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Abstract	The extracellular space of leaves is a highly dynamic compartment harboring a number of activities involved in signal recognition, import/export of organic and inorganic compounds, and defense against pathogens. Although this has not been extensively studied, there is evidence for the involvement of the extracellular space in signal perception and nutrient remobilization during senescence. Integration of the apoplast into the larger picture of cellular activities during senescence may help understand key events in the terminal phase of leaf development. Important events associated with senescence occur in the apoplast, and these events may offer targets for genetic manipulation to modulate senescence. In this paper we look into changes in the extracellular space of leaves accompanying senescence, with a special focus on apoplastic proteins and plasma membrane proteins related to signaling and export of amino acids. Other not less relevant senescence-related metabolic changes such as NH_4 accumulation and the oxidative burst are beyond the scope of this review.

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2

Senescence-Related Changes in the Leaf Apoplast

Dana E. Martínez · Juan J. Guiamet

Abstract The extracellular space of leaves is a highly dynamic compartment harboring a number of activities involved in signal recognition, import/export of organic and inorganic compounds, and defense against pathogens. Although this has not been extensively studied, there is evidence for the involvement of the extracellular space in signal perception and nutrient remobilization during senescence. Integration of the apoplast into the larger picture of cellular activities during senescence may help understand key events in the terminal phase of leaf development. Important events associated with senescence occur in the apoplast, and these events may offer targets for genetic manipulation to modulate senescence. In this paper we look into changes in the extracellular space of leaves accompanying senescence, with a special focus on apoplastic proteins and plasma membrane proteins related to signaling and export of amino acids. Other not less relevant senescence-related metabolic changes such as NH_4 accumulation and the oxidative burst are beyond the scope of this review.

The Plant Apoplast

The plant extracellular space (ES) is composed of the cell wall and the fluid matrix that fills the interstitial spaces within the cell wall skeleton and the spaces beyond the plasma membrane (PM). The fluid matrix, also called apoplastic fluid (APF), is a continuous extracellular compartment through which cell–cell signaling and a myriad of other biochemical and physiological processes occur (Sattelmacher 2001).

The ES is composed of substances of different natures, such as solutes (that is, ions, organic acids, sugars, and amino acids), celluloses and pectins (the main constituents of the cell wall), proteins and glycoproteins with a variety of functions, for example, proteases, protease inhibitors, hydrolases, oxidoreductases, and other enzymes related to carbohydrate and lipid metabolism, and signaling (Basu and others 2006; Jamet and others 2006). Over the last decade, secreted small proteins and peptides were shown to act as PM receptor-mediated signals involved in biotic and abiotic stress responses and developmental regulation of cell proliferation and meristem activity (Matsubayashi and others 2001; van Norman and others 2011; Nakamura and others 2012). Recent progress in proteomics techniques has uncovered the highly dynamic nature of the extracellular compartment.

The Extracellular Environment and its Versatile Proteome

Experimentally identified extracellular proteins are characterized by the lack, rather than the presence, of conserved moieties, that is, no specific localization signal, no endoplasmic reticulum (ER) retention signal, and no transmembrane domain (unless it is located within the first 40 amino acid residues). The presence of an N-terminal signal peptide (SP) and potential N-glycosylation site/s are hallmarks of secretory proteins that are first targeted to the ER

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on their way to reach either the ES or the central vacuole. In fact the presence of a SP is considered a feature to classify a protein as predicted to be secreted. However, it is estimated that SP-containing proteins represent only around half of the extracellular proteome, whereas the other half "leaderless secretory proteins" reach the ES by other mechanisms (Kaffarnik and others 2009; Regente and others 2012; Ding and others 2012).

In the *Arabidopsis* genome, 5,879 out of 32,825 proteins were predicted by TargetP to have a SP at the N-terminus, and approximately over 70 % of them contain putative N-glycosylation sites. Interestingly, the N-glycan structure of leaf glycoproteins varies with aging, but it is currently unclear whether this is due to changes in protein expression, post-translational modification patterns during leaf development, or N-glycan maturation of pre-existing proteins (Song and others 2011). State-of-the-art N-glycoprotein analytical techniques such as "secretion gene traps" (Groover and others 2003) or more recently Concanavalin A Sepharose affinity chromatography followed by 2D-electrophoresis and nanoHPLC–MS or LC–MS/MS have allowed the identification of N-linked glycoproteomes highly enriched in ES proteins from *Arabidopsis* stems (Minic and others 2007) and *Arabidopsis* and *Brassica oleracea* xylem sap (a fluid that can be understood as part of the apoplast compartment, Ligat and others 2011). Notably, secreted N-glycoproteomes are enriched in hydrolases (that is, glycoside hydrolases and proteases), oxidoreductases, transferases, and kinases, possibly involved in degradation, remodeling, and signaling related activities.

