Participation of Proteases in the Degradation of Chloroplast Proteins

Maria Ciesielska, Małgorzata Adamiec and Robert Luciński*

Adam Mickiewicz University in Poznań, Faculty of Biology, Institute of Experimental Biology, Department of Plant Physiology

Abstract: Degradation of chloroplast proteins within the organelle is supported by the observation that chloroplasts contain several proteases of the ClpP, FtsH, Deg, and Lon families. Clp proteases were among the first identified chloroplasts' proteases and may play an important role during chloroplast biogenesis. Some members of the ClpP family (*i.e.*, nclpP3 and nclpP5) are up-regulated during senescence, whereas the expression of other Clp proteases is constitutive, with no changes during leaf ontogeny. Interestingly, the mRNA levels of erd1, a Clp regulatory subunit are up-regulated during senescence of *Arabidopsis thaliana* leaves, but the levels of the corresponding ERD1 protein decline. Homologs of the bacterial FtsH protease are also found in plastids. At least 12 FtsH proteases have been identified in *Arabidopsis thaliana*, and some of them may play roles in thylakoid protein degradation. An FtsH protease is involved in the breakdown of the 23-kDa fragment of the D1 protein of the PSII reaction centre, which is formed upon photooxidative damage. Chloroplast DegP and FtsH proteases seem to cooperate in D1 degradation during photoinhibition, and it seems likely that they might also be responsible for D1 degradation during senescence. *In vitro* studies with thylakoids isolated from knock-out lines for FtsH6 have implicated the involvement of this protease in LHCII degradation during senescence. Other FtsH subunits may function in chloroplast biogenesis rather than senescence. In this article, we show which proteases are involved in the degradation of chloroplast proteins. We will focus on both: intrachloroplast and non-chloroplast proteases and their mechanism of the process.

Keywords: Arabidopsis thaliana, Chloroplast, Chloroplast proteases, Proteolysis.

1. INTRODUCTION

Proteases probably arose in the earliest stages of protein evolution as simple enzymes necessary for protein catabolism and amino acid production in primitive organisms. For many years, research on proteases has focused on their primary roles in protein disruption. However, the discovery that in addition to these non-specific degradation functions, proteases act as sharp scissors and catalyse highly specific proteolytic processing reactions to produce new protein products, beginning a new era in protease research. These enzymes regulate the activity of many proteins, modulate protein-protein interactions, create new bioactive molecules, contribute to the processing of cellular information, and generate, process, and amplify molecular signals. Proteases also play a key role in plants and contribute to the processing, maturation, or destruction of specific sets of proteins in response to developmental signals or changes in environmental conditions. In the article below, however, we will focus on the participation of proteases in the degradation of chloroplast proteins.

*Address correspondence to this author at the Adam Mickiewicz University in Poznań, Faculty of Biology, Institute of Experimental Biology, Department of Plant Physiology, Ul. Uniwersytetu Poznańskiego 6, 61-614 Poznań, Poland; Tel: +48 61829 58 89; E-mail: robert.lucinski@amu.edu.pl

Chloroplast proteins are largely degraded during the aging of leaves and other green plant organs. Chloroplasts contain the large amounts of proteins, and their breakdown provides the plant with nutrients used by other organs [1]. The mechanisms involved in the degradation of chloroplast proteins are, however, poorly understood. In general, they can be divided into two routes: inside the chloroplasts and outside the plastids. The intrachloroplast pathway is carried out by the chloroplast proteases present in the organelles. Outside the plastid, the route includes mainly the enzymes located into the central vacuole [1].

2. MECHANISMS OF THE CHLOROPLAST PROTEINS DEGRADATION

2.1. Proteases

Proteases are a large group of hydrolytic enzymes with a very diverse structure of the catalytic centre, which carry out the reaction of breaks the peptide bond.

According to the proteolytic enzyme classification system used in the MEROPS protease database, proteases are divided into classes, clans, families, and subfamilies [2]. The basis for distinguishing classes in this system are the features of the catalytic centre structure and mechanism of catalysis. According to this classification method, 6 classes of proteolytic enzymes

E-ISSN: 2311-858X/21

are distinguished: aspartic, cysteine, glutamine, serine, metalloprotease, threonine [3].

The basis for distinguishing clans is phylogenetic relatedness, which manifests itself in a similar tertiary structure or - when this structure is not known - the order in the polypeptide chain of amino acid residues performing catalytic functions, and the frequent occurrence of popular sequence motifs around catalytic residues.

The family is built by a set of homologous proteolytic enzymes, the homology of which is manifested by a significant similarity of the amino acid sequence to the holotype, representative of the type of protein belonging to a given family. The similarity of the amino acid sequence of a given enzyme to the holotype must relate at least to the protease domain of the compared proteins. Some families are divided into subfamilies due to the very distant time of divergence (more than 150 point mutations / 100 amino acid residues) within the family.

Proteolysis is considered to be an important factor influencing the structure and functions of the plant organism in changing environmental conditions and in a changing ontogenetic context [4]. Regulatory hydrolysis of proteins catalysed by proteolytic enzymes is directly or indirectly involved in most cellular processes in plants - it is an element of protein metabolic turnover, an important component of the protein quality control system, and plays an important role in the course of signalling pathways triggered in response to various stimuli exo- and endogenous and regulating many important cellular processes (including gene expression, cell cycle, differentiation, targeting and sorting of proteins, the course of programmed cell death). Protein quality control is mainly based on the hydrolysis of those proteins that have been damaged either by mutation or plant contact with stress factors, or are synthesized in excessive amounts, or are sorted into the wrong cell compartment. On the other hand, the control of protein metabolic turnover is manifested by the hydrolysis of proteins that, in a specific space-time context, are no longer needed [5].

2.2. Hypothetical Mechanisms of Chloroplast Proteins Degradation

These are three hypothetical mechanisms that might be involved in chloroplast breakdown during senescence [6]. These mechanisms are:

1) degradation by chloroplast-targeted, nuclear-encoded hydrolases - in this model,

The protein-pigment complexes (*e.g.*, LHCII) dissociate to render the apoprotein susceptible to proteolytic attack by chloroplast proteases (*e.g.*, FtsH6). Rubisco (RbCO) and other stromal proteins may be initially nicked by chloroplast proteases, then transferred to senescence-associated vacuoles (SAVs).

