

## REVIEW

# The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions<sup>OPEN</sup>

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**Peptides fulfill a plethora of functions in plant growth, development, and stress responses. They act as key components of cell-to-cell communication, interfere with signaling and response pathways, or display antimicrobial activity. Strikingly, both the diversity and amount of plant peptides have been largely underestimated. Most characterized plant peptides to date acting as small signaling peptides or antimicrobial peptides are derived from nonfunctional precursor proteins. However, evidence is emerging on peptides derived from a functional protein, directly translated from small open reading frames (without the involvement of a precursor) or even encoded by primary transcripts of microRNAs. These novel types of peptides further add to the complexity of the plant peptidome, even though their number is still limited and functional characterization as well as translational evidence are often controversial. Here, we provide a comprehensive overview of the reported types of plant peptides, including their described functional and structural properties. We propose a novel, unifying peptide classification system to emphasize the enormous diversity in peptide synthesis and consequent complexity of the still expanding knowledge on the plant peptidome.**

## INTRODUCTION

Peptides (from the Greek  $\pi\epsilon\pi\tau\omicron\varsigma$  meaning “digestive”) are the smallest biological molecules of the plant proteome, often arbitrarily restricted to proteins of 2 to 100 amino acids. They fulfill diverse roles in plant growth, development, reproduction, symbiotic interactions, and stress responses (Albert, 2013; Czyzewicz et al., 2013; Matsubayashi, 2014). On the one hand, peptides can interact directly with pathogens through their antimicrobial properties (Goyal and Mattoo, 2014). On the other hand, they function by interfering with signaling cascades or by representing important messages in cell-to-cell communication (Murphy et al., 2012; Tintor et al., 2013; Uchida and Tasaka, 2013; Araya et al., 2014; Costa et al., 2014; Farkas et al., 2014; Haruta et al., 2014; Qu et al., 2015). Despite many studies on their occurrence in various plant species and involvement in different plant processes, peptides are still often overlooked in gene predictions and mass spectrometric analyses (Yang et al., 2011; Guillén et al., 2013;

Niarchou et al., 2013). As a result, the total number of peptides in plants as well as the diversity of various peptide types is underestimated (Silverstein et al., 2007; De Coninck et al., 2013a). Here, we review the reported types of peptides starting from a more adapted classification to emphasize the enormous diversity in peptide synthesis, aiming at boosting research on the “shortest” members of the plant proteome.

## A NEW, COMPREHENSIVE, AND UNIFYING PEPTIDE CLASSIFICATION SYSTEM

Peptides are often referred to as proteins smaller than 100 amino acids. Although this length restriction is rather artificial, in this Review, we maintain this upper limit and, moreover, only discuss peptides that are ribosomally synthesized. Up to now, so-called secreted small signaling peptides and antimicrobial peptides (AMPs) have mainly been studied (Goyal and Mattoo, 2014; Matsubayashi, 2014). Although they strikingly differ in amino acid sequence, length, 3D structure, and function, they are all derived from nonfunctional precursor proteins (Figure 1). The latter can be a preprotein that results in the mature peptide upon removal of an N-terminal signal sequence (NSS) (also referred to as N-terminal signal peptide), which directs the precursor via the endoplasmic reticulum to the secretory pathway. Alternatively, such a precursor can be a proprotein that is enzymatically

