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Evolutionary advantages of secreted peptide signalling molecules in plants

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Abstract. Peptide signalling molecules create diverse modular signals in animal systems, but it is only relatively recently that an expanding array of peptide signalling groups has been identified in plants. Representatives occur in moss although most are in angiosperms (both monocot and dicot) including many agronomically important crops. Some groups show high diversity within a species, whereas other peptide signalling groups are small or represented by a single peptide or only found in a single family of plants. Plant peptide signals regulate meristem organogenesis and growth, modulate plant homeostasis and growth, and recognise damage or imminent danger from pathogen attack. The peptide signalling molecules are secreted into the apoplast where they are often further proteolytically processed before acting on receptors in nearby or adjacent cells with all the hallmarks of paracrine molecules. Where the receptors have been identified, they are receptor-like kinases that form oligomers upon peptide binding and relay messages via phosphorylation cascades. The use of nitrogen rich amino acids in the signalling peptides was analysed and nitrogen scores were obtained that are higher than the mean nitrogen score for the overall average of the *Arabidopsis* proteome. These findings are discussed in terms of nutritional availability and energy use.

Additional keywords: Clavata3 (CLV3), CLE peptides, C-terminally encoded peptide 1 (CEP1), Embryo Surrounding Region (ESR), Epidermal Patterning Factor (EPF), Inflorescence Deficient in Abscission (IDA), leucine-rich repeat receptor-like kinases (LRR-RLKs), PEP1 peptide, phytosulfokine (PSK), plant natriuretic peptide (PNP), Rapid Alkalisation Factor (RALF), S-locus cysteine rich (SCR) proteins, Tapetum Determinant1 (TPD1).

Introduction

Plants are highly complex sessile living organisms that have evolved many methods to respond rapidly to environmental changes to continue normal growth and development. This is, in part, achieved by complex signalling processes mediated through networks of regulatory proteins and hormones. Peptide signalling molecules create diverse modular signals in animal systems, but it is only relatively recently that this class of molecules has been recognised in plants. In the last 20 years, peptide signalling molecules have been shown to contribute to a wide variety of plant functions ranging from plant cell differentiation to host defence responses (some recent reviews include Farrokhi *et al*. 2008; Jun *et al*. 2008; Boller and Felix 2009; Butenko *et al*. 2009). This is in addition to the arsenal of plant defence peptides and proteins with anti-microbial activity or the many protease inhibitors with *Arabidopsis thaliana* (L.) alone having 41 proteinase inhibitors (for a review, see Farrokhi *et al*. 2008).

In this review, we examine peptide molecules that are secreted and act in the extracellular apoplastic space to regulate plant growth, development, defence and other stress responses. We focus on secreted peptide signalling molecules found across a range of species including *A. thaliana*. Many of these molecules are listed in Table 1 and a brief review on each peptide family is available in the Accessory Publication to this paper. In this report, we briefly review the role of the various peptide signalling molecule classes in development and stress responses, and discuss some generalised themes that have become evident from combinations of biochemical, genetic and molecular biology studies over recent years. Finally, we use peptide nitrogen and sulfur content analysis as a way to assess the importance of these peptides to the plant proteome where we argue that the peptide molecules form an energy efficient means to allow gradients of signalling molecules to occur in niche areas within the plant.

Systemin: the discovery of peptide signalling systems

The first peptide signalling molecule identified was systemin, which was isolated from wounded tomato leaves (*Solanum lycopersicum* L.), where it induces synthesis of proteinase inhibitors (Pearce *et al*. 1991). Systemin is an 18 amino acid peptide product processed from the C-terminal of prosystemin, a 200 amino acid precursor protein (Dombrowski *et al*. 1999). Along with systemin, jasmonic acid (JA) has also been associated with early wound response (Farmer *et al*. 1992). In their review, Schilmiller and Howe (2005) describe grafting experiments in tomato, which show that both systemin and JA synthesis are required in the wounded tissue for a systemic response

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Table 1. Summary of secreted plant peptide secretory molecules present in *A. thaliana*

Propeptide[A]	Gene family	Propeptide size	Processed peptide, size	Function	Site of action	Receptor	References
CEP	5	8.5–11.5 kDa	CEP1, 14 aa	Inhibits root growth	Lateral root primordia	Unknown	Ohyama *et al.* (2008)
CLE (and CLV3)	31	7.8–14.5 kDa	CLE or mCLV3, 12–14 aa	Stimulates organogeneses and inhibits meristematic growth; can stimulate vascular development	Floral, shoot and root meristems; vascular	CLV1, BAM, CLV2,	Clark *et al.* (1995, 1997), Cock and McCormack (2001), Fiers *et al.* (2006), Kondo *et al.* (2006), DeYoung and Clark (2008)
EPF	7	11.5–14.3 kDa	Unknown	Promotes epidermal cell division leading to guard cell (stomatal) formation	Epidermis and meristemoid mother cells	TMM, ER, ERL1, ERL2	Hara *et al.* (2007, 2009), Hunt and Gray (2009)
IDA and IDL	6	8.4–13 kDa	EPIP	Inhibits floral abscission	Abscission zone	HAS, HSL	Butenko *et al.* (2003), Stenvik *et al.* (2008)
PROPEP	7	9.3–12.3 kDa	Pep1, 23 aa	Promotes innate immune responses (a danger signal)	Widespread, leaves	Pep1R	Huffaker *et al.* (2006), Yamaguchi *et al.* (2006), Pearce *et al.* (2008)
PNP	2	13–14 kDa	Unknown	Extracellular, Cell expansion, water or ion movement, stomatal opening, inhibits ABA induced stomatal closure	Mesophyll protoplasts, guard cells, root stele, stem	Unknown	Gehring *et al.* (1996), Maryani *et al.* (2001), Ludidi *et al.* (2002), Rafudeen *et al.* (2003), Wang *et al.* (2007), Gottig *et al.* (2008)
PSK	6	8.7–9.7 kDa	PSK-α, 5 aa	Promotes cell proliferation and longevity, root elongation	Mesophyll cells, roots	PSKR1	Matsubayashi and Sakagami (1996), Matsubayashi *et al.* (2006, 2002), Kutschmar *et al.* (2009)
PSY	1	7.9 kDa	PSY1, 18 aa	Promotes cellular expansion and proliferation, upregulated by wounding	Mesophyll cells, roots	PSYR1	Amano *et al.* (2007)
RALF and RALFL	34	7–14 kDa	RALF, 25–30 aa	Associated with danger signals, affects growth – inhibits root growth	Widespread in plants	Unknown	Pearce *et al.* (2001), Silverstein *et al.* (2007), Wu *et al.* (2007)
SCRL	27	9.2–11.5 kDa	Not processed	Prevents self-fertilisation (but not in *A. thaliana*)	Pollen	SRK	Schopfer *et al.* (1999), Vanoosthuyse *et al.* (2001)
TPD	1	19.5 kDa	TPD1	Anther development promoting tapetum formation	Anthers	EMS1	Yang *et al.* (2003), Jia *et al.* (2008)

[A]Brief reviews about each peptide class are available in the Accessory Publication to this paper.

