# A ANNUAL REVIEWS

# Annual Review of Plant Biology Look Closely, the Beautiful May Be Small: Precursor-Derived Peptides in Plants

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#### **Keywords**

nonfunctional precursor-derived peptides, processing, receptor-like kinases, receptor-like proteins, cell signaling, evolution, peptide mimicry

#### Abstract

During the past decade, a flurry of research focusing on the role of peptides as short- and long-distance signaling molecules in plant cell communication has been undertaken. Here, we focus on peptides derived from nonfunctional precursors, and we address several key questions regarding peptide signaling. We provide an overview of the regulatory steps involved in producing a biologically active peptide ligand that can bind its corresponding receptor(s) and discuss how this binding and subsequent activation lead to specific cellular outputs. We discuss different experimental approaches that can be used to match peptide ligands with their receptors. Lastly, we explore how peptides evolved from basic signaling units regulating essential processes in plants to more complex signaling systems as new adaptive traits developed and how nonplant organisms exploit this signaling machinery by producing peptide mimics.

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#### **1. INTRODUCTION**

#### Peptide ligand:

protein-derived peptide perceived by a receptor or a receptor complex to elicit a biological response

#### **Precursor:**

prepropeptide that gives rise to a functional active peptide ligand after processing

#### Prepropeptide:

propeptide containing an N-terminal sorting signal directing it into the secretory pathway Until recently, phytohormones, such as auxin, held a central role in our understanding of the regulatory mechanisms governing plant development and stress response (197). A new view emerged when researchers discovered that plants, like animals, utilize peptide ligands as short- and longdistance signaling molecules to orchestrate plant development and integrate internal cues with external environmental stimuli (120, 132, 136, 184, 185). The function of peptide ligands spans from the regulation of developmental processes, such as meristem maintenance and organ growth, to defense against pathogens and abiotic sensing (120, 191). Recent research has shown that, in addition to local signaling, plants also use secreted peptides to mediate long-distance communication via their vascular systems (136, 184, 186). This is of particular importance for integrating information on nutrient availability and growth conditions between the local external environment and the plant's internal status (155).

Plant peptide ligands are classified into different groups: those that are processed from precursor proteins and those that do not require post-translational processing for function (non-precursor proteins). For those that are processed from precursor proteins, the transcript can encode either a nonfunctional or functional precursor protein. Most peptide ligands are processed and modified from nonfunctional prepropeptide precursors to produce mature functional peptides, and these are classified based on the specific characteristics of the mature peptide (191): (*a*) peptides rich in cysteine (Cys) residues; (*b*) peptides containing post-translational

modifications (PTMs), such as tyrosine (Tyr) sulfation and proline (Pro) hydroxylation, where the latter can be further modified with additional sugar moieties; and (c) peptides not rich in Cys and without PTMs, which contain specific amino acids important for peptide activity (106, 191) (**Supplemental Table 1**). With respect to non-precursor proteins, recent research has identified peptide ligands derived from open reading frames (ORFs) that are encoded within microRNA molecules, the 5' region of mRNA, or as part of other transcripts (89, 162, 191). Upon discovery of new peptide classes, novel roles for these signaling molecules as central regulators of plant development and as communicators between organisms will be uncovered.

In this review, we focus on peptides derived from nonfunctional precursors. Rather than providing a comprehensive overview of all peptide families and their function, we focus on areas that have enhanced our understanding of peptide processing, recognition, signaling output, evolution, and mimicry. We also highlight questions that remain to be addressed.

### 2. HOW ARE MATURE PEPTIDES PROCESSED FROM THEIR PRECURSORS?

Genes encoding nonfunctional precursor-derived peptides are expressed in response to environmental or developmental cues. The final mature peptide controlling the signaling output can also be post-translationally modified, contributing an additional level of control over production of the biologically active peptide. In this section, we explore the role of post-translational regulation of peptide signaling. The order of these events is highlighted in **Figure 1**.

#### 2.1. Post-Translational Processing

Most plant peptide ligands are initially translated into prepropeptides containing an N-terminal sorting sequence directing peptides into the secretory pathway. Throughout the secretory pathway and, in some cases, in the extracellular space, prepropeptides are further processed by proteolytic cleavage and addition of PTMs to produce biologically active, mature peptides (**Figure 1**). Common to all prepropeptides is the removal of the sorting sequence by an endoplasmic reticulum–localized signal peptidase, resulting in a propeptide. Further processing of the propeptide varies between different peptide families and individually between peptide ligands of the same family (106). As the crystal structures of peptide–receptor complexes indicate (see Section 3 in this review), correct length, folding, and PTMs of the peptide ligands are essential for peptide binding by receptor proteins (19, 64, 106, 217) (**Figure 1**).

#### 2.2. Formation of Disulfide Bonds in Cysteine-Rich Peptides

Cys-rich peptides contain between 2 and 16 Cys residues and usually require correct formation of disulfide bonds to achieve the correct fold of the active peptide ligand (191). Mechanisms regulating disulfide bond formation in the endoplasmic reticulum are not well understood in plants, but their formation in eukaryotes is generally understood to be catalyzed by protein disulfide isomerases located in the endoplasmic reticulum (48) (**Figure 1**). A plant disulfide isomerase isolated from *Oldenlandia affinis* (OaPDI) directly binds the precursor of the mature cyclotide peptide, a plant defense molecule, and chemical analysis suggests that OaPDI introduces disulfide bond formation necessary for the active peptide (55), indicating that plants also use protein disulfide isomerases for this purpose. The genome of *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) encodes 22 protein disulfide isomerases, but if and how these contribute to disulfide formation in other secreted peptide ligands remain unknown (69).

Mature peptide: biologically active, secreted peptide following processing and adding post-translational modifications

Peptide processing: proteolytic processing that is required to produce an active peptide

Nonfunctional precursor-derived peptides: peptides derived from a longer precursor that has no biological function as a preprotein, proprotein, or preproprotein

**Propeptide:** inactive version of the peptide ligand that requires further post-translational modifications and/or proteolytic cleavage to become fully active

Supplemental Material >

#### 2.3. Post-Translational Modification of Peptide Ligands

The secretory pathway contains various processing enzymes responsible for peptide PTMs, which are necessary for biological activity and downstream signaling (106). Three different PTMs occur on plant peptide ligands: Tyr sulfation, Pro hydroxylation, and hydroxyl arabinosylation. Each is catalyzed by enzymes in the secretory pathway.

**2.3.1. Tyrosine sulfation.** Tyr sulfation is catalyzed by plant TYROSYLPROTEIN SULFO-TRANSFERASE (TPST), a Golgi-localized protein (85) (**Figure 1**). Interestingly, TPST from *Arabidopsis* shares little sequence similarity with animal TPST proteins, suggesting independent



(Caption appears on following page)

#### Figure 1 (Figure appears on preceding page)

Processing of peptides containing PTMs and Cys-rich peptides throughout the secretory pathway. Most peptide ligands are synthetized as prepropeptides containing a sorting sequence directing the prepropeptide into the secretory pathway. Upon entry into the endoplasmic reticulum, the sorting sequence is cleaved off by a signal peptidase. For peptides carrying PTMs, modifications are introduced throughout the endoplasmic reticulum and the Golgi network: P4H enzymes introduce proline hydroxylation, TPST introduces Tyr sulfation and HPAT, XEG113, and RRA1–3 introduce Hyp arabinosylation. A general view is that peptide ligands are proteolytically processed throughout the secretory pathway and, in some cases, in the extracellular space to yield the active peptide ligand. For peptides in the Cys-rich family, disulfide bond formation and, in some cases, proteolytic processing occur throughout the secretory pathway and in the extracellular space to yield the active peptide. Whether enzymes such as protein disulfide isomerases are involved in the formation of disulfide bonds is largely unknown. For peptide ligands containing PTMs and Cys-rich peptides, the proteases that are responsible for proteolytic processing are also largely unknown. However, for some specific peptide ligands, proteases important for processing have been identified (see text for details). Abbreviations: Cys, cysteine; HPAT, Hyp O-arabinosyltransferase; Hyp, hydroxyproline; P4H, PROLYL-4-HYDROXYLASE; Pro, proline; PTM, post-translational modification; RRA, REDUCED RESIDUAL ARABINOSE; TPST, TYROSYLPROTEIN SULFOTRANSFERASE; Tyr, tyrosine; XEG, XYLOGLUCANASE.

evolution of Tyr sulfation in the plant and animal lineages (85). In *Arabidopsis*, TPST is encoded by a single gene, and loss-of-function mutants of TPST show a variety of phenotypes such as dwarfism, early senescence, reduced number of flowers and siliques, and extremely short roots, indicating that Tyr sulfation is important for multiple plant developmental processes (85, 108). TPST was first identified as the enzyme responsible for Tyr sulfation of two peptides modulating cell proliferation, PLANT PEPTIDE–CONTAINING SULFATED TYROSINE 1 (PSY1) and PHYTOSULFOKINE (PSK) (85, 107). TPST was subsequently shown to catalyze Tyr sulfation of CASPARIAN STRIP INTEGRITY FACTOR 1 (CIF1) and CIF2, two peptides that redundantly regulate formation of the Casparian strip, a boundary layer in the root separating the apoplast of the cortex from that of the vascular tissue (37, 122), and of members of the ROOT MERISTEM GROWTH FACTOR (RGF)/GOLVEN (GLV)/CLE-LIKE (CLEL) peptide family (108).