2) export of chloroplast components to lytic vacuoles - the central vacuole might be a site for transient storage of amino acids released from protein degradation, particularly because the concentration of amino acids in the vacuole increases during senescence [7], possibly placing the central vacuole as the endpoint of chloroplast protein degradation pathways operating during senescence [1].

3) re-location of vacuolar hydrolases to the chloroplast - there is not much evidence for redirection of vacuolar proteases to chloroplasts. As discussed above, there is evidence (albeit scant and fragmentary) for the operation of the first two mechanisms, whereas, as far as we know, there is not much evidence for the re-direction of vacuolar proteases to chloroplasts

2.3. Chloroplast Proteases

Chloroplasts belong - next to mitochondria, peroxisomes, and endoplasmic reticulum - to organelles inside which protein degradation takes place, catalysed by their proteases. Chloroplast proteases were discovered by the observation that some proteins were degraded depending on light and ATP during *in vitro* incubation of chloroplasts isolated from pea leaves. It has been shown that degradation in such an experimental set-up can also take place independently of ATP [8].

The chloroplast proteases on the example of A. thaliana include Clp, Deg, FtsH and Lon.

2.3.1. Chloroplast Clp Proteases

The most diverse family of proteins is the Clp proteases. A single complex particle in AtClp chloroplast proteases is made up of eleven individual proteins. The core responsible for the proteolytic activity is formed by the proteins AtClpP1 and AtClpR1-R4 and AtClpP3-ClpP6 in the form of a heptameric P ring, which interacts with the AtClpT1 and AtClpT2 proteins [9]. The P ring contains seven catalytic sites. According to commonly accepted views, the role of AtClpS1 is to facilitate the interaction of the substrate intended for degradation with chaperone molecules, which in turn in an ATP-dependent manner degrade

the substrate, thus allowing it to enter the catalytic core formed by the P ring, where proper degradation takes place [10].

Clp proteases are among the first identified chloroplast proteases [1]. Clp family proteins are mainly located in the chloroplast stroma; the exception is the AtClpC1 protease, which is a protein with a dual localization: stroma and inner membrane of the chloroplast envelope [11]. Most AtClp proteins are encoded by nuclear genes and synthesized on cytoplasmic ribosomes as precursor molecules containing an N-terminal transit peptide to direct precursor forms through the chloroplast sheath to the stroma. The exception is ClpP1 - this protein is encoded by the chloroplast genome and synthesized on the stroma ribosomes immediately in its mature form.

In search of the physiological functions of AtClpC1, the phenotype of *A. thaliana* insertional mutants was analysed. The results indicate that this protein plays a very important role in the proper function of the whole plant. The clpC1 mutants are stunted, shown strong chlorosis, and the significant changes in the photosynthetic apparatus, both in terms of the chloroplast ultrastructure (less developed thylakoid system), and the number of proteins associated with photosynthetic electron transport, primary carbon metabolism including the Calvin cycle, glycolysis and photorespiration, and increased levels of proteins involved in protein biogenesis and plastid gene expression), and photosynthetic activity of leaves [12].

AtClpD is a protein under normal environmental conditions represented by smaller amounts than AtClpC1. AtClpD mRNA is strongly induced during leaf aging [13], whereby older leaves have an increased level of AtClpD accumulation [14]. Cold stress also increases the accumulation of AtClpD [15].

AtClpB3 protease, belonging to family of chaperones, has been demonstrated to function in crucial processes of chloroplast development and probably in thylakoid membrane formation and protein translation in proplastid/chloroplast differentiation [16]. The current model for ClpB3 heat stress-related function in plants proposes that this chaperone confer thermotolerance by promoting disassembly of aggregates formed by denatured proteins and small heat shock proteins. The same mode of ClpB3 action may hold for acclimated recovery of proteins denatured by exposure to elevated irradiance.

AtClpB3 can function as a chaperone completely independent of the AtClp holocomplex. The action of AtClpB3 most likely consists in resolubilizing the interprotein aggregates formed during *A. thaliana* exposure to thermal stress [17] and light with increased intensity [18], and in stress-free environmental conditions, AtClpB3 participates in biogenesis chloroplasts from proplastids [19].

AtClpP3 plays an important role in plant life, as shown by the fact that *clpP3* mutants are characterized by a delay in embryogenesis, the embryos are white and smaller than in WT plants [20]. The development of mutant seedlings stops at the cotyledon stage, and only the addition of sugars to the medium results in the slow development of leaves, greening, flowering, and seed production [20].

AtClpP6 results in pleiotropic changes affecting mainly mature leaves, the most significant of which is chlorosis, slowing growth rate, wrinkling of the leaf edges, slowing down the rate of photosynthesis, and abnormal chloroplast development [21].

The phenotype of mutants lacking AtClpR1 is moderate and is characterized by weak chlorosis and slight changes in the ultrastructure of the chloroplast [22].

Lack of AtClpR2 and AtClpR4, delay of embryogenesis, no greening of seedlings (whose development stops at the cotyledon stage), and smaller seeds than in WT plants [23], which can mature in autotrophic conditions. Under heterotrophic conditions, pale green seedlings slowly develop, but plants produce non-viable seeds [22].

2.3.2. Chloroplast Proteases Deg

Sixteen genes coding for proteins homologous to DegP, Q, and S have been identified in Arabidopsis thaliana nuclear genome. It has been experimentally proved that five of these genes - *AtDEG1, 2, 5, 7*, and *AtDEG8* - encode proteins targeting to chloroplasts, *AtDEG10* - a protein with mitochondrial localization, and *AtDEG15* - a paroxysmal protein. The remaining nine *AtDEG* genes encode proteins whose localization has been determined only in silico as both mitochondrial and chloroplast, or their location has not been determined [24]. AtDeg is serine proteases that the MEROPS database places in the S1 family (holotype - ox chymotrypsin).

The Deg protease's primary structure consists of a protease domain (with the HDS catalytic triad) and a

single or multiple PDZ domains, which are responsible for the protein-protein interaction and substrate recognition [24].

AtDeg1 monomers associate into trimers through interactions between prosthetic domains, while the hexamer is formed by interactions between LA loops of the protease domain and the IC1 and IC2 motifs of the PDZ domain. The rigid walls of the catalytic chamber are formed by the six PDZ domains along with the six LA loops of protease domains, three distinct entrances leading to the interior of the chamber form in the walls [25].

