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REVIEW

Senescence-associated proteases in plants

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Senescence is the final developmental stage of every plant organ, which leads to cell death. It is a highly regulated process, involving differential gene expression and outstanding increment in the rate of protein degradation. Senescence-associated proteolysis enables the remobilization of nutrients, such as nitrogen (N), from senescent tissues to developing organs or seeds. In addition to the nutrient recycling function, senescence-associated proteases are also involved in the regulation of the senescence process. Nearly, all protease families have been associated with some aspects of plant senescence, and numerous reports addressing the new identification of senescence-associated proteases are published every year. Here, we provide an updated report with the most recent information published in the field, focusing on senescence-associated proteases presumably involved in N remobilization.

Introduction

Senescence is the final developmental phase of every plant organ, leading to death either of single cells, tissues or the whole plant. As for other types of programmed cell death (PCD), senescence proceeds in an orderly and regulated manner, involving *de novo* protein synthesis and up- or down-regulation of specific genes (Buchanan-Wollaston et al. 2003). Functionally, senescence is a recycling process, by which the nutrients accumulated in the senescing tissues are redirected to other areas of the plant, where they can be used for the production of new vegetative, reproductive or storage organs. Protein degradation, allowing the recycling of nitrogen (N) and other nutrients, is probably the most important degradation process that occurs during senescence. For this reason it is not surprising that many of the genes up-regulated during senescence are proteases (Bhalerao et al. 2003, Guo et al. 2004).

Chloroplasts are the first organelles to be disorganized, while the nucleus and mitochondria remain active for longer (Lim et al. 2007). Up to 75% of total leaf N is located in the chloroplasts (Hörtensteiner and Feller 2002), mostly in the form of D-ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) in the stroma, and light-harvesting complex of photosystem II (PSII) (LHCII) in the thylakoid membrane. In leaves of C_3 species, Rubisco can represent up to 50% of soluble proteins. This amount of Rubisco exceeds by far the requirements for photosynthesis and it has been postulated that it serves as the main N reserve in vegetative tissues. Degradation of chloroplastic proteins is an early event during senescence, resulting in decrease of the photosynthetic capacity of the cells (Hörtensteiner and Feller 2002). The numerous reports showing that Rubisco can be hydrolyzed in intact isolated chloroplasts or chloroplasts lysates (Feller et al. 2008 and references

Abbreviations – APs, aspartic proteases; CPs, cysteine proteases; LHCII, light-harvesting chlorophyll *a*/*b*-binding protein complex; MMPs, matrix metalloproteinases; MCs, metacaspases; MPs, metalloproteases; N, nitrogen; PSII, photosystem II; PCD, programmed cell death; Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; SAVs, senescence-associated vacuoles; SPs, serine proteases; VPE, vacuolar processing enzyme.

therein) together with the current knowledge on the complexity of the chloroplastic proteolytic machinery (Kato and Sakamoto 2010) support the idea that at least the first steps in the degradation of plastidial proteins occur inside the organelle. Later, degradation of these proteins would proceed by the action of vacuolar proteases either through a physical association between chloroplasts and the central vacuole or the mobilization of chloroplast components to the vacuole carried into senescence-specific vesicles (Hörtensteiner and Feller 2002, Martínez et al. 2008).

The current knowledge on senescence-associated proteases has been achieved largely by two opposite approaches. One is the large-scale gene expression analysis, allowing the identification of senescence-induced genes coding for known or predicted proteases, and the other one is the identification and characterization of enzymes with proteolytic activity from senescent plant tissues. Only for a few senescence-associated proteases both approaches have been investigated, while for the majority of them our knowledge remains incomplete or fragmented.

Several comprehensive reviews have addressed the subject of protein degradation at senescence in the past (Hörtensteiner and Feller 2002, Feller et al. 2008, Liu et al. 2008a). Here, we provide an updated report with the most recent information published in the field, with special interest on senescence-associated proteases presumably involved in N remobilization.

