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REVIEW

Arabidopsis thaliana intramembrane proteases

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Abstract Proteolysis is considered as a crucial factor determining the proper development of the plant and its efficient functioning in variable environmental conditions. The role of proteases in protein quality control and protein turnover processes is well documented. The results of studies performed in recent years reveal; however, that proteolytic enzymes also participate in signal transduction pathways by releasing membrane-anchored transcription factors in the process known as regulated intramembrane proteolysis (RIP). The first described intramembrane protease was identified in human cells in 1997. In turn, the first plant intramembrane protease was identified in 2005, in *Arabidopsis thaliana*. To date, most studies concerning the RIP process in plants have been performed on this model plant. The knowledge concerning the potential physiological role of RIP is very limited. However, continuously accumulating information concerning this issue indicates that RIP, like the other proteolytic mechanisms, has a significant effect on plant ontogenesis, acclimatization and fertility. The aim of this article is to gather and systemize the present knowledge concerning the intramembrane proteases in *A. thaliana*.

Keywords Regulatory intramembrane proteolysis · Intramembrane proteases · Site-2 proteases · Rhomboid · Presenilin

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Introduction

Plant development and adaptation to constantly changing environmental conditions require many precise supervising mechanisms. One of the most important factors controlling these processes is proteolysis. Proteases are involved in protein quality control and protein turnover processes. Protein quality control includes the hydrolysis of proteins, misfolded or damaged by the exposure of the plants on stress factors, as well as the hydrolysis of proteins synthesized in redundant quantities or sorted to an incorrect cell compartment. Protein turnover, in contrast, comprises hydrolysis of proteins which, in a given spatio-temporal context become unnecessary. However, knowledge accumulated in the last several years has revealed another, previously unknown mechanism of proteolytic control—regulated intramembrane proteolysis (RIP). RIP is performed by intramembrane proteases—an integral membrane proteins able to hydrolyze a transmembrane helix of their substrates and release them from the membrane. This relatively recently discovered class of proteases occurs ubiquitously in all living organisms from bacteria through archaea to eukarya (Adam 2013; Schneider and Glickman 2013; Knopf et al. 2012). The first described intramembrane protease was site-2 protease (S2P), identified in human cells in 1997 (Rawson et al. 1997). Homologous proteins were identified in the genomes of archaea, and Gram-positive and Gram-negative eubacteria (Rudner et al. 1999). The known substrates for intramembrane proteases are mostly membrane-bound transcription factors or anti-sigma factors, that both are common parts of mechanisms regulating gene expression in bacterial systems (Hughes and Mathee 1998). The intramembrane proteases have been shown to participate in numerous very divergent processes. In mammalian cells, S2P substrates are involved in lipid

metabolism (SREBPs—sterol regulatory element binding proteins), the endoplasmic reticulum stress response (ATF6α, ATF6β, CREBH) and dendritic cell activation (Rawson 2013). The prokaryotic substrates of S2P are involved in the stress response (RseA), cell division (PodJs), sporulation (pro-SigK) and pathogenesis (Schneider and Glickman 2013). The homologs of intramembrane proteases are also present in plants. To date, four families of intramembrane proteases have been identified in *Arabidopsis thaliana*: site-2 proteases (S2Ps), rhomboids, presenilins and signal peptide peptidases. Knowledge concerning the physiological functions of these proteases and their role in *Arabidopsis thaliana* development is constantly accumulating.

The S2P homolog from *A. thaliana*—EGY1, was the first intramembrane protease identified in plants (Chen et al. 2005). 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. Two other highly conserved motifs were also necessary for their proteolytic activity. The first of them, with the consensus GpxxN/S/G, is usually present in the second transmembrane domain (TM), and the second, with the consensus NxxPxxxxDG, is usually present in the third TM (Feng et al. 2007). 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 activation of the protease domain (Schuhmann et al. 2012; Kinch et al. 2006; Saras and Heldin 1996).

