.

Panel C: Ethidium bromide stained agarose gel carrying fractionated RNA from the leaves of light grown seedlings (GREEN) and three green pollen plants (G201, G202 and G203). 10μg total RNA was loaded per lane. The positions of the 25S rRNA (25S), the two plastid 23S rRNA breakdown products (23Sf1 and 23Sf2) and the 18S rRNA (18S) are indicated by arrows.


kb
GREEN
G202
G203
9.5
7.5
4.4
2.4
1.4
A
G202
G203
GREEN
1.7kb
1.4kb
B


GREEN
G201
G202
G203
25S
18S +
23Sf1
16S
23Sf2
C

From the data presented in Chapter 5, fragments encompassing the plastid genes for which transcript levels have been determined are often intact and present at relatively high abundance (see Figures 5.2 and 5.3). Even where this is the case, transcription of plastid genes is still reduced, eg. rRNAs and *psbD-psbC* (see Sections 6.3 and 6.5). This decrease in transcript abundance is often greater in proportion than the estimated reduction in the levels of the ptDNA fragments encompassing them, suggesting that the low abundance of plastid transcripts may be due to a reduction in the transcription of plastid genes rather than to limiting levels of template DNA. However, a decrease in stability or an increase in the turnover of these transcripts cannot be excluded at this level of analysis.

In some cases the under-representation or alteration of a ptDNA fragment encompassing a plastid gene can be correlated with the failure of a transcript to be detected, eg. *rbcL* (see Section 6.4). However, the significance of this, considering the generally low abundance of plastid transcripts, is unclear. Interestingly, in one case (albino A308), major alterations to the plastid rRNA operon, involving the apparent removal of sequences encompassing the 23S rDNA (see Section 6.3.4) have not eliminated the transcription of the remaining 16S rDNA, although the 16S rRNA is still present at reduced abundance.

Although in some cases plastid encoded transcripts do accumulate at different levels in individual albino plants (see Table 6.1), there appears to be no simple correlation between this and the overall structure of the plastid genome. For example, the rDNA appears to be transcribed in a plant which possesses an apparently intact ptDNA (A320) and another which has undergone major structural alterations to its plastid genome including deletion of portions of the rRNA operon (A308, see Figure 5.3). Furthermore, another plant also appearing to possess a full complement of intact ptDNA fragments at relatively high abundance (A301) fails to accumulate the plastid 23S rRNA. Another example in this context is the observation that *psbD-psbC* transcripts accumulate in all albinos for which ptDNA mapping has been carried out at a uniformly low level which appears to be independent of the overall structure of the plastid genome in these plants. It is interesting to note,

however, that albino A320, which does not appear to have undergone major structural alterations to its ptDNA, in contrast to other plants (see Table 6.1), accumulates transcripts from all the plastid genes studied. However, no major increase is seen in the abundance of any of these plastid RNAs compared to other albinos. Hence, it is difficult to determine if the presence of an intact ptDNA has a positive effect on plastid gene expression in A320.

The pale yellow pollen plant, V1, accumulates transcripts for *rbcL* and *psbD-psbC* at the levels found in albino plants, although the ptDNA of this plant appears to be intact and present at the abundance found in green leaves. The plastid 23S and 16S rRNAs are more abundant in V1, and are present at levels 10-50% of that found in green leaves.

Green pollen plants accumulate transcripts for all the plastid genes studied at the same level as that found in seed-derived green leaves.

Table 6.1. The estimated abundance of different plastid transcripts in albino pollen plants.

All values refer to the estimated level of each transcript as compared to that found in the leaves of green seedlings. A dash (–) indicates the absence of detectable transcript. ND = not determined Plants for which ptDNA mapping studies have been carried out are marked with an asterix (*).

	A301 *	A307 *	A308 *	A309 *	A320 *	A501	A502	A503	A504	V1
23S rRNA:	–	–	–	–	≤1%	–	–	–	–	10–50%
16S rRNA:	ND	–	≤1%	–	≤1%	–	–	–	–	10%
rbcL:	≤5%	–	–	–	≤5%	≤10%	≤5%	≤5%	≤5%	≤10%
psbD–psbC:	≤5%	≤5%	≤5%	≤5%	≤5%	10–20%	10–20%	≤5%	10–20%	≤5%

CHAPTER 7: EXPRESSION OF NUCLEAR GENES ENCODING CHLOROPLAST POLYPEPTIDES IN ALBINO POLLEN PLANTS.

7.1. Introduction.

It has been shown that light-grown albino pollen plants appear to possess developmentally arrested plastids containing variant ptDNA at reduced levels in addition to low levels of plastid-encoded RNAs. It has been demonstrated for barley that the lack of a functional chloroplast compartment can adversely affect the expression of nuclear genes encoding chloroplast polypeptides (Batschauer *et al.*, 1986; also see Section 1.3.7). To test whether this was the case in albino barley pollen plants the levels of mRNA for the nuclear genes *rbcS* and *cab* (encoding the small subunit of Rubisco (RbcS) and a chlorophyll a/b binding protein (Cab), respectively) were determined in total RNA samples from a number of albino individuals.

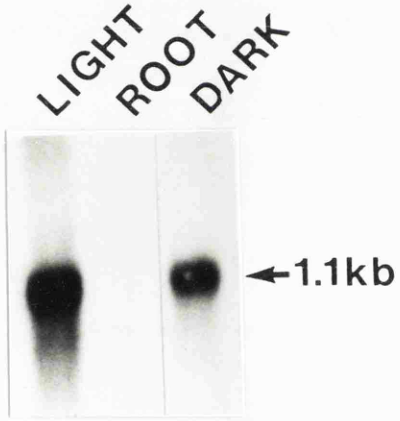
7.2. Levels of *rbcS* Transcripts in Albino Pollen Plants.

The plasmid pKG4626 contains a full length cDNA encoding *rbcS* from barley (Barkardottir *et al.*, 1987). The 0.9kb cDNA insert was present as a *Pst* I fragment in the vector pBR327 from which it was excised by restriction enzyme digestion, isolated by agarose gel electrophoresis and purified in preparation for radiolabelling as a probe in hybridisation experiments.

As a preliminary to determining the levels of *rbcS* mRNA levels in albino tissues the size of the mRNA detected with the pKG4626 cDNA insert and its pattern of abundance in normal barley tissues was investigated. Total RNA was extracted from the roots and leaves of 8 day old light grown barley seedlings and the leaves of seedlings grown in darkness. Equal amounts of RNA from each tissue were then fractionated by agarose gel electrophoresis, transferred and fixed onto a nylon filter and hybridised with the radiolabelled pKG4626 cDNA insert. The result of this hybridisation experiment is shown in Figure 7.1. The cDNA insert of pKG4626 hyridises to a transcript of 1.1kb. Maximum levels of *rbcS* mRNA are found in the leaves of

Figure 7.1. *Rbc*S mRNA Levels in Barley Seedlings.

Autoradiograph of Northern blot hybridised with a barley *rbc*S cDNA probe. Lanes carry total RNA from the leaves and roots of light grown seedlings (LIGHT and ROOT, respectively) and dark grown leaves (DARK). 12μg of total RNA was loaded per lane. The position of the 1.1kb *rbc*S mRNA is indicated. The hybridisation filter was washed under the conditions of moderate stringency detailed in Section 2.15. Autoradiograph represents a 16 hour exposure to X-ray film at -80°C.


LIGHT
ROOT
DARK
1.1kb

light-grown seedlings while lower levels (approximately 50% by laser densitometry) are found in those of dark-grown individuals. No detectable *rbcS* mRNA can be seen in root tissue. This illustrates two important aspects of *rbcS* expression in barley. Firstly, *rbcS* transcript levels are elevated by light treatment but are also relatively high in dark-grown tissues, therefore implicating some light-independent developmental control of *rbcS* gene expression. Secondly, *rbcS* transcripts are found only in leaves and not roots indicating tissue specificity for *rbcS* expression (see also Section 1.3.6). This data is consistent with similar observations made by Barkardottir *et al.* (1987).

To determine the level of *rbcS* transcripts in albino tissues, equal amounts of total RNA extracted from a number of albino individuals, in addition to two anther derived callus samples (C470 and C505) were fractionated by agarose gel elecrophoresis, transferred to nylon filters and hybridised with the cDNA insert of pKG4626. The results of these experiments are shown Figure 7.2. In both experiments equal amounts of total RNA from the green leaves and roots of barley seedlings were included as positive and negative controls respectively. The *rbcS* mRNA was not detected in lanes carrying RNA from anther-derived non-green callus tissue. Other studies have also suggested that *rbcS* gene expression is reduced in undifferentiated, non-green tissues (Herrera-Estrella *et al.*, 1984). Lanes carrying RNA from albino pollen plants also have a lower level of *rbcS* mRNA than does green tissue. However, the levels of *rbcS* transcript do vary from plant to plant. The *rbcS* transcript is barely detectable in plants A301, A307, A308, A309, A320 and A503 but is present at higher abundance in the plants A501, A502 and A504. Plant A501 exhibits the highest abundance of *rbcS* mRNA, which by laser densitometry scanning was estimated to be 20-30% of that found in green tissues. Similarly, the abundance of *rbcS* mRNA in plants A502 and A504 was estimated to be ≤15% of that found in green tissues. The very low levels of *rbcS* transcript seen in the remaining albino pollen plants were unable to be quantified since the low intensity of the corresponding bands made them unsuitable for laser densitmometry. However, it was estimated that *rbcS* mRNA was present in these plants at levels ≤5% of those found in green leaves.

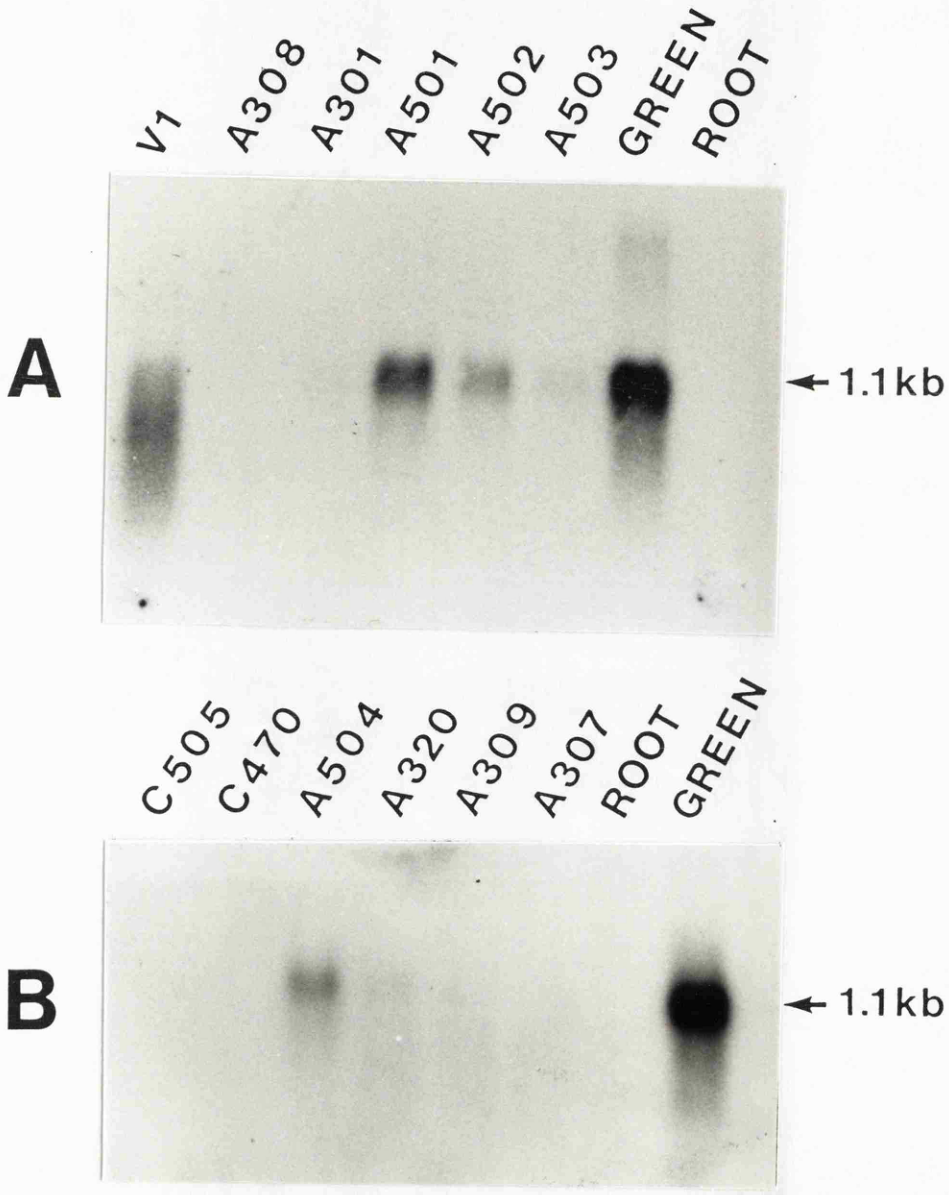
Figure 7.2. The Detection of *rbcS* mRNA in Albino Plants.