Serine proteases (SPs)

Serine proteases (SPs) are the largest class of proteases in plants (van der Hoorn 2008). Tripathi and Sowdhamini (2006) identified a total of 206 and 222 serine proteases and serine protease-like proteins in Arabidopsis and rice, respectively.

Several lines of evidence suggest that SPs play a crucial role in N remobilization (Table 1). In wheat, SPs were found to be the predominant proteases responsible for protein degradation during monocarpic senescence both under heat stress and in a non-stress environment (Chauhan et al. 2009). In addition, two subtilisin-like proteases (P1 and P2) belonging to the family S8 of SPs according to the MEROPS classification (Rawlings et al. 2010) were demonstrated to be strongly associated to senescence in wheat. Induction of both proteases was shown in wheat leaves under dark- and N starvation-induced senescence (Roberts et al. 2006) and more recently, in the flag leaf of naturally senescing plants, where they were suggested to participate in Rubisco degradation and N remobilization to developing grains (Roberts et al. 2011). Also in barley it has been shown that subtilases are highly expressed under natural and steam girdling-induced senescence (Parrott et al. 2007). In that study, phloem interruption by steam girdling at the leaf base was used to induce carbohydrate accumulation and senescence acceleration together with enhanced degradation of both soluble (Rubisco) and membrane (LHCII) proteins in the girdled leaves. Subtilisin-like proteases have also been involved in PCD triggered by both biotic and abiotic stress in tobacco (Chichkova et al. 2010). Interestingly, in oat, they have been shown to indirectly regulate Rubisco degradation during PCD induced by victorin or heat shock treatment (Coffeen and Wolpert 2004).

Chloroplasts contain a whole range of proteolytic enzymes, most of them highly homologous to prokaryotic proteases (Kato and Sakamoto 2010, Olinares et al. 2011). The most abundant of the stromal proteases is the Clp protease complex (family S14), homolog of the *Escherichia coli* Clp system. The functional Clp complex consists of two multi-subunit components: a catalytic core and a chaperone with ATPase activity responsible for the recognition, unfolding and translocation of the substrate into the proteolytic compartment. The family of Clp proteases in *Arabidopsis thaliana*, consist of nine chaperone/ATPases subunits (ClpC1-2, ClpD, ClpB1,3-4 and ClpX1-3), six ClpP (ClpP1-6) and four ClpP-like (ClpR1-4) proteolytic subunits, two accessory subunits (ClpT1-2), and one ClpS presumably functioning as a substrate regulator. Most of the Clp subunits are localized in plastids, while ClpB4, the three ClpX, and ClpP2 are found in the mitochondria and ClpB1 in the nucleus and cytoplasm. In chloroplasts, the active core is composed of two heptameric rings containing all the ClpP, ClpR and ClpT subunits, while the chaperone subunits are predicted to form hexameric rings attached to each end of the central core (Kato and Sakamoto 2010, Olinares et al. 2011).

On the basis of the similarity of structural arrangement and action mechanism among Clp protease and the eukaryotic proteasome, a role in degradation of abnormal, damaged or short-lived proteins has been proposed for Clp (Kato and Sakamoto 2010). Constitutive expression of several Clp catalytic subunits (ClpP1-3, ClpP5-6 and ClpR3-4) in dark-induced senescence in Arabidopsis supports this idea (Lin and Wu 2004). However, up- and down-regulation of different subunits have also been reported varying with age, tissue and type of senescence (Guo et al. 2004, Ruuska et al. 2008, Olinares et al. 2011) (Table 1). Induction of ClpD has been observed in autumn senescence in aspen leaves (Andersson et al. 2004) and in dark-induced senescence in Arabidopsis together with up-regulation of ClpC1 (Lin and Wu 2004). ClpP2, the only mitochondrial

Table 1. Plant senescence-associated proteases. Arrows indicate up- (↑) or down- (↓) regulation. ABA, abscisic acid; C, chloroplast; Cyt, cytosol; EC, extracellular; ET, ethylene; Fam, family according to MEROPS; HL, high light; JA, jasmonate; KT, kinetin; Loc, localization; M, mitochondria; N, nitrogen; NS, natural senescence; Nu, nucleus; S, stroma; SA, salicylic acid; SG, steam girdling; Sec, secretory pathway; TL, thylakoid lumen; TM, thylakoid membrane; V, vacuole; W, wounding.