To date, in *A. thaliana* six genes encoding homologs of S2P protease have been identified, and five proteins encoded by these genes are considered to be proteolytically active (see Table 1). EGY1, EGY2, ARASP, and S2P2 were shown to be targeted to chloroplasts. EGY2 was found to be located in the thylakoid membrane (Chen et al. 2012), while ARASP was identified as an inner-envelope membrane protein (Bötler et al. 2006). The precise chloroplast localization of EGY1 and S2P2 still remains unknown (Zybailov et al. 2008; Chen et al. 2005). The protein encoded by the AT4G20310 gene has been demonstrated to be directed to the Golgi membrane (Che et al. 2010). The localization of EGY3 proteins remains unconfirmed, but AramLocCon predicts localization of this protein in the chloroplast (Schwacke et al. 2007).

The S2P proteases were found to play important role in *A. thaliana* growth and development; however, knowledge of the exact physiological processes in which they participate remains very limited. The *egy1 A. thaliana* mutants displayed deficiency in ethylene-induced gravitropism (Guo et al. 2008) and chlorophyll accumulation, as well as reduced levels of grana stacking and light-harvesting complex (LHC) proteins, suggesting that this protease is required for proper chloroplast development (Chen et al. 2005).

In addition, ARASP has been demonstrated to be required for proper chloroplast biogenesis and essential for plant development. The most severe phenotype of *araSP* mutants was characterized by very small size, red color of cotyledons, underdeveloped roots, no apical meristem and life expectancy of less than 20 days (Bötler et al. 2006). The ARASP protein tightly coexpresses with S2P2 (encoded by the AT1G5140 gene; Aoki et al. 2016). There is; however, no direct evidence indicating cooperation of these proteins, or any experimental research indicating the potential role of S2P2.

EGY2, in turn, was found to be involved in regulation of the level of accumulation of several enzymes involved in fatty acid biosynthesis, namely ACP1 (acyl carrier protein 1), CAC2 [biotin carboxylase subunit of the plastidic acetyl-coenzyme A carboxylase (ACCase)] and BCCP1 (biotin carboxyl carrier protein, subunit of ACCase), but the physiological effects of its deficiency were far less severe than in the case of *araSP* or *egy1* mutants (Chen et al. 2012). The molecular mechanism leading to changes

Table 1 Localization of S2P proteins

Gene accession number	Protein name	Localization	Proteolytic activity	References
AT5G35220	EGY1	**Chloroplast**	**Active**	Chen et al. (2005)
AT5G05740	EGY2	**Chloroplast (thylakoid membrane)**	**Active**	Chen et al. (2012)
AT1G17870	EGY3	*Chloroplast*	*Inactive*	–
AT4G20310	–	**Golgi membrane**	**Active**	Che et al. 2010)
AT1G05140	S2P2	**Chloroplast**	*Active*	Zybailov et al. (2008)
AT2G32480	ARASP	**Chloroplast (envelope membrane)**	*Active*	Bötler et al. (2006)

The localization and activity confirmed experimentally are indicated in bold, while predicted in italic. Localization in italic are predicted by AraLoc Con algorithm (Schwacke et al. 2007), the prediction of protein activity was based on primary structure analysis with the use of MOTIF (sequence motif search tool from Kyoto University Bioinformatics Center; http://www.genome.jp/tools/motif/)

in the accumulation of enzymes involved in fatty acid biosynthesis remains; however, unknown, and the connection between EGY2 protease and those changes remains to be clarified. In non-stressing conditions no significant differences between *egy2* and wild type plants were observed in chloroplast number in cotyledons, chlorophyll content of leaves, length of inflorescent stem, or weight of seeds (Chen et al. 2012).