**2.3.2. Proline hydroxylation.** Pro hydroxylation is catalyzed by PROLYL-4-HYDROXYLASE (P4H), a 2-oxoglutarate-dependent dioxygenase, localized in the membrane of the endoplasmic reticulum and Golgi (62, 216) (**Figure 1**). To date, 13 genes encoding P4H enzymes have been identified in *Arabidopsis* (62, 194, 198). Owing to high protein similarity and consequent functional overlap in the *Arabidopsis* PH4 family, genetic analysis has yielded little information on the specific physiological roles of P4H enzymes (62, 199). Consequently, which P4H enzyme is responsible for specific hydroxylation events is largely unknown. Compared with animals, plant P4Hs are smaller in size, differ in their substrate specificity, and have largely divergent amino acid sequences (54, 62, 106). No consensus sequences have been determined for Pro hydroxylation of plant peptide ligands; however, some sequences are modified more efficiently than others, indicating that Pro hydroxylation is dependent on the sequence context of the Pro residues (54, 62).

**2.3.3.** Hydroxyproline arabinosylation. In some cases, hydroxyproline (Hyp) residues are further modified by the addition of an O-linked L-triarabinose chain, creating an arabinosylated Hyp residue (128) (Figure 1). As a PTM unique to plants, arabinosylation occurs in two separate steps mediated by individual enzymes. Addition of the first arabinose moiety to Hyp is catalyzed by Hyp O-arabinosyltransferase (HPAT) enzymes, of which three are encoded by the *Arabidopsis* genome (128, 140, 212). Loss of HPAT function in *Arabidopsis* has seemingly pleiotropic effects, including early flowering and senescence, impaired pollen tube growth, and defects in cell wall thickening, indicating an essential role for the Hyp O-arabinosylation modification in plant growth and development (128). In tomato, the HPAT3 homolog is encoded by *FASCLATED INFLORESCENCE* 

**Proteases:** enzymes that cleave peptide bonds, thus shortening the peptide sequence

(*FIN*), and a loss-of-function *fin* mutant has an enlarged shoot apical meristem reminiscent of the *Arabidopsis clavata3* (*clv3*) mutant. Adding exogenous arabinosylated tomato CLV3 peptide rescues the *fin* phenotype, indicating the importance of arabinosylation for tomato CLV3 peptide activity (212). In contrast to tomato, the *Arabidopsis hpat1 hpat2 hpat3* triple mutant does not have an enlarged meristem (101, 128), suggesting that a requirement for PTM of the CLV3 peptide may not be conserved across plant species or that arabinosylation of *Arabidopsis* CLV3 is not essential for its biological activity (134). Recent research showed a higher binding affinity of the arabinosylated *Arabidopsis* CLV3 to the CLV1 receptor; however, both the arabinosylated and the non-arabinosylated CLV3 peptides were able to rescue a *clv3-2* mutant phenotype at the same concentration, providing evidence that both CLV3 peptides are functional in *Arabidopsis* (82).

Once the first arabinose moiety is added, further extension of the arabinose chain is catalyzed by two arabinosyltransferases, REDUCED RESIDUAL ARABINOSE 3 (RRA3) and XYLOGLU-CANASE 113 (XEG113), which are located in the Golgi apparatus (168, 212). In *Arabidopsis*, arabinosylation of EXTENSIN proteins (plant cell wall Hyp-rich glycoproteins) may be performed by RRA3 and by XEG113. The same linkage between arabinose residues in EXTENSIN is also found in Hyp arabinosylated peptide ligands indicating that the same enzymatic machinery may be used to modify peptide ligands (128). Like the *fin* mutant, tomato plants harboring mutations in genes encoding arabinosyltransferases homologous to RRA3 and XEG113 show *clv3*-related phenotypes. Interestingly, plants harboring a mutation in the RRA3 homolog show a more severe phenotype than plants mutated in the XEG113 homolog, indicating that the addition of sequential arabinose residues is essential for CLV3 signaling in tomato (212).

### 2.4. Proteolytic Processing of Propeptides into Mature, Biologically Active Peptide Ligands

Proteolytic cleavage is necessary to produce a peptide of optimal length for receptor binding (Figure 1). The Arabidopsis genome encodes 56 subtilisin-like proteases, and even though redundancy and lack of visible phenotypes in mutant lines make assigning physiological function to these enzymes difficult (160), a small number of SUBTILASEs (SBTs) have been shown to function in peptide processing. Interestingly, several different SBTs can be required to process a single propeptide. For example, SITE 1 PROTEASE (S1P)/SBT6.1 and SBT6.2 process RGF/GLV/CLEL, which controls cell elongation (52), and SBT1.1 is required for processing PSK4 precursors (174). Also, some Cys-rich peptides are proteolytically processed to yield active peptide ligands (Figure 1), such as S1P processing the Cys-rich RAPID ALKALINIZATION FACTOR 23 (RALF23) peptide. S1P is localized in the endoplasmic reticulum and Golgi and requires an RRIL motif for substrate recognition (173, 176). Furthermore, tissue-specific expression of protease inhibitors indicates that the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) prepropeptide, the precursor for a peptide ligand controlling floral organ abscission in Arabidopsis together with its signaling receptors HAESA (HAE) and HAESA-LIKE 2 (HSL2) (18, 26, 87, 158, 177), is cleaved by SBT5.2, SBT4.13, and SBT4.12 to derive the 14-amino acid-long biologically active peptide (160). However, contra previously published results (18), weaker *ida* phenotypes are observed when inhibitors of SBTs are expressed under the IDA promoter, indicating that other proteases may also be important in processing mature IDA peptides or that the SBTs are incompletely inhibited in vivo (160). Similar to IDA, CLV3/EMBRYO SURROUND-ING REGION (CLE) peptides are also N-terminally processed. For these peptides a conserved arginine residue is required for efficient N-terminal cleavage. Based on inhibitor studies, the Nterminal processing of CLE has been proposed to be performed by secreted serine proteases (124).

C-terminal processing of peptide ligands may occur via a carboxypeptidase (124). SUPPRES-SOR OF LLP1 1 (SOL1), a putative membrane-bound Zn<sup>2+</sup> carboxypeptidase, removes the Cterminal arginine of the CLE19 propeptide to produce a functional CLE19 peptide (189). SOL1 contains a transmembrane domain, and it colocalizes mainly with ARA7-positive endosomes (189). Transmembrane domain topology prediction suggests that the catalytic domain of SOL1 resides within the endosome, indicating that proteolytic processing of CLE19 may occur in endosomes (189). Endosomal processing might also add another level of complexity to the regulation of active peptide ligands: PTM ligands may be stored in endosomes, awaiting a cellular stimulus to induce the final processing step and rapid formation of an active peptide ligand.

#### 2.5. Conclusion and Emerging Questions

Production of active peptide ligands during plant development and in response to biotic and abiotic stimuli is a complex, well-regulated process occurring in multiple steps from regulation of gene expression to post-translation that produces mature ligands. To date, information on specific enzyme functions important for peptide processing, localization of these enzymes, and enzyme recognition signals on peptide ligands is largely missing. To address these questions, genome editing (e.g., through CRISPR/Cas9) to make higher order mutations that overcome redundancy and the use of high-resolution imaging for cell-specific localization of proteins will enhance the knowledge of these processes. In vitro approaches, such as enzyme activity assays, can also be used to further understand specific functions of processing enzymes. Processing of several peptide ligands likely occurs in multiple steps throughout the secretory pathway and in the extracellular space. In the future, it will be necessary to explore the function of processing in species-, cell-, and ligandspecific manners and in response to different environmental stimuli.

#### 3. HOW CAN WE IDENTIFY PEPTIDE-RECEPTOR PAIRS?

Bioinformatics tools that inspect in silico genome sequences have led to the identification of multiple peptides (18, 111, 133) (**Figure 2***a*). Through in silico comparison of members within a peptide family, the length of mature peptides has been predicted by matching conserved regions. Unfortunately, the algorithms used for gene identification cannot always distinguish between genes encoding peptides and short, random ORFs (90, 138), and they additionally do not predict the various potential PTMs with which a peptide can be decorated. However, most methods utilized to subsequently identify receptors require a highly active form of the peptide ligand (see below), which would be equivalent in length and modifications to the one found in vivo.

