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RESEARCH PAPER

An Arabidopsis rhomboid protease has roles in the chloroplast and in flower development

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Abstract

Increasing numbers of cellular pathways are now recognized to be regulated via proteolytic processing events. The rhomboid family of serine proteases plays a pivotal role in a diverse range of pathways, activating and releasing proteins via regulated intramembrane proteolysis. The prototype rhomboid protease, rhomboid-1 in *Drosophila*, is the key activator of epidermal growth factor (EGF) receptor pathway signalling in the fly and thus affects multiple aspects of development. The role of the rhomboid family in plants is explored and another developmental phenotype, this time in a mutant of an *Arabidopsis* chloroplast-localized rhomboid, is reported here. It is confirmed by GFP-protein fusion that this protease is located in the envelope of chloroplasts and of chlorophyll-free plastids elsewhere in the plant. Mutant plants lacking this organellar rhomboid demonstrate reduced fertility, as documented previously with KOM—the one other *Arabidopsis* rhomboid mutant that has been reported in the literature—along with aberrant floral morphology.

Key words: *Arabidopsis*, chloroplast, flower development, membrane, pollen, protease, proteolysis, rhomboid.

Introduction

Our increasing knowledge of the sophisticated networks of cellular regulation shows that proteases are more than workhorses of protein degradation, acting as pivotal regulators of many cellular processes. Those proteins that allow intramembrane proteolysis are a prime example and include the rhomboid protease family (Urban and Freeman, 2002). This is a relatively newly discovered family, but it is already known that it is almost universal in both unicellular and multicellular eukaryotes and in prokaryotes, and genetic, cellular and biochemical data about rhomboids have accumulated quickly. The substrates of these 5–7 transmembrane-domain serine proteases are fellow transmembrane proteins, and they operate in an interesting range of pathways. The prototype member of the group and, crucially, its substrates, have been explored in some detail: Rhomboid-1 is the key activator of epidermal growth factor

(EGF) receptor pathway signalling in *Drosophila* and thus affects multiple aspects of development (Wasserman *et al.*, 2000). The mutant embryo and its unusually rhomboid-shaped head skeleton give the name to the class. The ligand for the EGF receptor in *Drosophila* is the tumour growth factor (TGF) α -like growth factor ‘Spitz’, which requires proteolysis of its endoplasmic-reticulum (ER)-tethered precursor for its active form to be released. Rhomboid-1 cleaves Spitz within its single transmembrane domain so that Spitz can be secreted as an extracellular protein and activate the EGF receptor (Urban *et al.*, 2001, 2002). The regulation of the process is unusual, via controlled intracellular trafficking. Because Rhomboid-1 is constitutively active (although its RNA has a short half-life), the enzyme and its substrate are kept separate until signalling occurs: a third protein, called Star, traffics Spitz from the

endoplasmic reticulum to the Golgi, where Rhomboid-1 is located. It is not yet known whether this signalling system is shared by other organisms: Spitz is a homologue of human TGF- α but no Star homologues have been identified outside the arthropods (Freeman, 2004).

As well as developmental signalling by this founding member of the group, other roles for rhomboids include mitochondrial dynamics, host cell infection in the apicomplexa and transport (processing TatA in the bacterium *Providencia*; Lee *et al.*, 2001; Gallio *et al.*, 2002; McQuibban *et al.*, 2003; Stevenson *et al.*, 2007). Meanwhile, there is interest in the human counterpart of *Drosophila* Rhomboid-1 and cancer, with regard to the EGF receptor and other related ErbB-receptor tyrosine kinases, since hyperactive receptor signalling allows deregulated growth control and altered developmental programmes (Casci and Freeman, 1999).

The mechanism of action of rhomboid proteases attracts interest because hydrolysis of a peptide bond requires water near the (membrane-located) active site. All substrates of these proteases so far identified have been single-transmembrane domain proteins, and seem to have some regions of similarity as well as helix-destabilizing residues that would presumably facilitate cleavage (Strisovsky *et al.*, 2009). A recognition motif for substrates of rhomboids has proved difficult to identify, but it has now been suggested that a specific arrangement of small and large amino acid residues in substrate proteins is necessary for cleavage, at least in non-mitochondrial members of the group (Strisovsky *et al.*, 2009). The active site of the enzyme itself is better established, with catalytic site and residues now being illuminated by crystal

structures (Baker *et al.*, 2007; Ben Shem *et al.*, 2007). There is a core of six transmembrane spans in a rhomboid protease, with two small helices dipping into the membrane between spans 1 and 2 (Fig. 1a; Lieberman and Wolfe, 2007). These domains are well-conserved and there is structural similarity across the predicted rhomboids of all organisms. Serine and histidine residues are proposed to form a catalytic dyad (the previously proposed catalytic-triad-forming asparagine is now thought less or variably important because it is less sensitive to mutation; see Supplementary Fig. S1 at *JXB* online) and there is a well-conserved motif (GxSx, often GASG in *Arabidopsis* rhomboids) containing the serine of the serine-protease, which is located in or near transmembrane span 4 of the six core rhomboid hydrophobic domains (Fig. 1a). Of those hydrophobic domains, 2, 4 and 6 were seen in the GlpG crystal structure (Baker *et al.*, 2007; Ben Shem *et al.*, 2007) to run in the same orientation and contribute the principal active site residues. The large loop between transmembrane domains 1 and 2 contains the often-conserved WR amino acids, which are not essential but reduce activity when mutated (Urban, 2010).

Beyond this structural similarity and a handful of key residues, the sequence similarity is low between rhomboids: determining the evolutionary history and relationships between these proteases can be a challenge. Whereas rhomboids are ubiquitous across all kingdoms of life and they demonstrate some conservation of function, they also present some intriguing differences. These give rise to possible subgroups, for example, according to the presence of an extra N-terminal sequence, the predicted number of transmembrane