Autoradiographs of Northern blots hybridised with a barley *rbcS* cDNA probe. The source of RNA is indicated above each lane. Position of the 1.1kb *rbcS* mRNA is indicated.

Panel A: V1 = RNA from pollen plant V1; A308, A301, A501, A502 and A503 = RNA from albino plants; GREEN = RNA from the leaves of light grown barley seedlings; ROOT = RNA from the roots of barley seedlings. 3.5μg of total RNA was loaded per lane.

Panel B: C505 and C470 = RNA from pollen derived callus samples; A504, A320, A309 and A307 = RNA from albino plants; GREEN and ROOT as panel A. 7μg of total RNA was loaded per lane.

Hybridisation filters were washed under the conditions of moderate stringency detailed in Section 2.15. Autoradiographs represent 72 hour exposures to X-ray film at -80°C.


A
V1
A308
A301
A501
A502
A503
GREEN
ROOT
1.1kb
B
C505
C470
A504
A320
A309
A307
ROOT
GREEN
1.1kb

7.3. Levels of *cab* Transcripts in Albino Pollen Plants.

The plasmid pHvLF2 contains a 0.6kb cDNA insert encoding a barley chlorophyll a/b binding protein cloned into the *Pst* I site of pBR322 with the subsequent elimination of one *Pst* I site (Gollmer and Apel, 1983). The cDNA was released from the vector by digestion with the restriction enzymes *Pst* I and *Bgl* I. *Bgl* I cuts in the region of pBR322 approximately 100bp distal to the eliminated *Pst* I site and therefore the fragment to be used as a probe in hybridisation experiments included some pBR322 DNA. This 0.7kb fragment was isolated by agarose gel electrophoresis and purified in preparation for radiolabelling as a probe in hybridisation experiments.

The size and pattern of expression of *cab* transcripts in barley seedlings was investigated using the pHvLF2 cDNA insert as a probe in Northern hybridisation experiments as described for *rbcS* in Section 7.2. The result of this hybridisation is shown in Figure 7.3. Maximum expression of a 1.2kb transcript is seen in the lane carrying total RNA from light-grown leaves. In dark-grown leaves the levels are much lower, approximately 10% of the level found in light grown leaves as estimated by laser densitometry scanning of the autoradiograph in Figure 7.3. This indicates that light stimulates a large increase in the levels of *cab* mRNA (see Section 1.3.6). No *cab* transcript can be detected in the lane carrying total RNA from roots illustrating the tissue specificity of *cab* gene expression. The size of the transcript detected using the pHvLF2 cDNA insert and the tissue specific and light dependent patterns of its expression described above agrees closely with data obtained by other workers (Gollmer and Apel, 1983; Batschauer *et al.*, 1986; Barkardottir *et al.*, 1987)

To determine the levels of *cab* transcript in albino tissues, equal amounts of total RNA from various albino pollen plants and two anther derived callus samples were fractionated by agarose gel electrophoresis, transferred and fixed to nylon filters and hybridised with the radiolabelled cDNA insert of pHvLF2. The results of these experiments are presented in Figure 7.4. In each experiment equal amounts of RNA extracted from the leaves and roots of light-grown barley seedlings were included as positive and

Figure 7.3. *Cab* mRNA Levels in Barley Tissues.

Autoradiograph of Northern blot hybridised with a barley *cab* cDNA probe. Lanes carry total RNA from the leaves and roots of light grown seedlings (LIGHT and ROOT, respectively) and dark grown leaves (DARK). 12μg of total RNA was loaded per lane. The position of the 1.2kb *cab* mRNA is indicated. The hybridisation filter was washed under the conditions of moderate stringency detailed in Section 2.15. Autoradiograph represents a 72 hour exposure to X-ray film at -80°C.

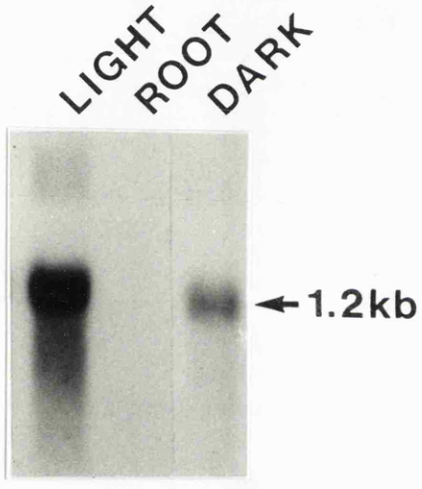

LIGHT
ROOT
DARK
1.2kb

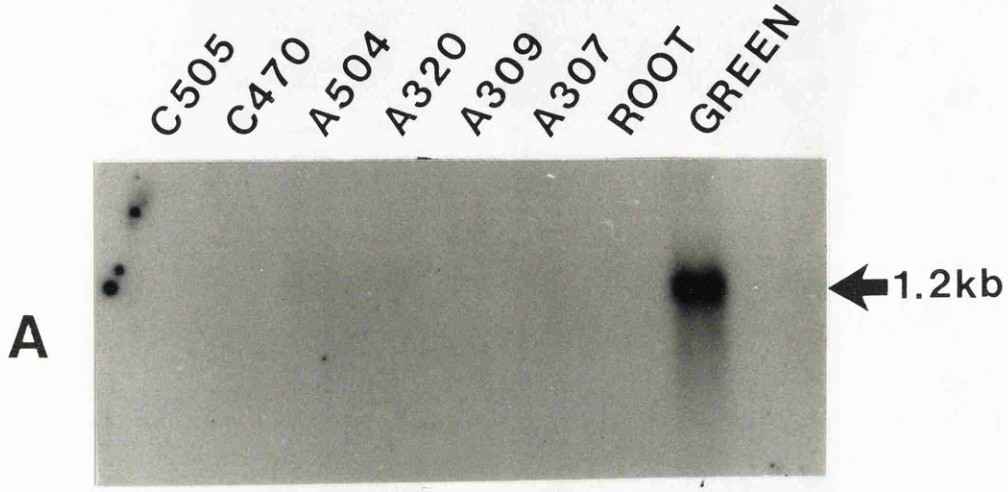
Figure 7.4. Detection of *cab* mRNA in Albino Pollen Plants.

Autoradiographs of Northern blots hybridised with a barley *cab* cDNA probe. The source of RNA is indicated above each lane. Position of the 1.2kb *cab* mRNA is indicated.

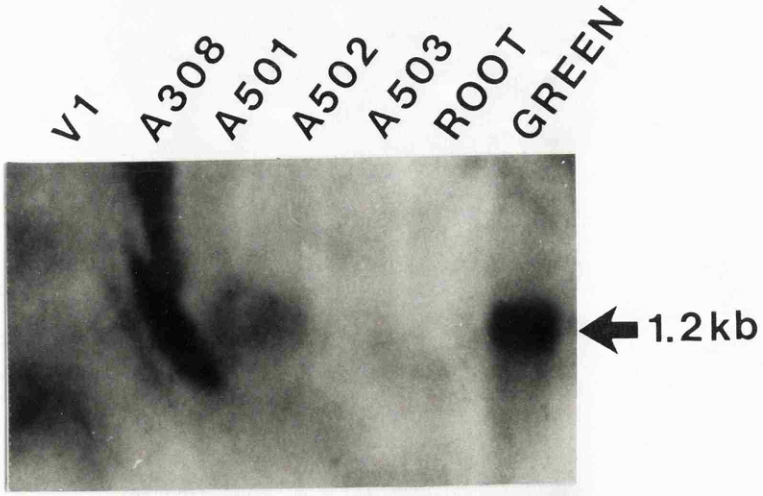
Panel A: C505 and C470 = RNA from pollen derived callus samples; A504, A320, A309 and A307 = RNA from albino plants; GREEN = RNA from the leaves of light grown barley seedlings; ROOT = RNA from the roots of barley seedlings. 7μg of total RNA was loaded per lane.

Panel B: V1 = RNA from pollen plant V1; A308, A501, A502 and A503 = RNA from albino plants; GREEN and ROOT as panel A. 3.5μg of total RNA was loaded per lane.

Hybridisation filters were washed under the conditions of moderate stringency detailed in Section 2.15. Autoradiographs represent 72 hour exposures to X-ray film at -80°C.


C505
C470
A504
A320
A309
A307
ROOT
GREEN
1.2kb

A


V1
A308
A501
A502
A503
ROOT
GREEN
1.2kb

B

negative controls respectively. As for *rbcS*, no *cab* mRNA can be detected in lanes carrying RNA from non-green, anther-derived callus samples. This suggests, as for *rbcS* (Herrera-Estrella *et al.*, 1984), that *cab* genes are not expressed at high levels in undifferentiated, non-photosynthetic tissues such as callus. Lanes carrying RNA from albino plants show reduced levels of *cab* transcript compared to the positive control. In most cases the band corresponding to the *cab* mRNA is barely visible. This is more clearly seen in Figure 7.4A. Although no accurate quantification was possible, it was estimated that *cab* mRNA was present in albino plants at levels <5% of those found in green leaves.

7.4. Levels of *rbcS* and *cab* Transcripts in Green Pollen Plants.

A number of green pollen plants were tested to determine whether they contained similar levels of *rbcS* and *cab* transcripts as compared to seed-derived green tissues. Equal amounts of total RNA extracted from two green pollen plants and control tissues were fractionated by agarose gel electrophoresis, transferred and fixed to nylon filters and hybridised with the cDNA inserts of pKG4626 and pHvLF2. The results of these experiments are shown in Figure 7.5 and indicate that green pollen plants accumulate *rbcS* and *cab* mRNAs at the same levels as found in seed-derived green leaves.

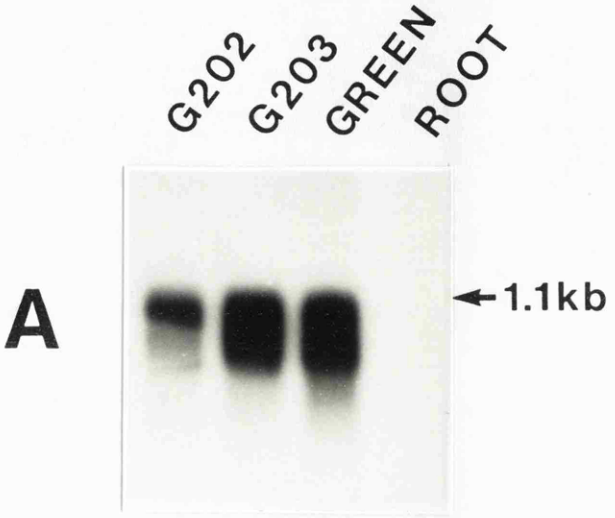
7.5. Structure of Nuclear DNA Sequences Encoding RbcS and Cab Polypeptides in Albino Pollen Plants.