Even less is known about the possible functions of EGY3 protein. According to the ARANETv2 platform the *EGY3* gene is probably involved in the response to heat, which is the feature clearly distinguishing it from the *EGY1* and *EGY2* genes (Lee et al. 2015). The transcript accumulates in roots and shoots after 1 h of heat stress. *EGY3* is also co-expressed tightly with several genes encoding chaperonins (e.g., AT1G54050, AT5G37670, AT4G10250) and heat shock proteins such as Hsp70b, Hsp23.6-Mito or Hsp21, which are known to be involved in ER-associated protein degradation (Aoki et al. 2016, Kenehisa and Goto 2000). The expression level of *EGY3* also increases in shoots during long-term osmotic stress (12 h) and in response to ABA treatment. In most development stages the expression level of *EGY3* remains at a relatively low level. Transcript accumulation is observed only in mature pollen and during seed maturation, where the transcript accumulates gradually from the curled stage of the embryo to achieve the highest accumulation level in dry seeds (Winter et al. 2007).

The S2P protease encoded by At4g20310 gene was shown to play an important role in ABA signaling during seed germination (Zhou et al. 2015). According to a proposed mechanism, the direct substrate for the AT4G20310 protease is bZIP17—a membrane-associated transcription factor that was shown to relocate from the endoplasmic reticulum (ER) to the Golgi (Che et al. 2010). In response to ABA, the bZIP17 is relocated from ER to the Golgi and its proteolytic cleavage by site-1 protease (S1P) and S2P is performed. The active form of the transcription factor released from the membrane is directed to the nucleus where it participates in regulation of the expression level of the AtHB7 transcription factor and protein phosphatases HAB1, HAB2, HAI1 and AHG3, which are known to be negative regulators of abscisic acid (Zhou et al. 2015). The S1P/S2P-dependent activation of bZIP17 also occurs in response to salt stress. It is thought that in this case the bZIP17 relocation and processing are activated by accumulation, in endoplasmic reticulum, of unfolded or misfolded proteins as part of a mechanism known as unfolded protein response (UPR) (Fu and Gao 2014; Liu et al. 2007). The bZIP17N-terminal domain released by proteolytic cleavage is translocated to the nucleus where in cooperation with bZIP60 it activates salt stress-responsive and ER stress-induced genes (Silva et al. 2015). The AT4G20310 encoded protease also participates in the heat stress response via activating bZIP28, which similarly to bZIP17 is a membrane-anchored transcription factor located in the ER (Gao et al. 2008) and in response to heat stress is relocated to the Golgi apparatus and cleaved by S1P and S2P (Silva et al. 2015). The released domain is transported to the nucleus where it integrates with other proteins to form a heterotrimeric NF-Y complex and recognizes promoters of genes regulated by UPR (Silva et al. 2015). The RIP-dependent activation of both bZIP17 and bZIP28 was also demonstrated to be involved in activation of brassinosteroid signaling, which, in turn, participates in regulation of many different physiological and developmental processes (Che et al. 2010).

Rhomboids in *A. thaliana*

The first described rhomboid protease was Rho-1 from *Drosophila melanogaster*. The protease was shown to be involved in activation of epidermal growth factor receptor (EGFR) ligands. A mutation within the gene encoding the protein resulted in skeleton deformations in *Drosophila* larvae, leading to a characteristic "rhombus-like" shape of their heads (Ha et al. 2013).

The rhomboid proteases belong to serine-type proteases, and their active site is constituted by a serine-histidine catalytic dyad (Erez et al. 2009). Both catalytic amino acid residues are located within transmembrane domains in evolutionarily conserved motifs. The consensus motif containing the catalytically active serine residue is GxSx, and in close proximity of catalytically active histidine two glycine residues are always present in the HxxGxxxG consensus (Lemberg and Freeman 2007). The rhomboid proteases contain a variable number of TMs, from six to eight, and relatively heterogeneous primary structure. Due to internal differentiation of this family two subfamilies were distinguished: PARLs and secretases. The secretases were further divided into A-and B-secretases and mixed other secretases. Proteolytically inactive proteins with high similarity to rhomboid proteases were also identified. These proteins were described as the rhomboid-like family and were divided into iRhom and mixed inactive homolog subfamilies (Lemberg and Freeman 2007).