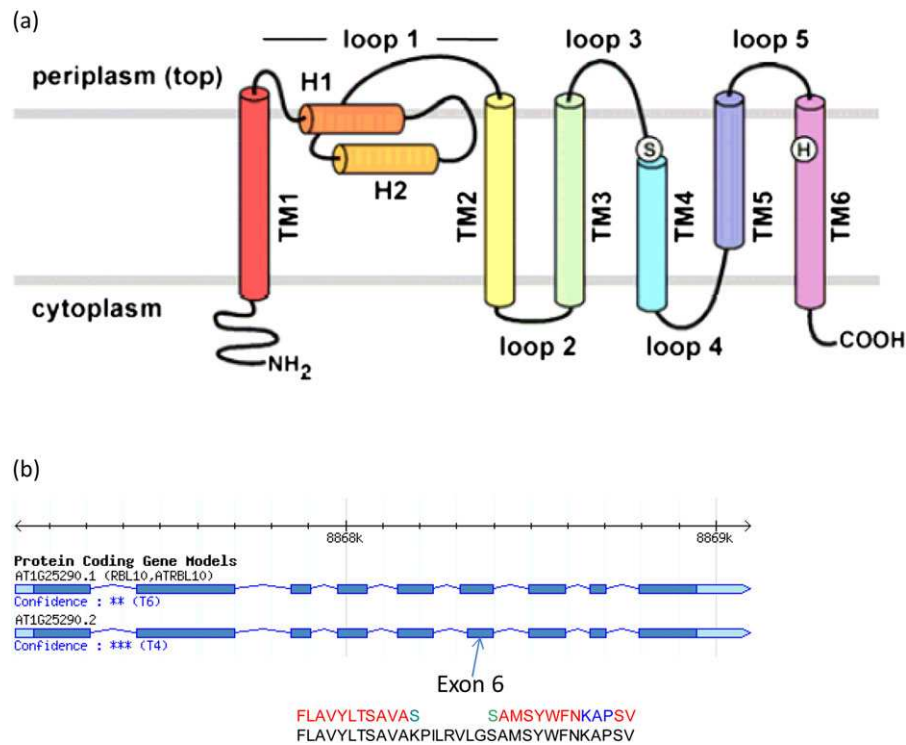


Fig. 1. Rhomboid protein structure. (a) The GxS motif at the top of the fourth hydrophobic span is thought to form the periplasmically exposed water-filled cavity (cartoon from Ben-Shem *et al.*, 2007). (b) The RBL10-encoding At1g25290 transcript (lower line) differs from predicted exon/intron structure (upper line). (This figure is available in colour at *JXB* online.)

spans, and conservation of the active site or other motifs. A mitochondrial subgroup has been defined, comprising proteases similar to the human PARL protease, which are characterized by a seventh hydrophobic span. Another group are the so-called secretase rhomboids, such as *Drosophila* Rhomboid-1, which have an additional transmembrane region but are located in the Golgi or plasma membrane and operate in the secretory pathway. Other members have been defined as 'active rhomboids', 'inactive rhomboid homologues', and the 'iRhoms', a structurally conserved group that are predicted to be inactive because of the absence of key catalytic residues in their sequences and the presence of a proline before the catalytic serine (Freeman, 2008).

In plants, there has been little work on rhomboids. Rice contains a family of approximately 20 members (Tripathi and Sowdhamini, 2006) and *Populus trichocarpa* is predicted to contain about 15 members (Garcia-Lorenzo *et al.*, 2006), suggesting that other plants will each contain a similar, small family. These groups, as so often seen in plants, are larger than those in other kingdoms: for example, *Drosophila* has a family of seven rhomboids. As mentioned above, the amino-acid sequences of diverse rhomboid proteins have low identity, but alignment of available plant cDNAs shows moderate similarity over much of their length, particularly transmembrane helices 2–6. In *Arabidopsis*, there seems to be one PARL-type rhomboid and 12 secretase-type rhomboids. A subgroup may be catalytically inactive because key residues are absent. Freeman and colleagues have found that *Arabidopsis* RBL2 (At1g63120) can cleave *Drosophila* ligands including Spitz (Kanaoka *et al.*, 2005) but some other *A. thaliana* rhomboids did not do so in their *in vitro* assays

(M Freeman, personal communication). About four *A. thaliana* rhomboids are predicted to have organellar locations according to analysis of putative presequence. The PARL-type At1g18600-encoded protein (RBL12), although confirmed by GFP to be directed to the organelle, did not recognize either of the two yeast mitochondrial substrates (Ccp1/ Mgm1) when used to complement a yeast deficient in its mitochondrial rhomboid (Kmiec-Wisniewska *et al.*, 2008). Meanwhile, the *Arabidopsis* translocon component Tic40 may be processed by a plastid rhomboid (At1g74130; Karakasis *et al.*, 2007).

The observation that an *Arabidopsis* rhomboid mutant named 'kom' (At1g77860) demonstrates altered morphology of the pollen exine wall (Kanaoka *et al.*, 2001; Fig. 2a, b) directed our search for homologues in the plant, and a study of several members of the family was undertaken (in preparation). We were particularly interested in the organellar members of the *Arabidopsis* rhomboid family and focused on At1g25290 (currently designated AtRBL10; UniProt Q9FRH8), which was predicted to localize to the chloroplast. The effects of loss of gene function in a null mutant line, together with analysis of expression and protein localization, are described here. It is confirmed that RBL10 is primarily chloroplast localized, but we report that it also plays a role in floral development.

Materials and methods

Plants and growth conditions

Wild-type (WT) and mutant lines were grown in compost in greenhouses maintained at 20 °C. For growth assays and

