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Review

Plastid intramembrane proteolysis☆

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ABSTRACT

Progress in the field of regulated intramembrane proteolysis (RIP) in recent years has not surpassed plant biology. Nevertheless, reports on RIP in plants, and especially in chloroplasts, are still scarce. Of the four different families of intramembrane proteases, only two have been linked to chloroplasts so far, rhomboids and site-2 proteases (S2Ps). The lack of chloroplast-located rhomboid proteases was associated with reduced fertility and aberrations in flower morphology, probably due to perturbations in jasmonic acid biosynthesis, which occurs in chloroplasts. Mutations in homologues of S2P resulted in chlorophyll deficiency and impaired chloroplast development, through a yet unknown mechanism. To date, the only known substrate of RIP in chloroplasts is a PHD transcription factor, located in the envelope. Upon proteolytic cleavage by an unknown protease, the soluble N-terminal domain of this protein is released from the membrane and relocates to the nucleus, where it activates the transcription of the ABA response gene ABI4. Continuing studies on these proteases and substrates, as well as identification of the genes responsible for different chloroplast mutant phenotypes, are expected to shed more light on the roles of intramembrane proteases in chloroplast biology.

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1. Introduction

Hydrolysis of a peptide bond in a transmembrane α-helix, within the hydrophobic core of a membrane, has seemed anomalous. However, this intramembrane proteolysis has been recognized in the past two decades or so as a ubiquitous process occurring in all forms of life. Such cleavage events are involved in numerous biological processes and regulate many different functions. Four different families of proteases have been demonstrated to mediate intramembrane proteolysis: rhomboid proteases, site-2 proteases, signal peptide peptidases and presenilin/γ-secretases. Together with the demonstration of their role in signaling, membrane remodeling, protein quality control, cell adhesion and communication, their fundamental role in development and physiology of eukaryotes and prokaryotes alike have been consolidated (for reviews, see [1–3].

The progress made in the field of regulated intramembrane proteolysis (RIP) in recent years has radiated into plant biology as well [4]. Although the number of reports on RIP in plants is still sparse, interesting observations have started to accumulate. The ubiquitous nature of RIP and the enzymes responsible for this process has prompted plant biologists to look for homologs of these enzymes in plants, and to incorporate the concepts associated with RIP into their hypotheses. Independently of these, analysis of specific mutants culminated in the identification of mutations in specific intramembrane proteases as responsible for certain mutant phenotypes. In yet another line of research, potential substrates for intramembrane proteolysis in plants have been identified, although the responsible proteases are still unknown. In the following, the limited available information on these subjects that is relevant to chloroplast biology is summarized and evaluated.

2. Intramembrane proteases in plastids

2.1. Rhomboid proteases

Rhomboids are widely spread intramembrane serine proteases that are found in nearly all sequenced organisms. They are involved in different biological functions such as signaling, development, apoptosis, organelle integrity, parasite invasion and more (for recent reviews, see [5,6]). The well-studied GlpG rhomboid protease of *Escherichia coli,* which can be considered as a paradigm for this family, contains six transmembrane helices, incorporating the catalytic dyad of Ser-His. These helices form a conical cavity that is open to the aqueous phase, providing the hydrophilic environment required for hydrolysis of a peptide bond within a transmembrane helix of the substrate protein (Fig. 1, [5,6].

Of the 16 genes related to rhomboid proteases found in the Arabidopsis genome, one belongs to the PARL-type (At1g18600), and three (At1g74130, At1g77860, At5g38510) are expected to be inactive due to lack of conservation in and around the active serine [6,7]. It should be noted that contradicting nomenclature already exists in the literature. Thus, we will use here the one of Lemberg and Freeman [7], along with the other published names where relevant.