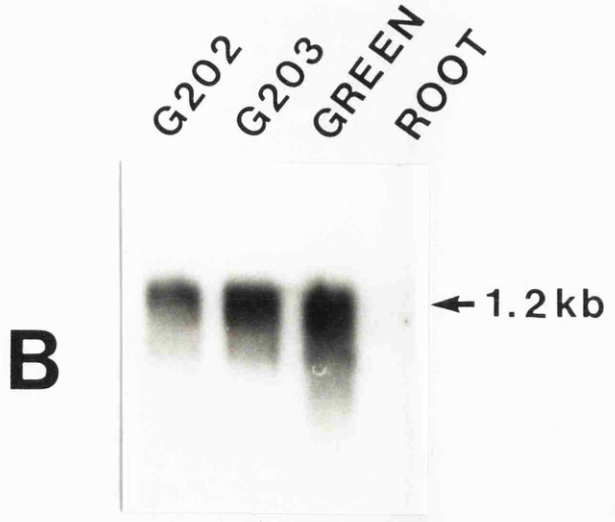
7.5.1. Rationale: Tissue Culture-Induced Variation.

The variation observed in plants regenerated from somatic tissues *in vitro* has been termed somaclonal variation. There is now strong evidence to suggest that somaclonal variation may be due to genetic modifications such as DNA base pair changes, chromosome translocations, inversions and deletions that are induced during the regeneration of plants from somatic explants (for a recent review see Evans and Sharp, 1986). Variation is also observed in plants regenerated from gametophytic tissues such as pollen and has been termed gametoclonal variation (Morrison and Evans, 1988).

Figure 7.5. The Levels of *rbcS* and *cab* mRNAs in Green Pollen Plants.

Autoradiographs of Northern blots hybridised with *rbcS* (Panel A) and *cab* (Panel B) cDNA probes. The source of RNA is indicated above each lane: G202 and G203 = RNA from green pollen plants; GREEN and ROOT = RNA from the leaves and roots of light grown barley seedlings respectively. The positions of the 1.1kb *rbcS* and 1.2kb *cab* mRNAs are indicated. Hybridisation filters were washed under the conditions of moderate stringency detailed in Section 2.15. Autoradiographs represent 16 hour exposures to X-ray film at -80°C.


G202
G203
GREEN
ROOT
A
1.1kb


G202
G203
GREEN
ROOT
B
1.2kb

Variation in agronomic characters such as plant height and tiller number has been observed in pollen derived plants of barley (Powell *et al.*, 1984). Although in this study no specific genetic changes were characterised, there is evidence from pollen plants of *Nicotiana tabacum* (Dhillon *et al.*, 1983) and *N. sylvestris* (De Paepe *et al.*, 1982) that the nuclear DNA of regenerated individuals differs qualitatively and quantitatively from that of the pollen donor plants. Such induced genetic changes might include the deletion or rearrangement of DNA sequences encompassing specific genes and their flanking regulatory regions leading to a reduction or abolition of gene expression. Albino pollen plants used in this study have been shown to accumulate transcripts encoding RbcS and Cab polypeptides at reduced levels compared to those in green tissues (see Sections 7.2 and 7.3). This reduction in transcript levels may be a manifestation of some induced deletion or rearrangement affecting the nuclear DNA sequences encoding RbcS and Cab in albino regenerants. Albino pollen plants accumulating low levels of *rbcS* and *cab* transcripts were therefore tested to determine whether they had undergone changes in the nuclear DNA sequences encompassing these genes.

7.5.2. Integrity of Sequences Encoding RbcS in Albinos.

Total DNA from two albino plants A301 and A309, a green regenerant, G301, an anther-derived callus, C470, and barley seedlings was digested with the restriction enzyme *Eco*R I. Digested DNA was fractionated by agarose gel electrophoresis, transferred and fixed to nylon filters and hybridised with the cDNA insert of plasmid pKG4626. The result of this experiment is shown in Figure 7.6. Also included in this figure for comparison is the result of a Southern hybridisation experiment to determine the arrangement of DNA fragments encompassing *rbcS* coding sequences in seed-derived tissues using the restriction enzymes *Eco*R I, *Bam*H I, *Hin*d III and *Sph* I. In these digests 3-8 bands are seen to hybridise to the *rbcS* cDNA. This is similar to the number of bands seen in the genomic Southern analyses of Barkardottir *et al.* (1987) and suggests that in barley like other plant species, eg. pea (Corruzi *et al.*, 1983), petunia (Dean *et al.*, 1985) and wheat (Broglie *et al.*, 1983), RbcS is encoded by a multi-gene family. From Figure 7.6A, the major bands hybridising to the *rbcS* cDNA in *Eco*R I digests of barley DNA

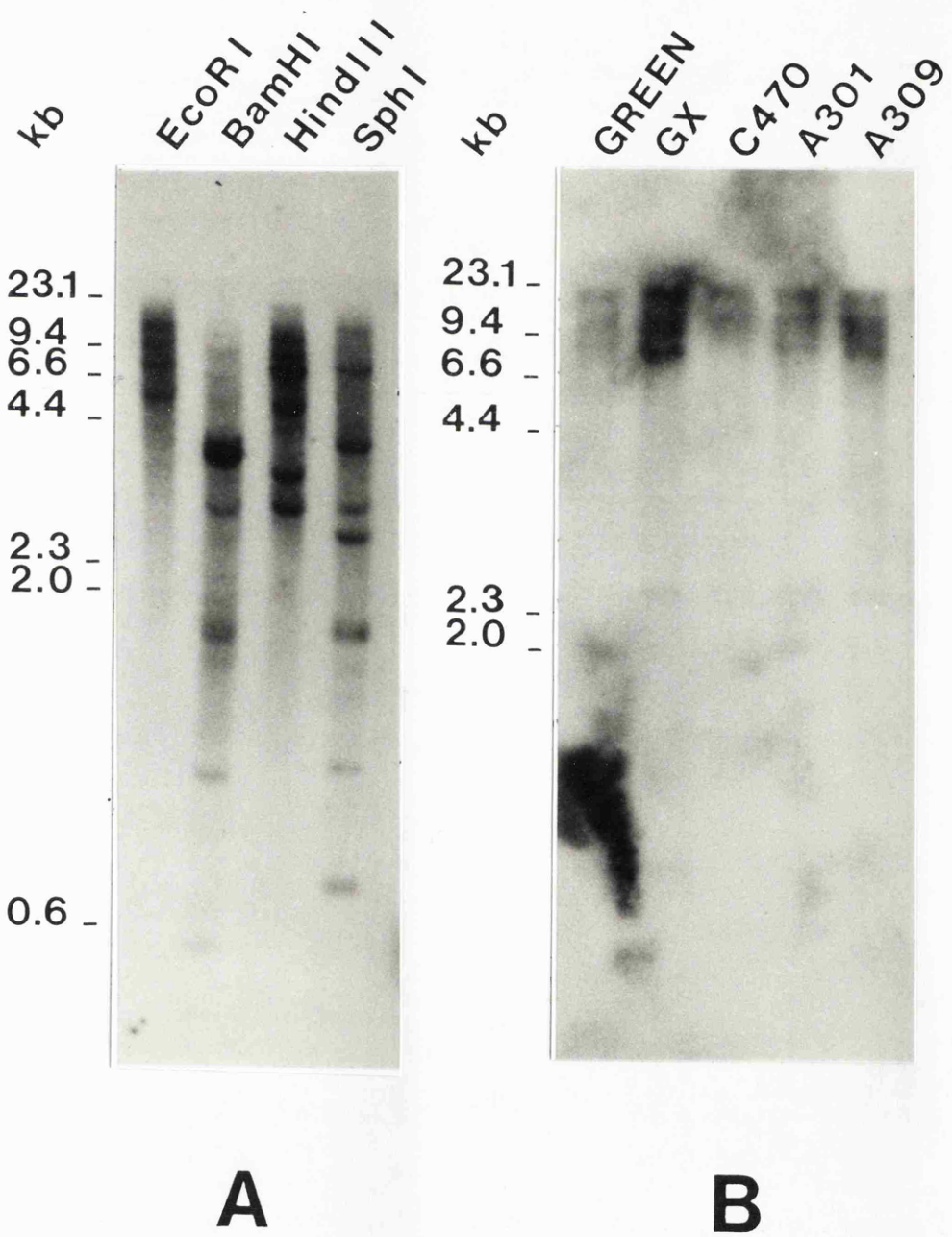
Figure 7.6. Southern Analyses of *rbcS* Genomic Sequences in Albino Plants.

Autoradiographs of Southern blots hybridised with a barley *rbcS* cDNA probe.

Panel A: Lanes carry digests of DNA from the leaves of barley seedlings. The identity of the restriction enzyme used is indicated above each lane. 10μg of digested total DNA was loaded per lane.

Panel B: Lanes carry *EcoR* I digests of DNA from barley seedlings (GREEN), a green pollen plant (GX), a pollen derived callus sample (C470) and two albino pollen plants (A301 and A309). 10μg of digested total DNA was loaded per lane.

The position and size, in kb, of the DNA molecular weight markers is shown on the left of each autoradiograph. Hybridisation filters were washed under the conditions of moderate stringency detailed in Section 2.12. Autoradiographs represent exposures of 72 hours to X-ray film at -80°C.


kb
EcoR I
BamHI
HindIII
Sph I
23.1
9.4
6.6
4.4
2.3
2.0
0.6
kb
GREEN
GX
C470
A301
A309
23.1
9.4
6.6
4.4
2.3
2.0
A
B

are approximately 14.5kb, 10.5kb, 7.8kb and 6.0kb in size. All these fragments, except the 6.0kb band, can also be seen in *Eco*R I digests of the various DNA samples in Figure 7.6B. The reason for the absence of the 6.0kb band in Figure 7.6B is unclear since both hybridisation filters were washed under similar conditions of moderate stringency. In addition to the larger bands, a less intense fragment of 2.3kb can also be seen in all the lanes shown in Figure 7.6B except that carrying DNA from barley seedlings. However, this minor band is visible on inspection of the original autoradiograph. The 2.3kb fragment is not visible in the *Eco*R I digest shown in Figure 7.6A, which may be due to the higher background present in this experiment.

The experiments detailed above indicate that the albino pollen plants A301 and A309 and other pollen derived tissues have DNA fragment patterns that are identical to that found in the seed-derived control when probed with a *rbc*S cDNA. This has also found to be the case for a number of other albino pollen plants including A307, A308 and A320 (data not shown). The genomic DNA analyses described above therefore suggest that loss or rearrangement of RbcS coding sequences is not responsible for the observed low levels of *rbc*S transcripts in these plants. This level of the analysis, however, did not allow a more stringent characterisation of the sequences encoding RbcS in these plants. It is likely that minor but undetectable changes such as small deletions and single base pair mutations could be equally effective in reducing or abolishing expression of these genes in albino pollen plants. It is also possible that structural changes in regulatory sequences important for the expression of *rbc*S genes might have similar effects on the abundance of *rbc*S mRNA in these individuals.

7.5.3. Integrity of Sequences Encoding Cab in Albino Plants.

To determine the arrangement of restriction fragments encompassing *cab* genes in seed-derived tissues aliquots of total barley seedling DNA were digested with the restriction enzymes *Bam*H I, *Bgl* II, *Eco*R I, and *Hind* III, transferred and fixed to nylon filters and hybridised with the cDNA insert of pHvLF2. The result of this

experiment is presented in Figure 7.7. Despite the high background, 7-12 bands can be seen to hybridise with the *cab* probe in each digest on the original autoradiograph. This data is consistent with other Southern blot analyses which indicate that Cab proteins are encoded by a multigene family in barley (Dunsmuir *et al.*, 1983; Barkardottir *et al.*, 1987).

To determine the arrangement of *cab* sequences in albino pollen plants, the filter described in Section 7.5.2 was treated to remove radiolabelled probe (see Section 2.12) and rehybridised with the DNA fragment containing the cDNA insert of pHvLF2. The result of this experiment is shown in Figure 7.8. A single band of 11kb, which also appears to be present in the *EcoR* I digest in Figure 7.7, can be seen in all lanes. To test the reproducibility of this finding a repeat hybridisation was carried out under identical conditions (see Figure 7.8) with the same result (data not shown). The only difference between the hybridisation experiments shown in Figures 7.7 and 7.8 was in the washing strategies used. The filter shown in Figure 7.8 was washed for longer (1 hour rather than 15-30 minutes) in both low stringency (3xSSC/0.1% SDS) and high stringency (0.5xSSC/0.1% SDS) buffers in an attempt to remove high levels of background. Therefore, one explanation for the appearance of single rather than multiple bands in Figure 7.8 could be that the pHvLF2 cDNA probe was behaving in a gene specific manner due to the use of extended high stringency washes.