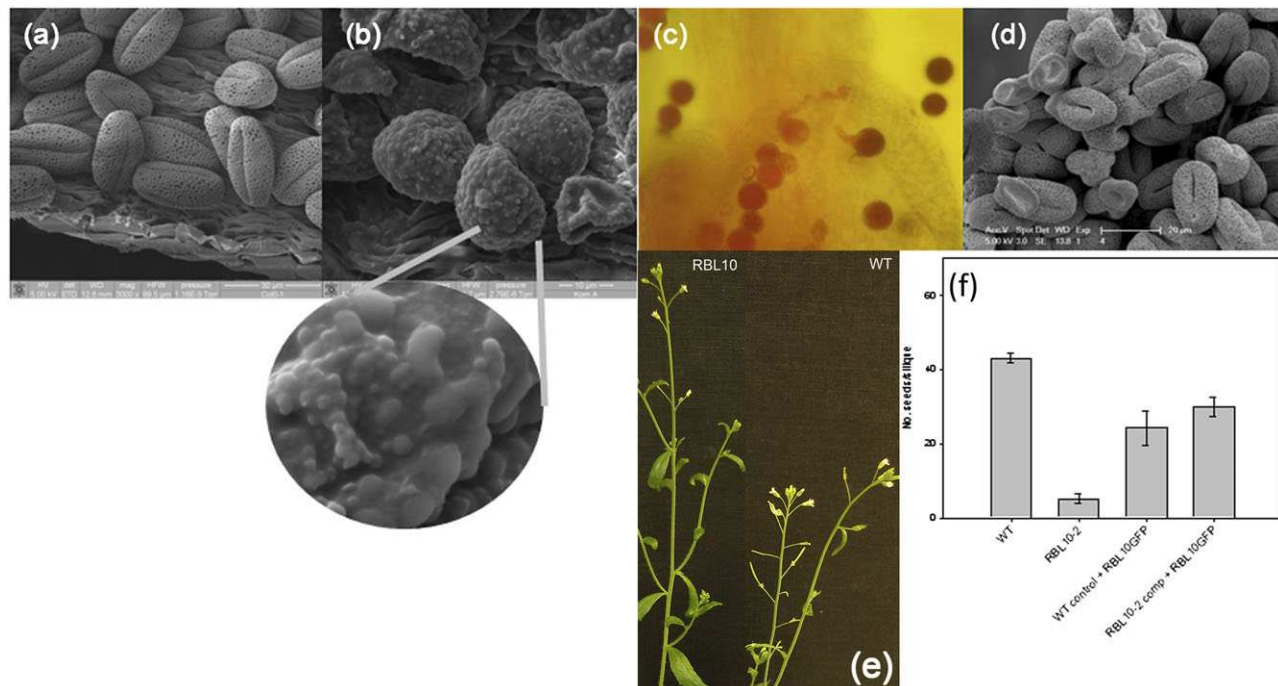


Fig. 2. Cryo-scanning electron microscopy (SEM) of *Arabidopsis thaliana* wild-type (Col-0; a) and KOM mutant (b). (c) Alexander stain showing viability and morphology, and cryo-SEM (d) in RBL10 mutant pollen. Reduced silique formation in the primary inflorescence of RBL10 mutant (e) and the consequent reduction in the number of seeds/silique (f; \pm standard error of the mean), which was restored following complementation (WT control and RBL10-2 mutant transformed with 35S-RBL10-GFP). (This figure is available in colour at JXB online.)

transformed plants, half-strength Murashige and Skoog (1/2MS) agar (pH 5.7; Duchefa, Haarlem, The Netherlands) was used, and plates or pots were incubated in a growth room at 20 °C with light of 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (16 h light cycles). For high-intensity light assays when testing photosynthetic parameters, a Fitotron growth chamber (Weiss-Gallenkamp, Loughborough, Leicestershire, UK) was used. Two T-DNA insertion lines (N537037, here designated RBL10-1 and N592293, RBL10-2) were purchased from the Nottingham *Arabidopsis* Stock Centre (NASC; Nottingham, UK) and segregated until homozygous. The presence and location of single T-DNA insertions were confirmed by Southern analysis according to Church and Gilbert (1984) and the absence of transcript by RT-PCR. Growth assays of mutant lines were conducted on plates in which one half of each plate was sown with mutant and the other half with WT seed, with 6–11 seeds on each half for germination counts. ImageJ (<http://rsbweb.nih.gov/ij/>) was used to record measurements from digital images of plates.

Molecular biology

DNA manipulation was performed essentially according to Sambrook *et al.* (1989). Primers for amplifying cDNA were 5'-ATGGTATCAGTGTCATTATCTCA-3' and 5'-TCAAAGCCGTCGCTGTTTCATT-3', along with 5'-GGATTTAAGCTCGAGTCTGA-3' and 5'-CACCAAGTAAGCCACCAA-3' for locating T-DNA insertions. For the RBL10-GFP fusion protein, primers were 5'-CGGGATCCGAAGATGGTATCAGTGTC-3' and 5'-GGAATCAAGCCGTCGCTGTTTCATT-3'. Primers for the RBL10 promoter were 5'-GCTCTAGACTTACATGTTACTAACTTCTA-3' and 5'-TTCCCGGATCTTCCCCTTCACGTTTC-3'. All constructs were based upon vectors from the John Innes Centre, Norwich, UK, or the gift of the J Haseloff group, University of Cambridge, Cambridge, UK, namely a CaMV 35S-eGFP-containing pGreen0029 vector for fusing RBL10 N-terminal of GFP, and pGreen0029 containing the β -glucuronidase gene for constructing the RBL10 promoter-GUS vector.

For transcript analysis via RT-PCR, primers were used that would generate product across introns to differentiate any genomic contamination in template from transcript. To investigate transcript levels in specific tissues, RT-PCR was carried out using control primers to actin8 (5'-AGAAAGATGCGTATGTTGTGA-3' and 5'-CTGCTGGAAAGTGCTGAGGGAA-3'), and the appropriate template concentration was used.

Binary vectors and plant transformation

Promoter- β -glucuronidase (GUS) and 35S-RBL10-GFP-encoding vectors were transformed into wild-type or RBL10-mutant *Arabidopsis* as required for localization studies or complementation, via *Agrobacterium tumefaciens* and floral dipping (Gilmartin and Bowler, 2002). Transformed plants (T_1) were grown on 1/2MS plates as above, supplemented with antibiotic as appropriate, and antibiotic-resistant plants were grown on in compost.

Mutant complementation

The RBL10-2 mutant was complemented to ensure a return of WT characteristics by transforming it with a WT copy of the RBL10 transcript fused N-terminal of GFP, under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Effects on seed and floral phenotypes were checked in three independent transformant lines.

GUS staining

For promoter-GUS localization, tissues were stained according to Jefferson *et al.* (1987). Tissues were fixed in 90% (v/v) ice-cold acetone for 20 min, rinsed twice in GUS working solution [0.1 M sodium phosphate buffer, 0.5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.5 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 10 mM Na_2EDTA , 0.1% (v/v) Triton X-100; pH 7.0]

and stained by adding 40 mg ml^{-1} stock 5-bromo-4-chloro-3-indolyl β -D-glucuronide cyclohexamine salt at 12.5 $\mu\text{g ml}^{-1}$ buffer, vacuum infiltrating the tissue, and incubating for 12–48 h as required at 37 °C. Tissues were cleared with 70% (v/v) ethanol.

Microscopy

Pollen and inflorescence tissues were examined using cryo-scanning electron microscopy (SEM; Oxford Instruments CT 1500, Abingdon, Oxfordshire, UK). The Alexander stain was carried out essentially according to Pline *et al.* (2002).

Initial visualization to identify GFP-expressing plants was by epifluorescence (Nikon Eclipse 50i, epifluorescence visualized using the UV-2A filter block). For confocal microscopy, tissues were excited at the peak of GFP absorbance at 488 nm and emission recorded at 500–520 by confocal microscopy [Leica (Milton Keynes, UK) TCS-SP1, or TCS-SP5, equipped with Coherent Enterprise (Santa Paula, CA, USA) UV laser]. Samples were examined with a $\times 64$ oil immersion objective lens with a numerical aperture of 1.3. Fluorescence intensity was measured in 10 nm windows and displayed in the TCS (Leica) software as spectra. Control plants were untransformed WT, Dr5-GFP auxin reporter transformed plants (gift of L Ostergaard, John Innes Centre, UK), and extensin-GFP-expressing lines (gift of J Haseloff, University of Cambridge).