Identification of the mature peptide in planta is not trivial. Peptidomics-based approaches to detect a mature peptide through mass spectrometry (133, 141, 153, 184), which include precise identification of proteolytic processing and PTMs, are not (yet) routinely applied when trying to identify peptide ligands because of the experimental limitations that are encountered, such as the amount of peptide present in the sample, the extraction method used, the presence of impurities, and the overall sensitivity of the mass spectrometer used. Furthermore, given that processing enzymes act specifically in certain cells, it is not guaranteed that the peptide structure identified from a whole plant or cell culture truly reflects the same processing the peptide would undergo in specific cell types.

In general, peptide ligands are perceived via plasma membrane–localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs), which transmit extracellular signals across membranes (64). An enormous number of peptide–receptor pairs are expected to exist given the large number of peptide ligands, RLKs, and RLPs in plant genomes (90, 167) and the possibility that one ligand

#### Receptor-like kinases (RLKs):

cell-surface receptors that are essential components of signal transduction pathways that mediate cell-to-cell communication

### Receptor-like proteins (RLPs):

cell-surface receptors that consist of an extracellular domain, a transmembrane domain, and a short cytoplasmic tail, with no kinase domain





#### Figure 2

How to identify a peptide–receptor pair. (a) The peptide can be identified through mass spectrometry or genome analyses ( $\bullet$ ). Subsequently, a synthetic or recombinant peptide can be used in a bioassay, where it induces a phenotype in WT plants, which is lost in mutants lacking a functional receptor ( $\bullet$ ). (b) Interaction between the peptide and receptor can be investigated by adding a synthetic peptide or by overexpressing it. The first step in the signaling cascade is the interaction between peptide and receptor, which can be investigated through protein–protein/peptide interaction assays ( $\bullet$ ) or using a structural approach ( $\bullet$ ). After the peptide has bound to the receptor, conformational changes and/or (auto)phosphorylation occurs ( $\bullet$ ). The (phosphorylated) receptor will interact with other proteins ( $\bullet$ ) and further relay the signal to a final cellular output ( $\bullet$ ). Abbreviations: m/z, mass-to-charge ratio; *rlk, receptor-like kinase*; WT, wild type.

may interact with multiple receptors and one receptor may recognize multiple ligands (32). However, to date, only a small portion of those possible pairs has been identified using various approaches (**Table 1**). In this section, we provide an up-to-date roadmap describing approaches for the identification of peptide–receptor pairs utilizing the different signaling steps from peptide maturation to cellular output as a guide, and we illustrate these approaches with suitable examples (**Figure 2**). Specifically, recent developments in high-throughput peptide–receptor interaction assays and structural biology are resulting in a major leap forward.

#### 3.1. Genetic and Physiological Approaches for Peptide–Receptor Pair Identification

Once the mature peptide has been identified (either through testing variants or based on mass spectrometry data), application of a synthetic or recombinant peptide giving rise to a measurable phenotype verifies a peptide's active form. Suitable bioassays that provide easy readout for peptide activity, such as root growth inhibition, changes in cytosolic calcium, or extracellular release of reactive oxygen species (ROS) (18, 19, 35, 154, 218) (**Figure 2***a*,*b*), allow confirmation of the mature peptide and determination of essential amino acids and/or PTMs in the sequence (e.g.,

		Receptor class	
Peptide	Receptor	and subfamily	Identification technique(s)
IDA	HAE	LRR-RLK X1	Genetics, synthetic peptide assay, cocrystallization
IDA	HSL2	LRR-RLK X1	Genetics, synthetic peptide assay
IDL6	HAE	LRR-RLK X1	Genetics
IDL6	HSL2	LRR-RLK X1	Genetics
CLE3	CLV1	LRR-RLK X1	Genetics
CLE8	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE9	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE9	BAM2	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE9	BAM3	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE9	CLV1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE10	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE11	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE12	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE13	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE14	BAM1	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling, co-IP
CLE40	CLV1	LRR-RLK X1	Genetics, co-IP, ligand-binding assay with photoaffinity labeling
CLE40	ACR4	CR4L	Genetics, synthetic peptide assay
CLE41/CLE44/TDIF	PXY/TDR	LRR-RLK X1	Synthetic peptide assay, ligand-binding assay with photoaffinity labeling, cocrystallization
CLE42	PXL2	LRR-RLK X1	Ligand binding assay by the use of gel-filtration chromatogram and MS, binding affinity by ITC
CLE45	BAM3	LRR-RLK X1	Genetics, synthetic peptide assay
CLE45	SKM1	LRR-RLK N.A	Ligand-binding assay with photoaffinity labeling
CLE45	SKM2	LRR-RLK X1	Ligand-binding assay with photoaffinity labeling
CLV3	CLV1	LRR-RLK X1	Genetics, ligand-binding assay with photoaffinity labeling
CLV3	CLV2	LRR-RLP	Genetics
CLV3	RPK2	LRR-RLK	Genetics, synthetic peptide assay
CEP1 + other members of CEP	CEPR1/XIP1	LRR-RLK X1	Binding assay with photoaffinity labeling
CEP1 + other members of CEP	CEPR2	LRR-RLK X1	Binding assay with photoaffinity labeling
CEP5	CEPR1	LRR/RLK X1	Genetics, synthetic peptide assay
EPF1	ER	LRR-RLK XIII	Co-IP, synthetic peptide assay, ligand-binding assay with biosensor platform
EPF1	ER	LRR-RLK XIII	Synthetic peptide assay, cocrystallization, pull-down assay
EPF1	ТММ	LRR-RLP	Synthetic peptide assay, cocrystallization, pull-down assay
EPF1	ERL1	LRR-RLK XIII	Co-IP, synthetic peptide assay, ligand-binding assay with biosensor platform

 Table 1
 List of peptides and putative receptors known in Arabidopsis (see Supplemental Table 2 for associated references)

Supplemental Material >

#### Table 1 (Continued)

		Receptor class	
Peptide	Receptor	and subfamily	Identification technique(s)
EPF2	ER	LRR-RLK XIII	Co-IP, synthetic peptide assay, ligand-binding assay
			with biosensor platform
EPF2	TMM	LRR-RLP	Synthetic peptide assay, cocrystallization, pull-down
			assay
EPF2	ERL1	LRR-RLK XIII	Co-IP, synthetic peptide assay, ligand-binding assay
	- DD		with biosensor platform
EPFL4	ER	LRR-RLK XIII	Genetics, co-IP
EPFL4	ERL1	LRR-RLK XIII	Genetics, co-IP
EPFL6	ER	LRR-RLK XIII	Genetics, co-IP
EPFL6	ERL1	LRR-RLK XIII	Genetics, co-IP
EPFL9 (STOMAGEN)	ER	LRR-RLK XIII	Genetics, co-IP, ligand-binding assay with biosensor
			platform
EPFL9 (STOMAGEN)	TMM	LRR-RLP	Genetics, co-IP, ligand-binding assay with biosensor
			platform
PIP1	RLK7	LRR-RLK XI	Genetics, pull-down assay, Y2H, cross-linking assay,
	DEDD /	I DD DI WIW	binding assay with photoaffinity labeling
PEP1	PEPR1	LRR-RLK XI	Genetics, bioassay, binding assay with photoaffinity
	DEDDA		
PEPI	PEPR2	LKK-KLK XI	Genetics, bioassay, ligand-binding assay with
DED1	DEDD 1		
PEP2	PEPKI	LKK-KLK AI	Genetics, bioassay, ligand-binding assay with
DED2	DEDD2		Constinue history abening
PEP2	PEPR2	LKK-KLK AI	chenetics, bioassay, ligand-binding assay with
GRI	PRK5	I BB-BI K III	Binding assay with photoaffinity labeling MS
	DSVD1		Binding assay with photoannity labeling, MS
ISK	I SKRI		corrystallization co-IP
PSK	PSKR2	LRR-RLK X	Genetics photoaffinity labeling
PSV	PSVR		Genetics
	FFRONIA	RIK-CrRIK1I	Ouantitative phosphoproteomics
RAL F4/10	BUPS1/2	REK-CrREKIE	Genetics pull-down assay microscale thermonhoresis
RAL F4/19	ANIX1/2	REK CrRIKIL	Genetics
TPD1	FMS1	I RR_RI K Y	V2H null-down assay co-IP cocrystallization
	MDIS1		Bull down assay, co-11, cocrystalization
	MIV1	I DD DI V VI	Pull down assay, co-IP, bloassay
		LRR-RLK AI	Pull-down assay, co-IP, bloassay
	MIK2	LRR-RLK AII	Pull-down assay, co-IP, bloassay
	PKK0	LRR-RLK III	Genetics, bioassay, BIFC, co-IP
	PRK8	LKK-KLK III	Genetics, bioassay, BIFC, co-IP
RGF1/2/3/4/5(GLV10)/10	RGFR1/RGI1-5	LRR-RLK XI	Pull-down assay, co-IP, cocrystallization,
			photoaffinity-labeled peptides to receptor library, MS

(Continued)

