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

Elinor P. Thompson1,*, Stefan G. Llewellyn Smith2,† and Beverley J. Glover1

1 Department of Plant Sciences, University of Cambridge, Cambridge CB2 3EA, UK
2 Institut de Mécanique des Fluides de Toulouse, UMR CNRS/INPT/UPS 5502, Allée Camille Soula, F-31400 Toulouse, France

† Permanent address: Department of Mechanical and Aerospace Engineering, Jacobs School of Engineering, UCSD, La Jolla CA 92093-0411, USA.
* Present address and to whom correspondence should be sent. School of Science, University of Greenwich, Medway Campus, Chatham Maritime, Kent ME4 4TB, UK; E-mail: te30@gre.ac.uk

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-β 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.)](image-url)
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 (García-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 (Ccpl1/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, ± 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; Ducha, Haarlem, The Netherlands) was used, and plates or pots were incubated in a growth room at 20 °C with light of 10 μmol m⁻² 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. For T-DNA insertion lines (NS37037, 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'-ATGGTATCGTGTATTCTCA-3' and 5'-TCAAGACCGGCTCGTGTATT-3', along with 5'-GGATTTAAGCTCAGGAGCT-3' and 5'-CACCAGTAGCACCACAAA-3' for locating T-DNA insertions. For the RBL10-GFP fusion protein, primers were 5'-GGTCCAGGATGTTATCGTGTA-3' and 5'-GAGATTCAAGCCGTCGCTGTTCATT-3', and 5'-TGCTCTAGACTTACATGTTACTAACTTCTA-GAGATGGTATCAGTGTCA-3'. For the RBL10 promoter primers were 5'-GGATTTAAGCTCGAG-3' and 5'-CACCAAGTAAGCCACCAAA-3' for locating T-DNA insertions. For the RBL10 promoter–GUS vector, primers were 5'-CGGGATCCGAAGATGGTATCAGTGTCA-3' and 5'-GGATTTAAGCTCAGGAGCT-3'.

DNA was extracted from above-ground tissue as described in Hipkins and Baker (1986) and Porra et al. (1989), extracting pigments 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_POPTR), which is 56% identical at the amino-acid level (see Supplementary Fig. S1 at JXB online). The Camelid 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 chlorophyll 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
but RT-PCR and microarray data suggested widespread

**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.)

saturating 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
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 μmol m⁻² s⁻¹ and 750 μmol m⁻² 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 μmol m⁻² 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 Fv/Fm, 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 μmol m⁻² s⁻¹, both Col-0 and RBL10-2 gave an Fv/Fm 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.
and 0.54±0.02 in the mutant (\(P=0.04, 10\) d.f.). Mean \(\varphi\text{PSII}\) 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 \(\mu\text{mol m}^{-2}\text{s}^{-1}\)), \(F_{v}/F_{m}\), \(\varphi\text{P}\), and \(\varphi\text{PSII}\) remained similar but, again, mean NPQ was markedly higher in mutant (mean ±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 flat 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 siliqua 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/siliqua 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 \textit{in vitro}; Brossier et al., 2005). In this apicomplexan parasite,
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 Rh1 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 releasimg 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-exitation NPQ of chlorophyll fluorescence (Mueller 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 photodamage 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 Yta10p/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.

Acknowledgements

We thank Matthew Freeman (LMB Cambridge) for helpful discussions, Julian Hibberd (University of Cambridge) for the loan of equipment, Jeremy Skepper (University of Cambridge) and Conrad Mullineaux (Queen Mary University London) for help with confocal microscopy, and Matthew Dorling (University of Cambridge) for excellent plant care. This work was funded by HFSP grant RGY0073/2005-C to BJG and SGLS.

References

Acosta IF, Farmer EE. 2009. The Arabidopsis book. Number 8. Rockville: American Society of Plant Biologists.

Bailey S, Thompson E, Nixon PJ, Horton P, Mullineaux CW, Robinson C, Mann NH. 2003. A critical role for the Var2 FtsH homologue of Arabidopsis thaliana in the Photosystem II repair cycle in vivo. Journal of Biological Chemistry 277, 2006–2011.

Baker NR. 2008. Chlorophyll fluorescence: a probe of photosynthesis in vivo. Annual Review of Plant Biology 59, 89–113.

Baker RP, Young K, Feng L, Shi Y, Urban S. 2007. Enzymatic analysis of a rhomboid intramembrane protease implicates transmembrane helix 5 as the lateral substrate gate. Proceedings of the National Academy of Sciences, USA 104, 8257–8262.

Baker SS, Wilhelm KS, Thomashow MF. 1994. The 5’-region of Arabidopsis thaliana cry1a has cis-acting elements that confer cold-, drought-, and ABA-regulated gene expression. Plant Molecular Biology 24, 701–713.