(high levels of proteinase inhibitors in upper leaves) and that this wound response required leaves to perceive but not synthesise jasmonate. The overexpression of prosystemin leads to expression of a systemic signal in tomato that induces the systemic response (McGurl *et al*. 1994). Although homologues of systemin have been found in other species such as potato (*Solanum tuberosum* L.), bell pepper (*Capsicum annuum* L.) and black nightshade (*Solanum nigrum* L.), it is restricted to species within the Solaneae subtribe (Ryan and Pearce 1998), which is suggestive of systemin developing after the divergence of Nicotiana and Solanum.

Surprisingly, systemin was shown to bind to SR160, which is the tomato homologue of AtBRI1, the brassinosteroid receptor (Scheer and Ryan 2002). AtBRI1 is a leucine-rich repeat receptor-like kinase (LRR-RLK) that activates a well characterised phosphorylation cascade beginning with receptor autophosphorylation in response to brassinosteroids (Wang *et al*. 2005, 2008). Subsequently, the brassinosteroid mutant *cu3* was found to be as sensitive to systemin as wild-type plants (Holton *et al*. 2007; Lanfermeijer *et al*. 2008). These conflicting results were recently clarified by Malinowski *et al*. (2009), who showed that while systemin does bind specifically to SR160, systemin does not activate the BRI1 receptor autophosphorylation cascade. However, the characteristic signalling responses of the systemin pathway are induced, indicating that the true systemin receptor is still to be identified. In addition, there was also no difference in the level of proteinase inhibitors between tomato plants that overexpressed prosystemin and those that had the SR160 silenced (Malinowski *et al*. 2009). These findings highlight the importance of using binding and phosphorylation assays in addition to combinations of knock-down interactions to determine the veracity of the receptor–ligand complex.

Phylogenic relationships between classes

Most of the peptide groups examined have representatives in agronomically important monocot and dicot lineages such as rice (*Oryza sativa* L.), maize (*Zea mays* L.), sorghum (*Sorghum bicolour* (L.) Moench), soybean (*Glycine max* (L.) Merr.), castor oil bean (*Ricinus communic* L.), wine grape (*Vitis Vinifera* L.) and the black cottonwood tree (*Populus balsamifera* L. ssp. *trichocarpa* (Torr. & A. Gray ex hook.) Brayshaw). Some peptide groups such as plant peptide containing sulfated tyrosine (PSY) and *S*-locus cysteine-rich-like (SCRL) are similar to systemin in that representatives have been found in fewer species. A smaller subset of peptide groups are also represented in the conifers namely: Rapid Alkalinisation Factor (RALF), Phytosulfokine (PSK) and Epidermal Patterning Factor (EPF). Plant Natriuretic Peptide (PNP; sometimes annotated as expansin-like), Tapetum Determinant (TPD) and EPF are found in moss, and the conserved six cysteine residues within the carboxy terminal of EPF peptides are also found in sea anemone sequences.

Closer examination of the 126 *A. thaliana* peptide sequences available shows that peptides of the same group are more similar to each other than any peptide from another group (see Fig. S1 available as an Accessory Publication to this paper). A single protein sequence was selected to represent each peptide group and a radial cobalt tree shows the peptides in relation to one another (Fig. 1) where, for instance, Clavata3 (CLV3) was chosen to represent the CLE family. CLV3 is the prototype member of the CLE family named after *Clv3* from *A. thaliana* (Fletcher *et al*. 1999) and *Embryo Surrounding Region* (*ESR*) from maize (Opsahl-Ferstad *et al*. 1997) and forms one of the largest families of plant peptide signalling molecules present throughout the plant kingdom (Cock and McCormack 2001; Oelkers *et al*. 2008), with over 30 annotated genes in *A. thaliana*.

The number of members of the peptide groups we examined varied from 1 (TPD1) to 34 (RALF and RAFL-like (RALFL)), and groups with many members did not necessarily correlate with ancient lineages. A TPD1 sequence homologue has been found in moss but the prolific SCRL family has 26 members in *A. thaliana* but is limited to the Brassicaceae (Schopfer *et al*. 1999). Within each peptide group, the members were often spread across the chromosomes but in some cases, the peptide-encoding genes were clustered (see Fig. S1). This is seen with PROPEP (the full length forms of the peptide signalling family involved in innate defence responses) where six genes are clustered in two groups on chromosome 5, and another gene (*PROPEP6*) occurs on chromosome 2, which is more similar to members of one of the clusters than the other. Similarly, the more expanded signalling peptide groups such as RALFL, SCRL and CLE also have clustered encoding genes. In the case of RALFL, several encoding gene groups are clustered and are very similar in sequence, suggesting recent duplication such as RALFL2 with RALFL3 and RALFL8 with RALFL9. However, close proximity of encoding genes does not necessarily mean there is a high level of sequence similarity (see Fig. S1).