The primary structure of AtDeg2 protein consists of a protease domain and two PDZ domains (PDZ1 and PDZ2) 12- or 24-mer are formed by joining together hexamers (probably proteolytically inactive). The AtDeg2 hexamer is a barrel consisting of two trimeric rings arranged on top of each other in such a way that they form an internal catalytic chamber; access to it is possible through six pores. The assembly of the monomers to the trimers is mediated by the protease domains and the PDZ1 domain, and the stabilization of the trimer association by all three domains [25].

The proteolytically inactive protease AtDeg5 most likely forms a hexamer linked to the AtDeg8 hexamer in a heterododecamer located on the thylakoid lumen side of the thylakoid membrane. In the linear structure of this protease molecule, we find a single prosthetic domain and no PDZ domain [26].

In the primary structure of AtDeg7, there are two protease domains, the second of which is a catalytically inactive (degenerate) form and four PDZ domains [27].

There is little information available about the functions of AtDeg proteases. Several studies have shown that they are essential for the degradation of PSII apoproteins under photoinhibition conditions, namely, they degrade PsbA, which is the primary target for high irradiance-induced photodamage. Most probably AtDeg1 is responsible for the cleavage of photodamaged PsbA at the luminal CD loop [28] and downstream of transmembrane helix E whereas AtDeg5/AtDeg8 heterocomplex cooperates by performing another cut at CD loop and AtDeg7 seemingly makes a cut at stromal BC loop of the photodamaged molecule. The action of the four proteases yields fragments that possibly undergo complete proteolysis catalysed by other, unidentified proteases. It has also been suggested that it is AtDeg7 that may cleave PsbB-D PSII reaction centre complex proteins as secondary targets for irradiance-dependent damage. There are indications that AtDeg1, AtDeg7, and AtDeg5/AtDeg8 may cooperate with unidentified proteolytical enzyme/enzymes belonging to the FtsH family inefficiently cleaving PsbA protein after photoinhibition. Furthermore, it was suggested that AtDeg5/AtDeg8 may be engaged in PsbA loss observed in leaves exposed to brief heat stress [29].

AtDeg2 proteolytic activity is required throughout the development of older leaves. Mutants lacking the deg2 protein exhibited a photoperiod-independent reduction in the area of leaves of older whorls [30]. Also noticed changes in the ultrastructure of the chloroplasts of palisade mesophyll cells within older (second-whorl) leaves of mutants relative to those of wild-type plants grown under long-day conditions. Over the 4-wk period, the appearance of moderate senescence symptoms was observed in wild-type chloroplasts, involving periodic undulations of the envelope and thylakoids as well as the appearance of numerous large plastoglobules. Mutants' leaves yielded chloroplasts with no undulations and with significant reductions in the number and size of plastoglobules. The plastoglobules of wild-type chloroplasts had a translucent appearance, in contrast to those of the mutant chloroplasts, which had strongly osmiophilic structures. In addition to increases in their number and size, a loss of the osmiophilic character of plastoglobules is associated with chloroplast senescence, reflecting an alteration in the lipid composition of the plastoglobules [30].

AtDeg2 is involved in the chloroplast senescence program. Some early symptoms of thylakoid system disintegration may have been observed in chloroplasts of second-whorl leaves of 4-wk-old wild-type plants; relative to those of *deg2* mutants, they had a lower number of thylakoids stacked per individual granum [30].

A lot of studies have also been conducted to determine if AtDeg2 is responsible for the degradation of Lhcb6 in leaves exposed to high salt, injury, heat, and high radiation intensity [30]. in wild-type plants, Lhcb6 decreased markedly in response to all four stresses, but this apoprotein resisted degradation in the mutant. AtDeg2 is involved in the Lhcb6 proteolysis occurring during brief treatment of A. thaliana leaves with high salt, wounding, heat, or high irradiance [30].

AtDeg5 is of seminal importance for normal plant development and degradation of PsbF apoprotein which occurs following brief wounding. It was shown

that whereas PsbA, C, and F apoproteins of photosystem II reaction centre undergo an extensive disappearance in response to a set of brief stresses *deg5* mutant was fully resistant to the disappearance of PsbF apoprotein which follows an exposition of leaves to wounding [31].

Downregulation of a chloroplast protease AtDeg5 might lead to an increase in leaf area [31]. When grown under non-stressing conditions *deg5* mutants showed an altered leaf morphology involving a photoperiod-independent increase in leaf area. No significant differences concerning leaf area were detected to exist for leaves of younger plants grown both at long and short day regimes [31].

AtDeg5 repression altered the chloroplast ontogenesis programmed by delaying the point of entering the senescence phase. Transmission electron microscopy observation showed that chloroplasts produced clear moderate senescence symptoms of which some waviness in thylakoids, as well as a presence of numerous, large plastoglobules, were the most remarkable ones. In striking contrast, mutant chloroplasts showed no signs of thylakoid undulating structure and yielded much less numerous plastoglobules of significantly reduced size [31].

Studies with the recombinant AtDeg7 protease show that this enzyme exhibits *in vitro* proteolytic activity against the non-physiological substrate β-casein, but no such activity was observed against the α and κ forms of casein [26]. Information on physiological AtDeg7 substrates is very scarce. Presumably, this protease interacts with AtDeg1, AtDeg2, and AtDeg5/AtDeg8 in the degradation of the photo-damaged PsbA protein and regulates the degradation of other PSII core proteins (PsbB, PsbC, and PsbD) under photo-inhibitory conditions [26].

2.3.3. Chloroplast Proteases FtsH

The *A. thaliana* nuclear genome contains 12 genes that code for AtFtsH proteins (Martinez *et al*. 2008). Proteins targeting chloroplasts are probably: AtFtsH1, 2, 5, 6, 7, 8, 9, 12. The AtFtsH11 protease targets both chloroplasts and mitochondria [32]. The products of the genes encoding chloroplast AtFtsH1, 2, 5 and 8 are integrally associated with the thylakoid membrane [32], where form a heterooligomeric complex (called the AtFtsH heterocomplex) containing AtFtsH1 / 5 (type A AtFtsH) and AtFtsH2 / 8 (type B AtFtsH). Under normal circumstances, FtsH2 is the most abundant chloroplast isoform of AtFtsH, FtsH5 is the second most abundant, and FtsH8 and FtsH1 are represented by very small amounts [32].

In the primary structure of AtFstH1, 2, 5, and 8 the AAA + domain can be found. It contains two major motifs, namely the Walker A and Walker B, which participate in the binding and hydrolysis of ATP necessary for unfolding the substrate, respectively. Otherwise, the SRH region, which acts auxiliary in the binding and hydrolysis of ATP and the C-terminal protease domain are the important part of the FtsH proteins. The catalytic centre with the HEXXH motif binding Zn2 + situates - according to the MEROPS classification - all AtFtsH in the M41 family (holotype - FtsH E coli) [33].