Gene specific hybridisation probes have been generated from sequences at the 5' and 3' ends of cDNAs encoding members of the maize *cab* multigene family (Sheen and Bogorad, 1986). These experiments relied on the extreme nucleotide sequence divergence seen between the 5' and 3' untranslated regions of different *cab* genes (Dunsmuir *et al.*, 1983; Dunsmuir, 1985). Size comparison of the pHvLF2 cDNA insert (0.6kb) to that of the *cab* transcript detected in Northern blot experiments (1.2kb) suggests that it represents a less than full-length cDNA clone of the *cab* mRNA. Inefficient first and second strand synthesis during cDNA cloning experiments can lead to the preferential retention of sequences corresponding to the 5' and 3' ends of the template mRNA in less than full length cDNA products (Buell *et al.*, 1978; Wickens *et al.*, 1978). It is therefore possible that 5' or 3' untranslated

Figure 7.7. Southern Analysis of *cab* Genomic Sequences in Barley Seedlings.

Autoradiograph of Southern blot hybridised with a barley *rbcS* cDNA probe. Lanes carry 10μg aliquots of total DNA from the leaves of barley seedlings digested with the enzymes indicated at the top of the autoradiograph. The positions of bands in *EcoR* I digested DNA are shown by arrows. The top arrow indicates the position of a band approximately 11.0kb in size that may correspond to that seen in Figure 7.8 (see text). The hybridisation filter was washed under the conditions of stringency detailed in the text. Autoradiograph represents a 72 hour exposure to X-ray film at -80°C.

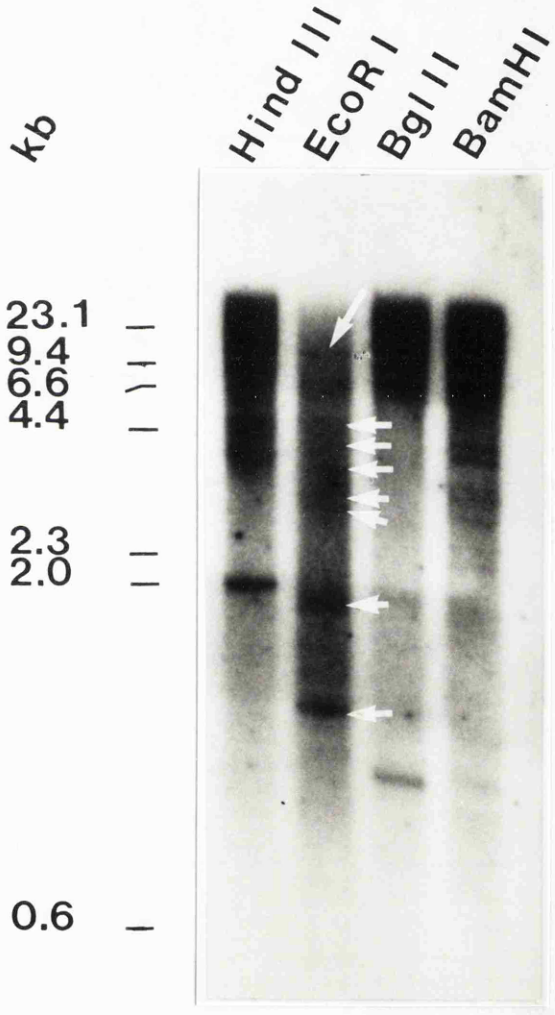
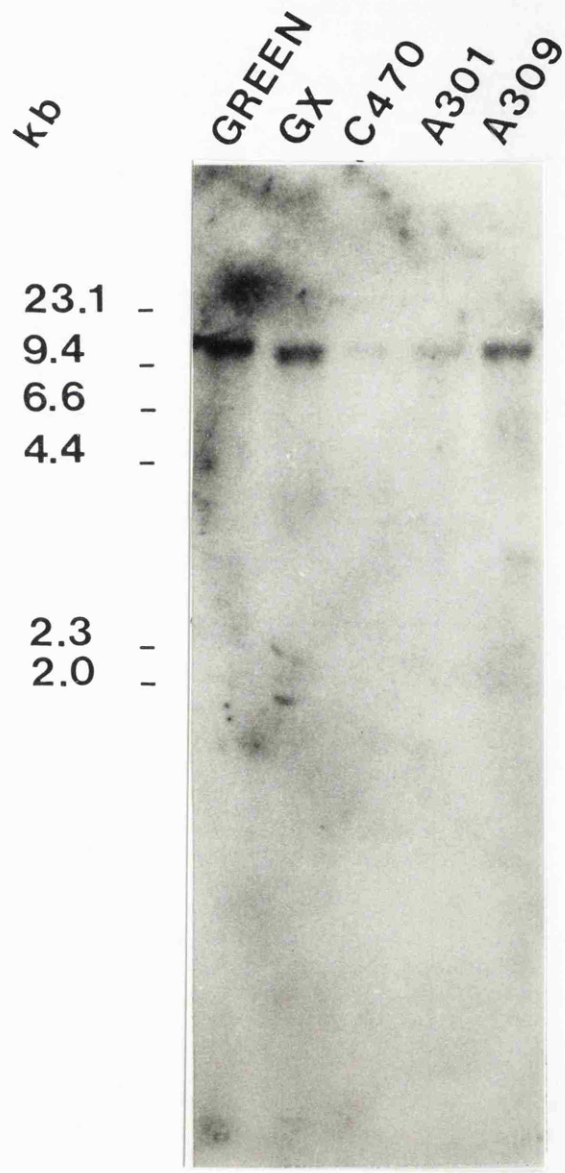

kb
Hind III
EcoR I
Bgl II
BamHI
23.1
9.4
6.6
4.4
2.3
2.0
0.6

Figure 7.8. Southern Analysis of *cab* Genomic Sequences in Albino Pollen Plants.

Autoradiograph of Southern blot hybridised with a barley *cab* cDNA probe. Lanes carry *Eco*R I digested total DNA from the leaves of barley seedlings (GREEN), a green pollen plant (GX), a pollen derived callus sample (C470), and two albino pollen plants (A301 and A309). 10μg of total DNA was loaded per lane. The position and size, in kb, of the DNA molecular weight markers is shown on the left. The hybridisation filter was washed under the conditions of stringency detailed in the text. The autoradiograph represents a 72 hour exposure to X-ray film at -80°C.


kb
GREEN
GX
C470
A301
A309
23.1
9.4
6.6
4.4
2.3
2.0

sequences have been preferentially retained in the pHvLF2 cDNA insert. If these regions are divergent in different members of the barley *cab* multigene family then this could cause the pHvLF2 cDNA to act in a gene specific manner at high washing stringencies. It should also be noted in this context that the restriction fragment used as probe contains approximately 100bp of pBR322 sequence which could further destabilise weak hybrids formed between the probe fragment and barley genomic DNA sequences under conditions of high stringency.

In conclusion, it is possible that the extended washes used to remove excess probe from the filter shown in Figure 7.8 might have been sufficient to destabilise any weak hybrids formed between the pHvLF2 cDNA and the barley DNA digests such that the most stable hybrid remaining was represented by the 11kb *EcoR* I band also seen in Figure 7.7. However, the result shown in Figure 7.8 indicates that the albino pollen plants A301 and A309 and other pollen derived tissues have DNA fragment patterns that are identical to that found in the seed-derived control when probed with the *cab* cDNA.

Presumably if the pHvLF2 cDNA is acting in a gene specific manner in Southern analyses of the barley nuclear genome then this may also be the case in Northern hybridisation experiments and therefore the 1.2kb mRNA detected with the pHvLF2 cDNA may be representative of transcription from a subset of the barley *cab* multigene family.

Taken together the Northern and Southern analyses using the pHvLF2 cDNA as a probe show that *cab* transcripts are present at greatly reduced levels in albino pollen plants but that the nuclear DNA restriction fragments encompassing *cab* sequences in at least two of these plants appear to be intact at this level of analysis. This suggests, as for *rbcS*, that major alteration of sequences encoding the *cab* genes in albino pollen plants are not responsible for the observed low levels of *cab* transcripts.

7.6. Summary.

The data described in the preceding sections indicate that transcripts from two nuclear genes that encode chloroplast localised polypeptides, namely RbcS and Cab, are present at reduced levels in light-grown albino tissues compared to those found in seed-derived green tissues. The levels of *rbcS* and *cab* mRNA seen in individual plants are summarised in Table 7.1, where they are also compared to the levels of the various plastid encoded transcripts described in Chapter 6. Southern analysis of these albino plants indicates that this reduction in transcript levels is probably not due to rearrangements or deletions of the nuclear DNA sequences encompassing the *rbcS* and *cab* genes. As mentioned above, however, the possible presence of minor but undetectable genetic changes with major effects on gene expression cannot be disregarded at this level of analysis.

Table 7.1. The Estimated Abundance of Different Plastid and Nuclear Encoded Transcripts in Albino Pollen Plants.

All values refer to the estimated level of each transcript as compared to that found in the leaves of green seedlings. A dash (–) indicates the absence of detectable transcript. ND = not determined Plants for which ptDNA mapping studies have been carried out are marked with an asterix (*).

	A301 *	A307 *	A308 *	A309 *	A320 *	A501	A502	A503	A504	V1
rbcS:	≤5%	≤5%	≤5%	≤5%	≤5%	20–30%	≤10%	≤5%	≤10%	≤15%
cab:	ND	≤5%	≤5%	≤5%	≤5%	≤5%	≤5%	≤5%	≤5%	≤5%
23S rRNA:	–	–	–	–	≤1%	–	–	–	–	10–50%
16S rRNA:	ND	–	≤1%	–	≤1%	–	–	–	–	10%
rbcL:	≤5%	–	–	–	≤5%	≤10%	≤5%	≤5%	≤5%	≤10%
psbD-*psbC*:	≤5%	≤5%	≤5%	≤5%	≤5%	10–20%	10–20%	≤5%	10–20%	≤5%

CHAPTER 8: DISCUSSION.

8.1. Introduction.

During the regeneration of double haploid plants from individual microspores both green and albino individuals are produced. Green double haploids are of potential use in plant breeding programmes whereas albinos are useless for this purpose. Although the high efficiency of green plant regeneration has reduced the practical importance of albino production during anther culture, relatively little is known about the fundemental basis of chlorophyll-deficiency in these plants. Preliminary studies have shown that albino pollen plants generally do not contain mature chloroplasts and lack major plastid gene products (Clapham, 1973; Sun *et al.*, 1974; Sun *et al.*, 1979). To date the most detailed efforts to characterise albino plants at the molecular level have focussed on the structure of the plastid genome (Day and Ellis, 1984; Day and Ellis, 1985; Ellis and Day, 1986; Day, 1985). The conclusion drawn from these studies was that albino pollen plants contain altered forms of ptDNA which have undergone deletion events removing large tracts of DNA sequence and which exist in both linear and circular conformations. I have termed these variant ptDNAs, ΔptDNAs (see Section 1.4).

The present study was undertaken to confirm and extend these observations and to study what effect the presence of ΔptDNAs might have on the expression of plastid and nuclear genes involved in photosynthesis. Preliminary ultrastructural studies showed that albino barley pollen plants possess plastids which are blocked in plastid development prior to the differentiation of proplastids to chloroplasts. Initial *in vitro* propagation experiments were performed with the aim of improving albino growth so that a good supply of tissue for molecular analysis could be maintained over long periods of time. Although these experiments were unsuccessful, sufficient material was obtained to allow the analysis of a number of albino barley plants which were also found to contain ΔptDNAs. Further studies indicated that transcripts from a number of plastid and nuclear genes encoding chloroplast components fail to accumulate to high levels in these individuals.