#### Table 1 (Continued)

		Receptor class	
Peptide	Receptor	and subfamily	Identification technique(s)
RGF1/2/3/4/5(GLV10)/10	RGFR2(RCH1)	LRR-RLK XI	Photoaffinity-labeled peptides to receptor library
RGF1/2/3/4/5(GLV10)/10	RGFR3	LRR-RLK XI	Binding assay with photoaffinity labeling, library
SCR/SP11	SRK	S-domain RLK	Bioassay, co-IP, binding assay, phosphorylation assay
CIF1/CIF2	GSO1/	LRR-RLK XI	Genetics, photoaffinity-labeled peptides to receptor
	SCHENGEN3		library, ITC
CIF1/CIF2	GSO2	LRR-RLK XI	Genetics, photoaffinity-labeled peptides to receptor
			library, ITC
Systemin	SYR1	LRR-RLK	Binding assay with acridinium-labelled peptide

Abbreviations: ACR4, ARABIDOPSIS CRINKLY4; ANX, ANXUR; BAM, BARELY ANY MERISTEM; BiFC, bimolecular fluorescence complementation; BUPS, BUDDHA'S PAPER SEAL; CEP, C-TERMINALLY ENCODED PEPTIDE; CEPR, C-TERMINALLY ENCODED PEPTIDE RE-CEPTOR; CIF, CASPARIAN STRIP INTEGRITY FACTOR; CLE, CLAVATA3/EMBRYO SURROUNDING REGION; CLV, CLAVATA; co-IP, coimmunoprecipitation; EMS, EXCESS MICROSPOROCYTES; EPFL, EPIDERMAL PATTERNING FACTOR-LIKE; ER, ERECTA; ERL, ERECTA-LIKE; GLV, GOLVEN; GRI, GRIM REAPER; GSO, GASSHO; HAE, HAESA; HSL, HAESA-LIKE; IDA, INFLORESCENCE DEFICIENT IN ABSCISSION; ITC, isothermal titration calorimetry; LRR, leucine-rich repeat; MDIS, MALE DISCOVERER; MIK, MDIS1-INTERACTING RECEPTOR-LIKE KINASE; PEP, PLANT ELICITOR PEPTIDE; PEPR, PLANT ELICITOR PEPTIDE RECEPTOR; PIP, PAMP-INDUCED SECRETED PEPTIDES; PRK, POLLEN-SPECIFIC RECEPTOR-LIKE KINASE; PSK, PHYTOSULFOKINE; PSRKR, PHYTOSULFOKINE RECEPTOR; PSY, PLANT PEPTIDE–CONTAINING SULFATED TYROSINE; PSYR, PLANT PEPTIDE–CONTAINING SULFATED TY ROSINE RECEPTOR; PXL, PHLOEM INTERCALATED WITH XYLEM-LIKE; PXY, PHLOEM INTERCALATED WITH XYLEM; RALF, RAPID ALKALINIZATION FACTOR; RGF, ROOT MERISTEM GROWTH FACTOR; RGFR, ROOT MERISTEM GROWTH FACTOR RECEP-TOR; RLK, RECEPTOR-LIKE KINASE; RLP, RECEPTOR-LIKE PROTEIN; RPK, RECEPTOR-LIKE PROTEIN KINASE; SKM, STERILITY-REGULATING KINASE MEMBER; SYR, SYSTEMIN RECEPTOR; TDIF, TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FAC-TOR; TDR, TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR RECEPTOR; TMM, TOO MANY MOUTHS; TPD, TAPE-TUM DETERMINANT; XIP, XYLEM INTERMIXED WITH PHLOEM; Y2H, yeast-2-hybrid. Table modified from Reference 210.

by introducing amino acid substitutions). However, application of large doses of a synthetic or recombinant peptide can result in phenotypes coming from unspecific signaling through nonnative receptors (19, 30, 177). Subsequently, forward or reverse genetic approaches can be employed to identify genes encoding candidate receptors (or other signaling components) for the peptide of interest, through either genetic interaction studies or insensitivity of the receptor mutant to the (synthetic) peptide (4, 18, 19, 27, 108, 120, 133, 153, 184, 209) (**Figure 2***a*) (**Table 1**). In the past, available T-DNA insertion lines and functional redundancy were limiting for genetic studies (19), but new approaches, such as CRISPR/Cas9-mediated mutagenesis, are now being employed to overcome this (215).

In combination with synthetic or recombinant peptide variants, genetics is a powerful method to identify and characterize peptide–receptor pairs. Identification of a highly active CLV3 peptide, which could subsequently be used for direct binding studies to the CLV1 receptor (127, 134), has enabled the verification of previous genetic observations (14, 27, 28). Furthermore, using a root growth assay, a synthetic CLE45 peptide, and RLK mutants, BARELY ANY MERISTEM 3 (BAM3) was identified as the CLE45 receptor (35). In addition, genetic analyses placed ANXUR 1 (ANX1) and ANX2 in the RALF4 and RALF19 pathways (51, 110). Also, phenotyping mutants with altered pollen tube attraction revealed that POLLEN-SPECIFIC RECEPTOR-LIKE KI-NASE 6 (PRK6) is responsible for sensing the LURE1 peptide (188). By using dominant negative versions of RLKs preferentially expressed in pollen, it was also shown that LURE1 could interact with MALE DISCOVERER1 (MDIS1) and MDIS1-INTERACTING RECEPTOR-LIKE KINASE1 (MIK1) and MIK2 (23, 205). Finally, genetic studies challenged SYSTEMIN RECEP-TOR 160/BRASSINOSTEROID INSENSITIVE 1 (BRI1) as a systemin receptor (66, 102, 161). Indeed, a collection of tomato (*Solanum lycopersicum*) introgression lines with specific parts of the

genome replaced by homologous parts of the wild tomato species *Solanum pennellii*, which lacked responsiveness to systemin, led to identification of SYSTEMIN RECEPTOR 1 as a receptor that binds systemin with high affinity and specificity (204).

### 3.2. Identification of Receptor–Ligand Pairs Through Direct Physical Interaction

A first step in the signaling cascade is the physical interaction of the peptide with its receptor (**Figure** *2b*). Therefore, physical interaction assays are emerging as a major approach to match ligands and receptors.

**3.2.1. Protein–protein interaction assays.** Classical methods such as yeast-2-hybrid and coimmunoprecipitation have been used to screen and verify peptide–receptor interactions, as they are easy to set up and relatively inexpensive. However, so far, such approaches seem to have been successful mainly for Cys-rich peptides that are larger than 50 amino acids. For example, these techniques were used to identify the interaction between the small protein ligand TAPETUM DETERMINANT 1 and the receptor EXCESS MICROSPOROCYTES 1 (78), between the pollen-expressed small protein LAT52 and the *Lycopersicon esculentum* pollen receptor kinase (190), and between the EPIDERMAL PATTERNING FACTORs (EPFs) and ERECTA (ER) family receptors (92). However, in these methods, proteins of interest are often overexpressed or expressed in a nonplant system (and subsequently not in the apoplast), which modifies the relative concentrations of interaction partners, possibly leading to false positives (88).

An enormous leap forward in ligand-receptor pairing resulted from photoaffinity-labeled peptides in combination with a library of a subset of Arabidopsis RLKs containing leucine-rich repeats (LRRs) in their extracellular domain expressed in tobacco BY-2 cells (184). This approach has been used to identify several ligand-receptor pairs, such as the RGF RECEPTORs (RGFRs) that directly interact with RGF, the GASSHO 1/SCHENGEN 3 receptor for CIF1 and CIF2 peptides, and C-TERMINALLY ENCODED PEPTIDEs (CEPs) interacting with the CEP RECEPTOR (CEPR) (122, 166, 184). One drawback of this approach is that it may expose interactions that are not biologically relevant since the ligand and receptor may not be coexpressed in planta. Nevertheless, this approach may help overcome limitations due to genetic redundancy. For example, photoaffinity labeling has allowed researchers to resolve ligand-receptor interactions involved in CLV3 perception. Genetic evidence showed that besides CLV1, other molecular components are involved in CLV3 perception (9, 77, 84, 118). Specifically, the RLP CLV2 (9, 77), the transmembrane pseudokinase CORYNE (CRN) (118), an additional RLK RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) (84), and CLV3-INSENSITIVE RECEPTOR KINASEs (CIKs), which function as coreceptors of CLV1, CLV2/CRN, and RPK2 (70), all contribute to CLV3 perception. Whether these proteins interacted directly with the CLV1 ligand was unknown. Indeed, photoaffinity labeling revealed that CLV2 and RPK2 do not directly bind CLV3 (165), suggesting that they function within the CLV1 signaling complex but do not participate directly in ligand perception. Photoaffinity labeling further showed that the BAM RLKs, which function oppositely to CLV1 in meristem maintenance (36), are also capable of direct perception of the CLV3 ligand (165).

Another in vitro approach incubated the purified extracellular LRR domain protein (from the subfamily XI containing an Arg-X-Arg motif) with a pool of chemically synthesized peptides (having a free C-terminal histidine or asparagine), separated the LRR-bound peptide or peptides, and used mass spectrometry to detect them. This approach allowed the validation of IDA-HSL2 and TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF)/CLE41/CLE44-PHLOEM INTERCALATED WITH XYLEM (PXY) pairs, identified CLE42 as a ligand for PXY-LIKE 2 (117), and detected the RGF1 receptor, RGFR1 (171).