Name	Fam	Loc	Species		Treatment	References
Serine proteases						
SPs	–	–	Wheat	↑	NS, heat stress, N	Ruuska et al. (2008) and Chauhan et al. (2009)
Carboxypeptidases	S10	V	Barley	↑	NS, SG	Parrott et al. (2007) and Jukanti and Fischer (2008)
Clp (chaperone)	S14	S	Wheat	↑	N	Ruuska et al. (2008)
ClpD	S14	S	Arabidopsis, barley, *Populus*	↑	NS, dark, SG	Andersson et al. (2004), Lin and Wu (2004), Parrott et al. (2007) and Jukanti and Fischer (2008)
ClpP4, ClpT1	S14	S	Arabidopsis	↓	Dark	Lin and Wu (2004)
ClpC1	S14	S	Arabidopsis	↑	Dark	Lin and Wu (2004)
ClpP2	S14	M	Rice	↑	NS, ABA, KT	Liu et al. (2008b)
Subtilisins P1, P2	S8	–	Wheat	↑	NS, dark, N	Roberts et al. (2006, 2011)
Subtilase	S8	–	Barley	↑	NS, SG	Parrott et al. (2007)
DegP	S1	C	*Populus*	↑	NS	Anderson et al. (2004)
Deg1, 5, 8	S1	C	Arabidopsis	↓	Dark	Lin and Wu (2004)
Lon2	S16	M	Arabidopsis	↑	Dark	Lin and Wu (2004)
Aspartic proteases						
AP	–	–	*Populus*, oilseed rape, wheat	↑	NS, N	Bhalerao et al. (2003), Andersson et al. (2004), Gregersen and Holm (2007) and Desclos et al. (2009)
OsAsp1	A1	–	Rice	↑	NS, ABA, KT	Liu et al. (2008b)
CND41	A1	C	Arabidopsis, barley, tobacco	↑	NS, SG, N	Kato et al. (2004, 2005), Parrott et al. (2007), Diaz et al. (2008) and Jukanti and Fischer (2008)
Cysteine proteases						
CPs	–	–	Arabidopsis, barley, *Populus*	↑	NS	Bhalerao et al. (2003), Guo et al. (2004), Andersson et al. (2004) and Jukanti and Fischer (2008)
CPs	–	V	Wheat	↑	NS, dark, drought	Martínez et al. (2007)
CPs	–	SAVs	Arabidopsis, tobacco, soybean	↑	NS, dark	Otegui et al. (2005) and Martínez et al. (2008)
AG12	C1	SAVs	Arabidopsis, wheat, barley, *Populus*	↑	NS, SG, N	Otegui et al. (2005), Guo et al. (2004), Parrott et al. (2007) and Ruuska et al. (2008)
cathepsin B/ B-like	C1	V/Cyt/Sec	Arabidopsis, barley, wheat	↑	NS, SG, dark, PCD	Guo et al. (2004), Parrott et al. (2007), McLellan et al. (2009) and Gregersen and Holm (2007)
Papain-like	C1A	–	Barley	↑	NS, SG, N	Parrott et al. (2007, 2010)
SPCP2	C1	V	Sweet potato	↑	NS, ABA, JA, ET	Chen et al. (2010)
GMCP3	C1A	–	Soybean	↑	NS, drought, ABA, JA, W	Esteban-Garcia et al. (2010)
Aleurain	C1	V	Rice, wheat	↑	NS, ABA, KT, N	Gregersen and Holm (2007), Liu et al. (2008b) and Ruuska et al. (2008)
αVPE	C13	V/Sec	Arabidopsis	↑	NS, ET, SA, W	Sanmartín et al. (2005)
γVPE	C13	V	Arabidopsis, wheat	↑	NS, ET, SA, W, N, pathogens	Sanmartín et al. (2005) and Ruuska et al. (2008)
MC6	C14	Cyt	Arabidopsis	↑	NS	Breeze et al. (2011)
MC9	C14	Cyt	Arabidopsis	↑	NS	Sanmartín et al. (2005) and Breeze et al. (2011)
MC3	C14	M	Arabidopsis	↑	NS	Sanmartín et al. (2005)
Putative MC	C14	–	Wheat	↑	N	Ruuska et al. (2008)
Metalloproteases						
FtsH	M41	–	Arabidopsis, wheat, oilseed rape	↑	NS, N	Guo et al. (2004), Gregersen and Holm (2007) and Desclos et al. (2009)
FtsH1,5	M41	TM	Arabidopsis	↓	Dark	Lin and Wu (2004)