8.2. The Plastid Genome of Albino Barley Pollen Plants.

8.2.1. The Structure of the ptDNA Found in Albino Pollen Plants.

Most of the albino plants examined in Chapter 5 (A301, A307, A308 and A309) appear to contain major forms of ptDNA that have undergone structural alteration. This conclusion is based on the observation that the ptDNA restriction fragment patterns in individual albino plants are different to that expected from the wild-type map of the barley plastid genome (see Chapter 5). However, the major form of ptDNA in one albino, A320, appeared to be intact, although some minor non-wild type bands were seen. These results confirm and extend the observations of Day and Ellis (1984, 1985) that albino cereal plants derived from anther culture often contain ΔptDNAs. In some cases these alterations appear to involve the deletion of large tracts of ptDNA. For example, the major form of ΔptDNA in albino plant A308 appears to have undergone a deletion event that has removed 75% of all the sequences found in the intact plastid genome including most of the large single copy region, all of the small single copy region and a portion of the inverted repeat (see Figure 5.3.). The observation that restriction fragments located in the LSC region are most often missing or at reduced abundance compared to other regions of ptDNA suggests that preferential deletion of sequences in this area of the genome have occurred in the plants examined in this study. Similarly, most of the albino barley pollen plants examined by Day and Ellis (1985) also appeared to have undergone deletion of restriction fragments located in the LSC region in addition to sequences in the SSC region and IR. However, the overall pattern of ptDNA deletion seen in plants A301, A307, A309 and A320 differed from that reported by these authors in that restriction fragments located in the SSC and IR regions were retained.

The possibility that the specificity of the events occuring to generate the ΔptDNAs found in albino pollen plants can differ both within and between individual plants is supported by two observations. Firstly, the altered ptDNA restriction patterns found in the five albino barley pollen plants examined in this study were all different from one another (see Figures 5.2 and 5.3) and from those found by Day and Ellis (1985; see above). Secondly, in

agreement with Day and Ellis (1984, 1985) individual albinos appear to contain heterogeneous populations of ΔptDNAs as demonstrated by the hybridisation of multiple restriction fragments of wild type and non-wild-type size to specific ptDNA probes (see Figures 5.2 and 5.3).

Despite the range of variant ptDNAs found in different albino pollen plants there is evidence to suggest that some areas of the plastid genome may be preferentially involved in the generation of these alterations. Many of the albino plants analysed in this study possess altered restriction fragments derived from sequences encompassing the borders between the the LSC and IR regions (P2 and P3; see Table 5.1 and Figures 5.2 and 5.3). Thus, sequences in these areas of the plastid genome may act as preferential targets for the mechanism (or mechanisms) operating to generate some of the ΔptDNAs found in albino pollen plants.

The generation of circular ΔptDNAs may involve recombination between directly repeated sequences (see Section 1.4) and the experiments described in Section 5.7 were carried out as a preliminary to defining the location of such sequences on the barley plastid genome. Weak cross-homology was observed between several distant regions of ptDNA (see Table 5.4) although the identity of the cross hybridising sequences was not determined. Further studies might include more precise mapping of the cross hybridising regions followed by cloning and sequence analysis to determine their structure. If the observed cross homologies are due to short repeated sequences then their involvement in the generation of ΔptDNAs could be investigated by comparing their position on the plastid genome to those of defined deletion endpoints in these molecules.

Although large regions of the plastid genome are deleted in albino barley pollen plants, some sequences appear to be preferentially retained. All the albino pollen plants examined in previous studies have retained DNA sequences located in the large single copy region, close to one inverted repeat and it has been suggested that this region may be involved in plastid genome replication (Day and Ellis, 1984; Day and Ellis, 1985; see Figure 5.1). Similarly, all the albino plants examined in this study have retained this same

region of ptDNA (Figures 5.2 and 5.3). In *Petunia*, sequences in an analogous region of ptDNA, close to one of the inverted repeats, have been shown to promote autonomous replication in yeast and to show significant homology to autonomously replicating sequences in *Chlamydononas* ptDNA (de Haas *et al.*, 1986). Although the presence of similar sequences in the retained region of barley ptDNA has yet to be demonstrated, it is possible that this area of DNA contains a functional origin of replication. It is also possible that this retained region contains *cis* acting sequences other than those involved in the replication of ptDNA. An example of this is the *cis* acting *STB* locus of the yeast 2μ plasmid. This region directs efficient plasmid partitioning at cell division thus leading to stable maintenance of the 2μ plasmid in a population of cells (Kikuchi, 1983). All plastids are thought to arise by division of preexisting organelles (Possingham, 1980). Thus, a similar function to that of *STB* could serve to direct efficient partitioning of ptDNA copies between the 'daughter' plastids generated by such a division during plant development. However, the presence of a functional replication origin would still be required to maintain ptDNA copy number at a constant level in a dividing population of organelles.

Since mixed populations of ptDNAs appear to exist in individual albino plants, it has not been possible to determine if all the molecular species present contain the sequences likely to be involved in ptDNA maintenance (see above). For example, the most abundant restriction fragments seen in albino A309 are located in the small single copy and inverted repeat regions and may therefore represent an independent form of ΔptDNA that lacks the putative replication origin (see Figure 5.3). If some ΔptDNA molecules lack this region then it suggests that other sequences on the intact plastid genome may also have a role in ptDNA maintenance. In support of this, another area of *Petunia* ptDNA located in the small single copy region has also been shown to possess functional and structural homology to yeast autonomously replicating sequences (de Haas *et al.*, 1986) raising the possibility that several different regions may be involved in plastid genome maintenance.

It should be emphasised that the condition of albinism in pollen plants may not necessarily be correlated with the presence of

ΔptDNAs. Restriction analysis has shown that the major form of ptDNA found in one of the albino pollen plants examined in this study (A320, see Chapter 5) appears to be intact, although non-wild type restriction fragments can be detected at low levels (see Figure 5.3). Several of the albino barley pollen plant examined by Day and Ellis (1985) also appear to contain intact plastid genomes. The presence of albinos with apparently intact ptDNA therefore makes the connection between ptDNA modification and albinism in these individuals unclear and raises the possibility that other factors may be involved in generating an albino phenotype in pollen plants. However, the presence of genetic lesions such as single base pair changes and small inversions and deletions, which may not be detectable at the level of analysis used, cannot be excluded.

8.2.2. The General Abundance of ptDNA in Albino Pollen Plants.

The ptDNA of albino barley pollen plants, in addition to having undergone changes in its structure, also appears to be present at reduced abundance. The levels of the major ptDNA restriction fragments found in albinos varies from 6%-20% of those found in green leaves (see Section 5.4). This general reduction in abundance also appears to be independent of any structural changes that have occurred in the plastid genome. For example, plant A320 appears to contain intact ptDNA but still accumulates ptDNA fragments at reduced abundance. One explanation for this could be that the procedure used to prepare total DNA (see Section 2.7) may have led to the preferential recovery of DNA species other than ptDNA in albino plants, eg. nuclear DNA, mtDNA. Alternatively, the ptDNA recovered from the albinos may have undergone specific degradation as an artefact of the extraction procedure. There is no evidence, however, to support the idea that reduced recovery or preferential degradation is responsible for the low levels of ptDNA found in albino tissues. Another untested possibility is that albino plants contain much larger amounts per cell of another DNA species, eg. mtDNA when compared to green seedlings. If this were the case then, using the method described in Chapter 5, the levels of ptDNA in albino plants would be an underestimate. Nevertheless, it is still likely that there may be a genuine reduction in the abundance of ptDNA in albino pollen plants. However, it is not known whether the

low abundance of ptDNA in albino plants is caused by a reduction in the numbers of plastids per cell or by a reduction in the ptDNA copy number per plastid or whether both have a contributory role. Electron microscopy of albino leaf sections has indicated that the proplastid-like organelles seen in the leaves of albino pollen plants do appear to be reduced in number compared to the chloroplasts found in green leaves, although this is only a preliminary observation (see Section 3.2). The levels of ptDNA in individual plastids could be measured *in situ* by the use of a DNA specific fluorochrome such as 4'6-diamidino-2-phenylindole (Coleman, 1979). However, in the absence of data such as this it is possible that some of the plastids found in albino pollen plants may lack ptDNA entirely. Support for this possibility comes from studies on the alga *Acetabularia* in which up to 80% of plastids were found to lack detectable levels of ptDNA (Woodcock and Bogorad, 1970; Coleman, 1979).

In contrast to the observations with albino pollen plants, it has been found that the leaves of some albino mutants of barley and *Pelargonium* contain normal numbers of undifferentiated plastids. Similarly, the abundance of ptDNA in these plants is the same as that found in green, wild type tissues (Hagemann and Borner, 1978; Steele-Scott *et al.*, 1982). This suggests that in some cases albinism is not necessarily correlated with reduced levels of ptDNA. Both the barley and *Pelargonium* albino mutants fail to synthesise plastid encoded proteins due to a lack of 70S ribosomes in these organelles. This has led to the suggestion that the control of plastid numbers and ptDNA abundance is determined by nuclear gene products (Hagemann and Borner, 1978; Steele-Scott *et al.*, 1982). This suggests that the nucleus may be acting to reduce the levels of ptDNA in albino pollen plants perhaps by limiting the abundance or activity of the plastid DNA polymerase or some other function involved in ptDNA replication.

Some albino pollen plants contain specific ptDNA restriction fragments which are present at an unusually high abundance compared to other regions of the plastid genome. Some of the fragments include a region of the plastid genome that may contain a functional origin of replication (see Section 8.2.1). Although further details of the structure of these fragments are unavailable

a number of possible models can be invoked to explain their unusually high abundance.

One explanation for the increased abundance of these fragments is that they are tandemly repeated in a specific form of ΔptDNA. A similar arrangement is found in the mitochondrial genome (mtDNA) of respiratory deficient petite mutants of the yeast *Saccharomyces cerevisiae* and senescing cultures of the slime mould *Podospora anserina*. In both examples the major form of mtDNA has suffered large deletions and the remaining sequences are composed of specific DNA fragments reiterated tandemly many times (de Zamaroczy *et al.*, 1981; Evans, 1983; Jamet-Vierny *et al.*, 1980). Thus, if some ΔptDNAs have such a structure then it may explain the apparent amplification of certain areas of the plastid genome in albino pollen plants. As mentioned above, some of the amplified ptDNA fragments include sequences that are thought to contain an origin of replication. Furthermore, the tandem reiteration of a replication origin might enable some ΔptDNAs to be replicated more efficiently than others thus leading to an increase in their relative copy number. Support for this idea comes from the observation that in crosses between so-called suppressive petite and wild type strains of yeast a large proportion of the resulting progeny possess mtDNA identical to that of the petite parent. This may be explained by the ability of the petite mtDNA to 'out-replicate' the wild type genome due to the presence of tandemly repeated segments of DNA containing a replication origin (de Zamaroczy *et al.*, 1981; Chambers and Gingold 1986).

An alternative explanation could be that the amplified restriction fragments are present as non-repeated segments of a smaller molecule present at high copy number. If these amplified fragments represent distinct forms of ΔptDNA then the basis of their increased copy number is unclear. One possibility is that they are generated continuously and at a high rate from larger ptDNAs, the autonomous replication of these molecules leading to a further increase in copy number. Alternatively, they may be the product of a rare event that generates a specific ΔptDNA followed by its preferential replication and amplification relative to other ΔptDNA molecules. Although the conformation of the amplified fragments or the ΔptDNAs they are part of are unknown it should be noted that

they hybridise to areas of the wild-type plastid genome that are known to give rise to linear molecules in other pollen plants (Day and Ellis, 1985; Ellis and Day, 1986). In some cases these linear molecules have been found to have a inverted repeat structure arranged in a hairpin conformation (see Section 1.4). The production of linear, palindromic DNA molecules such as these has been implicated in the amplification of a chromosomally located, single copy rDNA sequence found in the micronucleus of the protozoan, *Tetrahymena pyriformis* (Yao and Gall, 1977) and therefore raises the possibility that a similar mechanism might be operating to amplify specific ptDNA fragments in albino plants.

8.2.3. The Origin of ΔptDNAs.

The molecular mechanisms by which ΔptDNAs may be generated from the intact plastid genome are discussed in Section 1.4. However, the more general aspects of ΔptDNA formation, both in terms of when and how they arise in albino plants, remain unknown. Thus, alterations to the plastid genome may be induced by the anther culture process itself or could arise as a feature of normal pollen development.