The occurrence of rhomboid homologs in plants was first mentioned in 2001 [8]. Koonin et al. [9] have then identified Arabidopsis sequences

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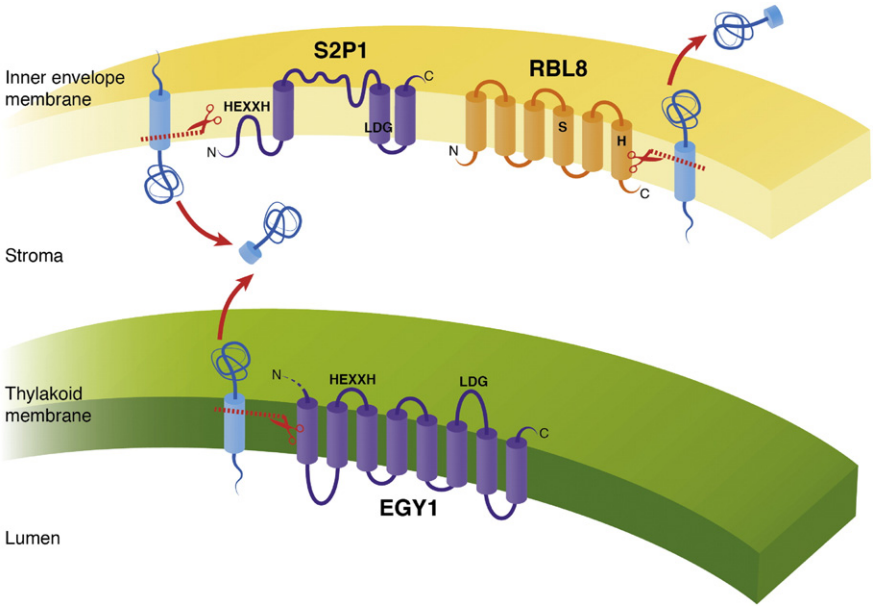

Fig. 1. Schematic model of three chloroplast intramembrane proteases. Two metalloproteases of the S2P family and one serine protease of the rhomboid family are colored in purple and orange, respectively. The HEXXH zinc-binding motif and the highly conserved LDG motif of S2Ps as well as the catalytic dyad of rhomboids (Ser and His) are indicated. As the structures of S2Ps are not yet determined, the location of transmembrane helices and the critical motifs are predicted based on hydropathy plots and the TMHMM (v. 2) program. Since the length of the N-terminus of mature EGY1 is not known, it is depicted as a broken line. Potential substrate proteins are colored in blue, and cleavage sites are indicated by red dotted lines. Predicted locations of the soluble products of proteolytic cleavages are also illustrated.

containing the conserved active serine and its surrounding residues (GA**S**GA), characteristic of rhomboid proteases. The first report on experimental work with plant rhomboids appeared shortly afterwards [10]. Based on their homology to the Drosophila Rho-1, eight sequences were identified in the Arabidopsis genome, although their overall sequence similarity was relatively low, less than 20%. Further characterization of two of these, AtRBL1 and AtRBL2 (for *Arabidopsis thaliana* rhomboid-like), revealed that their transcripts accumulated in all tissues, and transient expression assays of GFP fusions in protoplasts suggested that they located in the Golgi apparatus [10]. Testing their activity in a mammalian cell transfection system demonstrated that AtRBL2, but not AtRBL1, could cleave Drosophila substrates, suggesting that at least AtRBL2 is a *bona fide* rhomboid protease. However, since the known Drosophila substrates of Rho-1 do not have homologs in plants, it is believed that rhomboid proteases in plants have their own specific substrates.

The first report on the involvement of plant rhomboids in chloroplast biology was that of Karakasis and co-workers [11]. Reasoning that diverse rhomboid substrates have in common two features, a single transmembrane domain and a large soluble domain, they attempted to link between Tic40, a protein believed to be a component of the protein import machinery into chloroplasts, and one Arabidopsis rhomboid-like protein. Using a mitochondria-based system, they showed that Tic40 could be processed by the product of the At1g74130 gene, suggesting a role for this protein in the biogenesis of the chloroplast protein import machinery. Nevertheless, this suggestion is debatable, as the product of the aforementioned gene lacks the conserved catalytic serine and histidine residues, and thus is expected to be proteolytically inactive [6].

Two other Arabidopsis rhomboid-like proteases that were studied are AtRBL9 and AtPARL (designated AtRBL11 and AtRBL12 in the original paper) [12]). Transient expression assays of GFP fusions suggested that these proteins were targeted to chloroplasts and mitochondria, respectively [12]. However, the Arabidopsis mitochondrial rhomboid failed to complement the corresponding yeast mutant and did not recognize the yeast substrates cytochrome *c* peroxidase and a dynamin-like GTPase.