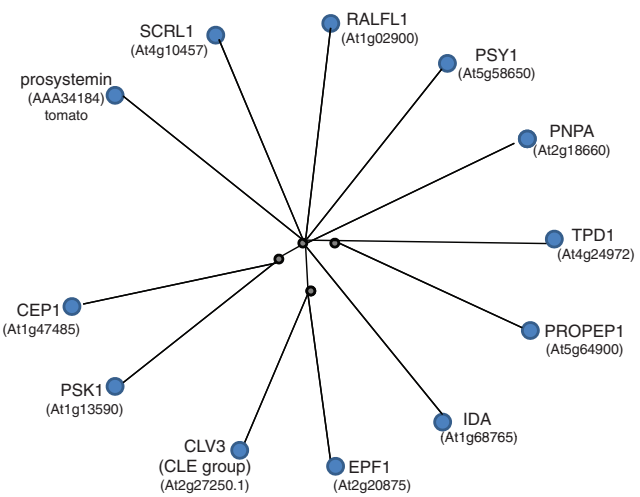

Fig. 1. Phylogenic diagram demonstrating that the different peptide classes are not related. This Radial Cobalt Tree was produced using National Centre for Biotechnology Information (NCBI) COBALT multiple alignment tool (Papadopoulos and Agarwala 2007). All 126 available *A. thaliana* amino acids sequences of the different peptide classes as well as the tomato prosystemin sequence were segregated into the different peptide classes. In the Radial Cobalt Tree shown, a single protein sequence was selected to represent each group: CEP (5 sequences), CLE (31), EPF (7), IDA (6), PROPEP (7), PNP (2), PSK (7), PSY (1), RALF (34), SCRL (26) and TPD1 (1). Refer to Table 1 for abbreviations and a description of each class is available in the Accessory Publication to this paper.

Processing of peptide signalling molecules

In general, peptide signalling molecules are relatively small proteins (ranging in size from ~60 to 180 amino acids) containing a N-terminal secretory signal sequence (pre-propeptide) that is cleaved off by endoplasmic reticulum proteases as the propeptide is translated and processed through the default secretory pathway (Denecke *et al*. 1990). In some cases, the propeptide (at least the active peptide region) is modified during this process. For instance, PSKs are sulfated on the tyrosine residues in the active pentapeptide region probably as the protein is processed through the Golgi network by enzymes such as tyrosylprotein sulfotransferase (Hanai *et al*. 2000) before being secreted. It is likely that it is during this processing stage that proCLV3 is hydroxylated on proline residues within its active region, as a modified 12 amino acid peptide (mCLV3) containing two hydroxyproline residues derived from the CLE region of CLV3 has been identified in *A. thaliana* tissues (Kondo *et al*. 2006). The CLE motif that forms the mature hydoxyproline-mCLV3 is all that is required for its activity (Fiers *et al*. 2006). Similarly, C-Terminally Encoded Peptide (CEP) 1 contains hydroxyproline residues in its 14 amino acid processed form (Ohyama *et al*. 2008), whereas the sulfation of the tyrosine residues is required to obtain high activity of PSK-α (Matsubayashi and Sakagami 1996).

Processing and secretion has only been described for a few of the peptides and is predicted for many of the other peptides from their sequence information. However, in most cases, evidence has been obtained that at least the prototype peptide of each class is found in the apoplast (Table 1; Fig. 2), indicating that the peptide has been secreted. PSK-α was originally isolated as a cell proliferation factor in the culture medium essential for low density cell cultures of asparagus (*Asparagus officinales* L.) (Matsubayashi and Sakagami 1996). Other peptides have been isolated from screens for factors in the extracellular medium

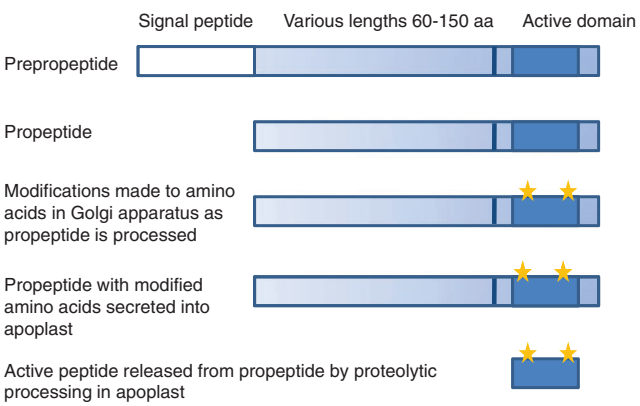

Fig. 2. Processing of peptide signalling molecules. After transcription, the pre-propeptide has its secretory signal sequence cleaved in the endoplasmic reticulum forming the propeptide. As the propeptide is processed through the Golgi apparatus, amino acid modifications are made (stars) before the propeptide is secreted into the apoplast, where the active peptide is released by further proteolytic processing as described in the text. The active domain is generally located in the C-terminal region of the peptide (indicated by the darker rectangle) with a dibasic region 5–20 amino acid residues upstream (dark line) that is a proteolytic cleavage point.

that stimulate defence responses such as alkalinisation of the extracellular medium; these include systemin (Pearce *et al*. 1991) and RALF (Pearce *et al*. 2001). Several of the factors were first identified from genetic screens where the knock-out mutants caused abnormal growth of particular regions such as the *clv3* mutant, which contains excess stem cells in shoot apical and floral meristems that continue to enlarge over time (Clark *et al*. 1995). ProCLV3 is secreted into the meristematic apoplast (Rojo *et al*. 2002) and PNP-A is also secreted from mesophyll cells (Y. H. Wang and H. R. Irving, unpubl. obs.). EPF1 and EPF2 are involved in determining epidermal cell division events that lead to stomatal formation in leaf and stem epidermis, and were identified from mutant screens that detected abnormal stomatal patterns (Hara *et al*. 2007, 2009; Hunt and Gray 2009). However, an approach based on an analogy to animal systems was used to identify and purify PNP, which was immunoreactive to antisera specific for the animal peptide factor atrial natriuretic peptide (Vesely and Giordano 1991; Gehring *et al*. 1996; Maryani *et al*. 2001). The genes for PNP have since been identified (Ludidi *et al*. 2002) and it appears from phylogenetic data that similarities between AtPNP-A and ANP may be the result of convergent evolution (Gehring and Irving 2003).

Once secreted into the apoplast, the propeptides can be further processed by specific extracellular proteases also secreted into the apoplast (Fig. 2). Processed peptide molecules have been identified using mass spectrometry for mCLV3, mCEP1, PSK-α and PSY1 (Matsubayashi and Sakagami 1996; Kondo *et al*. 2006; Amano *et al*. 2007; Ohyama *et al*. 2008; Table 1). In general, these active small peptides originate from conserved regions in the C-terminus of the propeptide molecule (Fig. 2) and this region has homology with other peptides of the same class but not between classes (Fig. 1). Alternatively, incubation of propeptide with cauliflower (*Brassica oleracea* L. var. *botrytis* L.) meristem extracts has been used to show that the propeptide is processed into active smaller peptides. This has been done with CLV3, CLE1 and Inflorescence Deficient in Abscission (IDA) to yield active mature peptide fractions (Ni and Clark 2006; Stenvik *et al*. 2008). Both PROPSK and PRORALF are cleaved by specific subtilisin type serine proteases in the apoplast at the dibasic amino acids upstream of the C-terminally encoded active peptide region to release the peptide (Srivastava *et al*. 2008, 2009). Further processing is still required to release the active pentapeptide in the case of PSK-α. Thus several enzymes and the propeptide are required to meet in the apoplast and presumably these need to be secreted from adjacent or the same cells for this to occur.