The FtsH1 protease is involved in the breakdown of the 23 kDa fragment of the D1 protein in the centre of the PSII reaction, which was caused by photooxidative damage [1]. The FtsH6 protease is involved in the degradation of LHCII during leaf aging. B-casein is the non-physiological substrate of the recombinant AtFtsH1 protease *in vitro* [34]. Numerous experimental data indicate that the AtFtsH heterocomplex is involved *in vivo* in the degradation of the photo-damaged PsbA protein and the biogenesis of chloroplasts [35].

Based on tests with var1-1 mutants (AtftsH1 mutants) it was proved that Lhcb1–3 apoproteins seem to be substrates for a stress-dependent degradation mediated by AtFtsH heterocomplex as the apoproteins in the mutant resisted the degradation which took place in wild-type plants in a stress-dependent manner [36].

The studies carried out with the use of ftsh6 mutants concluded that the physiological substrates of AtFtsH6 include two apoproteins of the main peripheral energy antenna PSII - Lhcb1 and Lhcb3. AtFtsH6 was considered to be responsible for the degradation of Lhcb1 under 24-h plant exposure to increased light intensity and the degradation of Lhcb3 associated with the aging of darkened leaves [37].

AtFtsH7 and AtFtsH9 form a heterocomplex in the chloroplast shell [38]. Each of these isoforms has two transmembrane domains [39].

In response to short-term (2.5 h) exposure to increased light intensity, the transcription of AtFTSH7 and AtFTSH9 is transiently amplified 2-4-fold [40].

A slight enhancement of the accumulation of AtFTSH8 transcripts was observed in response to short-term exposure to elevated temperature [40].

The phenotypic analysis of ftsh11 mutants shows that AtFtsH11 is involved in the development of plant resistance to elevated temperature and the acquisition of thermotolerance [41], although exposure to elevated temperature does not stimulate FTSH11 transcription [40].

No physiological substrates for AtFtsH11 have been identified so far.

AtFtsH12 is located in the chloroplast shell in the form of a complex co-formed with the inactive AtFtsHi isoform, which lacks all three amino acids necessary for zinc binding. AtFtsH12 has two transmembrane domains [39], although their topology in this membrane is still unknown.

The physiological AtFtsH12 substrates could not be identified.

2.3.4. Chloroplast Proteases Lon

Only one chloroplast protease belongs to the LON, ATP-dependent protease family in *A. thaliana*. The proteolytically active form of AtLon4 is a protein associated with strong interactions with the thylakoid membrane on the stroma side [42]; this protein is also assigned a mitochondrial location. In the primary structure of AtLon4, we find the AAA + domain, which contains the Walker A and Walker B motifs (involved in the binding and hydrolysis of ATP necessary for the unfolding of the substrate) and the C-terminal protease domain. The catalytic centre with the SK motif places - according to the MEROPS classification - AtLon4 in the S16 family (holotype - Lon E coli). There is no data on the structural organization and functions of AtLon4.

2.3.5. Chloroplast Proteases CND41

The CND41 protease is an aspartic chloroplast protease involved in the degradation of Rubisco during the aging of tobacco leaves [1]. CND41 has a proteolytic activity for the inactive form of Rubisco. The active form is resistant to its actions [1]. CND41 might be involved not only in the regulation of gene expression but also in the bio-genesis of the functional apparatus of chloroplasts and the degradation of denatured proteins. Only a few proteolytic enzyme that are involved in processing for the generation of functional components have been well characterized in chloroplasts. Although CND41 has an unusually low optimal pH and such a low pH would not occur in chloroplasts under normal physiological conditions. Certain environ-mental stresses, *e.g*. freeze-induced dehydration stress is known to lower the cytosolic pH due to disturbance of the tonoplast membranes. CND41 may function under such stress conditions [43].

2.3.6. Intramembrane Proteases

Proteases play a key role in the metabolism of proteins by degrading those proteins that have been damaged or are not needed at a given stage of life of a cell or the whole organism. They also play an important function in the process of protein quality control by hydrolysing these chains of polypeptides that have been mistakenly synthesized or targeted to improper cell compatibility. The results of the research carried out in recent years reveal that proteases also participate in pathways signal transduction by releasing membrane-anchored transcriptional factors. This process is known as regulatory intramembrane proteolysis (RIP) and it is catalysed by proteases, which carry out the hydrolysis of peptide-binding within cell membranes [44].

In plant cells, intramembrane proteases belonging to zinc metalloproteases (site-2-proteasses, S2P), rhomboids - belonging to serine proteases, and two families of aspartyl proteases: presenilins and signal peptide proteases have been identified so far [44].

The S2P homolog from *A. thaliana*-AtEgy1, was the first intramembrane protease identified in plants [45]. The S2Ps are zinc-containing metalloproteases comprising at least four hydrophobic regions characterized by the presence of a zinc-binding motif (HExxH) within the first of their transmembrane domains. They have two highly conserved motifs necessary for their proteolytic activity: GpxxN/S/G, which is usually present in the second transmembrane domain(TM), and NxxPxxxxDG, is usually present in the third TM [46].

The domain characteristic for S2P proteases containing those three motifs was named M50. In some of the M50 domains, also the presence of the PDZ domain was identified. The domain is known to mediate the interaction between protein molecules forming oligomeric complexes and may play a role in the activation of the protease domain [47, 48].

In *A. thaliana* have been identified six genes encoding homologs of S2P protease, and five proteins encoded by these genes are considered to be proteolytically active. AtEgy1, AtEgy2, AraSP, and S2P2 are located in chloroplasts. AtEgy2 was found to be located in the thylakoid membrane [48], while ARASP was identified as an inner-envelope membrane protein [50]. The precise chloroplast localization of

AtEgy1 and S2P2 remains unknown [51]. Recently, the chloroplastic localization, inside the thylakoid membrane, has been experimentally proven for AtEgy3 [52].

The role of the AtEgy1 protease has been the subject of several microscopic studies. Mature chloroplasts from wild-type plants and mature chloroplasts from *egy1* mutants were compared. Mutants showed a reduced number of chloroplasts, poorly developed internal thylakoid membrane system, smaller thylakoids, no gran, smaller starch grains, and plastoglobulins. Mutants lacking the AtEgy1 protein are also characterized by a reduced level of chlorophyll, which gives the leaves a yellow colour and disturbs the ethylene-induced gravitropism. The absence or deficiency of the AtEgy1 protein causes the disorder development of chloroplasts and possibly the development of amyloplasts, which causes a reduced perception of gravity and leads to an abnormal geotropic reaction of seedlings [51, 53].