In the *A. thaliana* genome, 20 genes encoding proteins containing the rhomboid domain have been identified (Tripathi and Sowdhamini 2006; Lemberg and Freeman 2007; Garcia-Lorenzo et al. 2006). Among these 20 proteins 13 may be considered as proteolytically active since they contain both evolutionarily conserved motifs with catalytically active amino acids, although proteolytic activity has been confirmed experimentally only in one of them (Kanaoka et al. 2005) (for details see Table 2). The remaining seven proteins lack at least one of the amino

Table 2 Localization and proteolytical activity of rhomboid homologs in *A. thaliana*

Gene accession number	Protein name	Rhomboid type	Localization	Proteolytic activity	References
AT2G29050	RBL1	Mixed-secretase	**Golgi apparatus**	*Active*	Kanaoka et al. (2005)
AT1G63120	RBL2	Mixed-secretase	**Golgi apparatus**	**Active**	Kanaoka et al. (2005)
AT5G07250	RBL3	Mixed-secretase	**Plasmodesmata**	*Active*	Fernandez-Calvino et al. (2011)
AT3G53780	RBL4	Mixed-secretase	**Plasma membrane, plasmodesmata**	*Active*	Benschop et al. (2007), Fernandez-Calvino et al. (2011)
AT1G52580	RBL5	Mixed-secretase	*Chloroplast*	*Active*	–
AT1G12750	RBL6	Mixed-secretase	*Mitochondria*	*Active*	–
AT4G23070	RBL7	Mixed-secretase	*Secretory pathway*	*Active*	–
AT1G77860	KOM	Mixed inactive homologs	*Chloroplast*	*Inactive*	–
AT5G38510	RBL9	Mixed inactive homologs	*Chloroplast*	*Inactive*	–
AT1G25290	RBL10	Mixed-secretase	**Chloroplast**	*Active*	Thompson et al. (2012)
AT5G25752	RBL11	Mixed-secretase	**Chloroplast, chloroplast inner membrane**	*Active*	Kmiec-Wisniewska et al. (2008), Knopf et al. (2012)
AT1G18600	RBL12	PARL	**Mitochondria**	*Active*	Kmiec-Wisniewska et al. (2008)
AT3G59520	RBL13	Secretase B	*Secretory pathway*	*Active*	–
AT3G17611	RBL14	Secretase B	**Plasma membrane**	*Active*	Inzé et al. (2012)
AT3G58460	RBL15	Secretase B	*Mitochondria/secretory pathway*	*Active*	–
AT1G74130	RBL16	Mixed inactive homologs	*Chloroplast/mitochondria*	*Inactive*	–
AT1G74140	–	Mixed inactive homologs	*Chloroplast*	*Inactive*	–
AT2G41160	–	–	*Secretory pathway*	*Inactive*	–
AT3G07950	–	–	*Secretory pathway*	*Inactive*	–
AT3G56740	–	–	*Secretory pathway*	*Inactive*	–

The experimentally confirmed localization and activity are marked in bold. The predicted localization and activity are marked in italic. The prediction of proteins localization was made with AraLoc Con (Schwacke et al. 2007), the prediction of protein activity was based on primary structure analysis with the use of MOTIF (sequence motif search tool from Kyoto University Bioinformatics Center; http://www.Genome.jp/tools/motif/)

The protein nomenclature was based on (Garcia-Lorenzo et al. 2006), and supplemented with (Kmiec-Wisniewska et al. 2008)

acids that constitute the catalytic dyad and are unable to perform the proteolytic cleavage. They are, therefore, referred to as rhomboid-like proteins (Tripathi and Sowdhamini 2006; Lemberg and Freeman 2007; Garcia-Lorenzo et al. 2006; Kanoka et al. 2005). According to Lemberg and Freeman (2007), one of the *A. thaliana* potentially active rhomboid homologs belong to PARL-type rhomboids, three others to B-secretases and nine to mixed secretases. The four rhomboid-like proteins were described as an inactive rhomboid-like homologs (for details see Table 2). The sequences of the three remaining proteins (encoded by AT2G41160, AT3G07950 and AT3G56740) were not analyzed in terms of subfamily or clade membership.