Photosynthesis and pigment content

Pulse amplitude modulation fluorimetry was used to measure the quantum yield of Photosystem II (Blankenship, 2002) according to the manufacturer's instructions (Hansatech, King's Lynn, Norfolk UK). Estimation of chlorophyll content was carried out according to Hipkins and Baker (1986) and Porra *et al.* (1989), extracting pigment with TRIS-Cl (pH 8.0)-buffered 80% (v/v) acetone. Anthocyanin extraction was with 1% HCl-methanol according to Mehrtens *et al.* (2005).

Results

Sequence analysis

The RBL10 protein encoded by At1g25290 contains six hydrophobic regions predicted to form transmembrane spans (Fig. 1a; see Supplementary Fig. S1 at *JXB* online), following a 26 AA predicted chloroplast targeting sequence (www.cbs.dtu.dk/services/TargetP; Emanuelsson *et al.*, 2007). This would produce an unmodified version of the protein of mass 38.2 kDa with the 343 AA sequence listed in most *Arabidopsis* genome databases (see comments on transcript, below). This predicted rhomboid protein fits best in the secretase subgroup (Lemberg and Freeman, 2007) and contains the catalytic site residues that suggest that this rhomboid type is enzymatically active. Similarity between rhomboids is high enough to make them easily identified in other plants, the most similar sequence to RBL10 among sequenced plants being a putative protein from *Populus trichocarpa* (Western balsam poplar; *Populus balsamifera* subsp. *trichocarpa*: B9GZF8_POPT), which is 56% identical at the amino-acid level (see Supplementary Fig. S1 at *JXB* online). The most similar non-land plant sequences are putative rhomboids from unicellular algae *Micromonas* and *Chlamydomonas*, at 41% and 34% identical, respectively. The *Clostridium carboxidivorans* genome encodes the most similar prokaryotic protease (37% identical).

Transcript

Translation of the sequence from cDNA produced in RT-PCR from various tissues in Col-0 *A. thaliana* showed that the RBL10-encoding open reading frame in *Arabidopsis* sequence databases may be variable or incorrect. In our work, two different possible transcripts were found. An alternative splice site in intron 5 increased its size from 73 nt to 86 nt, causing a loss of 13 nt from exon 6 and a stop codon-producing frameshift. With only one transmembrane domain encoded by the truncated mRNA, this transcript would not result in a functional protease. A second variant, however, encoded a polypeptide lacking seven amino acids in the predicted transmembrane helix 3, and would still produce a functional protease (Fig. 1b). From our sequence data, this appears to be the true encoded amino acid sequence, not that predicted by TAIR. This difference in the encoded protein does not alter the transmembrane domains but does make the RBL10 protein align better with its most closely related rhomboids, and the most similar protein in poplar, in particular (see Supplementary Fig. S1 at *JXB* online).

Promoter analysis

The short (351 nt) non-coding region upstream of RBL10 contains the core of the low-temperature responsive element of the *A. thaliana cor15a* gene which is repeated twice in the *cor15a* promoter (ppdb.gene.nagoya-u.ac.jp/cgi-bin/index.cgi; Baker *et al.*, 1994; Yamamoto and Obokata, 2008). No other sequence features are scored as reliable by prediction programs. Unfortunately, the usual means of investigating the timing and location of transcription, with a promoter–GUS fusion construct transformed into *A. thaliana*, did not produce any localization information. There was no X-gal staining under various conditions in roots, seedlings or floral tissues, all locations that were examined because they would have corresponded with the mutant phenotype or microarray data (see below). It is therefore likely that the introns contain regulatory information with respect to RBL10.

Protein localization

Transformation of a 35S–RBL10–GFP construct into *Arabidopsis* revealed that, as predicted by its putative signal peptide (see Supplementary Fig. S1 at *JXB* online), RBL10 protein is localized to chloroplast membranes (Fig. 3a, b, c). In epifluorescence microscopy this manifests itself as green fluorescence in emerging true leaves, with their high density of cells and chloroplasts (Fig. 3d, e). Using the confocal microscope, GFP can consistently be observed in the chloroplast membrane throughout development (Fig. 3a, b show day 4 cotyledons and root plastids, whereas Fig. 3c shows day 9), lending bright fluorescence even early on in a time-series, since emerging leaves have a high concentration of chloroplasts. Localization is seen in the plastid envelope in both leaf (Fig. 3c) and root (Fig. 3b), and is perhaps most clearly observed in the latter. In day 9 leaves, the localized GFP appeared clearly external to the chloro-

phyll fluorescence of the chloroplast (Fig. 3c). The single N-terminal transit peptide of RBL10 would suggest the location of the mature rhomboid in the chloroplast inner or outer membrane, but the exact location cannot currently be accurately predicted from the presequence, nor whether the latter is cleaved by a processing peptidase, if indeed the outer membrane is the final location (Hofmann and Theg, 2005). Also of interest are the GFP-fluorescent membranes emanating from and linking the organelles: these can be seen in both root plastids and chloroplasts (see Supplementary Fig. S2 at *JXB* online).

In silico analysis not only corresponds with the chloroplast localization of the protein and predicted chloroplast signal peptide but, interestingly, the ATTED coexpression database (atted.jp/data/cor/At1g25290.html) also identifies the top co-regulated proteins as being the chloroplast-thylakoid protein EGY2, a S2P-like putative intramembrane metalloprotease (Chen *et al.*, 2012), and CRR1 (chlororespiration reduction 1), a dihydrodipicolinate reductase essential for chloroplast NAD(P)H dehydrogenase activity (Fig. 4).