AtEgy2 is involved in hypocotyl elongation and may be involved in the synthesis of fatty acids [49]. Several enzymes are reduced in the *egy2* mutants involved in the biosynthesis of fatty acids. They are ACP1, CAC2, and BCCP1. However, the exact mechanism by which AtEgy2 affects the level of accumulation of these enzymes is not fully understood. Under non-stress conditions, no significant differences were observed between the *egy2* mutant and the wild-type plant. Parameters such as chlorophyll content in leaves, number of chloroplasts in cotyledons, stem length, and seed weight were taken into account [49].

Least is known about the possible functions of the AtEgy3 protein. It is proteolytically inactive because in its structure the lack of HExxH motif, necessary for the proteolytic activity, has been detected [49].

The AraSP protease is located in the inner membrane of chloroplasts and is essential for the proper development of plastids. Mutants lacking this protein show small size, red cotyledons, poorly developed roots, no apical meristem, and shortened life expectancy to about 20 days. Mutants lacking this protein had a disordered nature of irregularly shaped thylakoids and chloroplasts. AraSP is involved in seed germination. Mutants are also characterized by slow growth and development of inflorescences [50].

Protease S2P2 is the least studied protease. Based on the presence of the catalytic motif, it is inferred that it is proteolytically active. S2P2 plays an important role in the ABA-dependent signal transduction pathway during seed germination as it is involved in the regulation of the expression level of AtHB7, a transcription factor as well as protein phosphatases such as Hab1, Hab2, Hai1, and Ahg3, which are known to be ABA negative regulators [50].

3. EXTRAPLASTID PROTEASES

3.1. The Central Vacuole

In higher plant cells, most hydrolytic activities reside in the central vacuole, which typically contains 50–100% of acid nuclease and 80–100% of acid protease activity of the cell [7]. Various lines of evidence point to an important role of the central vacuole in the execution of programmed cell death in certain developmental scenarios, such as differentiation/death of immature tracheary elements. However, while rupture of the central vacuole and release of hydrolytic enzymes into the cytosol precedes terminal differentiation (death) of immature tracheary elements [54], the central vacuole remains intact, and compartmentation is maintained during senescence of leaves [7].

3.2. Autophagic

The central vacuole might function in the degradation of organelle proteins through the operation of an autophagic pathway. Different autophagic pathways can be distinguished morphologically, even if the underpinning molecular mechanisms overlap [55]. In microautophagy, the tonoplast membrane invaginates, enclosing a portion of the cytoplasm, and the invaginated tonoplast eventually pinches off, releasing a vesicle containing cytosol into the central vacuole. Macroautophagy involves the formation of an autophagosome, *i.e.*, a double membrane-bound structure enclosing portions of the cytoplasm that eventually fuses with the tonoplast and discharges a single membrane-bound autophagic vesicle into the vacuole. Alternatively, whole organelles, *e.g.*, peroxisomes, can be internalized by the central vacuole of yeast cells [1].

Vacuolar inclusions resembling chloroplasts were seen in cells of *A. thaliana* lines where chloroplast development was impaired through inactivation of a component of the chloroplast import machinery, suggesting the operation of an autophagic pathway to dispose of aberrant chloroplasts. Chloroplasts and immunologically detectable Rubisco were also seen in the central vacuole of chemically fixed sections taken from senescing leaves of French bean.

Homologous atg genes are found in the *A. thaliana* genome. Inactivation of A. thaliana AtATG9, AtATG7, and/or AtATG5 through T-DNA insertion interferes with nutrient recycling and renders plants more susceptible to N starvation. Autophagy may also operate in the disposal of oxidatively damaged proteins [1].

3.3. Novel lytic Compartments in Senescing Leaf Cells

Lytic organelles distinct from the central vacuole develop in heterotrophic and photosynthetic cells during processes of active cellular breakdown. In addition to protein storage vacuoles, these aleurone cells contain secondary vacuoles that are more acidic and show intense proteolytic activity when probed with a fluorescent protease substrate. Similarly, small lytic vesicles ('ricinosomes') accumulate in endosperm cells of germinating seeds of Ricinus communis, preceding endosperm cell death [1].

Furthermore, senescing leaf cells contain small (0.5–0.8 lm), senescence-associated vacuoles (SAVs) which are absent from mature, non-senescing leaves but appear in significant numbers during senescence. SAVs have an acid lumen, and their limiting membrane

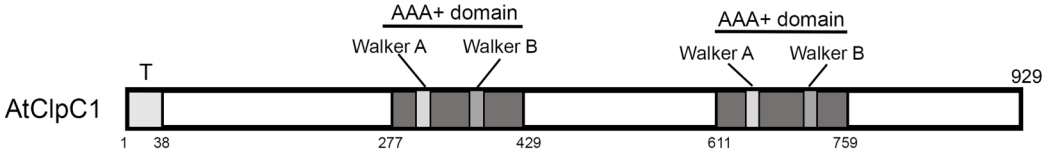

Figure 1: The linear structure of the AtClpC1 chaperone precursor molecule showing the location of the transit peptide and AAA+ domains along with Walker A and Walker B motifs. The data necessary to prepare the drawing was obtained from the NCBI database and the Target P 1.1 server (http://www.cbs.dtu.dk/services/TargetP).