Importantly, high-affinity peptide ligand-receptor interactions might only be apparent in the presence of a coreceptor (see Section 4 in this review). For example, SOMATIC EMBRYOGEN-ESIS RECEPTOR KINASE 1 (SERK1) acts as a coreceptor and increases HAE binding specificity and affinity for IDA (158). Similarly, CLE41 and CLE42 contribute to the interaction of SERK2 with PXY and PXY-LIKE 2, respectively (117), and RGF1 induced the interaction between RGFR1 or RGFR2 and SERK1/2/BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) (171). In this context, the above-described approaches could be further improved by coexpressing possible coreceptors.

**3.2.2.** Structural approaches. Structural biology can help make predictions that aid the identification of ligand–receptor pairs and elucidate the mechanistic basis of peptide perception and signaling (64). Although this approach has been applied to only a limited number of peptide ligands and ligand–receptor pairs from plants, it already resulted in new insights in peptide–receptor interactions (98, 158, 171, 217, 219, 221). For example, resolution of the IDA–HAE complex through cocrystallization suggested that IDA binds the ectodomain of HAE and that a conserved Hyp, identified by bioassay experiments, is crucial for the interaction (158). Another structural study revealed that PRK6 is a receptor of LURE1, which is in agreement with genetic data (188, 221). However, in contrast to other LRR-RLKs, PRK6 recognizes LURE1.2 through the C-terminal loop of its LRR domain rather than the LRR portion (221). Finally, crystal structures also guided the identification of RGFRs as receptors for RGF peptides (171). Unfortunately, crystallization of a protein is a tedious route, and although much knowledge about this technique has been gained in past decades, its success rate remains unpredictable.

#### 3.3. Identification of Peptide Receptors Through Phosphoproteomics

When the ligand binds to its receptor, conformational changes can occur at the receptor level (64, 158, 217). Mostly, recruitment of coreceptors results in multiple immediate biochemical changes, such as (auto)phosphorylation of the receptor, receptor-protein interactions, and phosphorylation of target proteins. Various cellular responses follow these immediate biochemical interactions (Figure 2b). Profiling peptide-induced changes in plasma membrane protein phosphorylation through quantitative phosphoproteomics is an elegant way to identify the receptor (59, 178). For example, a receptor for RALF1, a peptide that suppresses cell elongation in the primary root, was identified via this approach (59). Several plasma membrane proteins displayed a RALF-induced change in phosphorylation level, including the globally expressed RLK FERO-NIA (FER) (59). Subsequent genetic and biochemical studies confirmed FER as a receptor for RALF1 and other RALF peptides (59, 176). A limitation of such differential phosphoproteome analyses might include the low abundance and/or cell-specific expression of receptors. Phosphoproteomics approaches also revealed the inhibition of proton transport by RALF-induced phosphorylation of H<sup>+</sup>-ATPase2 (59). As such, phosphoproteomics following (short-term) peptide treatments can give insight into early signaling events beyond the actual receptor of the ligand.

#### 3.4. Conclusion and Emerging Questions

Various genetic, biochemical, and structural approaches have contributed and continue to contribute to the identification of peptide-receptor pairs and their downstream responses. However,

#### **Coreceptor:**

a receptor that interacts with the primary receptor to form a receptor complex often necessary for correct downstream signaling the receptor for many ligands is still unknown, and likewise, no ligands have been identified for many receptors. To gain comprehensive insight into the various possible combinations and their biological relevance, new high-throughput screening methods will have to be adopted, such as proteome-wide profiling of RLK phosphorylation status upon ligand treatment or photoaffinitylabeled peptides in the presence of RLKs and their coreceptors. In the near future, structural biology will provide us with more exciting observations and hypotheses, including from an evolutionary perspective.

## 4. HOW IS A SPECIFIC CELLULAR OUTPUT GENERATED AND MAINTAINED?

In the canonical mode of receptor kinase signaling, ligand binding by receptor kinase extracellular domains activates the intracellular protein kinase domain of the receptor. Auto- and transphosphorylation events occur between receptors and possible coreceptors, and give rise to activation of other downstream components, leading to a final cellular outcome (46, 64, 192, 217) (Figure 2b). Interestingly, growing numbers of receptors have been found to regulate multiple biological functions. For example, FER mediates RALF-dependent pollen tube reception by the ovule, growth inhibition, and immune responses and has recently been implicated in maintaining cell wall integrity during salt stress (43, 47, 71, 176). A single receptor can also perceive multiple peptides that may act in an antagonistic manner, as with EPF2 and STOMAGEN/EPF-LIKE 9 (EPFL9), both of which bind to ER and the RLP TOO MANY MOUTHS (TMM) to control stomatal patterning in the leaf epidermis (91, 98). Furthermore, different ligand-receptor pairs often share common downstream signaling components, such as coreceptors (e.g., BAK1), and identical mitogenactivated protein kinase (MAPK) signaling networks (46, 213). Given the commonality of signaling components, it becomes relevant to investigate how signal specificity and cellular output are derived for distinct biological processes. It is difficult to identify the specific role of signaling components shared between different pathways in genetic studies because pleiotropic phenotypes are observed. Despite these challenges, signaling specificity is observed at different levels throughout the signaling pathway (Figure 3). In this section, we illustrate how ligand-receptor pairs generate and maintain distinct cellular outputs.

#### 4.1. Ways to Establish Specificity

Specific cell responses are established out of the spatial and temporal regulation of peptide expression in addition to peptide processing, binding affinities, coreceptors and receptor localization, and other downstream components as detailed below.

**4.1.1. Expression patterns.** Studies in which genes encoding peptide ligands are misexpressed or in which synthetic peptides are applied exogenously to plant tissues show the importance of spatial and temporal regulation of peptide expression for proper function. One example is the spatial expression of peptide encoding genes in the male and female gametophytes necessary for successful sexual reproduction in plants (39, 63). Peptides secreted from distinct reproductive tissue fulfill critical roles during double fertilization in angiosperms (150, 151). Particularly, genes encoding Cys-rich peptides are differently expressed in reproductive cells ensuring proper fertilization. In dicot plants, such as *Torenia* and *Arabidopsis*, LURE peptides are secreted from synergid cells and function as pollen tube attractants (80, 137, 187). In *Arabidopsis*, the pollen tube tip grows in the correct orientation when LURE peptides are perceived by PRK6, MDIS1, and/or MIK1, thereby recruiting components of the core tip growth machinery (23, 188, 205, 221). After arriving



#### Figure 3

Acquisition of signal specificity. Specificity of a peptide ligand–induced response can arise at various stages of the signaling event. Abbreviations: MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; TF, transcription factor.

at the receptive synergid cells, the pollen tube bursts releasing the two sperm cells. Here, there is a delicate communication between tissues that ensures the proper timing of the event. FER, which localizes predominantly at the surface of synergid cells and is lacking in pollen tubes, has an essential role in growth arrest of the pollen tube (43). ANX1 and ANX2, two close homologs of FER, are expressed in pollen tubes and maintain pollen tube integrity during growth by forming a complex with BUDDHA'S PAPER SEAL 1 (BUPS1) and BUPS2 (51). The spatiotemporal regulation of the two antagonistic processes, pollen tube growth and disintegration, is regulated by RALF peptides (150). *RALF4* and *RALF19* are expressed in mature pollen grains and tubes, and autocrine signaling of RALF4 and RALF19 at the receptor complex BUPS1/2–ANX1/2

maintains pollen tube growth and integrity. *RALF34* is predominantly expressed in mature ovules and RALF34 serves as an ovule-derived paracrine signal to replace the autocrine signal of RALF4 and RALF19, enabling the pollen tube to respond by rupturing and releasing sperm cells (51).

Feedback systems can ensure correct and constrained expression of peptide encoding genes when restriction to a specific tissue or a few cells is necessary. For example, stem cell homeostasis depends on correct spatiotemporal expression of the gene encoding the CLV3 peptide in the central zone of the shoot meristem. In *Arabidopsis, CLV3* expression is directly regulated by HAIRY MERISTEM GRAS-domain transcription factors and the mobile transcription factor WUSCHEL (WUS), which in turn is restricted to the organizing center in the L3 layer by CLV3 and other CLE peptides, signaling through various RLKs and RLPs (125, 169, 214, 223). Recent work in maize has shown that signals from the differentiating organ primordia also contribute to the restriction of *WUS* expression. The RLP FASCIATED EAR 3, which is expressed in the L1 layer and in leaf primordia, is suggested to function as a receptor in perception of the maize FON2-LIKE CLE PROTEIN 1 peptide which is expressed in leaf primordia and in cells flanking the shoot apical meristem (76). A feedback signaling system from the developing tissue could be useful to provide control of stem cell proliferation and organ growth by integrating signals from the developing primordia (76).