Evidence that *in vitro* culture of plant tissues may induce structural changes in organellar DNAs comes from studies on fertile revertants of maize regenerated via callus from explants derived from male sterile individuals (Gengenbach *et al.*, 1977; Brettell *et al.*, 1980). These plants have been shown to contain mtDNA that has suffered deletions and other modifications such as frameshift mutations (Gengenbach *et al.*, 1981; Kemble *et al.*, 1982; Umbeck and Gengenbach, 1983; Rottmann *et al.*, 1987; Fauron *et al.*, 1987; Wise *et al.*, 1987). Although the cause of these alterations remains unknown it is possible that they could be induced by the *in vitro* culture process used to obtain the revertant plants. Alterations to the mtDNA could be induced by the developmental events occurring during the regeneration process and/or the *in vitro* culture conditions used. Thus, there is the possibility that similar factors may lead to the appearance of ΔptDNAs during the regeneration of albinos from individual microspores.

Alteration of the plastid genome has been proposed as a mechanism

to ensure the maternal inheritance of chloroplasts (Day and Ellis, 1984) and assumes that the formation of ΔptDNAs occurs during normal pollen development. Structural studies have shown that plastids undergo a phase of extreme simplification during microspore development which may be linked to the transition from sporophyte to gametophyte (Heslop-Harrison, 1972). However, these studies did not address the possibilty that structural alterations to the ptDNA may be occurring during this process. Southern analysis using specific ptDNA probes has revealed the presence of unique restriction fragments in DNA extracted from rye pollen. These fragments were absent in DNA prepared from the leaves of green rye plants (Day, 1985) leading to the tentative conclusion that plastid genome alteration may occur during rye pollen development. However, as the author points out, the presence of a specific restriction fragment in a mixed population of pollen grains requires the assumption that an identical modification event occurs during the development of every microspore. Furthermore, it was suggested that the apparently pollen specific restriction fragments may have been derived from sequences in rye mtDNA showing homology with ptDNA. The experiments described above were carried out with DNA extracted from mature pollen grains. Consequently, this would not necessarily reflect the integrity of the plastid genome at earlier stages of microspore development. For the ΔptDNAs seen in albino barley plants to originate from events occurring during normal pollen development would require their presence prior to the first microspore mitosis, the stage at which the induction of morphogenesis and subsequent plant regeneration are most efficient (Huang and Sunderland, 1982). Clearly, an analysis of plastid genome integrity throughout the development of the microspore, during both normal pollen formation and the induced regeneration of whole plants, is required to clarify when ΔptDNA formation occurs.

It should be noted that the procedures used to generate albino pollen plants in this study and those described previously (Day and Ellis, 1984) differ considerably. The albino plants analysed in the studies of Day and Ellis were obtained via a callus stage prior to regeneration whereas in this study plants were regenerated after the direct formation of embryos (Lyne *et al.*, 1984). Deleted plastid genomes are present in albino plants obtained using both

methods, suggesting that the formation of ΔptDNA is independent of the developmental pathway followed during the regeneration of plants from microspores. However, there are differences in the overall pattern of deletions found in these two groups of plants (see Section 8.2.1) which raises the possibility that the pathway used for regeneration may have a bearing on the types of deleted ptDNAs that are formed.

Although speculative models exist for some of the specific molecular events that give rise to ΔptDNAs, the more general ways by which these molecules are derived from intact plastid genomes remains unclear. ΔptDNAs could be generated by a number of initial events followed by replicative amplification of the altered molecules. The heterogeneity of the ptDNA restriction patterns seen in albino pollen plants suggests that a large number of different deletion events can occur. It is therefore possible that the patterns of ΔptDNAs seen in albino pollen plants are representatives of a larger initial population of altered molecules. Some of these molecules could be lost due to some selective process or by a failure to replicate, eg. if a deletion removed sequences such as a replication origin (see Section 8.2.1). The stoichiometry of different ptDNA fragments can also vary in individual albino plants (see Figures 5.2 and 5.3) which suggests that some forms of deleted molecules may be able to replicate more efficiently than others. In extreme cases, this may mean that some ΔptDNAs are present at very low or undetectable levels.

An alternative to the *de novo* generation of ΔptDNAs from intact plastid genomes could be the amplification of preexisting variants. This increase in the copy number of low abundance ΔptDNAs might be accompanied by a reduction in the levels of intact ptDNAs. The existence of low abundance variant organelle genomes has been suggested as the basis of rapid structural changes in the mtDNA of maize (Small *et al.*, 1987). These variants, termed sublimons, have been shown to be present at very low, substoichiometric levels compared to other species of mtDNA. Massive amplification of these molecules, possibly by preferential replication, with a concomitant reduction in the levels of previously abundant forms would result in a reorganisation of mtDNA sequences. If such a mechanism is responsible for the predominance of ΔptDNAs in albino pollen plants

then it implies the presence of these molecules in normal plants. To support this, recent evidence has shown that substoichiometric variant ptDNAs do exist naturally in some plant species. Structural analysis of the rice plastid genome has revealed the presence of two restriction fragments, of different size, containing a gene cluster comprised of *rbcL*, *atpA*, *trnM* and *trnV* (Moon *et al.*, 1987). Whereas one of the restriction fragments contains only genes that are potentially functional, the other includes a truncated version of *rbcL* and a frame shift mutated *atpA*. The restriction fragment carrying the defective genes is thought to be located on a ptDNA that is ten times less abundant than that which carries the intact versions. Furthermore, physical mapping of ptDNA from the brown alga, *Pylaiella littoralis* has revealed the presence of two circular molecules, one 133kb and the other 58kb in size, suggesting that heterogeneity can not only exist in terms of the structure of individual plastid genes, as in rice, but can extend to the structure of the whole genome (Loiseaux-de Goer *et al.*, 1988).

If the presence of ΔptDNAs can be explained in the context of preexisting variants, the range of different ptDNA restriction patterns seen in individual albinos could be reconciled in two ways. Firstly, segregation of specific preexisting ΔptDNAs into separate microspores would lead to the generation of plants containing different variant plastid genomes (Ellis and Day, 1985). Alternatively, if all microspores contain identical populations of such molecules selective amplification of different variants in individual microspores, or the plants regenerated from them, would again lead to the observed range of ptDNA structures seen in albinos.

In contrast to the albino plants studied, the Southern blot analyses described in Section 5.4 indicate that no non-wild type ptDNA restriction fragments are found in digests of total DNA from green pollen plants. However, the possibility that variant ptDNAs are present at undetectable levels in these individuals cannot be ruled out. Support for this comes from the observation that albinos can be seen in up to 1% of test plots of seed derived from individual green double haploid pollen plants (R. Lyne, personal communication). If the albino phenotype in these plants can be

correlated with the presence of ΔptDNAs then it implies that such variant molecules could be segregating out from a larger population, mainly composed of intact genomes, present in the green double haploid parent.

8.3. The Abundance of Plastid Transcripts in Albino Pollen Plants.

8.3.1. Summary.

In Chapter 6 it was shown that albino pollen plants and the pale yellow plant V1 fail to accumulate transcripts from the *rrn* operon and the genes *rbcL*, *psbD* and *psbC* or do so at reduced levels compared to green tissues. The restriction fragments encompassing these genes are often intact but are present at a reduced level compared to that found in green tissues. However, the apparent reduction in plastid transcript abundance is often greater in proportion than the reduction in the levels of the ptDNA fragments encompassing the corresponding genes. This could be explained by a reduction in the rate of transcription or decreased transcript stability. Recently, development of an *in vitro* plastid run-on transcription system in spinach has allowed the measurement of the relative transcriptional activities of individual plastid genes (Deng *et al.*, 1987). The availability of a similar system for barley might distinguish between the two possibilities mentioned above.

8.3.2. The Effect of ptDNA Deletions on Plastid Gene Expression.

Although all the albino pollen plants examined have very different ptDNA restriction patterns (see Chapter 5) the levels of plastid encoded transcripts are reduced to approximately the same extent in each (see Table 7.1). Thus, in the individuals tested, no conclusions can be drawn as to the differential effects, if any, one pattern of ptDNA alterations may have when compared with another. In fact the presence of one albino pollen plant, A320, whose ptDNA appears to be intact at the level of analysis used, suggests that deletion of a plastid gene or alteration of the sequences surrounding it cannot necessarily be correlated with its

reduced transcription. Again, this is evident in the case of the pale yellow plant V1, which although containing wild type ptDNA fragments at normal abundance failed to accumulate high levels of plastid transcripts. However, as mentioned in Chapter 5, the presence of minor genetic lesions, such as single base pair changes or small deletions and insertions, cannot be excluded at the level of analysis used in these studies.

Changes in the structure of the plastid genome may have an effect on its transcription at a number of levels. The deletion of a plastid gene will abolish its transcription directly but it is also possible that ptDNA alterations may have an indirect effect on plastid gene expression. Although the plastid DNA dependent RNA polymerase in thought to be encoded by the nucleus (Lerbs *et al.*, 1985), open reading frames bearing homology with *E. coli* RNA polymerase α, β and β' subunits have been located on ptDNA, eg. see Shinozaki *et al.*, 1986, raising the possibility that the plastid itself may be an alternative site of synthesis for this enzyme. Although the precise location of these genes on barley ptDNA is unknown, in tobacco they all lie within the large single copy region, an area of the plastid genome which includes sequences that are often underrepresented or deleted in albino pollen plants (see Figures 5.2 and 5.3). If the presence and expression of these genes is important for complete assembly or efficient activity of the plastid RNA polymerase, their deletion in albino plants could have a corresponding effect on the transcription of the remaining ptDNA. In cases where large ptDNA deletions have not occured, ie. A320 and plant V1 smaller genetic alterations could be equally effective in inactivating the plastid RNA polymerase.

The involvement of other factors which interact with the plastid RNA polymerase during transcription of plastid genes has been established by the characterisation of a sigma factor-like activity from mustard chloroplasts (Bulow and Link, 1988). A protein fraction bearing this activity was shown to confer ptDNA DNA binding and transcriptional specificity to *E. coli* core RNA polymerase. If this protein is plastid encoded then the deletion or alteration of the corresponding gene in albino pollen plants could have an effect on the transcription of the remaining sequences. In addition, plastid encoded functions could have a role in regulating

transcript processing and stability and their loss due to ptDNA alteration could have a corresponding effect on the abundance of plastid RNAs. In this respect, several unidentified open reading frames have been located on the plastid genomes of tobacco and *M. polymorpha* (Shinozaki *et al.*, 1986; Ohyama *et al.*, 1986) but whether their polypeptide products have a function in plastid gene expression remains unknown.

Albino wheat and barley pollen plants have been shown to contain ΔptDNA molecules with linear, open circular and supercoiled circular structures (Day, 1985) and it is likely that the ptDNA found in the albino plants analysed in this study has a similar range of conformations. *In vitro* experiments have demonstrated that the conformational state of a ptDNA template is an important determinant of its transcriptional activity (Stirdivant *et al.*, 1985; Lam and Chua, 1987). Furthermore, it has been shown that relaxed circular and linear ptDNA templates are not substrates for chloroplast transcription extracts (Lam and Chua, 1987) while an increase in superhelical density of circular template molecules leads to maximal transcriptional activity (Stirdivant *et al.*, 1985). If these observations reflect events *in vivo* then it suggests that the linear and open circular ptDNA molecules seen in albino pollen plants may have minimal transcriptional activity. Deleted circular and small linear ΔptDNA molecules are specifically associated with albino pollen plants and from the preceding argument may represent an example of how conformational alterations to the plastid genome could lead to reduced abundance of plastid transcripts in these individuals.

8.3.3. Possible Nuclear Control of Plastid Transcript Abundance.

As mentioned previously, albino pollen plants with apparently intact ptDNA also fail to accumulate plastid transcripts. Unless this is due to some undetectable lesion in the plastid genome, it implies that some extraplastidic factor may be involved in suppressing the abundance of plastid RNA levels in albinos. The concerted interaction of both plastid and nucleo-cytoplasmic genetic systems during chloroplast differentiation raises the possibility that this factor could be nuclear in origin. Two

possibilities are that plastid transcript abundance is reduced due to a specific defect in the nuclear genome or a developmental suppression of chloroplast function in albino pollen plants

The presence of variation within populations of anther culture regenerants has been well documented and in some cases has been shown to involve specific qualitative changes in the nuclear DNA (Dunwell, 1984; Morrison and Evans, 1988; De Paepe *et al.*, 1982). Thus, specific lesions in the nuclear genome could lead to the loss of a factor necessary for the accumulation of plastid RNA in albino plants. Alternatively, albino plants may have undergone a developmental suppression of chloroplast maturation. Anther culture relies on the diversion of a gametophytic mode of development to that of a sporophyte. Since microspores contain only proplastids (Mlodzianowski and Idzikowska, 1980) it is possible that a developmental programme is operating to block chloroplast maturation in these cells and that this effect persists when a sporophytic mode of development is induced during the anther culture process, thus resulting in an albino phenotype. Furthermore, this block to chloroplast maturation may include a specific reduction in the abundance of chloroplast RNAs. Green pollen plants would represent individuals that had escaped from this suppression of plastid differentiation.