More recently, AtRBL8 was also identified in chloroplasts and was located to the envelope membrane ([13] and Table 1). Interestingly, the Arabidopsis mutant lacking this protein demonstrated reduced fertility and aberrant flower morphology [14]. Proteomic analysis of a double mutant lacking both AtRBL8 and its homolog AtRBL9 revealed that in the absence of these two rhomboid proteases the level of allene oxide synthase (AOS) was affected, although it was not determined which of these two was responsible for this effect [13]. As AOS is involved in the synthesis of the plant hormone jasmonic acid, this observation provides a link between the lack of chloroplast rhomboid proteases and the morphologic phenotype. Another interesting observation was that AtRBL9 forms homo-oligomers [13]. Although the functional significance of rhomboid oligomerization is still unknown, it is interesting to note that bacterial rhomboids were recently reported to oligomerize as well [15].

Table 1
Identified plastid intramembrane proteases and substrates for intramembrane proteolysis in *Arabidopsis thaliana*.

Protein	Gene locus	Intraplastid location	Biological function	Reference
Proteases				
Rhomboids				
AtRBL8	At1g25290	Inner envelope	Jasmonic acid, biosynthesis, flower morphology	[13,14]
AtRBL9	At5g25752	Inner envelope	Unknown	[12,13]
S2Ps				
AtS2P1	At2g32480 (AraSP)	Inner envelope and/or thylakoid	Chloroplast development	[19]
AtS2P2	At1g05140	Thylakoid		[20]
AtEGY1	At5g35220	Thylakoid	Chloroplast development, ABA signaling	[20–23]
AtEGY2	At5g05740	Thylakoid	Hypocotyl elongation	[25]
Substrates				
AtPHD	At5g35210	Envelope	Chloroplast nucleus signaling	[38]

2.2. Site-2 protease (S2P)

S2Ps belong to a large family of metalloproteases, found in many different eukaryotic and prokaryotic organisms, where they are involved in stress response, cell division, bacterial mating, pathogenesis and more. The core structure of S2P proteases consists of at least four hydrophobic regions, with the conserved Zn^{2+} binding motif HEXXH of the active site located within or adjacent to two of them. A highly conserved LDG motif is also found within the corresponding membrane in all proteases of this family. The hydrophobic sequences around the active site create a favorable environment for cleavage of substrates within their transmembrane helices (Fig. 1). For most recent reviews, see [16–18].

The Arabidopsis genome contains a number of genes encoding homologs of the well-characterized bacterial S2Ps RseP and YluC from *E. coli* and *Bacillus subtilis*, respectively. Of these, AtS2P1 and AtS2P2 resemble most the cyanobacterial protein of *Synechocystis 6803* (Table 1 and Fig. 2). The former was previously designated AraSP [19], for Arabidopsis serine protease, but being clearly a metalloprotease, it is proposed here to name it AtS2P1. It was localized to the chloroplast inner envelope membrane (Fig. 1), and antisense and T-DNA insertion lines of this protease demonstrated severely impaired chloroplast biogenesis [19]. Similarly, the close homolog AtS2P2 was also identified in proteomic studies of chloroplasts.

A genetic screen for Arabidopsis mutants displaying reduced chlorophyll accumulation and deficiency in ethylene-induced gravitropism revealed AtEGY1, a 59-kDa membrane-bound metalloprotease homologous to S2P, located in the chloroplast [20]. It contains eight transmembrane α-helices (Fig. 1) and is proteolytically active. Although the intraorganellar location of AtEGY1 was not determined in that work, it was identified in thylakoid membranes in proteomic studies (see the Plant Proteomics Database, PPDB, [21]; http://ppdb.tc.cornell.edu). Mutant plants had reduced levels of grana stacking and light-harvesting complex (LHC) proteins, suggesting that this protease is required for proper chloroplast development [20]. In the absence of AtEGY1, pleiotropic effects were observed also in size and number of plastids, as well as in ethylene-dependent gravitropic growth [22]. More recently, analysis of an Arabidopsis mutant hypersensitive to NH_4^+ stress revealed a mutation in the gene encoding AtEGY1 as responsible for this phenotype [23]. Moreover, it appears that EGY1 integrates abscisic acid (ABA) signaling to regulate the expression of NH_4^+-responsive genes, although it is not known how. The chlorophyll-deficient phenotype of the tomato mutant *lutescent2* was recently also attributed to an EGY1 ortholog [24], supporting the proposed role of the protease in chloroplast development. However, mechanistic insights into this process are still missing.