Table 1. Continued

Name	Fam	Loc	Species		Treatment	References
FtsH8	M41	TM	oilseed rape, *Populus*	↑	NS, N	Andersson et al. (2004) and Desclos et al. (2009)
FtsH2	M41	TM	*Populus*	↑	NS	Bhalerao et al. (2003)
FtsH6	M41	TM	Arabidopsis, barley	↑	Detached leaves, dark, HL	Zelisko et al. (2004, 2005)
Neutral LAP	M17	–	Barley	↑	SG	Parrott et al. (2007)
At(1,3-5)-MMPs	M10	EC	Arabidopsis	↑	NS	Flinn et al. (2008)
SMEP1	M10	EC	Soybean	↑	NS	Graham et al. (1991) and Pak et al. (1997)
Cs1-MMP	M10	EC	Cucumber	↑	NS	Delorme et al. (2000)
At2-MMP	M10	EC	Arabidopsis	↑	NS	Golldack et al. (2002)
Threonine proteases/Proteasome						
AtRPT1a,3,5a,5b	–		Arabidopsis	↑	NS	Kurepa and Smalle (2008)
AtRPN6	–		Arabidopsis	↑	NS	Kurepa and Smalle (2008)
AtRPN2b,11,12a	–		Arabidopsis	↑	Dark	Lin and Wu (2004)
5 ATPase subunits	–	Nu/Cyt	Arabidopsis	↑	NS	Guo et al. (2004)
8 20S subunits	T1		Arabidopsis	↑	NS	Guo et al. (2004)
20S β-subunit A1	T1		Oilseed rape	↑	N	Desclos et al. (2009)
20S β5 subunit	T1		Wheat	↑	NS	Gregersen and Holm (2007)

proteolytic subunit, was shown to increase early at the onset of flag leaf senescence in rice, and to slowly decrease in advanced senescence (Liu et al. 2008b). In contrast, ClpP4 and ClpT1 (formerly ClpS1) were showed to be down-regulated during dark-induced senescence in Arabidopsis (Lin and Wu 2004). All these evidences suggest that in addition to a housekeeping role, Clp protease is also involved in controlling the development of senescence and response to various stresses. Moreover, the changes in the expression levels of the different subunits may function as a way to modulate its activity and substrate specificity.

Although degradation mechanism of LHCII remains largely unknown (Zienkiewicz et al. 2011), recent reports have suggested a role of Deg proteases (family S1). Deg proteases are ATP-independent SPs identified as the eukaryotic counterpart of the *E. coli* DegP. Five of the 16 Arabidopsis Deg proteases have been localized in the chloroplast; Deg1, 5 and 8 in the thylakoid lumen and Deg2 and 7 associated with the stromal side of the thylakoid membrane (Kato and Sakamoto 2010). Recently, the involvement of Deg1 in the degradation of CP29 (Lhcb4) and CP26 (Lhcb5) under photo-inhibition (Zienkiewicz et al. 2011) and of Deg2 in degradation of Lhcb6 under various stress conditions (Luciński et al. 2011) was demonstrated in Arabidopsis.