On the other hand, PNP has a region towards the N-terminus that is homologous with animal atrial natriuretic peptide and this region also contains its functional activity (Morse *et al*. 2004; Wang *et al*. 2007) but it is currently unknown if the protein is further processed. Also the low molecular weight cysteine rich (LCR) and SCRL proteins contain conserved cysteine residues throughout the secreted protein (Vanoosthuyse *et al*. 2001), indicating that extracellular processing may not be a universal feature of secretory peptides.

Paracrine and autocrine effects

In many cases, these signalling peptides are expressed in particular and restricted regions of the plant where they are

secreted, are processed further and act upon nearby cells (Fig. 3). The receptors that have been identified are generally members of receptor-like protein families that form oligomers and recognise particular patterns of the active peptide ligand (see next two sections). So even if the peptide ligands share common receptors, their action is limited to the areas that the peptides are actually secreted and processed. This type of action is similar to growth factors regulating development in animal cells where a compound acting on adjacent or nearby cells is said to have a paracrine effect to distinguish it from that of a long distance hormone (endocrine) effect. In some cases, the compounds act on the cell that generates them, which is referred to as an autocrine effect. One of the advantages of this type of signalling is that it allows organs to respond to a gradient of molecules and is a very ancient form of signalling evident throughout the development of multi-cellular organisms. Most of the prototype peptides listed in Table 1 demonstrate paracrine and possibly autocrine signalling. This is particularly marked with those directly affecting development such as CLE, EFP and IDA, which act at specific localised regions within the plant.

Peptide signalling molecules in development

Several of the peptide signalling molecules have distinct roles in development where they regulate cell differentiation and organogenesis, often by repressing cellular growth. In several instances, this has been determined from knock-out mutant studies that have identified phenotypic mutants displaying overgrowth of particular regions such as those occurring in the meristem with *clv3* (Clark *et al*. 1995), anthers with *tpd1* (Yang *et al*. 2003), or epidermal leaf surface and stomatal development with *epf1* and *epf2* mutants (Hara *et al*. 2007, 2009; Hunt and Gray 2009). To verify these interpretations, several groups have made use of overexpressing mutants that exhibit restricted development of the particular regions. Alternatively, peptide fractions have been directly (ectopically) applied and effects opposing those of the knock-out mutants but similar to the overexpressing mutants have been observed. For instance, overexpressing (ox) CEP1 is mainly found in the lateral root primordia and represses root growth, as does the ectopic application of synthetic mCEP1 (Ohyama *et al*. 2008). CLE19 is normally found in roots, and ectopic application of synthetic peptides corresponding to conserved CLE motifs of CLV3, CLE19 and CLE40 caused the termination of the root meristem, which is a similar phenotype to oxCLE19 mutants (Fiers *et al*. 2006). Most members of the CLE family (known as CLE-A) act to repress cell division in the meristematic regions (Whitford *et al*. 2008).

A useful phenomenon in genetic analysis is that similar phenotypes are associated with knock-out mutants of other members of the signalling pathway, such as the receptor and downstream signalling components. For instance, CLV3 encodes the secreted peptide that acts to repress apical and floral meristem cell division, as in its absence in the knock-out mutant *clv3*, the meristems continue to enlarge over time (Clark *et al*. 1995). While *clv1* exhibits a similar and weaker phenotype (Clark *et al*. 1993), it encodes an LRR-RLK (Clark *et al*. 1997). Similarly, the receptors for IDA, EPF and TPD1 have been identified using such genetic screens and are also members of the LRR-RLK family (Hara *et al*. 2007; Jia *et al*. 2008; Stenvik *et al*. 2008; Hara *et al*. 2009). However, where genetic redundancy is evident with multigene families expressed in the same tissues, mutant screens will be considerably less useful.

Another feature that is worth remarking on is that specific peptides from a particular class involved in regulating developmental responses are expressed in quite particular and restricted regions of the plant. In addition, there is considerable redundancy in the effects of the peptide class that is, to a certain extent, counterbalanced by the restricted expression patterns. For instance, although members of the CLE-A family act to repress cell division in the meristematic regions, members of the CLE-B family (CLE41–44) affect vessel development

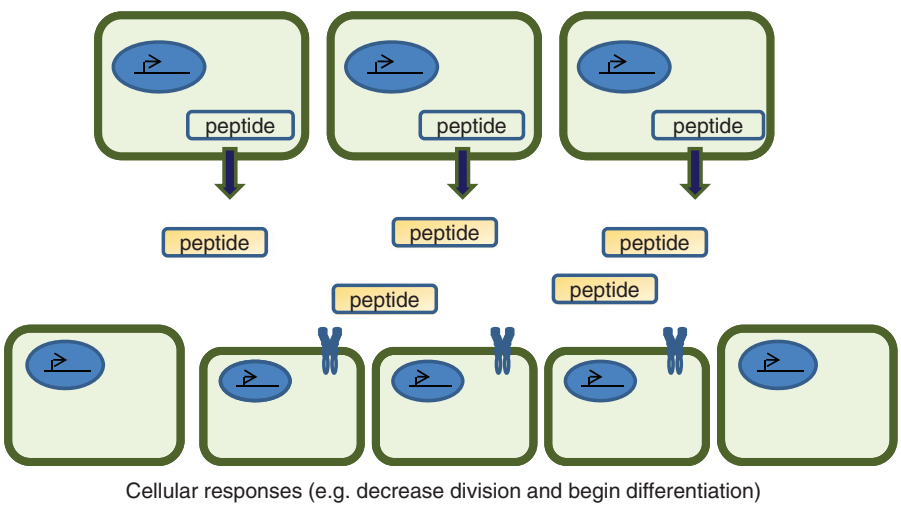

Fig. 3. Paracrine and restricted mode of peptide signalling. In many cases, peptide signalling molecules are released from a localised group of cells into the apoplast where they form a concentration gradient that acts most strongly on nearby cells that contain receptors and that, in the case of the meristem peptides, restricts growth.