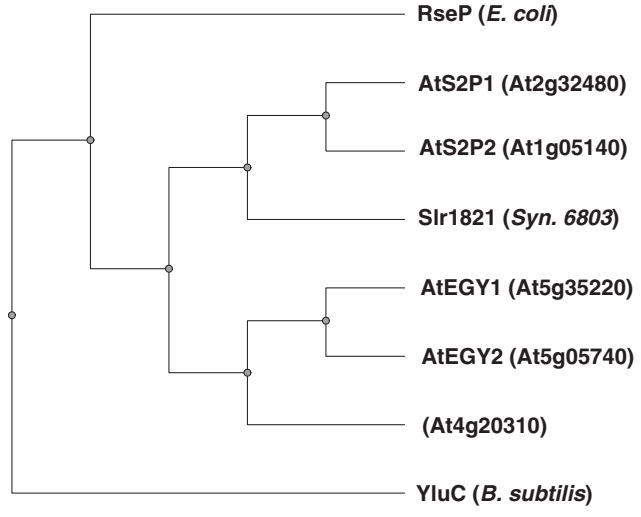

Fig. 2. A phylogenetic tree of Arabidopsis S2Ps. *Arabidopsis thaliana* sequences showing homology to *Escherichia coli* and *Bacillus subtilis* S2Ps (RseP and YluC, respectively) and their cyanobacterial homolog were retrieved from TAIR. Sequences lacking the characteristic and highly conserved motifs HEXXH and LDG were omitted from the analysis. Predicted chloroplast targeting sequences, according to the ChloroP program, were removed, and the remaining sequences were subjected to multiple sequence alignment with the MAFFT (v. 7) program, allowing large gaps. The phylogenetic tree was constructed using the PHYLIP program.

Sequence comparisons of the AtEGY1 protease revealed two other homologs: AtEGY2 and At4g20310 [25]. AtEGY2 resides in chloroplasts, contains seven transmembrane domains, and like AtEGY1, the recombinant protein degrades β-casein in vitro. Arabidopsis EGY2 knockout plants looked very similar to the wild type, however, their hypocotyls were somewhat shorter, and the level of their fatty acids was lower [25]. Nevertheless, how these proteases are involved in chloroplast biogenesis is not clear.

Although At4g20310 has all characteristics of S2P, there is currently no experimental evidence for its accumulation, at least not in chloroplasts. Other gene products showing similarities to S2Ps are found in the database, however, they should not be considered as true homologs as they lack conserved features of the protease. For instance, At1g17870, also designated EGY3, lacks the HEXXH motif, and At1g56180 does not contain the LDG motif.

3. Other intramembrane proteases

3.1. Signal peptide peptidase (SPP)

The aspartic protease SPP is another protein that belongs to the class of intramembrane proteases. In all eukaryotes studied, it is located in the ER membrane, with its N- and C-termini exposed to the lumen and the cytosol, respectively. Its active site sequences include YD and GXGD and its substrates are type II membrane signal peptides that need to be processed (see [19,26,27]). The Arabidopsis genome contains six orthologs of SPP, designated AtSPP and AtSPPL1-5 (for SPP-like) [26]. Transcripts of AtSPP and AtSPPL1-3 were found in all tissues whereas those of AtSPPL4 and 5 were undetectable. Expression of SPP-GFP fusions revealed that AtSPP localizes to the ER, and AtSPPL1 and 2 reside in endosomes [26]. Thus, there is no evidence to date for the presence of SPP in chloroplasts. The available information on the physiological roles of the above proteins was recently summarized in [4].

Chloroplasts do contain other peptidases that, due to their names, might be confused with SPP. These include the thylakoid processing peptidase (TPP), also known as type I signal peptidase (a serine protease) [28], the stromal processing peptidase (also abbreviated SPP) (a metalloprotease) [29] and the serine protease SppA [30]. However, none of these is an intramembrane protease as they do not cleave within transmembrane helices, and they do not share sequence homology with either SPP or any other intramembrane protease.