Aspartic proteases (APs)

Aspartic proteases (APs) are the second largest protease class in plants after SPs (van der Hoorn 2008). Most of the plant APs belong to the families A1 and A11 of clan AA (Rowlings et al. 2010). Proteomics studies in *Brassica napus* plants submitted to N starvation revealed increased levels of an AP starting after senescence onset and maintained until late senescence (Desclos et al. 2009). In addition, up-regulation of APs has been reported in autumn leaf senescence in *Populus* (Bhalerao et al. 2003), and during natural senescence in the flag leaves of rice (Liu et al. 2008b) and wheat (Gregersen and Holm 2007). However, the strongest evidence supporting a role of APs in plant senescence corresponds to the chloroplast located CND41 protease (family A1) from tobacco leaves. *In vitro*, it was shown to degrade denatured Rubisco at physiological pH (Kato et al. 2004). Silencing of CND41 resulted in delayed senescence and accumulation of Rubisco in the older leaves, suggesting a failure in N remobilization (Kato et al. 2004). In addition, overexpression of CND41 led to accelerated senescence and enhanced Rubisco degradation in the senescent leaves (Kato et al. 2005). In Arabidopsis plants senescing under low N availability, induction of a putative CND41 protease was followed by a decrease of Rubisco and correlated with senescence progress in five recombinant inbred lines exhibiting different leaf senescence rates (Diaz et al. 2008). In girdled barley leaves, increased expression of a CND41-like protease was observed progressing with time of treatment from 4 to 8 days (Parrott et al. 2007). However, a reduction in the large Rubisco subunit was evident only after 12 days of treatment and CND41 expression actually decreased in naturally senescing plants (Parrott et al. 2007). The expression of a CND41-like protease gene was also shown to decay over time after anthesis in two different lines of barley, one with high and one with low grain protein content (Jukanti and Fischer 2008).

Cysteine proteases (CPs)

Analysis of gene expression during natural or induced senescence in different plant species has shown that cysteine proteases (CPs) consistently appear as the most abundant class of proteases up-regulated (Bhalerao et al. 2003, Guo et al. 2004). The induction of at least four vacuolar CPs has been reported in wheat leaves senescing in continuous darkness (Martínez et al. 2007) and the formation of small vacuolar compartments [senescence-associated vacuoles (SAVs)] containing high CP activity has been observed in leaves of Arabidopsis and soybean (Otegui et al. 2005). The CP SAG12 highly expressed in naturally senescing tissues (Guo et al. 2004, Parrott et al. 2007, Ruuska et al. 2008) have also been found in SAVs (Otegui et al. 2005). Immunodetection of Rubisco and glutamine synthetase within these SAVs in naturally senescing tobacco leaves suggests SAVs to be important for the degradation of stromal proteins during senescence (Martínez et al. 2008). Additional evidence supporting CPs to be involved in the degradation of stromal proteins has been provided using specific protease inhibitors in wheat leaves (Thoenen et al. 2007), and expressing cystatin, a natural CP inhibitor from rice, in transgenic tobacco plants (Prins et al. 2008). In both experiments, impairment in Rubisco degradation was observed after inhibition of CPs activity.

In a recent report of Parrott et al. (2010), sugar accumulation induced by steam girdling of barley leaves was combined with different nitrate supplies to imposed varied C/N ratios. In this study, the analysis of the expression of several senescence-related proteases revealed that a papain-like protease (subfamily C1A) (MEROPS ID CO1.A13) correlated best with senescence markers like chlorophyll degradation or amino acids accumulation under high C/N ratio, suggesting an important role in bulk protein degradation.

The expression of two other papain-like CP genes, *SPCP2* in sweet potato (Chen et al. 2010) and *GMCP3* in soybean (Esteban-García et al. 2010) has been demonstrated in natural and stress-induced senescing tissues. Besides, transgenic Arabidopsis plants with constitutive expression of *SPCP2* showed developmental alterations such as earlier flowering time (Chen et al. 2010).