(Whitford *et al*. 2008) and are homologues of the *Zinnia elegans* Cav. (also known as *Zinnia elegans* Jacq.) tracheary element differentiation factor that suppresses xylem cell differentiation in cultured mesophyll cells (Ito *et al*. 2006). Combinations of CLE-A and -B peptides potentiated the B-type effect of proliferation of vascular development (Whitford *et al*. 2008). Thus in the meristem, a reciprocal gradient of CLE-A and CLE-B-type peptides will form that will regulate organogenesis and vascular development, and may be relayed by the same or similar classes of receptors recognising different combinations of CLE ligands. This is probably not surprising, as the CLE ligand is relatively conserved (Cock and McCormack 2001); however, it is likely that multiple combinations of CLE receptors are expressed in the developing vascular and meristematic regions (also see Fukuda *et al*. 2007; Jun *et al*. 2008). Such findings highlight the importance of spatial differentiation in the expression patterns of the CLE (and other) peptides to prevent developmental errors.

Peptide signalling molecules influencing growth

Other peptides appear to have subtle effects where they may be involved in modulating general growth and development in response to the environment. Although RALF and RALFL were first identified in a screen for plant defence proteins (Pearce *et al*. 2001), their role is considerably more diverse and they are also likely to influence development. Exogenous application of RALFL inhibits root growth (Pearce *et al*. 2001) and silencing of RALF disrupts root hair development (Wu *et al*. 2007). In another example, PSK-α appears to act in a cooperative manner with CLE41–44. PSK-α promotes tracheary element differentiation in *Z. elegans* mesophyll cell cultures in the presence of auxin and cytokinin (Matsubayashi *et al*. 1999; Motose *et al*. 2009), whereas CLE41–44 inhibits this process (Ito *et al*. 2006; see Fukuda *et al*. 2007, for a discussion). PSK-α has a general proliferative effect and was discovered as a cell proliferation agent essential for low density cell cultures (Matsubayashi *et al*. 1996). In a further example of redundancy, in *A. thaliana*, there are five preproPSK genes with overlapping expression patterns throughout the plant. These proteins also seem to promote cell longevity, as plants overexpressing the PSK receptor (oxPSKR1) exhibited delayed senescence and prolonged leaf expansion; root length was reduced in *pskr1* knock-out mutants (Matsubayashi *et al*. 2006). The effect of PSK-α on roots has recently been examined in more detail, where it was shown that PSK-α enhances root elongation by controlling cell size (Kutschmar *et al*. 2009). Even though the effects of PSK-α are proliferative and growth-enhancing as distinct from the growth-restricting effects of peptides affecting organogenesis, they still act in a paracrine fashion and built-in redundancy is evident.

PNP is an interesting molecule as it appears to have general effects on cellular homeostasis (Gehring and Irving 2003). PNPs represent a novel class of small proteins (~14 kDa) that are distantly related to expansions, which are regulators of cell wall extension (Ludidi *et al*. 2002; Kende *et al*. 2004). PNP also is likely to have a role in cell expansion as it enhances the volume of mesophyll protoplasts (Maryani *et al*. 2001; Morse *et al*. 2004; Wang *et al*. 2007). However, it appears to have many other properties as both PNP isolated from leaves and recombinant PNP-A also stimulated stomatal opening, activated the H^+-ATPase and modulated ion fluxes (Pharmawati *et al*. 1999; Maryani *et al*. 2001; Ludidi *et al*. 2004; Wang *et al*. 2007), although these effects could be part of the cell expansion process. In addition, PNP protein levels are increased in NaCl-stressed whole plants and *A. thaliana* suspension culture cells exposed to high salt or osmoticum (Rafudeen *et al*. 2003). Analysis of *A. thaliana* microarray data through Genevestigator (Zimmermann *et al*. 2004) also indicates that AtPNP-A transcripts are upregulated in response to abiotic stresses such as osmoticum, salt, mineral deficiencies and ozone exposure. Recombinant PNP-A directly increases stomatal conductance and transpiration rates, which are correlated with increases in photosynthetic rates where the efficiency of light use during photosynthetic CO_2 fixation was enhanced (Gottig *et al*. 2008). Furthermore, recombinant PNP-A modulates the effect of abscisic acid (ABA) on stomatal aperture (Wang *et al*. 2007). Since both compounds are upregulated in times of environmental stress (e.g. drought), it is conceivable that one of the physiological roles of PNP-A is to act as an antagonist to ABA and, in the case of stomata, promote limited gas exchange.

Peptide signalling molecules involved in defence responses

Perception of danger is a key part of the plant innate defence responses as argued by Boller and Felix (2009) in their recent review. Plants detect microbes via microbe-associated molecular signatures, which are molecules such as bacterial flagellin (flg22) with specific plant receptors that are generally members of the receptor-like proteins including members of the LRR-RLK family such as Flagellin Sensing (FLS) 2 (Boller and Felix 2009). Plants also seem to contain endogenous danger signals such as systemin, PEP1 and RALF/RALFL that are associated with responses to pathogen attack. These peptides were all identified in their active excreted processed form in alkalinisation screens (Pearce *et al*. 1991, 2001; Huffaker *et al*. 2006). Unlike systemin, both RALF and PROPEP are found throughout the plant kingdom (Pearce *et al*. 2001; Haruta and Constabel 2003; Germain *et al*. 2005; Huffaker *et al*. 2006; Silverstein *et al*. 2007). Since the processed forms of these peptides are more active, it is tempting to speculate that propeptides are found in the apoplast and the active peptides form degradation products of proteases released by damaged plant cells or the pathogens themselves. This is likely to be the case with proRALF23, which is cleaved by specific plant subtilisin serine proteases to release the active peptide (Srivastava *et al*. 2009). The receptor for AtPEP1 (PEPR1) has been identified through peptide crosslinking studies and it is an LRR-RLK (Yamaguchi *et al*. 2006) but the receptor for RALF has not yet been identified. Expression of PROPEP1 is upregulated by PEP1 itself as well as wounding, jasmonates, ethylene and bacterial flg22 (Huffaker *et al*. 2006), suggesting that PEP1 acts as an endogenous danger signal (Boller and Felix 2009).