Although some proteins are constitutively present in the ES, some others are expressed or secreted out of the cell upon specific scenarios or as part of specific responses. The constantly changing protein composition of the ES has lead to a new field of plant research called plant secretomics, that characterizes ES protein maps associated with a certain condition at a given time, and the underlying secretory mechanisms (for detailed reviews see Agrawal and others 2010; Alexandersson and others 2013). Proteomic techniques have allowed the study of protein changes in leaf and root apoplasts in response to varied stimuli in several species such as *Arabidopsis*, oilseed rape, tobacco, rice, and maize. Changes in tobacco leaf APF proteome in response to salt stress include decreases and increases in the levels of several proteins (Dani and others 2005). Among those showing accumulation, a germin-like protein and two chitinases markedly increased whereas two lipid transfer proteins were expressed de novo (Dani and others 2005). These three families of proteins are also involved in pathogen-induced responses, and some of their representatives are upregulated during leaf senescence (Yoshida and others 2001; Price and others 2008; Dunwell and others 2008). Chitinases are strongly affected in response to oxidative stress and were shown to be either up- or down-regulated in root APF proteomes of rice plants treated with hydrogen peroxide (H_2O_2) (Zhou and others 2011). In turn, extracellular H_2O_2 seems to accumulate in the apoplast in response to invading pathogenic fungi in combination with increases in whole leaf glutathione levels, as well as catalase, and peroxidase activities, slowing down or stopping the fungal invasion in barley leaves (Vanacker and others 2000). Extracellular H_2O_2 accumulation is part of the hypersensitive response (HR) which leads to cell death and prevents infection from spreading (Vanacker and others 2000). Elicited HR apoplast-mediated mechanisms involve a variety of enzymatic activities such as chitinases, glucanases, lipases, proteases of different types (aspartic, cysteine, and serine proteases), and protease inhibitors (Oh and others 2005; Ferreira and others 2007; van der Linde and others 2012). The Glip1 lipase was identified in a salicylic acid-induced secretome analysis (Oh and others 2005). Glip1 promotes HR and pathogen resistance in leaves in an ethylene-dependent manner and *glip1* plants are markedly more susceptible to fungal infection (Oh and others 2005; Kwon and others 2009). eFP Browser (Winter and others 2007) expression analysis of the gene coding for GLIP1, At5g40990, shows that this lipase is upregulated at the latest stages of embryo development and in senescing leaves.

CDR1 encodes an aspartic protease which accumulates in the APF in response to pathogen attack. Its overexpression causes dwarfing and resistance to virulent *Pseudomonas syringae*. CDR1 generates a small mobile signal involved in the activation of inducible resistance mechanisms in response to pathogens (Xia and others 2004). Some of the pathogen-related proteases are considered to be part of systemic signaling cascades (Gilroy and others 2007). These brief examples show the versatile nature of the apoplastic proteome, and the dynamic changes that may take place in different developmental stages, environmental conditions, or in response to pathogen challenge.

Leaf Senescence

Senescence can be defined as an internally controlled degradation process whereby cellular structures are dismantled and the products of macromolecule breakdown are redistributed to other parts of the plant (Krupinska 2007; Martínez and others 2008). In the context of leaf senescence, the most conspicuous change during senescence is the degradation of chloroplasts, which contain most of leaf N and P in the form of proteins and nucleic acids. Therefore, chloroplasts are important reservoirs of nutrients (for example, N, P, and S) that can be remobilized after

degradation of the corresponding macromolecules (for example, proteins and nucleic acids). In C_3 species with a monocarpic growth habit, N remobilization from senescing leaves may contribute to increase nitrogen use efficiency (Masclaux-Daubresse and others 2008).

Senescence of leaves has traditionally been divided into three phases: (I) initiation, (II) macromolecule degradation and export of breakdown products, and (III) a terminal phase dominated by programmed cell death (PCD) and, in many species, abscission (Noodén and others 1997). In the initiation phase (I), a number of different signals (developmental, hormonal, or environmental) re-program cell metabolism to shut down anabolic processes and enhance catabolism (Noodén and others 1997). The degradation phase (II) involves the apparently coordinated activity of a number of hydrolytic enzymes (proteases, nucleases, lipases, and so on) which break down macromolecules and bring about the desintegration of cellular structures. This is the phase when leaves display the typical symptoms of senescence, for example, leaf yellowing, decreased photosynthesis, and a decline in protein content. Although this degradation phase might be seen as a chaotic event, it actually appears as a fairly organized dismantling process. Chloroplasts break down before other organelles and cellular structures. Even as chloroplasts lose their contents and integrity, other parts of the cell are maintained relatively intact, and this allows for the export of materials (for example, amino acids) released from chloroplast protein breakdown. Finally (III), as the degradation phase comes to completion, cells die, either through programmed death, or because they can no longer maintain homeostasis, or leaves dry out following abscission.

Changes in the Apoplast as Part of the Regulation and Execution of Leaf Senescence

The apoplast and PM proteins appear to play no direct role in the dissasembly of chloroplasts, the main symptom of the senescence syndrome (Krupinska 2007; Martínez and others 2008; Gregersen and others 2008). However, apoplast and PM proteins may play important roles related to: a—signaling (Phase I but also Phase III), b—regulation of source–sink relations, c—extracellular trafficking of N compounds (both linked to Phase II), and d—cell death (Phase III).