The lack of some factor, whether due to nuclear mutation or developmental suppression, could act to decrease the abundance of plastid RNAs in albino plants at a number of levels. The reduced availability or activity of the nuclear-encoded plastid RNA polymerase would reduce ptDNA transcription. Alternatively, an effect may be exerted at the level of RNA processing and stability, two important determinants of plastid transcript abundance (Stern and Gruissem, 1987). The study of a number of mutants has enabled loci to be identified which may have a role in controlling plastid RNA abundance. The *hcf*#38 mutant of maize has been shown to have undergone quantitative and qualitative changes in the pattern of plastid transcription, including a reduction in the abundance of specific plastid RNAs and the accumulation of splicing intermediates (Barkan *et al.*, 1986; Taylor *et al.*, 1987). This locus may therefore specify a nuclear function important in plastid transcription and processing. Although the availability of

functions involved in synthesis, processing or stabilisation may profoundly affect the abundance of plastid RNAs in albino plants directly, it is equally possible that other factors may be involved.

As their name implies, albino pollen plants fail to accumulate chloroplast pigments or do so at levels undetectable by eye. One individual, plant V1, showed delayed accumulation of pigments resulting in it's original selection as an albino, although the pale yellow colour finally achieved indicates that they were not present at high abundance. In this context, a number of observations have demonstrated that a specific block in pigment accumulation in pollen plants may have profound effects on chloroplast development and in some cases plastid transcript abundance.

Albino nuclear mutants of maize specifically blocked in carotenoid biosynthesis, when exposed to high light intensities, undergo photooxidation of chlorophylls resulting in extensive damage to the plastid compartment and a consequent block to chloroplast maturation (Bachmann *et al.*, 1967). Under these conditions, carotenoid deficient maize plants fail to accumulate plastid RNAs (Mayfield *et al.*, 1986). Similar observations were made with a carotenoid deficient barley mutant (Batschauer *et al.*, 1986). Treatment of normal barley seedlings with the herbicide Norfluorozon, which also blocks carotenoid biosynthesis, leads to a similar reduction in the accumulation of plastid transcripts (Batschauer *et al.*, 1986). A number of barley mutants fail to accumulate chlorophylls (Holm, 1954; Von Wettstein *et al.*, 1971) and further analyses have demonstrated that some of the mutant loci specify functions involved in chlorophyll biosynthesis (Von Wettstein *et al.*, 1974). The absence of chlorophyll pigments in these plants leads to an arrest of chloroplast differentiation characterised by a lack of thylakoid membrane development (Von Wettstein *et al.*, 1971). However, to my knowledge no investigation has been made as to what effect a specific block in chlorophyll biosynthesis may have on ptDNA transcript abundance.

8.3.4. Possible Consequences of Reduced Plastid Transcript Abundance.

The specific RNAs examined in this study encode, or are, components of multisubunit complexes essential for chloroplast function, hence their reduced accumulation or absence might be expected to have profound effects on the organelle's development. As shown in Chapter 6, the levels of the plastid 23S and 16S rRNAs are greatly reduced in all the albino plants studied and to a lesser extent in plant V1. Since these RNA species are major components of the plastid ribosome, any shortfall might be expected to have an effect on the assembly of this structure in albino plants. Reduced levels of plastid ribosomes will in turn have an effect on translation within the organelle and a reduction in the accumulation of plastid encoded proteins. The recessive nuclear allele *iojap* of *Zea mays* appears to specify a programmed reduction in the abundance of plastid ribosomes with a concomitant failure of organellar protein synthesis in affected plants (Walbot and Coe, 1979). The action of *iojap* in maize plants leads to an albino phenotype and a block to the differentiation of proplastid to chloroplast at an early stage thus illustrating the importance of ribosome function during this process.

In addition to reduced levels of plastid encoded rRNAs, albino plants and plant V1 fail to accumulate mRNAs encoding components of at least two major chloroplast protein complexes. The low levels of *rbc*L mRNA in albino plants may suggest a similar reduction in the levels of the the RbcL polypeptide in these individuals. Although not established here, quantification of RbcL abundance could be achieved by detection of the polypeptide with specific antibodies (Western blotting; Burnette, 1981). The absence of RbcL in the plastids of albino plants would have an obvious impact on the assembly of the Rubisco holoenzyme even if RbcS continued to be synthesised and imported (for further discussion see Section 8.3). Since Rubisco catalyses the principal step in the fixation of atmospheric CO_2, non-assembly of this enzyme in albino plants would lead to a severe reduction in the synthesis of carbohydrate compounds via the photosynthetic carbon reduction cycle (Gibbs, 1971).

In addition to *rbcL* mRNA, most albino plants also fail to accumulate high levels of transcripts from the genes *psbC* and *psbD* which encode the P6 and D2 polypeptides, respectively, of PSII, a thylakoid membrane complex involved in light-harvesting, water-splitting reactions and photosynthetic electron transport (Rochaix and Erickson, 1988). As for RbcL, the quantities of P6 and D2 protein levels in albino plants has not been established. However, it is likely that the reduced abundance of the *psbD* and *psbC* transcripts also results in a parallel reduction in the levels of the corresponding proteins. Furthermore, reduced levels of individual PSII components has been shown to have major effects on the stability of PSII as a whole. For instance, mutants of *Chlamydomonas* which lack the *psbD* encoded D2 protein fail to assemble stable PSII complexes in the thylakoid membrane, and in addition specifically fail to accumulate another ptDNA encoded protein component of PSII, D1 (Erickson *et al.*, 1986). This evidence suggests that if albinos lack specific components of PSII, then they will also fail to assemble an active PSII complex and consequently suffer impaired photosynthetic function.

Although transcript levels were estimated for only four plastid genes, it is possible that the accumulation of RNAs from other regions of ptDNA are similarly affected in albino plants. This could lead to the perturbation of a wide range of organelle functions resulting in the block to chloroplast maturation seen in albino pollen plants. An example in this context comes from the apparent involvement of a plastid encoded tRNA in the synthesis of δ-aminolevulinate (ALA) from the amino acid, glutamate. ALA is one of the initial compounds formed during chlorophyll biosynthesis in the chloroplast stroma (Kannagara *et al.*, 1984). The conversion of glutamate to ALA occurs via a glutamyl-RNA intermediate, the RNA component originating from a region of ptDNA in the vicinity of the *psbC-psbD* gene cluster (Kannagara *et al.*, 1984; Schon *et al.*, 1986). In albino plants transcripts from this region of the plastid genome are present at much lower levels than those found in green tissues and may therefore result in a shortfall of the RNA component involved in ALA synthesis. Hence, reduced levels of this RNA may have a corresponding effect on the rate of ALA and ultimately chlorophyll synthesis. Lack of chlorophyll may then lead to an arrest of chloroplast development similar to that observed in

the pigment deficient mutants of barley studied by Von Wettstein *et al.* (1971).

In addition to showing that albino barley plants fail to accumulate plastid RNAs to high levels, further studies were carried out to determine what effect the absence of chloroplast development and the presence of ΔptDNAs might have on the the expression of two nuclear gene families encoding chloroplast polypeptides (see Chapter 7).

8.4. The Expression of Nuclear Genes Encoding Chloroplast Polypeptides in Albino Pollen Plants.

8.4.1. Summary.

Data was presented in Chapter 7 which indicates that transcripts from two nuclear gene families, namely those encoding the small subunit of Rubisco (RbcS) and chlorophyll a/b binding proteins (Cab), both chloroplast localised proteins, are present at reduced levels in light grown albino leaves and plant V1, as compared to those in green tissues. Southern analyses of these albino plants indicate that this reduction in mRNA levels is probably not due to major rearrangements or deletions of the nuclear DNA sequences encompassing the *rbcS* and *cab* genes. However, the possible presence of minor but undetectable changes with major effects on gene expression cannot be disregarded at this level of analysis. Interestingly, the failure of *rbcS* and *cab* mRNAs to accumulate in albino plants appears to be independent of the overall structure of the plastid genome. For all the albinos for which ptDNA mapping has been carried out the levels of both transcripts are uniformly low at ≤5% of that found in green leaves (see Table 7.1). This is the case even in albino plant A320 which appears to possess an intact plastid genome.

It is to be expected that the reduced abundance of *rbcS* and *cab* transcripts would be accompanied by a decrease in the levels of the corresponding proteins. This could be confirmed by the preparation of total protein from albino plants followed by Western blotting and immunodetection of RbcS and Cab proteins by the use of specific

antibodies (Burnette, 1981). The low levels of *rbcS* and *cab* transcripts in albino plants could be due to a reduction in mRNA stability or the rate of transcription from the corresponding genes. A discrimination between these two possibilities could be made by performing nuclear run-off transcription assays which measure the rate of transcription from a given gene sequence in isolated, radioactively pulse-labelled nuclei (Greenberg, 1987).

8.4.2. Possible Causes of Reduced Nuclear Transcript Abundance in Albino Pollen Plants.

There are a number of possible explanations for the reduced accumulation of *rbcS* and *cab* mRNAs in albino plants based on a knowledge of the regulatory pathways that modulate their expression in normal plants (Tobin and Silverthorne, 1985; Kuhlemeier *et al.*, 1987).

Although structural changes to the genes encoding RbcS and Cab may not be responsible for the failure of the corresponding mRNAs to accumulate, lesions elsewhere in the barley genome may be exerting an effect on the expression of these sequences. For instance, a nuclear mutation associated with albino pollen plants may affect a function involved in some specific regulatory aspect of *rbcS* or *cab* gene expression. This might include factors involved in the perception of light stimuli or transduction of this signal within the cell (Kuhlemeier *et al.*, 1987). Alternatively, a specific genetic lesion may affect the function or availabilty of a *trans*-acting factor that interacts with regulatory sequences flanking the *rbcS* and *cab* genes (Green *et al.*, 1987). Clearly, it would be difficult to pinpoint the loss of any single function that may be responsible for the observed failure of *rbcS* and *cab* mRNAs to accumulate in albino pollen plants. Further Southern analysis of albino pollen plants, perhaps by the use of restriction fragment length polymorphism (RFLP) probes, could reveal whether structural changes have occurred in the nuclear DNA of albinos and, if present, whether these alterations were identical or different from plant to plant.

A large body of recent evidence has indicated that the presence of

a functional chloroplast compartment is required for the transcriptional activation of *cab* gene expression in a number of plant species, including barley (Batschauer *et al.*, 1986; see also Section 1.3.9). These studies suggest that an as yet unidentified chloroplast derived factor is involved in the positive regulation of certain nuclear genes, including those in the *cab* multigene family. The absence of mature chloroplasts in light-grown albino pollen plants could therefore explain the low levels of *cab* mRNA in these individuals.

Although barley seedlings which have undergone a block in chloroplast maturation as a result of the photooxidative destruction of chlorophyll accumulate low levels of *cab* mRNA, the levels of *rbcS* transcript remain largely unaffected (Batshauer *et al.*, 1986), suggesting that, unlike *cab*, the presence of a functional chloroplast is not required for the transcriptional activation of *rbcS* genes. In contrast, albino pollen plants containing developmentally arrested plastids also fail to accumulate *rbcS* mRNA. However, it should be emphasised in this context that the experimental conditions used by Batschauer *et al.* (1986) are very different from those used in this study. These workers determined the levels of *rbcS* mRNA in the leaves of week-old barley seedlings. In contrast, the plants used in this study were often maintained *in vitro* for several months prior to analysis. It is therefore difficult to make a direct comparison of data obtained from these two sets of experiments. For instance, the presence of a functional chloroplast may be required for the long term maintenance of high levels of *rbcS* mRNA. This could be due to a requirement for continued stimulation of transcription or maintenance of transcript stability by some chloroplast derived factor. However, it is still possible that the reduced levels of *rbcS* mRNA observed in albino pollen plants may be independent of the absence of mature chloroplasts in these individuals and is instead due to some other factor.