Vacuolar processing enzyme (VPE, family C13), also called legumain, is a CP responsible for the maturation of vacuolar proteins. VPE exhibits caspase 1-like activity and has been shown to mediate cell death in response to a variety of stress inducers and during development of different organs (Hara-Nishimura et al. 2005). In Arabidopsis, it has been shown that αVPE and γVPE are up-regulated in vegetative organs during senescence (Sanmartín et al. 2005), and a role for γVPE in the activation of downstream proteases involved in amino acids recycling during senescence has been proposed (Rojo et al. 2003).

Cathepsins are a family of lysosomal proteolytic enzymes involved in protein turnover and apoptosis of mammalian cells. Most of them belong to the CP class, but some members of this family are APs and SPs. Increased expression of Cathepsin B and Cathepsin B-like CPs (family C1) has been reported in senescent leaves of barley (Parrott et al. 2007) and in naturally (Guo et al. 2004) or dark-induced (McLellan et al. 2009) senescent leaves of Arabidopsis.

Plant metacaspases (MCs, family C14) are CPs structurally related to animal caspases but exhibiting different substrate specificity. Caspases cleave after Asp residues and MCs after Arg and Lys. *A. thaliana* genome contains nine genes for MCs, *AtMCP1a-c*/MC1-3 and *AtMCP2a-f*/MC4-9. Several reports have demonstrated that MCs play a role in controlling cell death (Coll et al. 2010, Watanabe and Lam 2011), and up-regulation of some MCs genes have been observed in senescent leaves (MC6, MC9) and flowers (MC3, MC9) of Arabidopsis (Sanmartín et al. 2005, Breeze et al. 2011) (Table 1). However, experimental evidence demonstrating a functional role not only for MCs but also cathepsins and VPE in senescence is still lacking.

Metalloproteases (MPs)

Although there are approximately 100 different metalloproteases (MPs) in the plant genome (van der Hoorn 2008) there are few reports about their participation in senescence (Table 1). FtsH protease (family M41) is the best studied MP in plants. FtsHs are membrane-bound ATP-dependent MPs with a zinc-binding domain. The nuclear genome of *A. thaliana* encodes 12 active FtsH proteases. While nine of them are predicted to localize in the chloroplast, the presence of four FtsH proteases (FtsH1, 2, 5 and 8) in the thylakoid membrane has been demonstrated (Kato and Sakamoto 2010). Hexameric complexes of FtsH proteases are known to participate in plastid differentiation and in PSII repair cycle by degrading the core protein D1 (Nixon et al. 2010).

FtsH6, one of the plastidic members of this family, has been associated with degradation of LHCII in Arabidopsis. Żelisko and Jackowski (2004) reported a thylakoid membrane-bound zinc MP to be involved in the degradation of the Lhcb3 in detached barley leaves senescing in the dark. The authors (Żelisko et al. 2005) showed that a similar mechanism was operating in dark-induced senescing leaves of *A. thaliana* and

using reversed genetics identified FtsH6 as the protease responsible for Lhcb3 degradation. However, Wagner et al. (2011) reported no significant differences in the levels of Lhcb3 when comparing three different FtsH6 deletion mutants to wild type plants during age dependent senescence or high light acclimation, suggesting that this protease is not relevant for the degradation of LHCII *in vivo*. Increased gene expression of different FtsH isoforms has been reported in senescent leaves of Arabidopsis (Guo et al. 2004), and early senescent autumn leaves of aspen, in which FtsH2 was found among the 20 most abundant expressed sequence tags (ESTs) (Bhalerao et al. 2003). On the contrary, FtsH1 and 5 were down-regulated in dark-induced leaf senescence in Arabidopsis (Lin and Wu 2004). Proteomics studies in oilseed rape senescing under nitrate starvation showed transient induction of a chloroplast FtsH protease at early stages of N remobilization while decreasing at the end of senescence, suggesting a role in early degradation of chloroplastic proteins (Desclos et al. 2009).