PNPs may also have a role in plant defence, as coexpression and promoter content analyses indicate that PNP-A may function alongside other pathogenesis-related proteins as a component of

plant defence responses (Meier *et al*. 2008). The functionally uncharacterised transcript from citrus CjBAp12 is similar to PNP-B and was initially isolated as a mobile peptide associated with the plant response to citrus blight, which is a disease of unknown aetiology (Ceccardi *et al*. 1998). Interestingly, a PNP-like gene occurs uniquely in the bacterial pathogen that causes citrus canker, *Xanthomonas axonopodis* pv. *citri* (Nembaware *et al*. 2004). This bacterial protein alters the plant host homeostasis responses where it increases stomatal conductance, transpiration and photosynthetic rates, and enhances the efficiency of light use during photosynthetic CO_2 fixation (Gottig *et al*. 2008). It is speculated that expression of XacPNP allows the pathogen to create a favourable environment within the host for its growth and that it is an example of horizontal gene transfer (Gottig *et al*. 2008).

Receptors for peptide ligands

For ligands to communicate a message effectively, receptors need to exist that relay the message, so receptors and their ligands are thought to have evolved in parallel (Fryxell 1996). So far, the receptors for peptide ligands that have been identified are members of the receptor protein-like family and several of them are either LRR-RLKs or leucine rich repeat receptor-like proteins (LRR-RLPs). Several recent reviews have discussed the different types of receptor like proteins in relation to their interactions and signalling mechanisms (see Butenko *et al*. 2003; Afzal *et al*. 2008; Boller and Felix 2009; Tör *et al*. 2009). LRR-RLKs contain a large leucine rich motif that is repeated several times in the extracellular domain, a single transmembrane domain and a cytoplasmic serine-threonine kinase domain, whereas the LRR-RLPs lack the intracellular kinase domain (Fig. 4). In addition, these receptor-like proteins are sometimes associated with another group of serine-threonine kinase molecules that lack the extracellular domain (Fig. 4). Members of the LRR-RLK family that are well characterised include the brassinosteroid (AtBRI1) and flagellin (FLS2) receptors. An important part of their activation is the ability to form oligomers rapidly (within minutes) with other LRR-RLKs such as Brassinosteroid Associated Kinase 1 (BAK1) and this in turn stimulates receptor autophosphorylation and a phosphorylation cascade (Wang *et al*. 2005, 2008; Chinchilla *et al*. 2007).

Oligomer formation has been identified as part of the signalling cascade in response to CLV3. CLV1 is a full length LRR-RLK (Clark *et al*. 1997) that directly interacts with modified

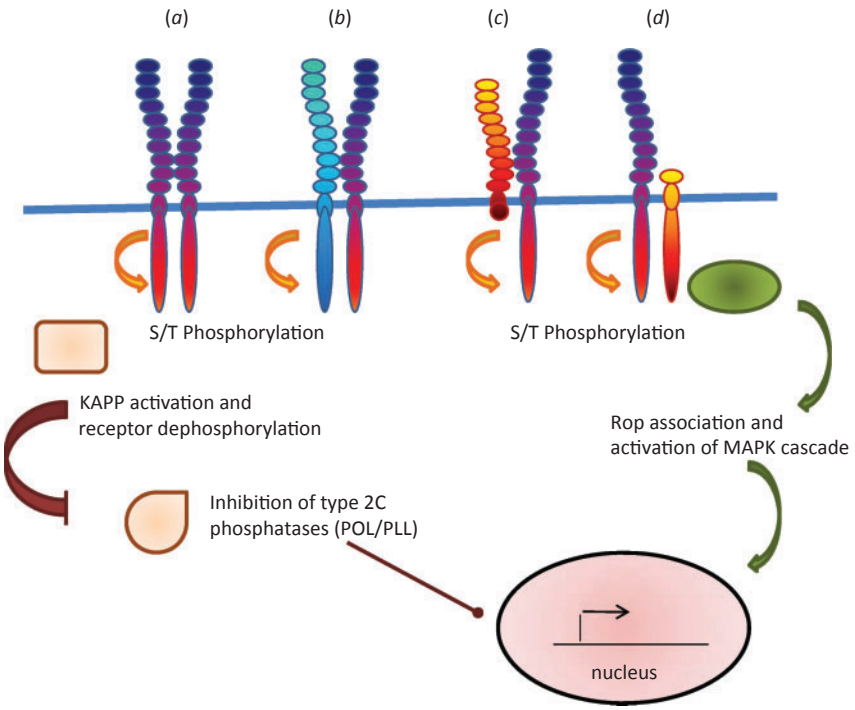

Fig. 4. Models of receptor oligomers and activated pathways. The functional receptors form oligomers containing a leucine-rich repeat external domain, a single transmembrane spanning domain and an intracellular (cytoplasmic) kinase domain. Oligomers form between (*a*) the same or (*b*) different LRR-RLKs where both receptors contain all of the functional domains. Alternatively oligomers can form with one LRR-RLK and either (*c*) a LRR-RLP that contains no kinase domain or (*d*) membrane associated kinase protein containing a limited extracellular domain. Upon ligand binding and oligomer formation, the kinase domains autophosphorylate serine and threonine residues and initiate a phosphorylation cascade, perhaps through small G proteins such as Rop, which then activates the mitogen activated protein kinase (MAPK) cascade to modify transcription. Various type 2C protein phosphatases such as KAPP are also stimulated that dephosphorylate proteins and act to repress the signalling pathway.

CLV3 in the external leucine rich domain (Ogawa *et al*. 2008). CLV1 forms dimers with other closely related LRR-RLKs such as Barely Any Meristem (BAM) to bind CLV3 (DeYoung and Clark 2008) while CLV2 is receptor like molecule that lacks any kinase domain (Jeong *et al*. 1999) but associates with the intracellular kinase Coryne (CRN) rather than CLV1 (Müller *et al*. 2008; see Butenko *et al*. 2009 for a review). Thus two parallel receptor pathways appear to be acting in the shoot and floral meristems that are receptive to CLV3. The other peptide receptors are not so well characterised. However, receptors for PSK-α, PSY1 and PEP1 have been identified by affinity-crosslinking studies and these receptors are all LRR-RLKs (Matsubayashi *et al*. 2002, 2006; Yamaguchi *et al*. 2006; Amano *et al*. 2007). Mutant studies have been useful in identifying potential receptors such as the LRR-RLKs Hasea (HAS) and HAS-Like (HSL) 2 for IDA (Stenvik *et al*. 2008). TPD1 binds to a specific site within the extracellular leucine rich domain of Excess Microsporocytes1 (EMS1) (also known as Extra Sporogenous Cells (EXS)), and this in turn activates EMS1 receptor auto-phosphorylation (Jia *et al*. 2008). Similarly, Too Many Mouths (TMM), which is an LRR-RLP, and the LRR-RLKs Erecta (ER) and ER-Like (ERL) 1 and 2 were identified by mutant studies as the receptors for EPF and are thought to form an oligomer complex (Hara *et al*. 2007; Bhave *et al*. 2009; Hara *et al*. 2009). Several of these LRR-RLKs (PEPR1, CLV1 and ER) also contain a putative guanylate cyclase domain within the general kinase domain region (Kwezi *et al*. 2007) and it is of interest to speculate that production of cyclic GMP may form part of the signalling pathway in addition to the phosphorylation cascade. Indeed, *in vitro* studies have revealed that AtBRI1, which also contains this domain, does have guanylate cyclase activity (Kwezi *et al*. 2007).