Another possibility emerges from studies on the regulation of Rubisco accumulation in the unicellular alga *Chlorogonium* which indicate that the addition of an external organic carbon source can inhibit the appearance of Rubisco subunit mRNAs (Boege *et al.*, 1981; Westhoff and Zetsche, 1981; Steinbiss and Zetsche, 1986). The

non-photosynthetic albino plants used in this study were maintained for extended periods on media containing sucrose as an external source of carbon. It is therefore possible that these growth conditions have an inhibitory effect on the accumulation of *rbcS* and *rbcL* mRNAs, although the high levels of these transcripts in green pollen plants grown under indentical conditions would argue against this.

It was suggested in Section 8.3.3 that albinism in pollen derived plants could be due to a developmental programme that blocks chloroplast maturation in the male gametophyte and that continues to operate in a proportion of the plants regenerated from microspores. Hence, it is possible that this could extend to a specific reduction in the expression of nuclear genes encoding chloroplast associated proteins such as *rbcS* and *cab*.

The mechanism that is responsible for the reduced abundance of *rbcS* mRNA in albino plants remains unknown and could be operating at a number of levels. However, the variable levels of *rbcS* mRNA seen in some albinos, ie. compare A309 and A320 with A501, A502 and A504; see Table 7.1, demonstrates that the accumulation of this transcript may be regulated to different extents from plant to plant.

8.4.3. Comparison of *rbcS* and *rbcL* mRNA Levels in Albino Plants.

Since both plastid and nuclear genomes cooperate in the synthesis of the Rubisco holoenzyme, the levels of *rbcS* and *rbcL* mRNAs were compared to determine if any coordination in their accumulation was apparent in albino pollen plants (see Table 7.1). Albino pollen plants accumulate low levels of *rbcL* mRNA and a number of possible explanations could account for this (see Section 8.3). Interestingly, comparison of Figures 6.7 and 7.2 show that the albino plant A501 and plant V1 which both accumulate the highest levels of *rbcS* mRNA also accumulate the most *rbcL* mRNA. In contrast, albino plants A502 and A504 show relatively high levels of *rbcS* transcripts compared to other albinos, but this appears not to be reflected by increased levels of *rbcL* mRNA in the same comparison (see Table 7.1). From these observations it is therefore

unclear whether, in albinos, there is any degree of coordination between the appearance of *rbcS* and *rbcL* mRNAs and by extension it would be difficult to say if the failure of one subunit mRNA to accumulate was having an effect on the appearance of the other. To investigate this it would be necessary to determine if the accumulation of either the *rbcS* or *rbcL* subunit mRNA were specifically affected in albino plants. Unfortunately, no distinction can be made between this possibility and an independent block in the accumulation of both transcripts.

Several lines of evidence suggest that the appearance of the RbcS and RbcL polypeptides is tightly coordinated at the translational level during chloroplast development (Dean and Leech, 1982; Steinbiss and Zetsche, 1986; Oelmuller *et al.*, 1986b). However, this coordination does not extend to the accumulation of the corresponding mRNAs which often appear with different kinetics (Steinbiss and Zetsche, 1986; Oelmuller *et al.*, 1986b). Furthermore, transgenic tobacco plants carrying *rbcS* antisense genes, which specifically reduce the levels of *rbcS* mRNA and protein, have been shown to accumulate *rbcL* transcripts to normal levels (Rodermel *et al.*, 1988). Thus, the observed low levels of *rbcL* mRNAs may be independent of the failure of the *rbcS* to accumulate in albino pollen plants, and possibly *vice versa*.

8.5. The Significance of Pollen Plant V1.

Pollen plant V1 was originally selected as an albino but after 3-4 weeks in culture developed a pale yellow colour. However, because the small size of this plant limited the quantity of tissue available for analysis no identification was made of the pigments present. Further analysis has shown that V1 possesses an apparently intact plastid genome which is present at normal abundance. Plant V1 accumulates several plastid encoded RNAs at reduced levels compared to those found in green tissues. The levels of *RbcL* and *psbD-psbC* transcripts in V1 are similar to those found in albino plants, although the 23S and 16S rRNAs accumulate to a slightly greater extent (see Table 7.1). In addition, transcripts from the nuclear gene families, *rbcS* and *cab* similarly accumulate at very low levels in plant V1.

The presence of chlorophyll deficient, pale yellow plants arising from barley anther culture has previously been reported by Clapham (1973). These so called *xantha* plants were shown to contain both chloroplasts of almost normal appearance and those at the proplastid stage. Unfortunately, the absence of ultrastructural data for V1 makes it difficult to assess the developmental state of plastids in this plant. However, it is possible that V1 represents a pollen plant which has been able to undergo partial plastid differentiation to the extent that it has accumulated low levels of pigment, has ptDNA of normal structure and abundance and has accumulated low levels of plastid rRNAs.

8.6. Summary, Conclusions and Further Work.

In agreement with previous workers, the albino barley pollen plants examined in this study appear to possess plastids which are blocked at a stage of development prior to the differentiation of proplastids to chloroplasts (Chapter 3). Attempts to formulate *in vitro* growth media with the objective of improving albino growth in terms of the yield of tissue per plant were unsuccessful. Thus, the quantities of albino tissue available allowed only certain aspects of the albino phenotype to be examined at the molecular level. In agreement with the general findings of Day (1985), the majority of these plants contain variant forms of the plastid genome that have suffered the loss of large tracts of DNA (Chapter 5). However, in one case, the presence of an apparently intact ptDNA indicates that albinism cannot necessarily be correlated with major structural alterations to the plastid genome. In all cases the level of ptDNA was estimated to be 6-20% of that found in green tissues. It was found that the levels of plastid encoded RNAs (16S and 23S rRNAs, *rbcL*, *psbC* and *psbD* mRNAs) are greatly reduced in albino pollen plants and there appears to be no simple correlation between the observed pattern of plastid RNA abundance and the overall structure of the plastid genome (Chapter 6). I have also shown that the levels of transcripts from two nuclear gene families encoding chloroplast polypeptides, *rbcS* and *cab*, also fail to accumulate to high levels in albino pollen plants. In addition, the level of transcripts that do accumulate appears to be independent of the

extent of the deletions seen in the plastid genome. Albinism in barley pollen plants therefore appears to involve a block to chloroplast differentiation accompanied by a general reduction in the accumulation of chloroplast associated transcripts. Green pollen plants were found, at the molecular level, to be indistinguishable from green seedlings.

The primary cause of albinism in pollen derived plants remains unclear. There is evidence to suggest that loss of photosynthetic function, presumably due to the lack of mature chloroplasts, can be induced when sucrose is used as a external carbon source *in vitro* (Dalton and Street, 1977). Since sucrose is the carbon source added to the media used in this study the lack of chloroplast development in albinos could result directly from the *in vitro* conditions used to generate plants from microspores. However, the prolonged maintenance of green plants under identical conditions would argue against this. Alternatively, it is possible that albinism may be due to anther culture induced lesions in the plastid or nuclear genomes of some regenerated plants, or a developmental block to chloroplast function specific to a proportion of plants regenerated from male gametophytes. The presence of apparently intact plastid genomes in some albino pollen plants suggests that structural alterations to ptDNA may not be the primary cause of albinism. Hence, it is likely that a number of diverse factors, including the presence of ΔptDNAs, could be acting together to generate the albino phenotype.

Although major alterations to the plastid genome may not be soley responsible for albinism in pollen plants, the intact ptDNA found in some individuals would require careful analysis to exclude the presence of small genetic changes which could be equally effective in blocking chloroplast maturation. The detection of single base substitutions, for instance, would require the sequencing of the entire ptDNA from these plants. Whether ptDNA deletions are induced by anther culture or whether they are part of normal pollen development, as suggested by Day and Ellis (1984), remains to be established. One way to resolve this would be to monitor ptDNA structure throughout pollen development. Barley anthers containing pollen at various developmental stages could be excised and DNA extracted prior to Southern analysis using specific ptDNA probes.

However, if microspores contain ΔptDNA, then the evidence from albino pollen plants suggests that the structure of these molecules may show heterogeneity both within and between individuals. Therefore, the ptDNA hybridisation patterns seen in these experiments may be too complex to allow specific ptDNA alterations to characterised. Some areas of the plastid genome, eg. sequences located in the large single copy region, appear to be preferentially lost during the deletion process. This may offer an alternative way to detect the presence of ΔptDNA in developing pollen. Equal amounts of total DNA from anthers at different stages of development could be hybridised with two radiolabelled probes: one representing sequences that are retained in albino plants and the other sequences that are commonly lost. The ratio of hybridisation between the two probes may then be calculated. Decreasing levels of hybridisation to the probe representing commonly lost sequences might indicate that they had been deleted in a proportion of the ptDNAs present in the original DNA sample.

Structural alterations to the nuclear genome may also be responsible for some aspects of albinism and there is evidence that qualitative changes in the nuclear DNA can occur during anther culture (see Chapter 7). Lesions in the nuclear genome may affect functions important for chloroplast development and the expression of plastid and nuclear genes encoding chloroplast components. Further efforts to characterise such changes might include the use of probes representing dispersed repeated sequences (Ananiev *et al.*, 1986) to identify structural variation in the nuclear genome of individual pollen plants by restriction analysis. If these repeated sequences had defined positions on the barley genome they might help to identify nuclear determinants important for chloroplast development.

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ABSTRACT.

MOLECULAR ASPECTS OF ALBINISM IN ANTHER CULTURE DERIVED BARLEY PLANTS.
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Haploid cereal plants can be regenerated from single pollen grains via the process of anther culture. Anther culture of cereals is of potential use in crop improvement programmes. One problem associated with anther culture of cereal plants is a high incidence of albino individuals which cannot be used in crop breeding schemes. Albinos derived from barley anther culture (albino pollen plants) are severely pigment deficient and from electron microscopy studies appear to possess plastids that are developmentally arrested at a stage prior to the differentiation of proplastids to mature chloroplasts. The aim of the project has been to investigate some of the molecular aspects of albinism in these individuals.

In vitro propagation experiments were carried out to find the conditions necessary to improve the growth and maintenance of albino pollen plants with the objective of producing a continuous supply of albino tissue for molecular analysis. However, use of various media containing organic and inorganic supplements including a number of plant growth regulators failed to improve the growth of albino plants.

Southern analysis revealed that four out of the five albino plants studied exhibit ptDNA restriction patterns that are different to that expected from the wild type map of the barley plastid genome due to the alteration or deletion of specific ptDNA fragments. One plant appears to contain a major form of ptDNA that has undergone a deletion event removing 75% of all sequences. This confirms that the albino pollen plants examined in this study contain forms of the plastid genome that have undergone structural alteration. I have termed these variant plastid genomes ΔptDNAs. Most of the albino plants studied appear to contain heterogenous populations of ΔptDNAs. One albino barley pollen plant appears to possess an intact plastid genome. For all the albinos studied the overall levels of ptDNA are reduced 5-15 fold compared to the levels found in normal green tissues.

Northern analyses revealed that the transcripts from the ptDNA genes *rbcL* and *psbD-psbC* do not accumulate or are present in albino tissues at ≤5-10% the level found in seed-derived green shoots. Levels of the plastid encoded 16S and 23S rRNAs are similarly reduced in albino tissues. Further Northern analysis revealed that the abundance of transcripts from the nuclear genes *rbcS* and *cab* are present in most albino plants at <10% the level found in normal green tissues. Southern analysis indicated that the nuclear DNA restriction fragments encompassing the *cab* and *rbcS* genes in two albino plants had not been altered or deleted during the anther culture process.

Analysis of green pollen plants indicated that they contain ptDNA of apparently normal structure and abundance and accumulate transcripts from plastid genes and nuclear genes encoding chloroplast polypeptides to the same levels found in the leaves of light grown seedlings. These results represent the first determination of the levels of photosynthetic gene expression in both albino and green pollen plants.