The intracellular localization of *A. thaliana* rhomboids is diverse. Experimentally it has been demonstrated that proteases RBL10 and RBL11 are located in chloroplasts (Thompson et al. 2012; Kmiec-Wisniewska et al. 2008), RBL12 in mitochondria (Kmiec-Wisniewska et al. 2008), RBL1 and RBL2 in the Golgi apparatus (Kanaoka et al. 2005) and RBL4 and RBL14 in the plasma membrane (Benschop et al. 2007; Inzé et al. 2012). RBL4 was additionally found in the plasmodesmata as well as RBL3 (Fernandez-Calvino et al. 2011). The AramLocCon prediction program (URL http://aramemnon.botanik.uni-koeln.de.) indicates that six other proteins may be targeted to locations within secretory pathways, five to chloroplasts and three to mitochondria (for details see Table 2). Knowledge concerning the physiological role of rhomboid proteases in higher plants remains elusive. The insertion mutant lacking chloroplast protein RBL10 displayed several phenotypic changes such as an elongated root system, increased number of lateral roots and reduced fertility due to aberrant flower development and reduced silique formation. The abnormalities leading to decreased fertility are most likely associated with an impaired jasmonate

Table 3 Localization of S2P proteins

Gene accession number	Protein name	Localization	References
At2g03120	SPP	**Endoplasmic reticulum membrane**	Tamura et al. (2008)
AT4G33410	SPPL1	**Golgi apparatus endosome/trans-Golgi network**	Tamura et al. (2008) and Nikolovski et al. (2012)
AT1G63690	SPPL2	**Plasma membrane/endosome**	Benschop et al. (2007) and Tamura et al. (2008)
AT2G43070	SPPL3	**Vacuolar membrane**	Jaquinod et al. (2007)
AT1G01650	SPPL4	*Secretory pathways*	
AT1G05820	SPPL5	*Secretory pathways*	
AT1G08700	PRESENILIN-1, PS1	**Vesicular compartment/reticular structures**	Smolarkiewicz et al. (2014)
AT2G29900	PRESENILIN-2, PS2	**Vesicular compartment/reticular structures**	Smolarkiewicz et al. (2014)

The localization confirmed experimentally was indicated in bold. The predicted localization is indicated in italic. The prediction of proteins localization was made with AraLoc Con (Schwacke et al. 2007)

biosynthesis pathway since the double knockout mutants *rbl10/rbl11* demonstrated a reduced level of accumulation of allene oxide synthase (AOS)—an enzyme present in the chloroplast envelope and involved in the biosynthesis of jasmonic acid (Thompson et al. 2012; Knopf et al. 2012). Another rhomboid protein involved in generative processes is KOM, which was also predicted to occur in chloroplasts. The mutant lacking this protein displayed abnormal flower and pollen morphology (Thompson et al. 2012). It is not known; however, whether also in this case the observed phenotypic changes are related to a defective jasmonate biosynthesis pathway. It has been suggested that RBL10 may also participate in photoprotective mechanisms and in response to cold (Thompson et al. 2012). According to AraNetv2, RBL14 (At3g17611) may be in turn involved in the heat response (Lee et al. 2015). This prediction is consistent with the transcription profile of the gene, whose expression increases significantly in response to elevated temperature in both roots (eightfold increase) and shoots (ninefold increase) (Winter et al. 2007). It has also been demonstrated that a rhomboid protease participates in mitochondrial retrograde signaling by releasing from the endoplasmic reticulum membrane ANAC017, a transcription factor considered as a primary response regulator in H_2O_2-mediated stress signaling (Ng et al. 2013). However, the exact protease involved in this process remains to be determined.