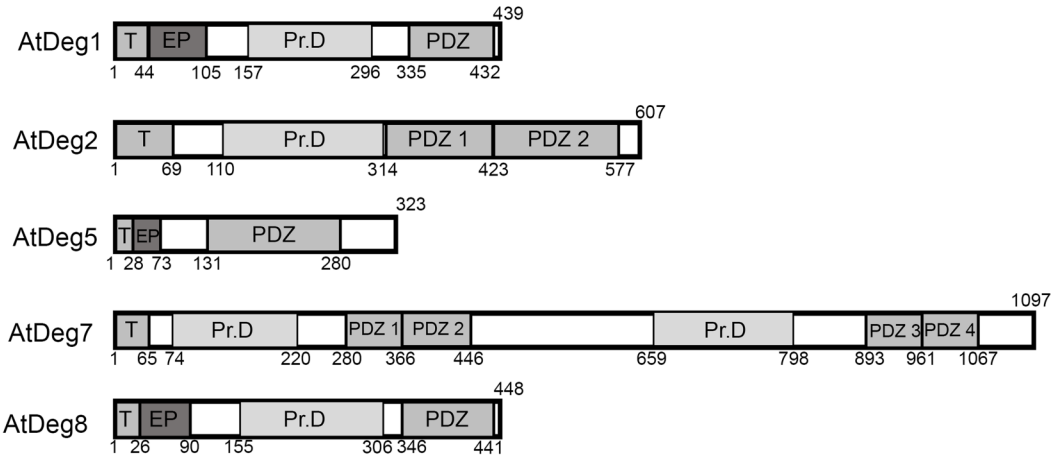

Figure 2: The linear structure of AtDeg chloroplast protease precursor molecules showing the location of transit (T) and export (EP) peptides, protease (Pr.D) and PDZ domains. For AtDeg7, no transit peptide has been identified so far, therefore it is not indicated in the diagram. The data necessary for the drawing was obtained from the NCBI database, the Target P 1.1 server (http://www.cbs.dtu.dk/services/TargetP).

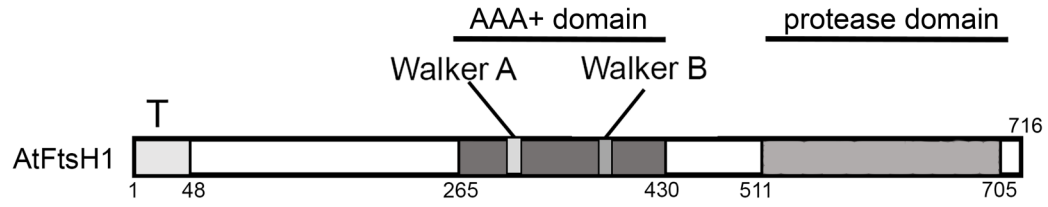

Figure 3: The linear structure of the AtFtsH1 chloroplast protease precursor molecule showing the location of the transit peptide (T), protease and AAA + domains with the Walker A, Walker B motifs. The data necessary to prepare the drawing was obtained from the NCBI database and the Target P 1.1 server (http://www.cbs.dtu.dk/services/TargetP).

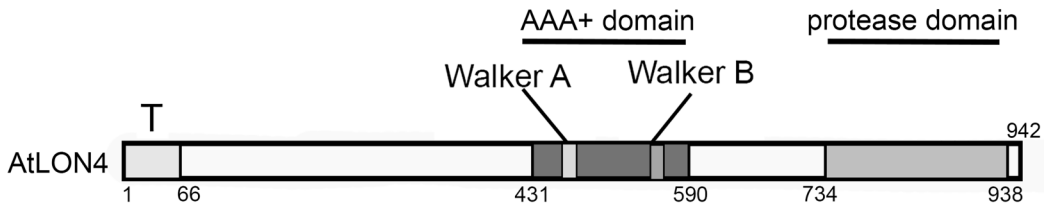

Figure 4: The linear structure of the AtLon4 chloroplast protease precursor molecule showing the location of the transit peptide (T), protease domain and AAA + domains along with Walker A and Walker B motifs. The data necessary to prepare the drawing was obtained from the NCBI database and the Target P 1.1 server (http://www.cbs.dtu.dk/services/TargetP).

contains vacuolar H+-pyrophosphatase, indicating that SAVs are *bona fide* vacuoles. SAVs are more acidic from the central vacuole and lack the aquaporin c-TIP, a marker for the central vacuole tonoplast. *In vivo* studies show that SAVs have intense proteolytic activity, apparently more than any other compartment of the cell, and they also contain the senescence-associated protease, SAG12 [1].

REFERENCES

[1] Martínez DE, Costa ML, Gomez FM, Otegui MS, Guiamet JJ. Senescence-associated vacuoles are involved in the degradation of chloroplast proteins in tobacco leaves. Plant J 2008; 56: 196-206 https://doi.org/10.1111/j.1365-313X.2008.03585.x

[2] Rawlings ND, Barrett AJ. Evolutionaty families of peptidases. Biochem J 2003; 290: 205-218. https://doi.org/10.1042/bj2900205

[3] Lopez-Otin C, Bond JS. Proteases: multifunctional enzymes in life and disease. J. Biol. Chem. 2008; 283: 30433-30437. https://doi.org/10.1074/jbc.R800035200

[4] Pesquet E. Plant proteases - from detection to function. Physiol Plant 2012; 145: 1-4. https://doi.org/10.1111/j.1399-3054.2012.01614.x

[5] Schaller A. A cut above the rest: the regulatory function of plant proteases. Planta 2004; 220: 183-97. https://doi.org/10.1007/s00425-004-1407-2

[6] Hörtensteiner S, Feller U. Nitrogen metabolism and remobilization during senescence. J Exp Bot 2002; 53: 927-937 https://doi.org/10.1093/jexbot/53.370.927

[7] Matile P. The vacuole and cell senescence. Advances in Botanical Research 1997; 25: 87-112. https://doi.org/10.1016/S0065-2296(08)60149-4

[8] Liu X, Jagendorf A. Neutral peptidases in the stroma of pea chloroplasts. Plant Physiol 1986; 81: 603-608. https://doi.org/10.1104/pp.81.2.603

[9] Olinares PD, Kim J, van Wijk KJ. The Clp protease system; a central component of the chloroplast protease network. Biochim Biophys Acta 2011; 1807: 999-1011. https://doi.org/10.1016/j.bbabio.2010.12.003

[10] Nishimura K, van Wijk KJ. Organization, function and substrates of the essential Clp protease system in plastics. Biochim Biophys Acta 2014; S0005-2728(14)00657-4.