For example, expression of PM receptor-like kinases (RLK), which are probably involved in signaling (a), increases during senescence (Hajouj and others 2000; van der Graaff and others 2006). Efficient use of N depends on remobilization of N compounds released from protein degradation (c), and this was most probably one of the selective pressures that shaped leaf senescence in the

course of evolution. The PM and apoplast are crucial for the export of amino acids and other organic compounds from senescing leaves, particularly in species where the route for phloem loading is apoplastic. There is evidence that PM transporter proteins are upregulated during senescence (van der Graaff and others 2006), suggesting an increased traffic of molecules through the apoplast. Interestingly, transcriptome analysis of senescence-associated transporter profiles reveals predicted PM transporters to be preferentially upregulated whereas plastidic transporters are downregulated (van der Graaff and others 2006). Many extracellular proteases belonging to all the four main clases of proteases (serine, cysteine, aspartic, and metalloproteases) are upregulated during leaf senescence (Delannoy and others 2008). Whether the main function of senescence-associated apoplastic proteases is processing/removal of signaling peptides, bulk protein degradation, defense against pathogens, and/or cell death is unknown. Finally, as mentioned above, the apoplast plays an important role in triggering PCD in response to pathogen invasion (Gadjev and others 2008), and it seems likely that it might also be involved in cell death as the last stage (d) of developmental senescence.

In the following sections, we will review apoplast and PM protein changes presumably associated with leaf senescence. Where possible, we will attempt to place these changes within the framework of the senescence processes described above.

Signaling

PM Receptors

RLK are PM proteins made up of an extracellular domain that recognizes specific ligands, and relays this information to a cytoplasmic kinase domain which transduces the signal through protein phosphorylation reactions (Becraft 1998). RLKs are involved in pathogen recognition, control of morphogenesis, and so on (De Smet and others 2009; Osakabe and others 2013). A few senescence-associated receptor kinases (SARK) have been shown to be upregulated during leaf senescence in bean, soybean, and *Arabidopsis* (Hajouj and others 2000; Li and others 2006; Xu and others 2011), and, consistent with their involvement in senescence, their expression is attenuated or delayed by cytokinins. Inducible expression of GmSARK in *Arabidopsis* results in upregulation of senescence-associated genes (SAGs) and accelerated leaf senescence (Xu and others 2011). In contrast, senescence is delayed in lines where GmSARK is silenced, suggesting a key role for SARK in the control of senescence of leaves. In part, this may be due to alterations of the hormonal balance, because induced expression of GmSARK results in downregulation

of cytokinin biosynthetic genes and upregulation of cytokinin oxidase. Ligands recognized by SARK are presently unknown. SARK, and possibly other RLKs, may recognize the as yet unknown extracellular ligands regulating senescence of leaves.

RPK1 is a leucine-rich repeat RLK involved in ABA early perception in relation to germination, growth, and stomatal closure in *Arabidopsis* (Osakabe and others 2005). RPK1 is a positive regulator of ABA signaling pathways and it is upregulated by this hormone. It was recently shown that RPK1 also plays a promotive role in ABA-mediated age-related senescence (Lee and others 2011). Mutant plants for RPK1 show delayed chlorophyll degradation and cell death, whereas RPK1 overexpression accelerates these symptoms. Interestingly, RPK1 induction in young plants leads to growth retardation but not senescence, suggesting that RPK1 may operate downstream of the age-related senescence program. In turn, SAGs such as WRKY transcription factor 6 (WRKY6), senescence-induced receptor-like kinase (SIRK), cis epoxycarotenoid dioxygenase 2 (AtNCED2), ACC synthase 2 (ACS2), phosphate transporter2 (AtPT2), and glutathione *S*-transferase 21 (GST21) are upregulated in response to the induction of RPK1 (Lee and others 2011). RPK1 is itself highly upregulated in senescing leaves (van der Graaff and others 2006, Supplemental Fig. 10).

Besides transcriptional regulation, many RLK as well as other surface receptors and transport proteins undergo constitutive or ligand-induced endocytosis. Indeed, PM protein internalization represents a tight, fine-tuned regulatory mechanism of signaling and PM protein degradation (Murphy and others 2005). Endocytosed PM proteins are sorted into membrane delimited vesicles called endosomes, that is, early or late (multivesicular) endosomes, that mediate PM protein and lipid recycling and degradation, respectively (Reyes and others 2011). The endosomal machinery is involved in many cell processes such as citokinesis, stomatal closure, and auxin transport (Otegui and Spitzer 2008). In plants, the endosomal machinery is composed of around 30 proteins organized in three protein-sorting complexes, plus one AAA ATPase. To date there is no evidence of particular endosomal trafficking related to senescence; however, some of the proteins comprising this machinery (for example, SKD1 and Snf7) are upregulated during leaf senescence, suggesting that active remodeling of PM composition might take place in senescing leaves (data obtained from inspection of gene expression at Bio-Analytic Arrays Resource efpBrowser, Winter and others 2007).