In contrast to FtsH, little is known about matrix metalloproteinases (MMPs, family M10) in plants. MMPs are extracellular zinc-dependent endopeptidases very well characterized in vertebrates, where they function in the degradation and remodeling of the extracellular matrix. Global analyses of Arabidopsis microarray data showed that most MMPs are highly expressed in senescent leaves (Flinn 2008). In agreement, SMEP1 from soybean, Cs1-MMP from cucumber and At2-MMP from Arabidopsis were also shown to be preferentially expressed during senescence (Graham et al. 1991, Pak et al. 1997, Delorme et al. 2000, Golldack et al. 2002), while loss of function of At2-MMP led to late flowering and early senescence compared to the wild type (Golldack et al. 2002), suggesting an active role of MMPs in senescence.

Finally, leucine aminopeptidases (LAPs, family M17) are ubiquitous metallopeptidases cleaving N-terminal amino acids from proteins and peptides. LAPs have been mainly associated to cell maintenance functions, peptides turnover and stress responses. However, in Arabidopsis it has been shown that suppression of LAP2 function led to reduced vegetative growth and accelerated senescence (Waditee-Sirisattha et al. 2011).

Threonine proteases: proteasome

The ubiquitin-proteasome 26S proteolytic system (family T1) is responsible for the specific degradation of abnormal, short-lived and regulatory proteins in the cytoplasm and nucleus of eukaryotic cells. The 26S complex is composed of a catalytic particle, the 20S proteasome, and one or two regulatory particles (19S) which requires ATP to unfold the substrates and direct them into the catalytic core (Kurepa and Smalle 2008). Substrate selectivity is controlled at the ubiquitination step. Conjugation of the ubiquitin molecules to target proteins is performed by a family of enzymes (E3 ligases) in a highly specific way, with each family member controlling the ubiquitination of one or a few target proteins (Kurepa and Smalle 2008).

Protein degradation through the ubiquitin/proteasome mechanism takes place in nearly all phases of plant growth and development. Its active role during senescence is suggested by biochemical analyses as well as expression data of genes coding for components of the pathway (Bhalerao et al. 2003, Lin and Wu 2004, Raab et al. 2009). In wheat, it has been shown that detached leaves senescing in the dark keep constant protein levels and activity of proteasome 20S as well as of ubiquitin conjugates in advanced senescent tissues (Roberts et al. 2002). The ubiquitin-proteasome pathway therefore may remain functional until late senescence participating in regulatory aspects of the process rather than in bulk protein breakdown. However, a potential role of the proteasome in N remobilization from carbonylated proteins accumulated under oxidative stress in detached leaves of Arabidopsis has been suggested (Jain et al. 2008). The authors reported that degradation of carbonylated proteins was prevented by MG132, a specific inhibitor of proteasome 26S. In addition, Arabidopsis mutants defective in proteasome function showed increased levels of carbonylated proteins.

Large-scale analysis of Arabidopsis microarray data showed that gene expression of most of the catalytic proteasome subunits did not change or was down-regulated in senescent leaves, while only 5 of 22 regulatory subunits were up-regulated (Kurepa and Smalle 2008). The proteasome activity might be regulated through changes in the holoenzyme subunit composition. In addition, it has been suggested recently that variations in the ratio of proteasome 26S to 20S occurring throughout the lifespan of the plant, would be also an important factor affecting plant growth and development (Kurepa et al. 2009).