It is also possible for external factors to modulate the expression of peptide encoding genes when peptides function to integrate environmental fluctuations or as sensors of biotic stress (24, 68, 185, 201). For example, some members of the CEP family are transcriptionally induced upon nitrogen starvation to control root architecture (184). In addition, expression of the genes encoding endogenous plant peptides PLANT ELICITOR PEPTIDE 2 (PEP2), PEP3, PAMP-INDUCED PEPTIDE 1 (PIP1), PIP-LIKE, and IDA-LIKE (IDL) is enhanced in response to pathogen infections thereby leading to an amplification of the immune response (68, 72, 200).

**4.1.2. Prepropeptide processing.** Peptide processing may be involved in regulating specific peptide functions (**Figure 3**). The *Arabidopsis* genome encodes about 35 RALF peptides (59, 114), but only 11 of them have a S1P cleavage site (176) (see Section 2). When testing the ability of different RALF peptides to act as negative regulators of immunity by monitoring pathogen-associated molecular pattern-induced ROS production in the *s1p* mutant background, only those containing a S1P cleavage site, such as RALF23, RALF33, and RALF34, suppressed the enhanced ROS production of *s1p* mutants. This indicated that inability to cleave PRORALF23, PRORALF33, and PRORALF34 causes enhanced immune responses in *s1p* mutants (176). By comparison, RALFs with and without an S1P cleavage site inhibit seedling growth in a manner similar to that of RALF1, indicating that proteolytic cleavage of RALFs is needed to inhibit immunity but is not needed for developmental responses or that other proteases are required (176).

**4.1.3. Competitive binding.** Combined with the spatial and temporal regulation of peptide expression, competitive binding of different ligands to receptor binding sites and binding to different receptor complexes can impact the signaling output (**Figure 3**). A prime example of competitive peptide binding and differences in the use of RLPs is found in the regulation of stomata formation. During stomatal development, cell fate decisions within the stomatal lineage are tightly controlled. The differentiation of protodermal cells into stomata is regulated by three homologous basic helix-loop-helix transcription factors: SPEECHLESS (SPCH), MUTE, and FAMA. Here, SPCH ensures the correct spacing and pattering of the meristemoids (stomatal meristem precursor cells), MUTE drives cells through the lineage to become stomata, and FAMA ultimately

determines the guard cell identity (100, 105, 130, 147). In leaves, this fate decision is in part regulated by three main peptide ligands, EPF1, EPF2, and STOMAGEN/EPFL9, which compete for binding to the ER family of receptors to fine-tune stomatal initiation (57, 58, 73, 74, 182). EPF1 and EPF2 negatively regulate stomatal development by activating a receptor complex consisting of TMM, ER, and SERKs that ultimately suppresses SPCH activity, while EPFL9 is a positive regulator that competes with EPF1/2 (91, 112). Structural nuclear magnetic resonance analysis of the EPFL9 and EPF2 peptides showed a variable loop region in between the fourth and fifth conserved Cys, although they share structural homology. This variable loop structure provides specificity to the antagonistic actions of the peptides (131). Interestingly, other members of the EPF family, such as EPFL4 and EPFL6 (also called CHALLAH), signal through ER and ER-LIKE without TMM (1, 2, 98, 196). Apoplastic mobile EPFL6 (and other EPFL6-related family members) and EPF1/2 ligands can encounter ER receptors in both the stomatal lineage and nonstomatal cells, and it is the presence of TMM in the stomatal lineage that differentially regulates these two ligand classes (2). Thus, EPFL6 signaling does not affect stomatal development in nonstomatal lineage cells because it is dampened by TMM, while EPF1/2 signaling does not affect growth because it is potentiated by TMM (2). The ligand-receptor interactions that regulate SPCH do so by activating an MPK cascade, including MAPKKs and MAPKs that are fundamental for many other developmental and stress responses. How they activate specific downstream targets remains unknown, but cell type-specific scaffold proteins that associate with MPK cascade components could provide specificity (38).

4.1.4. Specificity in the use of coreceptors. Another level of regulation can be obtained through interactions between receptor and coreceptor (Figure 3). The SERK family of LRR-RLKs functions as coreceptors for several plant LRR-RLKs, and SERKs interact with receptors that include PSK RECEPTOR 1 (PSKR1), PEP RECEPTOR 1, BRI1, and FLAGELLIN SENSITIVE 2 (FLS2) (25, 60, 94, 123, 159, 183), raising the question of how a common coreceptor can give rise to a wide range of cellular outputs. A way to achieve this is by employing a large range of residues in the SERK extracellular domain to interact with different receptors, in some cases binding directly to both ligand and receptor, as was first shown for FSL2 and BAK1, SERK1 and BRI1, and in other cases interacting with only the receptor, such as the PSK-PSKR1-SERK1 complex (46, 64, 113, 159, 183, 203). Besides the SERKs, there are other RLKs in Arabidopsis that have similar short extracellular structures. A reverse genetic approach and biochemical studies were employed to show that the CIKs not only function as coreceptors for CLV1, CLV2/CRN, and RPK2 to regulate stem cell homeostasis (70) but also interact with BAM1, BAM2, and RPK2 to control somatic cell fate determination during early anther development (29). Interestingly, even if CIKs displayed structures similar to BAK1, they were not able to bind FLS2, indicating that coreceptor interaction provides a level of signaling specificity (29).

It is largely unknown how the intracellular signaling is dependent on specific receptor or coreceptor complexes, but the use of chimeras and phosphoproteomics is shedding some light on this (65, 143). A combination of phosphoproteomics and targeted mutagenesis identified phosphosites that are required for the immune function of BAK1 but not for the BAK1-dependent brassinosteroid-regulated growth (143). It was also shown that a conserved Tyr residue present in FSL2 and the EF-TU RECEPTOR but not in BRI1 and the analogous residue in BAK1 require phosphorylation for the signaling complex to be active. This is a mechanism by which the common coreceptor BAK1 and other SERK members differentially regulate at least two classes of ligandbinding RLKs (143). Possibly, as shown in nonplant organisms (3), differences in phosphorylation of the receptors may be important for the regulation of specific downstream components in order Scaffold protein: a protein that binds multiple signaling components, thus creating a signaling complex of specific intracellular components, often important for a specific cellular response to recognize specific phosphorylation patterns. Another possibility is that a common coreceptor transphosphorylates different residues on the primary receptor allowing for activation of different downstream regulators and inhibitors.

4.1.5. Recruitment of different signaling components. For some signaling systems, where the receptors are expressed in a variety of plant tissues and organs, the signaling specificity depends on the cell type and the availability of downstream components (Figure 3). For example, FER that contributes to perception of RALF1 and RALF23 (59, 176) integrates several regulatory pathways targeting cell growth and stress responses (96). In this context, the FER-dependent ROS production could be a link that allows FER to regulate stress and developmental processes. The ROS production during root hair growth depends on the Rho-like GTPase-guanine nucleotide exchange factor (RopGEF) that in turn activates the RopGEF/Arabidopsis RAC by switching them from the GDP-bound inactive state into the GTP-bound active state in the plasma membrane to further modulate the activity of NADPH oxidases (42). The finding that the receptor-like cytoplasmic kinase RPM1-INDUCED PROTEIN KINASE directly interacts with and is phosphorylated by FER in a RALF1 peptide-dependent manner (41) makes it possible that RPM1-INDUCED PRO-TEIN KINASE functions similarly to BOTRYTIS-INDUCED KINASE 1, which, when phosphorylated and activated by FLS2, activates RESPIRATORY BURST OXIDASE HOMOLOG PROTEIN D and ROS production in plant immunity (79, 95). This would provide an alternative mechanism for FER-dependent ROS production (96).

Cell type–specific scaffold proteins associating with signaling components could also be important to obtain specific cellular responses as is seen in the stomatal lineage where BREAK-ING OF ASYMMETRY IN THE STOMATAL LINEAGE is phosphorylated by MPK3/6 and functions as a scaffold protein; this helps MPK6 to phosphorylate and enhance SPCH degradation (220, 222). Moreover, chaperone proteins in the signaling event can aid in activation of correct downstream components. For example, FER signaling depends on two homologous glycosylphosphatidylinositol-anchored proteins (GPI-APs), LORELEI and LORELEI-LIKE GLYCOSYLPHOSPHATIDYLINOSITOL 1, both of which interact physically with FER. LORELEI-LIKE GLYCOSYLPHOSPHATIDYLINOSITOL 1 forms a complex with the downstream components RopGEF1 and RAC/ROP (described above), and it has been suggested that FER is able to perform different roles in cells by recruiting different members of the GPI-AP family (93).