Expression analysis

Our own transcript analysis and published microarray data for RBL10 suggest low-level expression in many tissues and circumstances (data not shown). In RT-PCR, RBL10 transcript was amplified from RNA prepared from tissues as follows: seedling [day 5 and day 6 post-stratification (p.s.)], mature rosette leaf (stage 3; Boyes *et al.*, 2001), bolt stem, petiole, open flowers (stage 13; Smyth *et al.*, 1990), and immature siliques. Transcript could not be detected in day-2 seedling tissues at radical emergence. Various stresses were also looked at in order to examine any change in transcription levels. Infection with cucumber mosaic virus did not increase the levels of the RBL10 transcript but, whereas normally transcript was found to be difficult to amplify from the root, it was detected in roots if seedlings had been subjected to cold stress (4 °C). Microarray data compiled using Genevestigator (www.genevestigator.com/gv/index.jsp) also suggest transcription in all developmental stages but a peak of transcription at early flowering, and anatomical peaks in the leaf primordia, juvenile leaf, cotyledons, pedicel, and shoot apex (see Supplementary Fig. S3 at *JXB* online). Similarly, *Arabidopsis* eFP browser records flower stage 15, pedicel and vegetative rosette as having the highest levels of transcription, but with transcript again present in all tissues (www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). In roots, transcription is recorded as similar in all regions but Genevestigator puts the peak in the root hair zone and Arexdb in the elongation zone. Transcription is reported as being most upregulated in the *agl66* mutant (*agamous-like 66*, involved in pollen development).

Mutant analysis

A null mutant line of RBL10 (a Salk T-DNA line; supplied by NASC, Nottingham, UK) was segregated to homozygosity. To confirm that the line was null, RT-PCR at

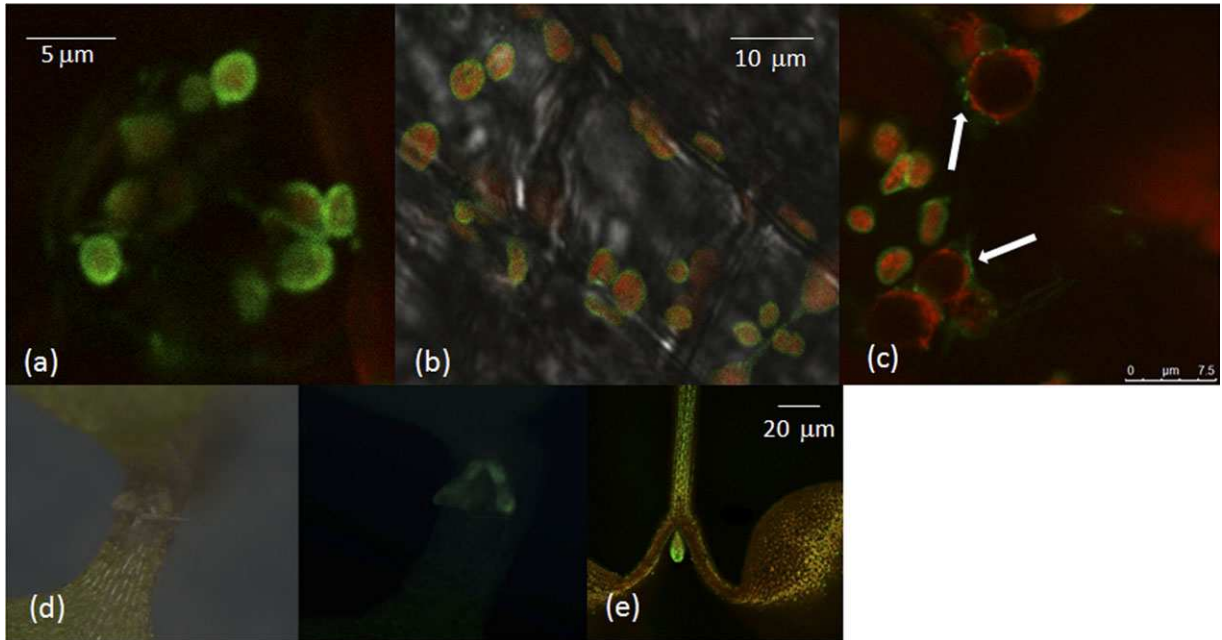


Fig. 3. RBL10-GFP protein localization. (a, b) Confocal microscopy of RBL10-GFP fusion protein expression in the chloroplast membrane throughout development (here at day 4 post-stratification in cotyledons and root plastids). (c) Plastid RBL10-GFP showing membrane localization at day 9. (d) Expression concentrated in seedling emerging leaves, viewed with epifluorescence (with bright field, left) and (e) confocal microscope. (This figure is available in colour at JXB online.)

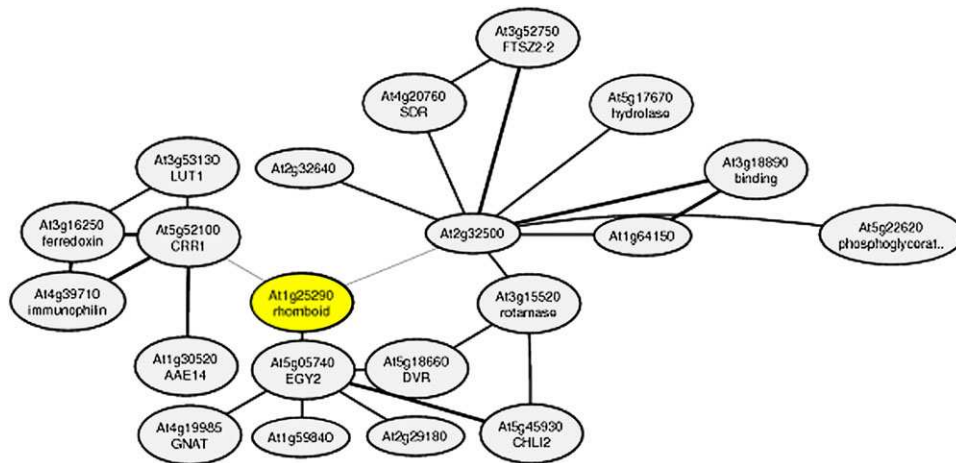


Fig. 4. ATTED database analysis of transcriptional coregulation (atted.jp/data/cor/At1g25290.html; Obayashi *et al.*, 2009) with RBL10 (At1g25290) identifies two chloroplast proteins: EGY2, a S2P-like putative membrane-located metalloprotease; and CRR1 (chlororespiration reduction 1), a dihydrodipicolinate reductase essential for chloroplast NAD(P)H dehydrogenase activity. (This figure is available in colour at JXB online.)

saturation cycle numbers was used to check that the transcript could not be amplified from tissues in which cDNA was routinely isolated in the wild type (WT; data not shown), and the presence of a single T-DNA insertion was confirmed by Southern analysis. PCR also allowed the insertion of the T-DNA to be located. A second null line was included to confirm aspects of the phenotype and treated as above. T-DNA insertions were found in exon 3 for Salk N537037 (RBL10-1) and in intron 6 for N592293 (RBL10-2; see Supplementary Fig. S4 at JXB online).