Ben-Shem A, Fass D, Bibi E. 2007. Structural basis for intramembrane proteolysis by rhomboid serine proteases. Proceedings of the National Academy of Sciences, USA 104, 462–466.
Bier E, Jan LY, Jan YN. 1990. rhomboid, a gene required for dorsoventral axis establishment and peripheral nervous system development in Drosophila melanogaster. Genes and Development 4, 190–203.

Blankenship RE. 2002. Molecular mechanisms of photosynthesis. Oxford: Blackwell Science.

Boyes DC, Zayed AM, Ascenzi R, McCaskill AJ, Hoffman NE, Davis KR, Görlich J. 2001. Growth stage–based phenotypic analysis of arabidopsis: a model for high throughput functional genomics in plants. The Plant Cell 13, 1499–1510.

Brossier F, Jewett TJ, Sibley LD, Urban S. 2005. A spatially localized rhomboid protease cleaves cell surface adhesins essential for invasion by Toxoplasma. Proceedings of the National Academy of Sciences, USA 102, 4146–4151.

Casci T, Freeman M. 1999. Control of EGF receptor signalling: lessons from fruitflies. Cancer Metastasis Review 18, 181–201.

Chao JR, Parganas E, Boyd K, Hong CY, Opferman JT, Ihle JN. 2008. Hax1-mediated processing of HtrA2 by Parl allows survival of lymphocytes and neurons. Nature 452, 98–102.

Chen G, Law K, Ho P, Zhang X, Li N. 2004. Genomic sequencing. In: Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP, and related tools. Annual Review of Genetics 42, 188–197.

Church GM, Gilbert W. 1984. Genomic sequencing. Proceedings of the National Academy of Sciences, USA 81, 1991–1995.

Delker C, Raschke A, Quint M. 2007. Auxin dynamics: the dazzling complexity of a small molecule’s message. Planta 227, 929–941.

Emanuelsson O, Brunak S, von Heijne G, Nielsen H. 2007. Locating proteins in the cell using TargetP, SignalP, and related tools. Nature Protocols 2, 953–971.

Esser K, Tursun B, Ingenhoven M, Michaelis G, Pratje E. 2002. A novel two-step mechanism for removal of a mitochondrial rhomboid signal sequence involves the mAAA complex and the putative rhomboid protease Pcp1. Journal of Molecular Biology 323, 835–843.

Freeman M. 2004. Proteolysis within the membrane: rhomboids revealed. Nature Reviews Molecular Cell Biology 5, 188–197.

Freeman M. 2008. Rhomboid proteases and their biological functions. Annual Review of Genetics 42, 191–210.

Gallio M, Sturgill G, Rather P, Kyisten P. 2002. A conserved mechanism for extracellular signaling in eukaryotes and prokaryotes. Proceedings of the National Academy of Sciences, USA 99, 12208–12213.

Garcia-Lorenzo M, Sjodin A, Jansson S, Funk C. 2006. Protease gene families in Populus and Arabidopsis. BMC Plant Biology 6, 30.

Gilmartin PM, Bowler C. 2002. Molecular plant biology: a practical approach. Oxford: Oxford University Press.

Herlan M, Vogel F, Bornhovd C, Neupert W, Reichert AS. 2003. Processing of Mgm1 by the rhomboid-type protease Pcp1 is required for maintenance of mitochondrial morphology and of mitochondrial DNA. Journal of Biological Chemistry 278, 27781–27788.

Hipkins MF, Baker NR. 1986. Spectroscopy. In: Hipkins MF, Baker NR, eds. Photosynthesis energy transduction: a practical approach. Oxford: IRL Press. 51–102.

Hofmann NR, Theg SM. 2005. Chloroplast outer membrane protein targeting and insertion. Trends in Plant Science 10, 450–457.

Horton P, Ruban AV, Walters RG. 1994. Regulation of light harvesting in green plants (indication by non-photochemical quenching of chlorophyll fluorescence). Plant Physiology 106, 415–420.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. Gus fusions: betaglucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO Journal 6, 3901–3907.

Kanaoka M, Shimizu KK, Okada K. 2001. KOMPEITO is required for exine formation and pollen–stigma adhesion in Arabidopsis. 12th International Conference on Arabidopsis Research, Madison, USA.

Kanaoka MM, Urban S, Freeman M, Okada K. 2005. An Arabidopsis rhomboid homolog is an intramembrane protease in plants. FEBS Letters 579, 5723–5728.

Kapri-Pardes E, Naveh L, Adam Z. 2007. The thylakoid lumen protease Deg1 is involved in the repair of photosystem II from photoinhibition in Arabidopsis. The Plant Cell 19, 1039–1047.

Karakashis K, Taylor D, Ko K. 2007. Uncovering a link between a plastid translocon component and rhomboid proteases using yeast mitochondria-based assays. Plant and Cell Physiology 48, 655–661.