Transcription factors ultimately regulated downstream of peptide ligand perception impact the final cellular outcome (**Figure 3**). The peptide ligand TDIF (CLE41/CLE44) and its receptor PXY regulate vascular cell division, cell organization, and xylem differentiation via two genetically separable pathways, resulting in a model where the transcription factors WUS RELATED HOMEOBOX 4 (WOX4) and WOX14 act redundantly to promote vascular cell division downstream of TDIF (CLE41/CLE44)–PXY but are not required for vascular organization (44, 181). The *wox4 wox14* double mutant shows similar cell division phenotypes as those of the *pxy wox4* mutant, but no vascular organization defects are observed in the *wox4 wox14* mutant (44). In contrast, the ER receptor is important for control of vascular cell organization, as displayed in the phenotypes of *pxy er* mutant plants, which show an increased defect in vascular cell organization compared with the *pxy* single mutant (44), making it possible that other transcription factors are involved in regulating vascular organization.

**4.1.6. Receptor localization.** Finally, polarity patterns or clustering of receptors and other signaling components in plasma membrane microdomains may play an important role in controlling

the intracellular response and promoting correct interactions between molecules in the signaling pathway (15) (**Figure 3**). For example, the localization of the RLK SCHENGEN 3 to a band in the transversal and anticlinal membrane domains of endodermal cells allows the embedding of CASPARIAN STRIP DOMAIN PROTEINs into a ring-like domain encircling the root cells where the Casparian strip forms (146).

Different receptors form into spatiotemporally separated signaling platforms on the plasma membrane. The specificity of signaling events using the same downstream components may thus be explained by spatial separation of different receptors between nanodomains (17). For example, live cell imaging shows that FLS2 and BRI1 form distinct plasma membrane-localized nanoclusters (17). The dynamics of receptor complexes may also have an impact on signal specificity.

Both FLS2 and CLV1 form complexes with additional receptors on the plasma membrane. Through the use of in vivo visualization of protein complexes and ligand–receptor pairs by multiparameter fluorescence imaging spectroscopy, which provides high spatial and temporal resolution of the interaction states of the receptors over time in individual cells, it was possible to monitor the dynamics of the receptor complex of CLV1, CRN, and CLV2 compared to that of FLS2 and BAK1. This revealed that the CLV receptor complexes are preformed and present prior to activation by CVL3 but that ligand binding stimulates their clustering, whereas FLS2 and BAK1 form a complex in response to treatment with the FLS2 ligand flg22 (170).

Differences in receptor behavior may reflect the biological function of the pathways. The CLV pathway is activated throughout plant development, and its activation is more or less continuous, whereas the flg22 pathway is activated only upon bacterial infection (170). Further studies are needed to fully understand how receptor clustering and plasma membrane–formed complexes affect peptide signaling.

#### 4.2. Conclusion and Emerging Questions

It is becoming clear that related peptides can bind to shared receptor complexes often employing the same coreceptor(s) and that peptides can modulate receptor complex dynamics and interactions. Most peptide ligands have been studied by their specific roles in defined cellular processes within given cell types. In the future, it will be crucial to understand how ligand–receptor mediated signaling is restricted spatially and temporally. These restrictions are of particular importance for cell fate–defining processes that are irreversible, such as the formation of the Casparian strip in the root endodermis, where the spatial and temporal localization of the RLK SCHENGEN 3/GASSHO 1 and the receptor-like cytoplasmic kinase SCHENGEN 1 are essential for CIF1- and CIF2-mediated lignification and suberization in given domains (37). How these and other receptors are restricted to specific cells and domains should be the focus of future research.

#### 5. EVOLUTION OF PLANT PRECURSOR-DERIVED PEPTIDES

As plants increased in complexity from single-celled green algae to the complex multicellular organisms that grow on land, more complex and more diverse signaling mechanisms were required. Various traits developed along this evolutionary path as organisms moved out of the water (**Figure 4**). In this section, we summarize when various peptide families appeared in the green lineage, how they diversified, and how this correlates with the evolution of different plant traits and the putative ancestral peptide function (**Figure 4**). In addition, signaling components involved in plant parasitism and pathogenicity appear to have coevolved with plant signaling systems.

**Evolutionary traits** 

#### **Representative species**

Peptide family



Correlation between the evolution of peptide families and different plant traits. A simplified plant evolution map illustrates the appearance of several peptide families and the peptide diversity of their representative species. Blue dots indicate key evolutionary plant innovations. Green check marks and red crosses respectively represent peptides present and not yet found (likely absent). The number of identified peptides in each representative species is indicated in parentheses. Question marks indicate that no search has been conducted against the genomes of these species. The asterisk indicates that a CLE was present but was possibly a false positive. Abbreviations: CEP, C-TERMINALLY ENCODED PEPTIDE; CLE, CLAVATA3/EMBRYO SURROUNDING REGION; EPFL, EPIDERMAL PATTERNING FACTOR-LIKE; IDL, INFLORESCENCE DEFICIENT IN ABSCISSION-LIKE; RALF, RAPID ALKALINIZATION FACTOR; RGF, ROOT MERISTEM GROWTH FACTOR.

#### 5.1. Is There a Link Between Evolution of Plant Traits and Peptide Families?

From single-celled green algae, multicellular algae and land plants with specific traits evolved, and this development seems to coincide with the appearance of several peptide families (**Figure 4**). By allowing control of gas exchange with the environment, stomata were central to

the adaptation of plants to a terrestrial environment, and they are considered to have evolved after the divergence of liverworts and mosses from their common ancestor (22). In *Arabidopsis*, members of the EPFL peptide family regulate stomatal density: EPF1, EPF2, and EPFL6 negatively regulate stomatal density (1, 57, 58), whereas STOMAGEN/EPFL9 acts as a positive regulator (86, 182). Consistent with the evolution of stomata, EPFL peptides are absent from single-celled algae (186). However, researchers have identified various EPFLs across early land plants, including the lycophyte *Selaginella moellendorffii*, the liverwort *Marchantia polymorpha*, and the bryophyte *Physcomitrella patens*, the last of which develops its stomata on sporophytes (13, 145, 157, 182, 186) (**Figure 4**). The EPFL gene family from several land plants is divided into four clades (186). The clade containing negative regulators includes orthologs from moss and vascular plants, whereas the clade with positive regulators consists of homologs only from vascular plants. Possibly, the acquisition of STOMAGEN/EPFL9, a potent inducer of leaf stomata, resulted in the dramatic stomatal density increase in early vascular plants (109, 186). With respect to the EPF and EPFL receptor, ER is also found across land plants which have developed stomata, including *P. patens* and *M. polymorpha* (13, 157).

Following the evolution of bryophytes, land plants developed vascular tissues for conducting water and nutrients within their multicellular bodies. Well-developed water and nutrientconducting cells constitute the tracheary and sieve elements found in xylem and phloem, respectively. Several members of the CLE family of peptides, including TDIF (CLE41/CLE44), CLE42, and CLE45 (referred to as vascular-related CLEs), have important roles in vascular development (35, 75, 208) (Figure 4). Bryophytes, which lack vascular tissues, also contain genes encoding for CLE peptides, but do not have direct orthologs of higher plant CLEs known to be involved in vascular tissue development (53). In contrast, though lycophytes developed xylem and phloem, no orthologs of TDIF (CLE41/CLE44) are present (53), suggesting that other CLE family peptides may have contributed to vascular tissue development in early land plants. Finally, though excluded as a putative false positive by stricter filtering criteria, CLE genes are also found in the green alga Chlamydomonas reinhardtii (53, 126). In green algae, which do not have vasculature, ancestral CLEs likely have roles unrelated to some of their functions characterized in land plants. This further supports that their role in vascular development is not the ancestral function. Regarding CLE receptors, several of these have been identified and studied in various dicots and monocots (9, 12, 35, 36, 76, 77, 84, 97, 115–117, 135, 165, 188). Furthermore, CLV1 and CLV2 orthologs are found in the S. moellendorffii and M. polymorpha genomes (6, 13).

A root system evolved from simple rhizoid (root-like) structures, which are widespread in bryophytes and early vascular plants (81, 164). Several peptide families are involved (directly or indirectly) in regulating aspects of root development, including members from CEP, RALF, RGF/GLV/CLEL, CLE, and IDL families (**Figure 4**). In addition to an important role in vascular development and in agreement with their presence in a wide range of plant species (see above), CLE peptides also regulate root development and root-associated processes in various plant species (30, 53, 99, 116, 135, 175). *RGF/GLV/CLEL* genes have key roles in root meristem maintenance (108, 111) and were identified in several species with well-developed root systems (180, 209). In addition, CEP family peptides regulate various aspects of root architecture, including lateral root initiation and nitrate-dependent lateral root elongation (153, 184). Overall, CEPs are widely distributed among seed plants (34, 129, 152), and the CEP domain of seed plants has diversified between dicots and monocots (129). However, *CEP* genes are absent in green algae and land plants that lack vasculature, like the moss *P. patens*, or true branching roots, like the lycophyte *S. moellendorffii* (34, 129, 152).