Polyamines

Polyamines are ubiquitous polycations bearing two or more primary amino groups, which are present in plants, animals, fungi, and bacteria (Takahashi and Kakhei 2010). Polyamines (for example, spermidine) are essential for plant growth, and they are also involved in responses to biotic and abiotic stress (Takahashi and Kakhei 2010). The involvement of polyamines in the regulation of leaf senescence is substantiated by numerous works where exogenous application of polyamines delayed chlorophyll and protein degradation (for example, Altman 1982; Noodén 1988). Variable levels of spermidine may be found in the apoplast, particularly in plants subjected to abiotic stresses, and apoplastic oxidation of polyamines in the apoplast, with the ensuing production of H_2O_2, may trigger defense responses against stress, for example salinity (Muschou and others 2008). Given the senescence-delaying effects of polyamines, on one hand, and the senescence-related signaling activity proposed for H_2O_2 (for example, Bieker and others 2012), it is interesting that one of the Cu-containing amine oxidases of *Arabidopsis* (At-CuAO1) is an apoplastic protein which increases in expression with plant age, and in response to senescence-accelerating hormones (salycilic acid and methyl jasmonate, Planas-Portell and others 2013). eFP Browser data show that AtCuAO1 expression increases about tenfold during senescence, compared to mature, rosette leaves. In transgenic lines overexpressing or downregulating apoplastic polyamine oxidase, apoplastic levels of polyamines decrease or increase accordingly (Muschou and others 2008). These lines might help to elucidate the role of apoplastic polyamines in signaling of senescence events in leaves.

Carbohydrate Transport and Regulation of Source–Sink Relations

Although it is generally assumed that senescence evolved mostly as a nutrient (for example, N) salvage mechanism, import/export of carbohydrates and source–sink relations for C may regulate senescence. For example, sugars accumulate in senescing leaves, and they can accelerate senescence, particularly under light and low N supply (Pourtau and others 2006). Not surprisingly, a number of sucrose and monosaccharide transporters, some presumably localized to the PM, are upregulated in leaves senescing attached to the plant (van der Graaff and others 2006); however, their specific functions during senescence remain unknown.

In addition to sugar transporters, extracellular invertase plays an important part in sugar transport and, therefore, source–sink relations in plants. Long-distance movement of sucrose is greatly facilitated by efficient phloem unloading at sink tissues, which is aided by the activity of extracellular invertase by maintaining a steep sucrose concentration gradient between sieve elements and the

apoplast. Extracellular invertase activity declines with leaf age (Balibrea Lara and others 2004), but this decline is prevented, and even reverted, in plants expressing the IPT gene under control of the senescence-specific SAG12 promoter (Gan and Amasino 1995); this implies that extracellular invertase activity is controlled by cytokinins (Balibrea Lara and others 2004). Inducible expression of extracellular invertase is sufficient to delay leaf senescence, whereas inhibition of extracellular invertase activity abolishes the senescence-delaying effects of cytokinins (Balibrea Lara and others 2004). Thus, by increasing extracellular invertase activity, cytokinins may help to direct assimilate flux toward young (sink) leaves, and the decrease in both cytokinin and invertase activity with age may be part of the sink–source transition which sets the stage for later senescence. On the contrary, abscisic acid (ABA) increases the expression of a cell wall invertase inhibitor (INVINH), leading to decreased cell wall invertase activity; silencing INVINH delays senescence and abolishes the senescence-promoting effect of ABA (Jin and others 2009). This evidence convincingly shows that extracellular invertase plays a crucial role in the regulation of senescence, possibly by controlling source–sink relations.

Export of Degradation Products: N Compounds

Transporter Proteins: Amino Acid Permeases (AAPs) and Related Transporters

Long-distance transport of N released from protein degradation occurs mostly in the form of amino acids and peptides (Caputo and Barneix 1999; Masclaux-Daubresse and others 2008). The transport of N-containing compounds across cellular membranes and their distribution along the plant is mediated by selective transporters. These are classified in families by functional categories, that is, nitrate transporters and ammonium transporters, ATP-binding cassette transporters (ABC family), oligopeptide (OPTs) and peptide (PTR) transporters for peptides, and two superfamilies: amino acid transporters (ATFs) and AAPs related to amino acid transport. The given names do not always relate to the nature of the transported substance, for instance OPTs were shown to transport not only small peptides, but also amino acids bound to metals, and glutathione (Lubkowitz 2011). A current understanding of N membrane transport mechanisms is reviewed in detail elsewhere (Stacey and others 2002; Liu and Bush 2006; Rentsch and others 2007; Tegeder and Rentsch 2010; Lubkowitz 2011). Masclaux-Daubresse and others (2008) published a detailed review of the senescence association of several ATFs in the context of leaf N remobilization. Here we will update the information on peptide and ATFs

localized to the PM, and, therefore, probably directly involved in extracellular and long-distance N movement, with emphasis on experimental and microarray database (eFP Browser) evidence of their participation in senescence.