Although the receptor for PNP has not been identified, the signalling cascade in response to application of PNP involves a very rapid (within seconds) increase in cGMP levels (Pharmawati *et al*. 1998; Pharmawati *et al*. 2001; Wang *et al*. 2007), indicating that the receptor either contains or is very closely associated with a guanylate cyclase. Rapid increases in cytoplasmic calcium occur in the surface cells of seedling roots in response to RALF1 (Haruta *et al*. 2008) and there is also a rapid alkalinisation of the external media, indicating that ion fluxes have been activated and ATPase-dependent proton pumps are inhibited (Pearce *et al*. 2001). Much remains to be discovered about the details of the signalling networks activated by the peptide signalling molecules. However, based on what is known about the BRI1–BAK interaction (Wang *et al*. 2005, 2008), they are likely to involve oligomerisation, phosphorylation and dephosphorylation cascades. Indeed, the CLV3–CLV1 cascade involves inhibition of the type 2C protein phosphatases Poltergeist (POL) and POL-Like (PLL), which eventually represses the transcription factor Wushel (WUS) and so inhibits stem cell formation (Mayer *et al*. 1998; Yu *et al*. 2000). A Rho-type GTPase molecule is also incorporated into the activated CLV3–CLV1 receptor complex and is thought to activate a kinase cascade (Trotochaud *et al*. 1999). The activity of CLV1 is reduced by a feedback system where CLV1 is dephosphorylated by the type 2C phosphatase, Kinase Associated Protein Phosphatase (KAPP) (Stone *et al*. 1998; Jun *et al*. 2008; Butenko *et al*. 2009). Cessation of the signal response could be further enhanced by events such as receptor mediated endocytosis, which has been reported to occur with FLS2 following 10–20 min stimulation with bacterial flagellin (Robatzek *et al*. 2006).

Expression of the receptors involved in organogenesis is restricted to localised areas, as occurs with CLV1, CLV2 and CRN (Müller *et al*. 2008). However, the receptor oligomers respond to ectopically applied ligands, which is paramount as a gradient of responses between different members of the CLE family (Whitford *et al*. 2008). This indicates that there is some overlapping redundancy in the receptor specificity, which is hardly surprising as they are recognising the relatively small but highly similar active peptide fragment. By the very nature of the reported actions of the ligands, it would be expected that receptors for PSK-α, PEP1, RALF and PNP are much more widely expressed. This is indeed the case for PSKR1 and 2 and PSYR1, which are widely expressed and appear to have overlapping and redundant functions, as triple mutants exhibit dwarfism due to decreases in both cell size and number (Amano *et al*. 2007).

Nitrogen and sulfur content of peptide signalling molecules

At first glance, it would appear counterintuitive for plants to invest heavily in nitrogen-rich molecules such as peptides and proteins for signalling molecules. Nitrogen and phosphorous are major nutrients limiting plant growth (Elser *et al*. 2007), and in Australian situations where ancient soils are present, phosphorous is more limiting (Lambers *et al*. 2009). However, a recent study revealed that plants have adapted to ecological nitrogen limitations so that crop plants have higher nitrogen contents in their transcribed RNA compared with undomesticated plants (Acquisti *et al*. 2009*a*). This phenomenon is carried through to the proteome, where crop plants and nitrogen fixing plants have proteins containing more amino acids with nitrogen-rich side chains than undomesticated plants (Acquisti *et al*. 2009*a*). Sulfur is another nutrient that, in many instances, is limiting, which will constrain the use of cysteine and methionine amino acid pools (Hawkesford and De Kok 2006).

With these findings in mind, we were curious to determine if the signalling peptides (prepropeptide) were relatively nitrogen-rich, as we reasoned that their nitrogen levels may reflect their level of importance to the plant. We assessed the importance of the signalling peptides in *A. thaliana* (an undomesticated species) using the nitrogen proteome criteria of Acquisti *et al*. (2009*a*), who found that the average nitrogen score for all proteins in the *A. thaliana* proteome was 0.3637 ± 0.0788 (mean ± s.d.). Surprisingly, many of the peptides have nitrogen scores that are higher than the mean nitrogen score for the overall average of *A. thaliana* (Fig. 5; Table S1 (the latter is available as an Accessory Publication to this paper)). With an average of 0.413 ± 0.096 across all prepropeptides, the N value is closer to the N value reported for cellular anabolic machinery (0.482 ± 0.164) than catabolic machinery (0.313 ± 0.061) in *A. thaliana* (Acquisti *et al*. 2009*b*). Several of the peptides such as SCRL and RALF contain highly conserved cysteine residues (Pearce *et al*. 2001; Vanoosthuyse *et al*. 2001; Silverstein *et al*. 2007) and since cysteine is a structurally