Interestingly, the common ancestor of all extant vascular plants was rootless, and roots with caps had at least two independent origins among lycophytes and euphyllophytes (61). This convergent

#### **Peptide mimic:**

a non-plant-derived peptide recognized by the plant receptor kinase that usually binds the endogenous peptide evolution might explain the absence of CEPs in lycophytes. Since the CEP receptor XYLEM INTERMIXED WITH PHLOEM 1/CEPR1 also controls vascular development (16), it is difficult to speculate on the precise ancestral role of CEPs: regulating root architecture or vascular development, a combination of both, or something else entirely.

Similarly, RALFs are involved in primary and lateral root development (121, 142), likely through their general roles in regulating alkalinization and cell size in various contexts (119). Following their discovery in tobacco leaves (142), RALF family members and members of the associated *Catharanthus roseus* RECEPTOR KINASE 1-LIKE (CrRLK1L) family have been identified within many species across the plant kingdom (20, 21, 49, 119). However, RALFs occur in species that do not develop a root system, such as the moss *P. patens* and the liverwort *M. polymorpha* (13, 20), further supporting that their ancestral function is not related to root architecture. In this context, it should be noted that CrRLK1L family members FER and ANX1/2 were first identified for their roles in reproduction and specifically in pollen rupture (11, 71). In addition, a mutant in *MpTHESEUS*, the only *M. polymorpha* CrRLK1L family member, displays impaired rhizoid elongation and rhizoid rupture (67). It is therefore likely that cell elongation and/or cell wall integrity sensing are the ancestral functions and that this was co-opted during the evolution of pollen tubes. While, thus far, no RALF orthologs have been found in green algae (20), there seems to be a CrRLK1L family member in charophytes (49).

At some point, flowering plants began to dominate terrestrial habitats, and various peptides have important roles during plant reproduction and flower development and maturation. Several Cys-rich peptide family members contribute to the plant reproductive process during self-incompatibility, pollen tube growth, guidance and reception, and gamete activation, and although they are found in multiple species, some appear to be Gramineae-specific (40, 103, 150). Furthermore, Cys-rich peptides involved in reproductive processes are proposed to have evolved from polymorphic peptides with antibacterial and antifungal activity after gene duplication and neofunctionalization (7). It is likely that the reproductive isolation and speciation in plants resulted in the fast evolution of new Cys-rich peptides regulating fertilization processes (7). Finally, IDLs, which play a major role in floral abscission, are found across angiosperms, gymnosperms, and the liverwort *M. polymorpha* (13, 179, 200), and the genes encoding putative orthologs of HAE and HSL2 were found across angiosperms and in the *M. polymorpha* genome (13, 179). This, together with the fact that IDA and IDL peptides are responsible for regulation of cell separation during both floral abscission and lateral root emergence (18, 87, 177), supports an ancestral role in cell separation rather than a direct association with flower evolution.

#### 5.2. Peptide Mimics from Outside the Plant Lineage

Specific plant receptors recognize nonplant peptides, for example, in the case of immune responses (163). Peptide mimicry by nonplant organisms also occurs and is an important component of nematode parasitism and plant–pathogen interactions. Several precursor-derived peptide families are found in a wide range of parasitic species associated with plants, including cyst, root-knot, and reniform nematodes (45, 56, 83, 99, 156, 195, 206, 211). The first cyst nematode *CLE* gene was identified from the soybean pest *Heterodera glycines*, and others soon followed (50, 202, 206). CLE mimics from nematodes are secreted into plant cells as proproteins (202). Importantly, ectopic expression of *HgCLE2* from *H. glycines* in *Arabidopsis* can complement the phenotype of the *clv3 Arabidopsis* knockout mutant (202, 207), which supports the finding that nematode peptides mimic endogenous host-plant peptides. Besides cyst nematode CLE peptides, *Meloidogyne* root-knot nematode species and the reniform nematode *Rotylenchulus reniformis* encode and/or secrete CLEs (156, 211). Beyond plant-parasitic nematodes, *CLE* sequences have not been identified in symbiotic or pathogenic bacteria and fungi (126). One hypothesis to explain this difference is that CLE-like motifs from nematodes may have arisen through convergent evolution with their host (5); another possibility is that *CLE* genes from parasitic organisms may have evolved through horizontal gene transfer (31). CEPs have also been identified in plant-parasitic nematodes (8, 10, 45). Interestingly, because *R. reniformis CEP* genes share no sequence similarity with any other plant or animal CEPs except the conserved CEP domains and because *R. reniformis* is only distantly related to root-knot nematodes, *R. reniformis* CEPs may have evolved independently from both plant and root-knot nematode CEPs (45).

Other precursor-derived plant peptide mimics have been identified in fungi and bacteria. For example, RALFs are detected in several phytopathogenic fungi and some species of bacteria, including plant-pathogenic species like *Streptomyces acidiscabies* (104, 193). Similar to plant-produced RALF, a synthetic RALF peptide based on orthologs from the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici* can be perceived by plants and inhibits plant growth (104). In addition, a *Fusarium RALF* mutant fails to induce alkalinization in plants and is less virulent (104).

Finally, the phytopathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* produces the sulfated peptide RaxX, which mimics PSY1 and which is recognized by the rice immune receptor XA21 (148, 149). Synthetic RaxX enhances root growth in *Arabidopsis* and rice, and a *Xanthomonas oryzae* pv. *oryzae* strain lacking RaxX has limited ability to infect rice (148).

#### 5.3. Conclusion and Emerging Questions

As the need for precise regulation of cell-to-cell communication increased, peptide families diversified through gene and whole-genome duplication. The evolution of peptide ligands is generally consistent with the emergence of developmental processes in which these peptides exert their regulatory functions. In some cases, members of peptide families are present in plant species that do not have the organs or tissues that have been associated with the respective peptides, and it remains to be investigated if these peptides have different ancestral-related functions. To the extent this has been investigated, several peptide families appear to be absent from bryophytes, lycophytes, and green algae. In the future, it will be necessary to explore the extent to which corresponding receptor families are also absent, and, if they are present, to determine to what signaling partners they bind.

In the context of beneficial bacteria–plant interactions, for example, during nodule development upon inoculation with *Sinorhizobium meliloti*, upregulation of *CLE* expression occurs at the plant level (115, 116, 139). However, nonplant species also produce precursor-derived plant peptide mimics, and these, as far as we know, mainly evolved in phytopathogenic species to hijack the plant signaling machinery and assist parasitic success of nematodes or pathogenic infection of hosts. Whether this is a general mechanism of pathogenicity remains unclear, and addressing this issue will require more detailed genome mining.

#### 6. FUTURE DIRECTIONS

To rephrase Immanuel Kant, it is important to look closely in order to identify beautiful, small things, and we argue such is the case for secreted, nonfunctional precursor-derived peptides. Not only are they (structurally) beautiful, but they also fulfill important roles in plant growth, development, and interactions with the environment.

Knowledge regarding the roles of nonfunctional precursor-derived peptides is gradually growing, and current biochemical tools allow easier matching of peptides to their receptors. Nevertheless, a number of aspects have been explored only limitedly. For example, genome editing by CRISPR/Cas9 approaches can be used to create specific knockout lines that can partially solve the issue of genetic redundancy and may result in the identification of novel peptide-receptor pairs in the near future (144, 215). Specificity in the expression patterns of proteins and the available signaling components in spatiotemporal contexts are largely responsible for regulating overall specificity. However, very little is known about the transcriptional regulation of nonfunctional precursor-derived peptides. In addition, peptide, receptor, coreceptor(s), and downstream signaling components must be available at the same time and place to induce signaling specific to the corresponding peptide. It is also becoming increasingly apparent that there is no simple one peptide, one receptor, and one function rule; instead, a complex and tightly regulated signaling potential is present. When we increase our understanding of the physical interactions among peptides, receptors, and coreceptors, we will gain insight into how this process arises. Other inputs will need to be integrated in addition to regulation within a peptide's own signaling cascade. Understanding such crosstalk with environmental and developmental signaling, e.g., mediation through plant hormones, will be crucial to capture the full complexity of specific peptide signaling. Plant peptides have previously been used in several antifungal and medical applications (33, 172). However, to our knowledge, there have not been any agricultural plant peptide applications to promote growth. This seems an exciting area to explore, especially since manipulation of peptide signaling can be used to increase tomato fruit size or seed yield in maize (76, 212). We are slowly starting to understand the roles and complex signaling of small and beautiful peptides. As our search for them continues, exciting discoveries await.

#### SUMMARY POINTS

- 1. Proteolytic processing of prepropeptides to yield biologically active peptide ligand occurs in multiple steps throughout the secretory pathway and in extracellular space.
- 2. Various methods have been applied to identify peptide–receptor pairs, but many orphan receptors and ligands remain to be matched.
- 3. Specific cellular output of a peptide ligand–activated response is established at various steps in a signal transduction pathway.
- Diversification and expansion of peptide genes and families seem to coincide with increasing complexity of the plant body and various environmental changes to which the plant is exposed.
- 5. Nonplant organisms also produce and secrete small peptides to hijack the plant machinery and, mainly, to facilitate infections.

#### **DISCLOSURE STATEMENT**

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