Since the *A. thaliana kom* mutant has a floral phenotype, but RT-PCR and microarray data suggested widespread

transcription of RBL10 and the ORF contained a likely chloroplast targeting sequence, our observation of the RBL10 mutants included growth characteristics, floral development, and photosynthetic parameters such as F_v/F_m and pigment content (chlorophylls and anthocyanins).

Growth characteristics

Inactivational insertion of RBL10 resulted in slightly faster root growth than in the WT (Col0). Roots were longer than in Col-0 seedlings in duplicate assays from day 4 to day 11 p.s. (RBL10-1 and RBL10-2 at day 6 p.s. were significant at

$P=0.09$, 43 degrees of freedom (d.f.), and $P < 0.01$, 39 d.f., respectively; Fig. 5a). There was another small difference, in the rate of germination, which might account for roots being longer in the first week [e.g. in RBL10-2 seedlings, 93% had radicle emergence 10 h p.s. versus 71% in WT ($P < 0.001$, 10 d.f.)]. The most dramatic feature of root growth, which corresponds with the faster rate of growth in the RBL10 mutants, was the formation of lateral roots. This was clearly evident when plants were grown on plates and was again statistically significant: mutant developed twice as many lateral roots at around 1 week p.s. (e.g. RBL10-1 and RBL10-2 both $P < 0.01$, with 39 d.f. and 28 d.f., respectively, at day 9 p.s.; Fig. 5b). There was no dramatic impact on the appearance of plants or time to flowering, despite these differences in root growth.

Pigment content

Chlorophyll *a* and *b* and total chlorophyll levels were the same in the mutant and the WT (Fig. 6a), despite the growth differences seen and the likely chloroplast membrane location of the RBL10 protein. A tendency for the mutant to contain more anthocyanin than the WT at the moderately high light intensity of $590 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $750 \mu\text{mol m}^{-2} \text{s}^{-1}$ was noted but the conditions causing this require further investigation since the results were variable.

Chlorophyll fluorescence parameters

Pulse amplitude modulation (PAM) fluorimetry was utilized to investigate the effect of insertional inactivation of RBL10 on protective photosynthetic response. At low light levels ($14 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the growth chamber), there were no significant differences between dark-adapted Col-0 and the mutant in standard parameters. In the case of F_v/F_m ,

Photosystem II (PSII) maximum efficiency, both WT and mutant yielded figures of 0.87 ($P=0.72$; indicative of healthy plants' photosynthetic performance: a normal value is about 0.85). The same plants showed no indication of abnormal or damaged photosynthetic pathways, in terms of photochemical quenching (qP, usually approaching 1.0, was 0.88 in both WT and mutant; $P=0.76$) and non-photochemical quenching (NPQ).

Feedback de-excitation dissipates excess absorbed light energy as heat, thereby protecting plants from high-light stress, which is quantified by the parameter NPQ (Müller *et al.*, 2001; Baker, 2008; which normally varies between 0.35–0.5 and here was 0.33 in the WT and 0.36 in the mutant; $P=0.29$); and quantum yield of PSII (ϕPSII , 0.55 in the WT and 0.54 in the mutant; $P=0.44$). The tendency to a difference in NPQ was amplified in the mutant in higher intensity light, however. At $212 \mu\text{mol m}^{-2} \text{s}^{-1}$, both Col-0 and RBL10-2 gave an F_v/F_m of 0.87 ($P=0.65$) and qP (both 0.83; $P=0.93$), but the mean NPQ was 0.41 ± 0.05 in the WT

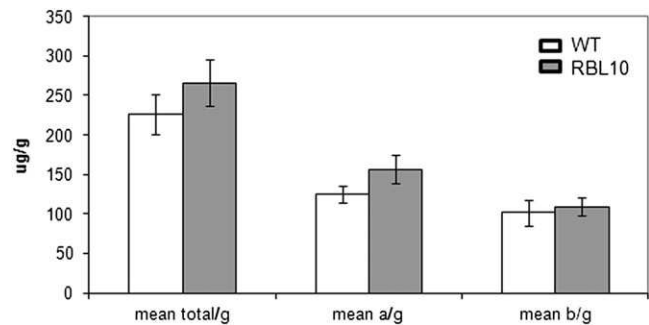


Fig. 6. Mean pigment content of the RBL10-2 mutant. Total chlorophyll and chlorophyll *a* and *b* per gram (\pm standard error of the mean), under standard growth conditions versus Col-0 (WT).

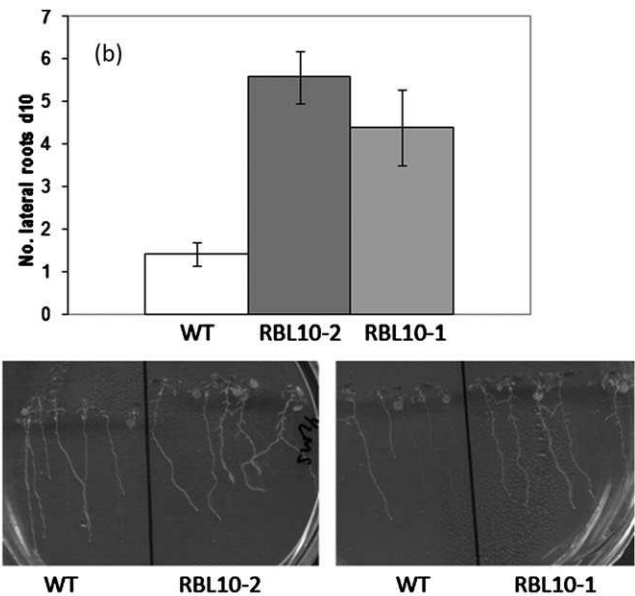
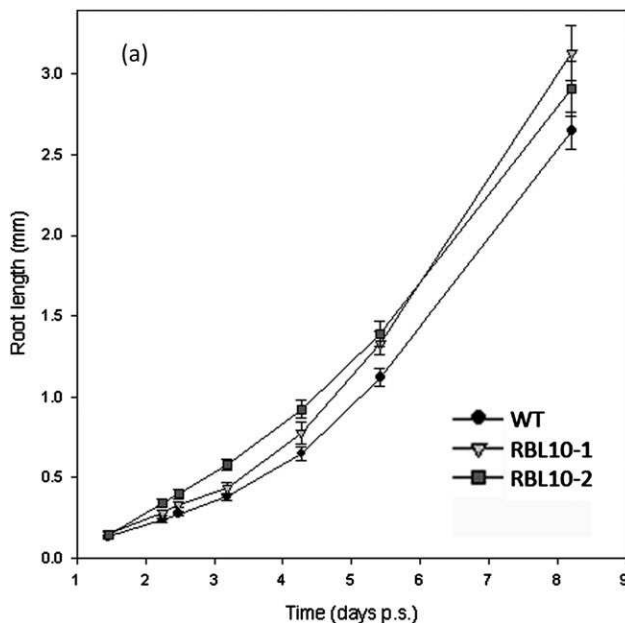