3.2. Presenilin/γ-secretase

The least studied intramembrane protease in plants to date is presenilin/γ-secretase. Presenilin/γ-secretases are aspartic proteases that cleave type I substrates within their transmembrane domains to release C- and N-terminal peptides. Their catalytic aspartate residues are found within the conserved sequence of YD/GXGD [31,32]. The Arabidopsis genome contains two genes encoding presinilins, whereas the genome of the moss *Physcomitrella patens* contains only one. However, there is no evidence for the localization of any of these gene products to chloroplasts. Knocking out the moss gene resulted in pleotropic phenotypic defects, including straight instead of curly filament growth, which could be linked to impaired function of the cytoskeleton [33]. The WT phenotype could be rescued by expression of either the WT presinilin protein or its proteolytically inactive variant, suggesting that the activity of presinilin in this model is independent of γ-secretase [33]. However, the mechanistic details of this phenomenon

are yet to be deciphered, and there is no reasonable way to link it to chloroplasts.

4. RIP substrates

In 2007, Liu et al. have reported on what appears to be the first two documented cases in plants where RIP substrates, their subcellular location and the relevant protease were described [34,35]. Upon treatment of Arabidopsis plants with tunicamycin, an inhibitor of N-linked protein glycosylation, they observed in vivo processing of the ER membrane-bound bZIP28 transcription factor. This cleavage releasing the N-terminal half of the protein into the cytoplasm allowed its translocation to the nucleus. This in turn elicited the upregulation of expression of ER stress response genes [34]. More details on this process as well as on other bZIP transcription factors operating in a similar manner have been recently reviewed [4].

This paradigm, of a membrane-anchored transcription factor, that is proteolytically cleaved within the hydrophobic core of the membrane, releasing a soluble factor that migrates to the nucleus and activates transcription, appears to be relevant also to chloroplasts. Communication between the chloroplast and the nucleus is essential for the biogenesis and function of the chloroplast. This has been the subject of intensive studies over the past two decades; however, the nature of the molecular relay has remained elusive (for reviews, see [36,37]). A major breakthrough in this field was the recent identification of a chloroplast envelope-bound PHD transcription factor that possesses transmembrane domains. Upon proteolytic cleavage, the soluble N-terminal domain of this protein is released from the membrane and relocates to the nucleus, where it activates the transcription of the ABA response gene ABI4 [38]. The protease involved in this process is still unknown, but it is most likely one of the intramembrane proteases capable of cleaving within transmembrane α-helices. It will not be surprising if similar chloroplast-bound transcription factors are identified in the future.

5. Conclusion

The expanding field of RIP has not surpassed plant biology. Nevertheless, reports on RIP in plants are still scarce, and even more so on such processes in chloroplasts. Of the four different families of intramembrane proteases, only two have been linked to chloroplasts so far, rhomboids and S2Ps. The first has been linked to jasmonic acid biosynthesis and the second to chloroplast development. However, it is not clear yet how they exert their effects on these processes.

It appears that the best-characterized RIP process in plants is the ER stress response. It is quite well established now that upon such stress, bZIP transcription factors embedded in the ER membrane are released, relocated to the nucleus, and activate the transcription of BiP chaperones that function in the lumen of the ER. Circumstantial evidence suggests that S2P is the protease involved in this process, but this needs to be further confirmed. Furthermore, how the presence of ill-folded protein in the ER is sensed, and the signal transduced to induce proteolytic cleavage is an enigma. It will be interesting to see whether an analogous RIP process operates in response to folding stress also in chloroplasts.

Other membrane-bound transcription factors, of the NAC and PHD families, have also been documented to be involved in intracellular signaling in plants. Of particular interest is the PHD transcription factor, apparently involved in communication between the chloroplast and the nucleus. However, the identity of the protease involved in the cleavage and what initiates it await deciphering.

The occurrence of S2Ps in plants and their importance for proper development of chloroplasts is also established, but mechanistic details of their involvement are obscure. Similarly, the presence of rhomboid proteases in chloroplast envelope membranes was demonstrated, but their link with developmental processes or other responses is still missing. These are expected to be the subjects of future studies, and together with studies originating from deciphering the genetic and molecular basis for specific phenotypes in different mutants, will most likely shed more light on the roles of intramembrane proteases in chloroplast biology.

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