[11] Paila YD, Richardson LG, Schnell DJ. New insights into the mechanism of chloroplast protein import and its integration with protein quality control, organelle biogenesis and development. J Mol Biol 2014; S0022-2836(14)00459-8

[12] Sjögren LLE, MacDonald TM, Sutinen S, Clarke AK. Inactivation of the clpC1 gene encoding a chloroplast Hsp100 molecular chaperone causes growth retardation, leaf chlorosis, lower photosynthetic activity, and a specific reduction in photosystem content. Plant Physiol 2004; 136: 4114-4126. https://doi.org/10.1104/pp.104.053835

[13] Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K. A nuclear gene, erd1, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in Arabidopsis thaliana. Plant J 1997; 12: 851-861. https://doi.org/10.1046/j.1365-313X.1997.12040851.x

[14] Sjögren LL, Tanabe N, Lymperopoulos P, Khan NZ, Rodermel SR, Aronsson H, Clarke AK. Quantitative analysis of the chloroplast molecular chaperone ClpC/Hsp93 in Arabidopsis reveals new insights into its localization, interaction with the Clp proteolytic core, and functional importance. J Biol Chem 2014; 289: 11318-11330. https://doi.org/10.1074/jbc.M113.534552

[15] Zheng B, Halperin T, Hruskova-Heidingsfeldova O, Adam Z, Clarke AK. Characterization of chloroplast Clp proteins in Arabidopsis: localization, tissue specificity and stress responses. Physiol Plant 2002; 114: 92-101. https://doi.org/10.1034/j.1399-3054.2002.1140113.x

[16] Adamiec M, Luciński R, Jackowski G. The irradiance dependent transcriptional regulation of AtCLPB3 expression. Plant Science 2011; 181: 449-456. https://doi.org/10.1016/j.plantsci.2011.07.004

[17] Lee U, Wie C, Escobar M, Williams B, Hong SW, Vierling E. Genetic analyses reveals domain interactions of Arabidopsis Hsp/ClpB and cooperation with the sHsp chaperone system. Plant Cell 2005; 17: 559-571. https://doi.org/10.1105/tpc.104.027540

[18] Adamiec M, Luciński R, Jackowski G. The irradiance dependent transcriptional regulation of AtCLPB3 expression. Plant Science 2011; 181: 449-456. https://doi.org/10.1016/j.plantsci.2011.07.004

[19] Myouga F, Motohashi R, Kuromori T, Nagata N, Shinozaki K. An Arabidopsis chloroplast-targeted Hsp101 homologue, APG6, has an essential role in chloroplast development as well as heat-stress response. Plant J 2006; 48: 249-260. https://doi.org/10.1111/j.1365-313X.2006.02873.x

[20] Kim J, Olinares PD, Oh SH, Ghisaura S, Poliakov A, Ponnala L, van Wijk KJ. Modified Clp protease complex in the ClpP3 null mutant and consequences for chloroplast development and function in Arabidopsis. Plant Physiol 2013; 162: 157-179. https://doi.org/10.1104/pp.113.215699

[21] Sjögren LLE, StanneTM, Zheng B, Sutinen S, Clarke AK. Structural and Functional Insights into the Chloroplast ATP-Dependent Clp Protease in Arabidopsis. Plant Cell 2006; 18: 2635-2649. https://doi.org/10.1105/tpc.106.044594

[22] Kim J, Rudella A, Ramirez Rodriguez V, Zybailov B, Olinares PD, van Wijk KJ. Subunits of the Plastid ClpPR Protease Complex Have Differential Contributions to Embryogenesis, Plastid Biogenesis, and Plant Development in Arabidopsis.

Plant Cell 2009; 21: 1669-1692.
https://doi.org/10.1105/tpc.108.063784

[23] Rudella A, Friso G, Alonso JM, Ecker JR, van Wijk KJ. Downregulation of ClpR2 leads to reduced accumulation of the ClppRs protease complex and defects in chloroplast biogenesis in Arabidopsis. Plant Cell 2006; 18: 1704-1721.
https://doi.org/10.1105/tpc.106.042861

[24] Schuhmann H, Adamska I. Deg proteases and their role in protein quality control and processing in different subcellular compartments of the plant cell. Physiol Plant 2012; 145: 224-234.
https://doi.org/10.1111/j.1399-3054.2011.01533.x

[25] Sun R, Fan H, Gao F, Lin Y, Zhang L, Gong W, Liu L. Crystal structure of Arabidopsis Deg2 protein reveals an internal PDZ ligand locking the hexameric resting state. J Biol Chem 2012; 287: 37564-37569.
https://doi.org/10.1074/jbc.M112.394585

[26] Sun X, Fu T, Chen N, Guo J, Ma J, Zou M, Lu C, Zhang L. The stromal chloroplast Deg7 protease participates in the repair of photosystem II after photoinhibition in Arabidopsis. Plant Physiol 2010; 152: 1263-1273.
https://doi.org/10.1104/pp.109.150722

[27] Schuhmann H, Mogg U, Adamska I. A new principle of oligomerization of plant DEG7 protease based on interactions of degenerated protease domains. Biochem J 2011; 435: 167-174.
https://doi.org/10.1042/BJ20101613

[28] Kapri-Pardes E, Naveh L, Adam Z. The thylakoid lumen protease Deg1 is involved in the repair of photosystem II from photoinhibition in Arabidopsis. Plant Cell 2007; 19: 1039-1047.
https://doi.org/10.1105/tpc.106.046573

[29] Sun X, Peng L, Guo J, Chi W, Ma J, Lu C, Zhang L. Formation of DEG5 and DEG8 complexes and their involvement in the degradation of photodamaged photosystem II reaction centre D1 protein in Arabidopsis. Plant Cell 2007; 19: 1347-1361.
https://doi.org/10.1105/tpc.106.049510

[30] Luciński R, Misztal L, Samardakiewicz S, Jackowski G. The thylakoid protease Deg2 is involved in stress-related degradation of the photosystem II light-harvesting protein Lhcb6 in Arabidopsis thaliana. New Phytol 2011; 192: 74-86.
https://doi.org/10.1111/j.1469-8137.2011.03782.x

[31] Luciński R, Misztal L, Samardakiewicz S, Jackowski G. The involvement of Deg5 protease in wounding-related disposal of PsbF apoprotein. Plant Physiol Biochem 2011; 49: 311-320.
https://doi.org/10.1016/j.plaphy.2011.01.001

[32] Sakamoto W, Zaltsman A, Adam Z, Takahashi Y. Coordinated regulation of and complex formation of yellow-variegated1 and yellow variegated2 chloroplastic FtsH metalloproteases involved in the repair cycle of photosystem II in Arabidopsis thylakoid membranes. Plant Cell 2003; 15: 2843-2855.
https://doi.org/10.1105/tpc.017319

[33] Sakamoto W, Tamura T, Hanba-Tomita Y, Murata M, Sodmergen. The VAR1 locus of Arabidopsis encodes a chloroplastic FtsH and is responsible for leaf variegation in the mutant alleles. Genes Cells 2002; 7: 769-780.
https://doi.org/10.1046/j.1365-2443.2002.00558.x