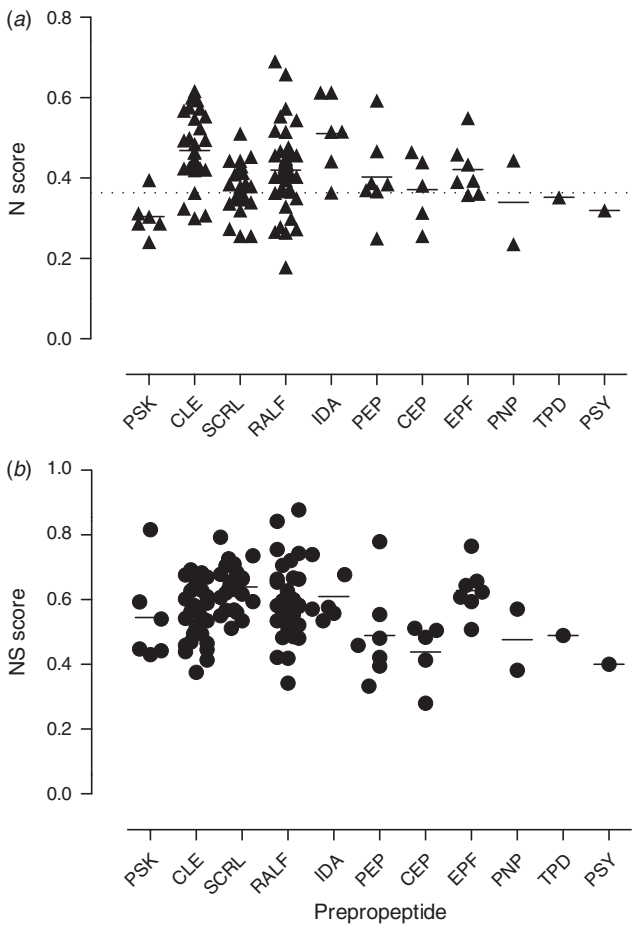

Fig. 5. Nitrogen and sulfur use of prepropeptide signalling molecules. (*a*) The nitrogen (N) score was assessed for prepropeptides using the formula $\Sigma(n_i \times p_i)$ devised by Acquisti *et al*. (2009*a*), where n_i is the number of N atoms in the side chain and p_i is the proportion of these in the final prepropeptide. The amino acids with nitrogen-rich side chains were scored: $n = 1$ for asparagine, glutamine, lysine and tryptophan; $n = 2$ for histidine; $n = 3$ for arginine; and $n = 0$ for the remainder. The dotted line is set at the average value (0.3637) obtained for the *A. thaliana* proteome by Acquisti *et al*. (2009*a*). (*b*) The use of amino acids containing sulfur was assessed in addition to the nitrogen-rich amino acids where $\Sigma(s_i \times p_i)$ with $s = 1$ for cysteine and methionine and $s = 0$ for the remainder. The calculated number was added to the N score to form the N–S score.

important amino acid and sulfur is a limiting nutrient (Hawkesford and De Kok 2006), we included sulfur containing amino acids in the analysis using a simple score based on the number of cysteine and methionine residues present, which was added to the nitrogen score to obtain a combined N–S score (Fig. 5). However, this N–S score does not include the extra processing of the propeptides such as proPSKs that are sulfated on tyrosine residues (Hanai *et al*. 2000). Another example of processing is the hydroxylation of proline residues in CLE and CEP (Kondo *et al*. 2006; Ohyama *et al*. 2008). It is evident that the plant peptide signalling molecules are the result of a considerable expenditure of energy and presumably represent a worthwhile investment.

A further factor that should be considered in this analysis is that amino acids found in proteins can be readily recycled through the cellular proteolytic machinery (e.g. proteosomes) and thus can be considered a renewable resource. In the last few years, the importance of protein degradation in regulating plant hormones has become apparent with the discovery of the auxin receptor TIR1 being the F-box subunit of the ubiquitin ligase complex SCF^{TIR1} (for a review, see Mockaitis and Estelle 2008). Moreover, plants can rapidly reprogramme their protein expression in response to the cellular energy status coordinated by the kinases KIN 10/11 (the *A. thaliana* orthologues of mammalian AMP activated kinase), where synthesis of biosynthetic genes is rapidly switched to catabolic enzymes in response to environmental cues such as dark or hypoxia (for a review, see Baena-González and Sheen 2008).

We argue that the expression of peptides as signalling molecules in localised restricted areas (e.g. the meristem) makes efficient use of limited nitrogen and sulfur resources that can be rapidly regulated in response to environmental cues by activation of gene transcription. We suggest that this process can be considered as a fine regional control mechanism that works in conjunction with classical hormones such as auxin and ABA to control growth and development. However, hormones such as ABA are not only synthesised *de novo* but ABA is also found as an inactive glucose ester conjugated ABA that circulates throughout the plant and that can be released by the action of β-glucosidases in the apoplast (Wasilewska *et al*. 2008) to produce an extremely rapid burst of the hormone in an immediate response to stressful environmental cues. In the case of peptide signalling molecules, such rapid responses are also partially cued, as propeptides are present in the apoplast. When the propeptide is digested by proteolytic enzymes, it releases the more active peptide ligand that triggers plant responses. We speculate that the propeptide is either inactively conjugated to another molecule in the apoplast or is present at such low concentrations that it does not activate signalling responses. However, upon cleavage, the mature active peptide can be recognised by its receptors at very low concentrations (e.g. CLE41/44, PEP1 and PSK-α) are active at subnanomolar concentrations (Matsubayashi and Sakagami 1996; Ito *et al*. 2006; Pearce *et al*. 2008). Peptide signalling molecules are thus dependent on the presence of additional enzymes to reach full activity, which provides a further level of control.

Conclusions and future perspectives

Peptide signalling molecules are used across all the kingdoms and form a relatively ancient evolutionary adaptation to the task of communicating between cells that has withstood the test of time and selection as they have several evolutionary advantages. First, the release of restricted amounts of material from specialised cells such as meristems to create signalling gradients can be used to regulate development. This is a feature of embryonic development that is carried through to adult multicellular organisms, which use paracrine signalling to ensure that particular regions or organs respond to the signal. Second, modular combinations of receptors can be used that allow flexibility in regulating responses to the peptide signalling molecules. Another advantage that secretion of

peptide signals may offer over other molecules is that relatively rapid and controlled release can be achieved by secreting not only the propeptide but also the processing enzymes that ensure the mature peptide is released. Although the cost of synthesising these separate proteins may be relatively high, it is likely to be economical from a nitrogen and energy use perspective at least, as often only a few cells make this demand on nitrogen and energy resources.

Although much has been uncovered about the roles of peptide signalling molecules in plants over the last 20 years, there is still a great deal to discover. It is likely that more peptide molecules will be found, as many small peptides are not annotated in the databases (Silverstein *et al*. 2007). At this stage relatively few receptor–ligand pairs are known and it is likely that further receptors will be discovered for many of the peptide ligands (see Butenko *et al*. 2009). Relatively little is known about the downstream signalling pathways leading to gene expression and cellular responses, and this is an area particularly worth exploring as it will reveal fundamental insights into the control mechanisms regulating plant growth and development.

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