Fig. 5. (a) Root growth over time post-stratification (p.s.) of lines RBL10-1 and -2 versus WT (Col-0) and (b) lateral root number at day 10 p.s. (\pm standard error of the mean).

and 0.54 ± 0.02 in the mutant ($P=0.04$, 10 d.f.). Mean ϕ PSII was only slightly different at 0.48 ± 0.02 in Col-0 and 0.43 ± 0.02 in the mutant but the difference was just significant ($P=0.09$). In very high-intensity light ($750 \mu\text{mol m}^{-2} \text{s}^{-1}$), F_v/F_m , qP, and ϕ PSII remained similar but, again, mean NPQ was markedly higher in mutant (mean \pm SEM, Col-0, 1.42 ± 0.19 versus RBL10-2, 2.04 ± 0.29).

Fertility

The aberrant-pollen phenotype of the *kom* rhomboid mutant and the presence of RBL10 transcript in floral tissues also prompted investigation of the fertility of the mutant lines compared with the WT, under both normal and high-intensity light conditions. Pollen and floral development were also examined using staining, light microscopy, and scanning electron microscopy.

Within the inflorescences, the two independent mutant lines showed their most profound phenotypic abnormalities (Fig. 2c–f). A proportion of pollen developed poorly. Viewed using cryo-SEM, the heterogeneous pollen grains were half the length of WT, with some collapsed grains and flatter surface topology (Fig. 2f), although this was not as severe as the effects seen in the *kom* mutant (Fig. 2b). Alexander staining confirmed that some pollen was malformed or unviable (Fig. 2e). Flowers also occurred in mutant lines with other abnormalities (Fig. 7a–c) such as five petals, and the stigma in particular was commonly affected, appearing as a double stigma or with other distortions in shape and size (Fig. 7b; arrowed). As a result, few siliques develop successfully on the plant, especially early in inflorescence development, and the number of seed per silique on the primary inflorescence was significantly lower than in the WT plant (Fig. 2d). As the inflorescence

developed (as observed before with fertility phenotypes: Thompson *et al.*, 2010), the mutant plants showed improved siliques and seed production over time (see Supplementary Fig. S5 at JXB online).

The RBL10–GFP construct was transformed into the RBL10-2 mutant line to discover if returning the WT ORF would restore a wild-type phenotype. Antibiotic-resistant seedlings expressing GFP (viewed with epifluorescence microscopy) were selected and grown on to produce seed and for further investigation. Complementing the RBL10 mutant with the GFP construct did indeed correct the mutant phenotype. There was no significant difference between the complemented line and the WT in the major aspects of the mutant phenotype, namely number of seeds/silique on the primary inflorescence (Fig. 2d), and previously aberrant stigma, anther, and petal morphology was corrected (Fig. 7c).

Discussion

The function of the RBL10 protein has not previously been analysed and seems to be dependent on organ, tissue, and cellular localization, given the plastid location and the varied fertility and developmental aspects of the mutant phenotype. The presence of transcript encoding RBL10 throughout *Arabidopsis* in different tissues and stages of development was suggested both by transcriptomics data and by our experiments. A pattern of regulated expression in varied but specific tissues during development has also been shown for rhomboids from other species. For example, the most similar rhomboid in *Toxoplasma gondii* is TgROM2, expressed in sporozoites (and able to cleave Spitz *in vitro*; Brossier *et al.*, 2005). In this apicomplexan parasite,



Fig. 7. Aberrant floral development in RBL10-2 mutants. (a) WT flowers. (b) Mutant flowers showed various abnormalities (arrowed), the stigma particularly commonly affected. (c) A return to normal morphology was observed when the mutant line was complemented with the RBL10–GFP construct. (This figure is available in colour at JXB online.)

the rhomboids are expressed differentially according to stage of life cycle and infection, an interesting possibility to consider in the regulation of rhomboid activity during development of *Arabidopsis*. A *Drosophila* rhomboid Rho1 is similarly differentially regulated during development (Bier *et al.*, 1990). Treatment with cold during seedling germination did induce transcription of RBL10 in our hands (data not shown). This result is consistent with the identification of the low-temperature responsive element of the *A. thaliana cor15a* gene (Baker *et al.*, 1994) in the promoter region of RBL10. Induction of RBL10 during cold stress might be important in releasing membrane-bound proteins for cold-sensitive pathways, or simply to moderate the fluidity of the membrane under low-temperature conditions. A detailed study using quantitative RT-PCR might help identify more exactly the peaks of transcription, which could be compared with those of the other rhomboid family members in the genome. Co-transcription analysis did link RBL10 with another chloroplast protease (the EGY2 metalloprotease), suggesting multiple proteases acting in regulation within the organelle, which is already known to occur within the thylakoid membrane during the repair of the photosynthetic apparatus (e.g. of the Photosystem II D1 protein; Bailey *et al.*, 2003; Kapri-Pardes *et al.*, 2007).

Previous work on rhomboids in a range of systems suggests, however, that an understanding of how they are sequestered and how their activity is regulated (which can be via movement of the substrate as in the prototype *Drosophila* rhomboid) would probably be more informative than analysis of transcription patterns. Our data show that RBL10 is localized to the chloroplast, and most likely to the chloroplast outer membrane. This localization persists through development and is consistent in a variety of organs, including the roots (in the non-photosynthetic plastids). Although little is known about plant organellar rhomboids, Karakasis *et al.* (2007) speculate that another predicted *Arabidopsis*-organellar rhomboid, At1g74130, may be involved with the plastid translocation machinery, namely the Tic40 component. A chloroplast membrane location limits the accessibility of RBL10 to a range of substrates, and might provide a useful starting point for the identification of target polypeptides. Our observations did not indicate any change in RBL10 localization as development proceeds, or in different organs, suggesting that RBL10 activity is not regulated by movement of the protease itself. The proteolytic activity of all rhomboids characterized to date seems to be controlled via some form of compartmentalization. This can be achieved by transporting the substrate from one location to another, as in the case of Star-Spitz in *Drosophila* (for a review see Freeman, 2008). The specialized organelles of the apicomplexa allow action of proteins at the right time and place (cleavage of adhesins in this case) to effect host-cell invasion (Brossier *et al.*, 2005). Examining expression of RBL10-GFP from germination to maturity shows that RBL10 does not move to different locations during development and that its transcription pattern is fairly uniform: it therefore seems likely that regulation of its catalytic activity would again be by transport of the, as yet unknown, substrate.