Peptide Transporters

Promoter-driven GUS expression analysis of six of the nine known AtOPTs provides evidence for strong OPT activity in prevascular tissues, suggesting AtOPTs participation in N remobilization during germination (Stacey and others 2006). eFP Browser analysis of AtOPTs shows that the extracellular/PM AtOPT4 is strongly active in senescing and cauline leaves. AtOPT4-mediated transport is pH sensitive, being detectable in vitro at pH 5.0, but not at higher pH (Osawa and others 2006). PTRs transport a broad spectrum of di/tripeptides and localize to the PM or the tonoplast (Weichert and others 2012). Sequence comparison analysis identified 51 putative PTR genes in the *Arabidopsis* genome (Stacey and others 2002). Some of them have been experimentally examined. The wound-inducible transporter AtPTR3 (Karim and others 2007) and AtPTR1 (Fluckiger and others 2004) resemble AtOPT4 in their PM localization and eFP Browser developmental map predictions (that is, senescence upregulation). AtPTR1 mutant plants display reduce growth when grown with dipeptides as the only source of N (Komarova and others 2008), but the phenotypic analysis has not further explored a possible senescence-related phenotype.

ATFs

The ATF superfamily includes LHTs transporters (Lys/His), ProTs (Pro), ANTs (aromatic and neutral ATFs), GATs (gamma amino butyric acid and related molecules transporters), AUXs (indol 3-acetic acid), and AAP (AAPs, for Glu and neutral amino acid). The APC (polyamine and choline transport) family groups 14 members distributed among CATs (cationic transporters), LATs (L-type amino acids), and GAPs (GABA permeases). All together 63 and 80 genes were predicted to belong to these groups in *Arabidopsis* and rice, respectively (Kohl and others 2012).

LHTs localize to the PM and import amino acids into the cell. Based on eFP Browser data, AtLHTs show distinctive expression patterns during plant development, including high expression in senescing leaves. *Arabidopsis lht1* mutant plants display reduced uptake of some amino acids, and early leaf senescence symptoms but not before entering the flowering stage (Svennerstam and others 2007).

All AAPs analyzed to date in *Arabidopsis* also localize to the PM and function as H^+-coupled amino acid uptakers

(reviewed in Tegeder and Rentsch 2010). Interestingly, all the AAPs experimentally examined so far are restricted to vascular plants (Tegeder and Ward 2012). The majority of AtAAPs analyzed with eFP-Browser show upregulated expression in senescing and cauline leaves to some extent. AtAAP2, 3, 4, 5, and 6 are involved in phloem loading (Tegeder 2012). AtAAP6 is the only AAP transporter with affinity for aspartate (Fischer and others 2002). Mutant plants for AtAAP6 develop larger rosettes and more cauline leaves and also larger seeds that the WT (Hunt and others 2010). AAP2 is phloem-localized throughout the whole plant and is involved in xylem–phloem transfer affecting amino acid distribution to the embryo. A detailed phenotypic analysis of AtAAP2 mutants reveals delayed leaf senescence and more branched inflorescences than WT and higher seed yield. *aap2* seeds are reduced in N content but contain higher levels of fatty acids (Zhang and others 2010). This suggests that AtAAP2 may be involved in amino acid export from leaves to growing seeds, thereby controlling source–sink relations and senescence.

The GAT transporter AtGAT1 is a PM H^+-driven, high-affinity γ-aminobutyric acid (GABA) importer from the apoplast (Meyer and others 2006). High GABA concentrations are related to abiotic stresses (Bouché and Fromm 2004). eFP Browser analysis depicts the highest level of *AtGAT1* expression in senescing leaves. AtGAT1 expression correlates with the expression of SAG genes, for example, pheophorpide *a* oxygenase (Pruzinska and others 2005), suggesting a role for this transporter in GABA-mediated regulation of leaf senescence.

Three genes represent the *Arabidopsis* ProTs family (Lehmann and others 2011). According to ProTs promoter analysis, AtProT1 is expressed in the phloem or neighboring parenchyma cells along the whole plant, AtProT2 promoter activity is detected in the root epidermis, and also in wounded leaves, whereas AtProT3 promoter activity is restricted to leaf epidermal cells (Grallath and others 2007). eFP Browser analysis of these 3 genes shows that AtProT2 and AtProT3 expression levels are slightly increased in senescing leaves. The *Arabidopsis* genome codes for 9 CAT genes, CAT1 through 9, with distinctive expression patterns, PM located with the exception of tonoplast CAT2 (Su and others 2004). CAT1, formerly AAT1, is expressed in major veins of leaves, in roots and floral tissues (Frommer and others 1995), and according to eFP Brower microarray data it is highly upregulated in senescing leaves. Seasonal nitrogen cycling is characteristic of deciduous trees. The poplar CAT transporter—Pt-CAT11 transports glutamine into phloem vessels, is upregulated during senescence, and seems to play a key role in amino acids transport from senescing leaves to perennial tissues (Couturier and others 2010).