Kmiec-Wisniewska B, Krumpe K, Urantowka A, Sakamoto W, Pratje E, Janska S. 2008. Plant mitochondrial rhomboid, AtRBL12, has different substrate specificity from its yeast counterpart. Plant Molecular Biology 68, 159–171.

Lee JR, Urban S, Garvey CF, Freeman M. 2001. Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell 107, 161–171.

Lemberg MK, Freeman M. 2007. Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. Genome Research 17, 1634–1646.

Lieberman RL, Wolfe MS. 2007. From rhomboid function to structure and back again. Proceedings of the National Academy of Sciences, USA 104, 8199–8200.

McQuibban GA, Saurya S, Freeman M. 2003. Mitochondrial membrane remodelling regulated by a conserved rhomboid protease. Nature 423, 537–541.

Mehrtens F, Krumpe K, Urantowka A, Sakamoto W, Pratje E, Janska S. 2008. Plant mitochondrial rhomboid, AtRBL12, has different substrate specificity from its yeast counterpart. Plant Molecular Biology 68, 159–171.

Mehler F, Kranz H, Bednarek P, Weisshaar B. 2005. The Arabidopsis transcription factor Myb32 is a flavonol-specific regulator of phenylpropanoid biosynthesis. Plant Physiology 138, 1083–1096.

Muller P, Li X, Niyogi KK. 2001. Non-photochemical quenching. A response to excess light energy. Plant Physiology 125, 1558–1566.

Niyogi KK. 1999. Photoprotection revisited: genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology 50, 333–359.

Obayashi T, Hayashi S, Saeki M, Ohta H, Kinoshita K. 2009. ATTED-II provides coexpressed gene networks for Arabidopsis. Nucleic Acids Research 37, D987–D991.

Pline WA, Edmisten KL, Oliver T, Wilcut JW, Wells R, Allen NS. 2002. Use of digital image analysis, viability stains, and germination assays to estimate conventional and glyphosate resistant cotton pollen viability. Crop Science 42, 2193–2200.

Porra RJ, Thompson WA, Kriedemann PE. 1989. Determination of accurate extinction coefficients and simultaneous equations for
assaying chlorophylls a and b extracted with four different solvent: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta 975, 384–394.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Smyth DR, Bowman JL, Meyerowitz EM. 1990. Early flower development in Arabidopsis. The Plant Cell 2, 755–767.

Stevenson LG, Strisovsky K, Clemmer KM, Bhatt S, Freeman M, Rather PN. 2007. Rhomboid protease AarA mediates quorum-sensing in Providencia stuartii by activating TatA of the twin-arginine translocase. Proceedings of the National Academy of Sciences, USA 104, 1003–1008.

Strisovsky K, Sharpe HJ, Freeman M. 2009. Sequence-specific intramembrane proteolysis: identification of a recognition motif in rhomboid substrates. Molecular Cell 36, 922–923.

Sun J, Xu Y, Ye S, et al. 2009. Arabidopsis ASA1 is important for jasmonate-mediated regulation of auxin biosynthesis and transport during lateral root formation. The Plant Cell 21, 1495–1511.

Tatsuta T, Augustin S, Nolden M, Friedrichs B, Langer T. 2007. m-AAA protease-driven membrane dislocation allows intramembrane cleavage by rhomboid in mitochondria. EMBO Journal 26, 325–335.

Thompson E, Wilkins C, Demidchik V, Davies JM, Glover BJ. 2010. An Arabidopsis flavonoid transporter is required for anther dehiscence and pollen development. Journal of Experimental Botany 61, 439–451.

Tripathi LP, Sowdhamini R. 2006. Cross genome comparisons of serine proteases in Arabidopsis and rice. BMC Genomics 7, 200–231.

Urban S. 2010. Taking the plunge: integrating structural, enzymatic and computational insights into a unified model for membrane-immersed rhomboid proteolysis. Biochemical Journal 425, 501–512.

Urban S, Freeman M. 2002. Intramembrane proteolysis controls diverse signalling pathways throughout evolution. Current Opinion in Genetics and Development 12, 512–518.

Urban S, Lee JR, Freeman M. 2001. Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107, 173–182.

Urban S, Lee JR, Freeman M. 2002. A family of Rhomboid intramembrane proteases activates all Drosophila membrane-tethered EGF ligands. EMBO Journal 21, 4277–4286.

Wasserman JD, Urban S, Freeman M. 2000. A family of rhomboid-like genes: Drosophila rhomboid-1 and roughoid/rhomboid-3 cooperate to activate EGF receptor signaling. Genes and Development 14, 1651–1663.

Wu K, Zhang L, Zhou C, Yu CW, Chaikam V. 2008. HDA6 is required for jasmonate response, senescence and flowering in Arabidopsis. Journal of Experimental Botany 59, 225–234.

Yamamoto YY, Obokata J. 2008. ppdb, a plant promoter database. Nucleic Acids Research 36, D977–D981.