An interesting aspect of the RBL10-null phenotype is the reduced fertility of the mutant, whereas the RBL10-GFP expression was localized in plastids and transcript found in leaves, inflorescence, and non-photosynthetic tissues. Since jasmonate synthesis begins in plastids and affects pollen maturation and other aspects of fertility (Wu *et al.*, 2008), the question arises as to whether this rhomboid affects that biosynthetic pathway.

The roles of rhomboids in the mitochondrial compartment are being investigated, and might provide models for the function of chloroplast rhomboids. The yeast mitochondrial Pcp1 (processing of cytochrome *c* peroxidase; Ccp) rhomboid has two substrates: along with Ccp1 it is needed for proteolytic maturation of the short and long populations of the Mgm1 GTPase, which are somehow responsible for maintaining WT mitochondrial morphology (Herlan *et al.*, 2003). Mammalian PARL rhomboids seem to have related, although possibly not identical, roles in processing the mammalian OPA1 equivalent of yeast Mgm1, but also additional functions: another mitochondrial protein is cleaved by PARL, namely, HtrA2, which functions in lymphocyte apoptosis pathways (Chao *et al.*, 2008).

Analysis of RBL10 mutants revealed phenotypes with links to the chloroplast localization of the protein: absence of this chloroplast protease resulted in altered photoprotection in mutant plants. Excess absorbed light energy can be dissipated as heat, protecting plants from high-light stress (Horton *et al.*, 1994; Niyogi, 1999), i.e. feedback de-excitation NPQ of chlorophyll fluorescence (Müller *et al.*, 2001; Baker, 2008). Analysis of the interaction between RBL10 and other chloroplast-localized proteins should shed light on the role RBL10 plays in photoprotection. Again, the metalloprotease apparently co-regulated according to microarray data is pertinent here, since proteases have been recorded to work sequentially in other pathways. The lack of an effect on chlorophyll levels makes it likely that the role of RBL10 protease is distant from chlorophyll synthesis and assembly, and that its absence does not result in photo-damage sufficient to alter the chlorophyll *a:b* ratio, which was similar in the WT and the mutant.

Alongside the apparently minor photosynthesis-related phenotype, more marked developmental abnormalities were also observed in the RBL10 mutant. Root growth was enhanced in the mutant lines and lateral root growth increased significantly. These phenotypes are suggestive of a relationship between RBL10 and plant growth regulators, with auxin in particular implicated in both root elongation and lateral root outgrowth. Recent studies suggest that lateral roots grow out in positions determined by local maxima (or waves) of combined auxin sensitivity and auxin content (for a review see Delker *et al.*, 2007). Jasmonate is involved in the regulation of auxin biosynthesis and transport (Sun *et al.*, 2009), further circumstantial evidence linking this hormonal pathway and the multiple aspects of the RBL10 mutant phenotype. That the loss of RBL10 function enhances lateral root growth suggests that RBL10 acts as a negative regulator to this pathway, perhaps interfering with the release or localization of auxin

transporters or a transcription factor in membranes, for example, controlling jasmonic acid levels.

The most striking developmental phenotype was floral, corresponding with transcript being amplified from the shoot apex and in flowering stage plants. The floral phenotypes were widespread and diffuse, with incomplete penetrance. Deficiencies were observed in petal number, in stamen development, in pollen exine patterning, in stigma formation, and in fertility and seed set. All floral phenotypes were stronger in early inflorescences, with late bolts producing the same number of seeds per silique as WT plants, despite initially showing almost complete failure in fertilization. This wide range of abnormalities is indicative of a protein with a central role in regulating the cellular machinery, rather than in any single aspect of tissue or organ development. It is hypothesized that RBL10 operates to release one or a number of key regulatory proteins from membrane tethers and, in doing so, ensures normal cell function. In the mutants, the abnormal cellular functioning might be compensated for later in development either by a gradual build-up of cellular machinery or by enhanced expression and function of a different intramembrane protease, perhaps even one of the other *Arabidopsis* rhomboid proteins, since the multiprotein family includes others predicted to localize to the plastid.

To explain our mutant phenotypes fully it will be necessary to identify the targets of RBL10. Rhomboid proteases are almost ubiquitous across living organisms, suggesting an important but not essential role in cells. Regulated intramembrane proteolysis (RIP) is an emerging mechanism for controlling signalling pathways, for example, cholesterol biosynthesis in animals and Spitz-EGFR signalling from the Golgi, where a membrane-tethered transcription factor or a protein required for the activation of a transcription factor is cleaved and released. The first-discovered proteases acting in RIP were aspartyl and metalloproteases, but rhomboid serine proteases, another large protein family, have now been added to the list. RIP has not been demonstrated in plants, although the ability of RBL2 (At1g63120; but not RBL1/At2g29050) to cleave two *Drosophila* ligands (Kanaoka *et al.*, 2005) does suggest some conservation of function. In plants, there is also a growing list of, and interest in, membrane-tethered transcription factors, which present a logical target for rhomboids. This possibility is currently being explored further. The possible link between the EGY2 metalloprotease and RBL10 suggested by their co-localization to the chloroplast and predicted co-regulation would also be fascinating to explore. These families of proteases have previously been noted to operate in the same network (Tatsuta *et al.*, 2007): Ccp1 is a nuclear-encoded protein targeted to the mitochondrion intermembrane space by a bipartite presequence, the latter being cleaved first by ATP-dependent, intramembrane Yta10/12 peptidases (Esser *et al.*, 2002), after which maturation of Ccp1 is achieved by Pcp1, a rhomboid-like protease, in the organelle's inner membrane.

It is concluded that RBL10 is a plastid-localized plant rhomboid protease necessary for correct root growth, floral

development, fertility and photoprotection. Further analysis of its substrate and mode of activity will provide greater understanding of the function and significance of this broadly important protein.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Sequence features of RBL10 and alignment with representative rhomboids.

Supplementary Fig. S2. Root expression of RBL10-GFP and plastid-linking membranes; visualization of extensin-GFP control plants.

Supplementary Fig. S3. Collected microarray data for RBL10, tissue localization, and cotranscribed genes.

Supplementary Fig. S4. Position of T-DNA insertions in Salk mutant lines.

Supplementary Fig. S5. Seeds/silique in later inflorescences returns to wild-type levels.

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