Senescence-Associated Apoplastic Proteases and PCD

Since chloroplast proteins constitute the largest pool of nitrogen in a leaf, and show a dramatic loss during senescence, understandably the emphasis in senescence research was placed on intracellular (mainly chloroplast or vacuolar) proteases. However, a few studies indicate that there are also senescence-associated changes in the expression and activity of extracellular proteases during senescence. Whether the main function of senescence-associated apoplastic proteases is bulk degradation of proteins, defense against pathogens, or processing/removal of signaling peptides is unknown. Delannoy and others (2008) listed 46 proteases which were identified in the proteome of the ES of *Arabidopsis* by various authors. We examined the changes in their expression patterns associated with senescence using the eFP Browser software (Winter and others 2007, Table 1). Of these proteases, only 17 (37 %) show no change during senescence, whereas 21 (45 %) are downregulated (>2-fold decrease), and transcript levels for the remaining 8 (17 %) proteases increase during senescence (Table 1; Fig. 1). The upregulated, extracellular proteases include two aspartic proteases, one cysteine protease and five serine proteases, with 2- to 6-fold increases in their transcript levels. Thus, there seem to be profound changes in the expression of extracellular proteases during senescence, with increases and decreases in expression. Moreover, this analysis only includes those proteases that were identified in extracellular fluids of young and mature, non-senescing leaves. Therefore, other senescence-specific or highly senescence-upregulated proteases were probably absent or present in undetectable concentrations to be identified in those experiments and await further research to be discovered.

MMPs are a family of Zn^{2+}-dependent proteinases involved in remodeling of the extracellular matrix in animal cells, where they play important roles in many physiological processes (Vu and Werb 2000). Identified plant MMPs shared structural similarity with mammalian MMPs. They show diverse expression patterns, but their activity and specific function is less clear (plant MMPs were recently reviewed by Flinn 2008). Fragmentary evidence shows that some matrix metalloproteinases (MMP) are upregulated in senescing leaves. For example, in cucumber, a matrix metalloprotease gene (Cs1-MMP) is expressed at non-detectable levels in mature leaves and at early stages of senescence, but transcript levels increase many fold in completely yellow leaves just prior to PCD (Delorme and others 2000). Likewise, the activity of a Zn^{2+} metalloprotease localized to the extracellular fluid of soybean leaves increases several fold as leaves age (Graham and others 1991). In *Arabidopsis*, the expression of At2-MMP increases with leaf age (Golldack and others

Table 1 Senescence-associated changes in the expression (mRNA levels) of apoplastic *Arabidopsis* leaf proteases

	Aspartic peptidases A1	Cysteine peptidases C1	Serine peptidases S8	Serine peptidases S10	Metallopeptidases M10
Senescence upregulated (>2-fold increase)	At1g44130, At5g10770	At1g47128	At4g34980, At1g32960, At3g14067	At4g12910, At2g22920	
Senescence downregulated (>2-fold decrease)	At3g52500, At4g16563, At3g61820, At1g01300, At1g09750, At5g07030, At3g54400	At1g20850, At4g11310	At1g62340, At2g05920, At1g01900, At4g02300, At1g04110, At1g30600	At1g28110, At5g22980, At5g42240, At4g30610, At5g08260, At5g23210	
Unchanged	At1g08210, At4g33490, At5g33340	At5g43060	At5g67360, At5g59090, At1g20160, At5g03620, At5g59130, At2g04160, At2g39850, At4g21630, At4g21650	At2g33530, At3g30810, At2g22970	At1g70170

Proteases were compiled from data shown in Delannoy and others (2008) and subjected to expression analysis using eFP Browser (Winter and others 2007). Proteases are indicated by their locus names. Proteases were considered upregulated or downregulated when their expression levels increased or decreased, respectively, more than twofold in leaves from 35-day-old plants compared to 17-day-old plants