Concluding remarks

Proteases are involved in all aspects of plant life from germination to death. The accumulated experimental evidence shows that senescence involves the participation of proteases belonging to nearly all the different families and catalytic types (Table 1). Although, most of

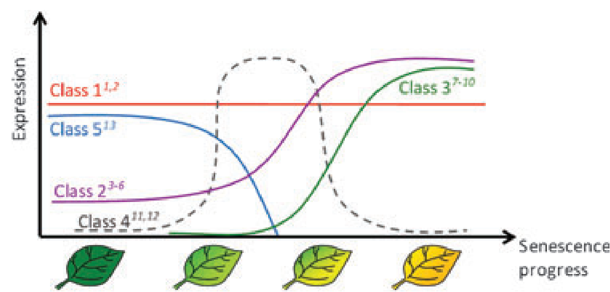

Fig. 1. Different classes of proteases according to their expression profile along the senescence progress. Examples of proteases for each class are denoted in superscript and correspond to: (1) ClpP1-3, ClpP5,6, ClpR3,4 (Lin and Wu 2004); (2) 20S proteasome (Roberts et al. 2002); (3) subtilisin P2 (Roberts et al. 2006, 2011); (4) CPs (Martınez et al. 2007); (5) SPs (Chauhan et al. 2009); (6) aleurain (Liu et al. 2008b); (7) subtilisin P1 (Roberts et al. 2006, 2011); (8) OsAsp1 (Liu et al. 2008b); (9) SAG12 (Parrott et al. 2007); (10) CPs (Martínez et al. 2007); (11) FtsH (Desclos et al. 2009); (12) ClpP2 (Liu et al. 2008b); (13) ClpP4, ClpT1, FtsH1, 5, Deg1, 5, 8 (Lin and Wu 2004).

the proteases here discussed are up-regulated during natural or stress-induced senescence, their participation in other phases of plant development cannot be discarded. According to their expression profile, senescence-associated proteases can be divided in groups for which different biological roles can be predicted (Fig. 1). Class 1 comprises all those proteases that are expressed both in green (non-senescent) and senescent tissues, maintaining almost invariable levels along the senescence progress. Although they are not specific of the senescence phase, the energy cost to sustain their synthesis in a clearly catabolic environment highlights their significance for the normal development of senescence. They are most likely needed to fulfill housekeeping functions in order to maintain cell viability. The proteasome system and the Clp protease fit well within this group, despite senescence-associated expression have been reported for some of their subunits (Roberts et al. 2002, Lin and Wu 2004, Kurepa and Smalle 2008). Class 2 includes those proteases that are present in green tissues at low levels and are induced soon after senescence has been onset. Proteases showing early and sustained induction along senescence are probably directed to bulk protein degradation. The fact that these proteases have already been synthesized in young tissues may facilitate their fast induction in the first stages of senescence. The subtilisin P2 from wheat (Roberts et al. 2006, 2011), the vacuolar CPs described in Martínez et al. (2007), and AP described in (Desclos et al. 2009) are good examples of proteases belonging to this group. Class 3 clusters those proteases that are specific of the senescence phase. These proteases are synthesized *de novo* only in senescent tissues, and are predicted to play a significant role in the late stages of senescence and cell death execution. The protease P1 from wheat is included in this group (Roberts et al. 2006, 2011) as well as still unidentified CPs in tobacco (Prins et al. 2008) and wheat (Martínez et al. 2007). Class 4 comprises those proteases that are transiently expressed during senescence and could be involved in the early breakdown of chloroplastic proteins, as it has been suggested for FtsH (Desclos et al. 2009). Finally, proteases negatively associated to senescence can be included all together in Class 5. In this group, we locate all those proteases that are normally expressed in green tissues but are repressed in senescent tissues. They likely fulfill proteolytic functions that are no longer needed once the cell enters the senescence phase, or they can act repressing senescence promoting signals. Since we have focused this review on the proteases involved in senescence-associated proteolysis, proteases belonging to this group are not discussed here.

In the last decade, the extensive use of genomic and proteomic approaches have allowed the massive identification of senescence-associated proteases expanding significantly our knowledge in this field. However, in order to include more proteases into each proposed class and validate our classification their time-based expression/activity profile during senescence is required. Besides, the fact that it is not always possible to establish a direct correlation between transcripts and protein levels with enzymatic activity, mainly due to post-translational modifications and the presence of positive and negative activity regulators, highlights the need to develop new techniques for large-scale analysis of protease activity and identification of target proteins. This information is critical to establish the physiological role of each protease or protease group in senescence development.

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