[34] Lindahl M, Spetea C, Hundal T, Oppenheim AB, Adam Z, Andersson B. The thylakoid FtsH protease plays a role in the light-induced turnover of the photosystem II D1 protein. Plant Cell 2000; 12: 419-431.
https://doi.org/10.1105/tpc.12.3.419

[35] Bailey S, Thompson E, Nixon PJ, Horton P, Mullineaux CW, Robinson C, Mann NH. A critical role for the Var2 FtsH homogue of Arabidopsis thaliana in the photosystem II repair cycle in vivo. J Biol Chem 2002; 277: 2006-2011.
https://doi.org/10.1074/jbc.M105878200

[36] Luciński R, Jackowski G. AtFtsH heterocomplex-mediated degradation of apoproteins of the major light harvesting complex of photosystem II (LHCII) in response to stresses. J Plant Phys 2013; 170: 1082-1089.
https://doi.org/10.1016/j.jplph.2013.03.008

[37] Garcia-Lorenzo M, Żelisko A, Jackowski G, Funk C. Degradation of the main photosystem II light-harvesting complex. Photochem Photobiol Sci 2005; 4: 1065-1071.
https://doi.org/10.1039/b506625e

[38] Wagner R, Aigner H, Pružinská A, Jänkänpää HJ, Jansson S, Funk C. Fitness analyses of Arabidopsis thaliana mutants depleted of FtsH metalloproteases and characterization of three FtsH6 deletion mutants exposed to high light stress, senescence and chilling. New Phytol 2011; 191: 449-458.
https://doi.org/10.1111/j.1469-8137.2011.03684.x

[39] Ferro M, Brugière S, Salvi D, Seigneurin-Berny D, Court M, Moyet L, Ramus C, Miras S, Mellal M, Le Gall S, Kieffer-Jaquinod S, Bruley C, Garin J, Joyard J, Masselon C, Rolland N. AT CHLORO, a comprehensive chloroplast proteome database with subplastidial localization and curated information on envelope proteins. Mol Cell Proteomics 2010; 9: 1063-1084.
https://doi.org/10.1074/mcp.M900325-MCP200

[40] Sinvany-Villalobo G, Davydov O, Ben-Ari G, Zaltsman A, Raskind A, Adam Z. Expression in multigene families. Analysis of chloroplast and mitochondria proteases. Plant Physiol 2004; 135: 1336-1345.
https://doi.org/10.1104/pp.104.043299

[41] Chen J, Burke JJ, Velten J, Xin Z. FtsH11 plays a critical role in Arabidopsis thermotolerance. Plant J 2006; 48: 73-84.
https://doi.org/10.1111/j.1365-313X.2006.02855.x

[42] Ostersetzer O, Kato Y, Adam Z, Sakamoto W. Multiple intracellular locations of Lon protease in Arabidopsis: evidence for the localization of AtLon4 to chloroplasts. Plant Cell Physiol 2007; 48: 881-885.
https://doi.org/10.1093/pcp/pcm052

[43] Murakami S, Kondo Y, Nakano T, Sato F. Protease activity of CND41, a chloroplast nucleoid DNA-binding protein, isolated from cultured tobacco cells. FEBS Letters 2000; 468(1): 15-8.
https://doi.org/10.1016/S0014-5793(00)01186-8

[44] Brown MS, Ye J, Rawson RB, Goldstein JL. Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. Cell 2011; 100: 391-398.
https://doi.org/10.1016/S0092-8674(00)80675-3

[45] Adamiec M, Ciesielska M, Zalaś P, Luciński R. Arabidopsis thaliana intramembrane proteases. Acta Physiologiae Plantarum 2017; 39: 146.
https://doi.org/10.1007/s11738-017-2445-2

[46] Feng L, Yan H, Wu Z, Yan N, Wang Z, Jeffrey PD, Shi Y. Structure of site-2-protease family intermembrane metalloproteases. Science 2007; 318(5856): 1608: 12.
https://doi.org/10.1126/science.1150755

[47] Schuhmann H, Huesgen PF, Adamska I. The family of Deg/HtrA proteases in plants. BMC Plant Biol 2012; 12: 52.
https://doi.org/10.1186/1471-2229-12-52

[48] Kinch LN, Ginalski K, Grishin NV. Site-2 protease regulated intramembrane proteolysis: Sequence homologs suggest an ancient signalling cascade. Protein Science: A Publication of the Protein Society 2006; 15(1): 84-93.
https://doi.org/10.1110/ps.051766506

[49] Chen G, Law K, Ho P, Zhang X, Li N. EGY2, a chloroplast membrane metalloprotease, plays a role in hypocotyl elongation in Arabidopsis. Mol. Biol. Rep. 2012; 39: 2147-2155.
https://doi.org/10.1007/s11033-011-0962-4

[50] Botler B, Nada A, Fulgosi H, Soll J. A chloroplastic inner envelope membrane protease is essential for plant development. FEBS Lett 2006; 580: 789-794.
https://doi.org/10.1016/j.febslet.2005.12.098

[51] Chen G, Bi YR, Li N. EGY1 encodes a membrane-associated and ATP independent metalloprotease that is required for chloroplast development. The Plant J 2005; 41: 364-375. https://doi.org/10.1111/j.1365-313X.2004.02308.x

[52] Adamiec M, Misztal L, Kasprowicz-Maluśki A, Luciński R. EGY3: homologue of S2P protease located in chloroplasts. Plant Biol 2020; 22: 735-743. https://doi.org/10.1111/plb.13087

[53] Guo D, Gao X, Li, H, Zhang T, Chen G, Huang P, An L, Li N. EGY1 plays a role in regulation of endodermal plastid size and number that are involved in ethylene-dependent gravitropism of light-grown Arabidopsis hypocotyls. Plant Mol Biol 2008; 66: 345-360. https://doi.org/10.1007/s11103-007-9273-5

[54] Kuriyama H, Fukuda H. Developmental programmed cell death in plants. Curr Opin Plant Biol 2002; 5: 568-573. https://doi.org/10.1016/S1369-5266(02)00305-9

[55] Thompson AR, Vierstra RD. Autophagic recycling: lessons from yeast help define the process in plants. Curr. Opin Plant Biol 2005; 8: 165-173. https://doi.org/10.1016/j.pbi.2005.01.013

Received on 28-02-2021 Accepted on 09-10-2021 Published on 29-10-2021

DOI: https://doi.org/10.12974/2311-858X.2021.09.5