Fig. 1 A representation of the proportion of apoplastic proteases whose mRNA levels are up- or down-regulated during senescence (*center circle*), and the proportions of apoplastic proteases that are up- or down-regulated or proteases with unchanged mRNA levels corresponding to each of the mechanistic classes of proteases: *C* cysteine-, *S* serine-, *A* aspartic-, and *M* metalloproteases (*external circles*)
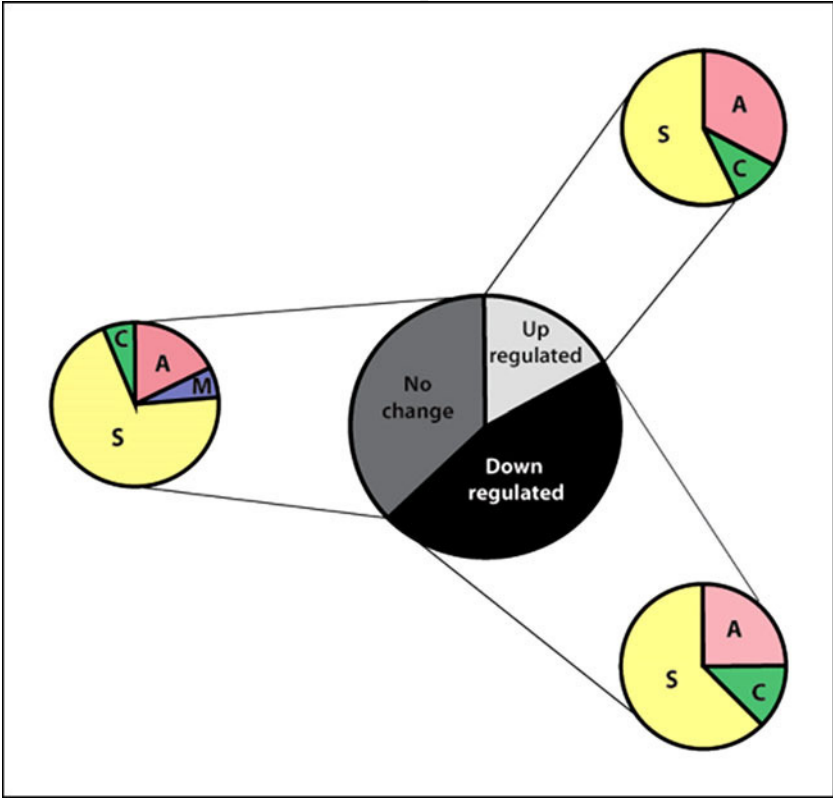
2001); knockout plants for At2-MMP show stunted growth, early flowering, and accelerated senescence (Golldack and others 2001). Although earlier senescence in a knockout for a senescence-upregulated protease seems counterintuitive, this suggests that At2-MMP might somehow negatively regulate senescence, and highlights the complexity of regulatory networks controlling this process. Plant MMPs are typically active against collagen in vitro (Graham and others 1991; Delorme and others 2000), but their in vivo function and endogenous substrates are unknown. In contrast, expression of Slti14, a matrix metalloprotease from soybean, increases in response to abiotic stress (low
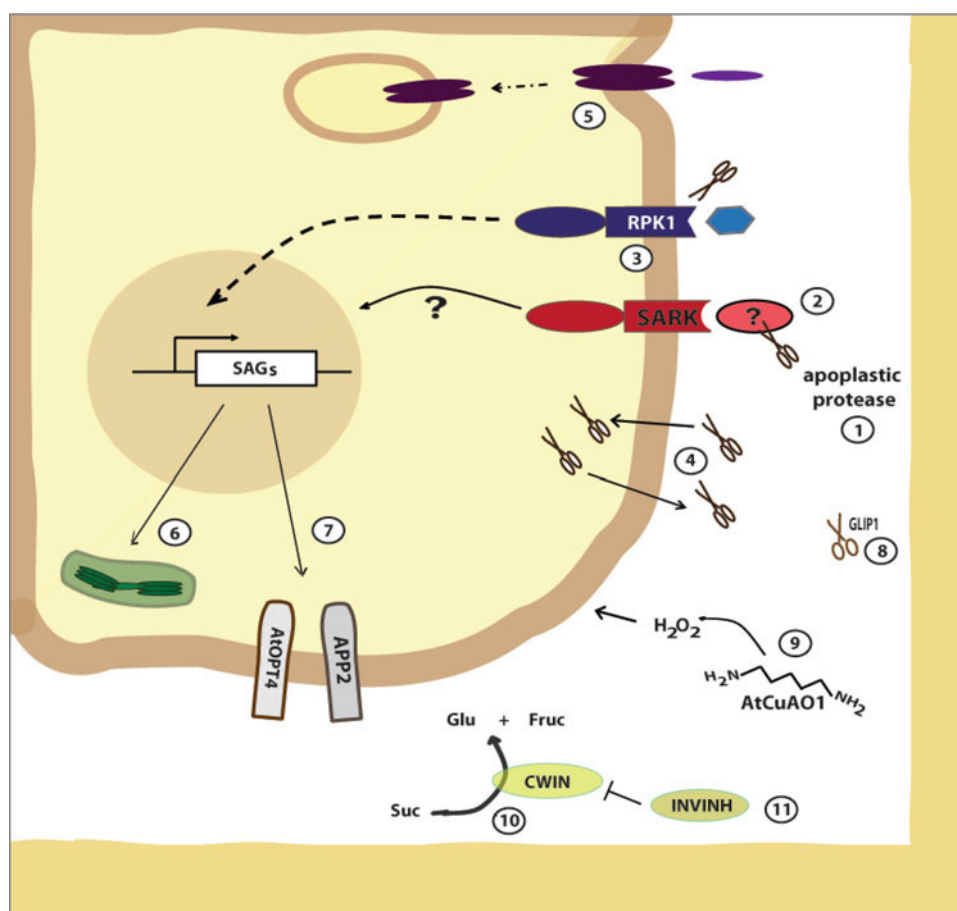
Fig. 2 A schematic summary of the most important changes occurring in the apoplast of senescing leaves. The apoplast may have an important signaling role. (*1*) Upregulated apoplastic proteases may process (*cleave*) apoplastic signals, or degrade extracellular ligands, plasma membrane proteins and receptors. Senescence-associated receptor kinases (SARK, *2*) and RPK1 (*3*) may bind specific ligands and relay this information intracellularly, to increase the expression of downstream senescence-associated genes (SAGs), probably involved in chloroplast breakdown (*6*). Other proteases may be regulated by their transport in and out of the cell (*4*), as shown for phytaspase in connection with pathogen resistance. Yet another level of regulation of plasma membrane proteins (for example, receptors and transporters) may involve their specific internalization by the endosomal machinery (*5*), which may be upregulated during senescence. The possibility of signaling by lipid molecules (*8*) should be explored in greater detail. Finally, apoplastic oxidation of polyamines (*9*) may have important regulatory consequences, because of both the decrease of apoplastic levels of polyamines and generation of H_2O_2. The apoplast is an important pathway for export of N compounds released from chloroplast protein degradation, as evidence by the upregulation of plasma membrane amino acid and peptide transporters (*7*). Apoplastic invertase (*10*) and invertase inhibitors (*11*) are implicated in the control of source–sink relations during senescence, thereby regulating the rate of senescence progression