Presenilins and other aspartic intramembrane proteases in *A. thaliana*

The presenilins (PSENs) were discovered in human cells during studies concerning Alzheimer's disease, when it was demonstrated that a gene bearing a missense mutation is involved in formation of senile plaques. Shortly after identification of presenilin (PSEN) another family of aspartic proteases, signal peptide peptidases (SPPs), was discovered. Both protease families contain proteins with 9 TMs and share similar construction of the catalytic center based not only on two catalytically active aspartates, but also on an additional PAL motif (Wang et al. 2006; Tomita et al. 2001). SPPs and PSENs differ; however, in orientation within the membrane. In PSENs the N-terminus is exposed to cytosol, while SPPs face the cytosol with their C-terminus. PSENs were also shown to be a subunit of the γ-secretase complex, whereas the SPPs seem to act independently (Erez et al. 2009). The SPPL2b protein identified in human cells was found to be proteolytically active (Fluhrer 2006; Carpenter et al. 2008).

In the *A. thaliana* genome two genes encoding presenilins, one gene encoding SPP, and five genes encoding SPP-like proteins, are present. The proteins encoded by these genes contain, in their primary structure, all motifs necessary to perform proteolytic cleavage, but the proteolytic activity of these proteins has not yet been experimentally confirmed. The localization of six *A. thaliana* intramembrane aspartic proteases was confirmed experimentally. All of these proteins were found within the secretory pathways. Similar location was predicted for the remaining two proteins (for details see Table 3).

The knowledge concerning the physiological functions of the aspartic intramembrane proteases and their homologs is very limited. Research performed on *A. thaliana* indicates that the γ-secretase complex may be involved in protein trafficking (Smolarkiewicz et al. 2014).

Presenilin itself was found to participate in cytoskeletal related responses, since a mutation in its gene resulted in curly filament growth and impaired chloroplast movement

in *Physcomitrella patens*. The interesting fact is that this effect was reversed by the expression of both active and inactive presenilin variants, suggesting that the protease acts independently from the γ-secretase complex (Khandelwal et al. 2007).

SPP is a proteolytically active protein crucial for *A. thaliana* development, since knockout of the *SPP* gene resulted in a lethal phenotype (Han et al. 2009, Hoshi et al. 2013). Studies performed on *spp* heterozygotes indicate that the protein is involved in pollen development and germination (Hoshi et al. 2013). The SPPL1, SPPL2 and SPPL3 transcripts were detected in the tissues, roots, rosette leaves, cauline leaves, stems, flower-bud clusters, siliques and dry seeds (Han et al. 2009) The significant accumulation of all three transcripts was; however, observed during seed germination. The SPPL3 transcript accumulates additionally during seed stratification and under dark treatment in response to sucrose (Grennan 2006). The level of SPPL4 gene expression increased from the curled stage of the seed embryo to achieve the highest accumulation level in dry seeds. Accumulation of the SPPL4 transcript was also observed during pollen tube growth in a *semi* in vivo experiment (Grennan 2006). Information concerning expression of the *SPPL5* gene is unavailable in databases.

Conclusion

Knowledge concerning the functions of intramembrane proteins in plant development and physiology remains elusive. Continuously accumulating data indicate; however, that their role in development should not be underestimated. A mutation in the SPP gene was demonstrated to be lethal (Hoshi et al. 2013). Many studies also indicate that intramembrane protease performs crucial functions in providing plant fertility. Aberrations in flower, pollen or silique development were observed in *rbl10* and *atkom* mutants as well as in *spp* (Thompson et al. 2012, Hoshi et al. 2013). The transcripts of SPPL1, SPPL2 and SPPL3 proteins accumulate in turn during seed germination, but their role in this process remains unexplored. The intramembrane proteases also have an influence on photosynthetic process efficiency and are involved in the process of acclimation to light conditions, since the role of EGY1 and ARASP in chloroplast biogenesis was experimentally confirmed and presenilin was shown to participate in chloroplast movement (Chen et al. 2005; Bötler et al. 2006). The mechanisms leading from gene mutation to phenotypic changes and development aberrations have not been discovered yet. This makes the plant transmembrane proteases an extremely interesting field for future research.

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