temperatures) and wounding, but appears to be downregulated during senescence of cotyledons (Cho and others 2009). Many cysteine-type proteases are upregulated during senescence (Buchanan-Wollaston and others 2005). Whereas most of them localize to vacuolar compartments, either the central vacuole (Martinez and others 2007) or senescence-associated vacuoles (Otegui and others 2005), some of them are delivered to the apopast. Some extracellular cysteine proteases are involved in inducing PCD in a salicylic acid-dependent defense pathway (Doehlemann and Hemetsberger 2013), including the senescence-associated cathepsin B and the *Nicotiana benthamiana* ortholog of RD21 (Gilroy and others 2007; Bozkurt and others 2011). These proteases are not exclusively located to the apoplast but may translocate to the ES upon pathogen infection. Both reduced levels of proteases degrading positive regulators of senescence or increased expression of proteases breaking down negative regulators might be essential to trigger senescence or modulate its progression, but so far no comprehensive functional analysis of senescence-associated, extracellular proteases, nor of their substrates, has been carried out.

PCD

PCD is a ubiquitous phenomenom that takes place under many different scenarios, for example, as part of the HR to

biotrophic pathogens or developmental PCD during tracheary element formation or leaf development (Kuriyama and Fukuda 2002; Gunawardena and others 2004; Gadjev and others 2008). PCD also occurs at advanced stages of leaf senescence, as shown by the detection of DNA laddering, a hallmark of PCD (Yen and Yang 1998; Cao and others 2003). However, our understanding of the regulation and mechanism of PCD as the last event of leaf senescence is rudimentary.

PCD associated with the HR is much better understood. It is clear that an oxidative burst, dependent to a large extent on the activity of the PM NADPH oxidase, triggers PCD in many cases (Gadjev and others 2008). H_2O_2 generated by polyamine oxidation in the apoplast may also direct a defense response against pathogens (Takahashi and Kakhei 2010). Other events, such as depletion of extracellular ATP (Chivasa and others 2005) and secretion of cathepsin B into the apoplast (Gilroy and others 2007) play important roles positively regulating PCD. However, the occurrence and relevance of such regulatory events in PCD during the terminal phase of senescence are unknown. Some PR proteases resemble mammalian caspases due to their specific type of substrates. Subtilisin-type proteases with caspase-like activity are known as saspases. Oat saspases SAS-1 and -2 are constitutively present inside the cell in a mature-active form and are released into the APF upon induction of PCD by either the fungal toxin victorin or heat shock (Coffeen and Wolpert 2004). A tobacco saspase-like protease (phytaspase) exemplifies an opposite PCD-related change in protease redistribution. Phytaspase is constitutively secreted into the leaf apoplast and it is re-imported inside the cell as part of the response against mosaic virus and abiotic stresses. Phytaspase gene over-expression demonstrated that this protease plays an essential role in HR-related PCD (Chichkova and others 2010). The extra- or intra-cellular localization of subtilisin caspase-like proteases may play a critical regulatory role preventing the untimely execution of an otherwise active mechanism of protein degradation and stress-induced PCD (Vartapetian and others 2011). Whether similar changes occur during developmental PCD is presently unknown.

Concluding Remarks

As reviewed here, the apoplast is a versatile compartment, which undergoes important changes during leaf senescence. Some of these changes involve components of signaling pathways presumably regulating senescence, whereas others affect transporter proteins that play a role in the export of degradation products (for example, amino acids) out of senescing leaves (for a summary, see Fig. 2). Indeed, manipulating the expression of extracellular or PM components (for example, extracellular invertase, RLK, or AAPs) alters senescence progression. Our understanding of senescence-associated changes of the apoplast would be much fostered by the availability of a proteomic map of the ES and PM from senescing leaves. In this regard, selecting apoplastic proteins with significant senescence-associated changes in concentrations for further functional studies might expand our understanding of the regulation of senescence and the execution of nutrient remobilization. In addition, by analogy to the HR, examination of late changes in the apoplast may shed light on the mechanism of PCD as the last stage of developmental senescence. Integration of changes in apoplast composition and function into the larger pìcture of cellular activities during senescence may help understand key events in the final stages of leaf development.

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