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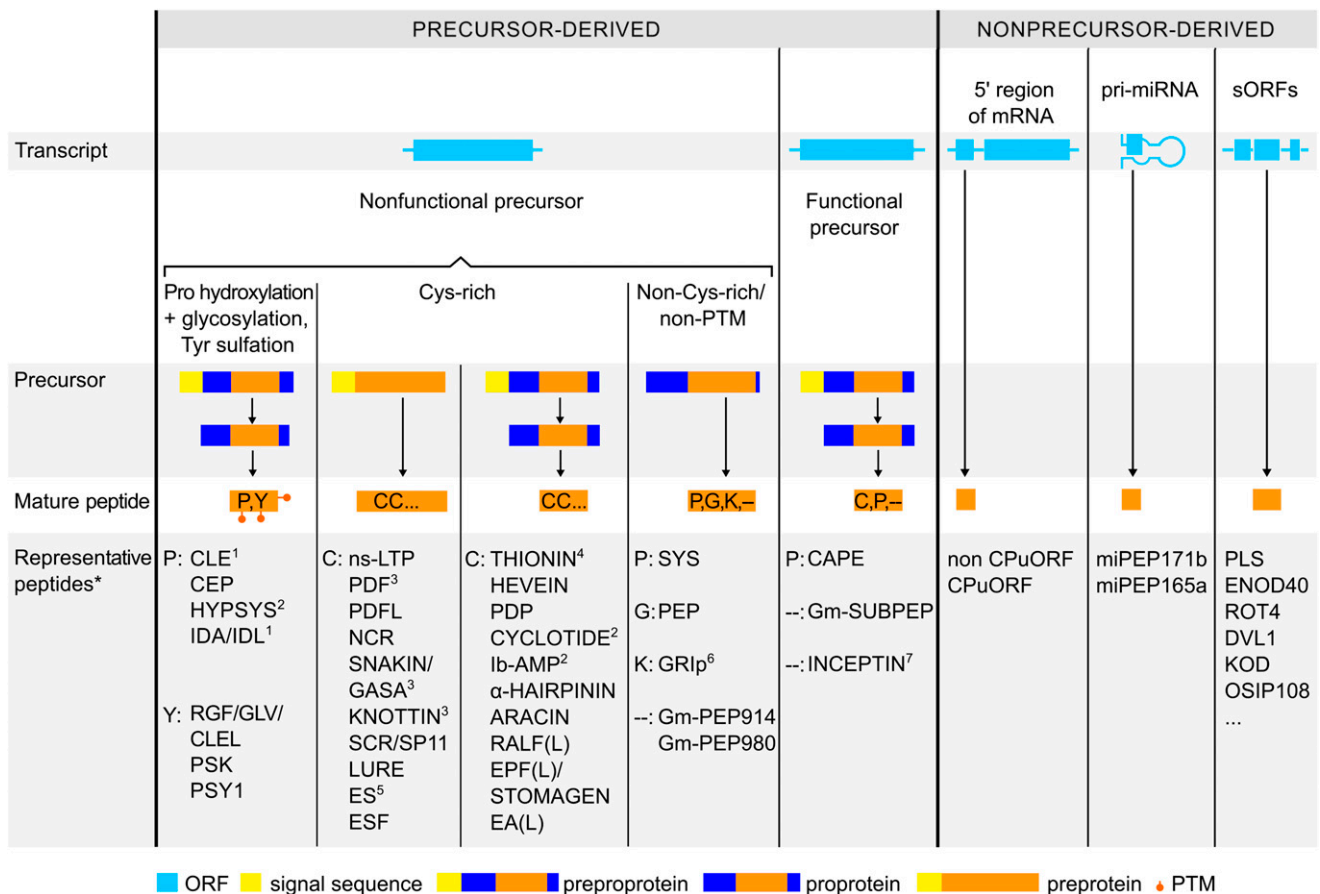
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modified to the mature peptide and is further termed pre-protein when also harboring an NSS.

Despite the majority of reported plant peptides being derived from such nonfunctional precursors, different studies also point to the existence of peptides originating from a functional protein but with an activity differing from that of the precursor (Schmelz et al., 2006; Pearce et al., 2010a; Chen et al., 2014) (Figure 1). This phenomenon raises the question whether many other well-known protein-encoding genes could have a double function, both as parent protein and as derived peptide. In addition, an increasing number of recent reports indicate that peptides can also be translated directly from a short open reading frame (sORF) (<100

codons) that is present in the 5' leader sequence of an mRNA, in primary transcripts of microRNAs (miRNAs) or in other transcripts not encoding longer (>100 amino acids) proteins (Hanada et al., 2013; von Arnim et al., 2014; Laressergues et al., 2015) (Figure 1). No precursor protein appears to be involved in their synthesis, but their maturation process has not yet been investigated in detail. It was shown that this type of peptides can influence plant morphogenesis or has a regulatory function (Hanada et al., 2013; von Arnim et al., 2014; Laressergues et al., 2015). Additionally, one must note that features of the transcripts encoding this type of peptides overlap with long and short noncoding RNA transcripts, as they also contain one or more sORFs (Dinger et al., 2008). This



**Figure 1.** The Diversity of Plant Peptide Synthesis.

Plant peptides are synthesized from precursor proteins or directly translated from sORFs embedded in transcripts. The former type of peptides is derived from nonfunctional precursors or functional precursors. Such a precursor can be a preprotein that results in the mature peptide upon removal of an NSS (yellow rectangle). Alternatively, such a precursor can be a proprotein that contains a prodomain (blue rectangle) and is enzymatically modified to the mature peptide (orange rectangle). If the proprotein also harbors an NSS, it is termed preproprotein. So far, peptides derived from nonfunctional precursors can be posttranslationally modified (PTM), Cys-rich, or non-Cys-rich and non-posttranslationally modified (non-Cys-rich/non-PTM). Representative peptides of these three subgroups are classified by their dominant residues where appropriate (\*), though sometimes a single dominant amino acid cannot be identified (-). Nonprecursor-derived peptides are encoded by sORFs (<100 amino acids) that are located (1) upstream of the main ORF in the 5' leader sequence of a gene, (2) in primary transcripts of miRNA (pri-miRNAs), or (3) in other transcripts not encoding longer (>100 amino acids) proteins. Most peptides in this group follow the presented scheme, but some exceptions may arise, including <sup>1</sup>some members do not contain an NSS, <sup>2</sup>multiple mature peptides are released from one single precursor, <sup>3</sup>some members can contain a prodomain, <sup>4</sup>an unprocessed THIONIN of 15 kD was reported, <sup>5</sup>an internal signal sequence is present instead of an NSS, <sup>6</sup>an NSS is present and proteolytic cleavage is executed by a metacaspase, and <sup>7</sup>proteolytic cleavage is executed by herbivorous insect processing machinery.

controversial phenomenon leads to questioning the distinctive border between peptide/protein-coding and noncoding transcripts.

The above-mentioned examples of novel types of peptides could be added to the short list of exceptions, but we rather expect that they represent a glimpse of completely new classes of peptides that are underexplored so far. We perceive that the plant peptidome as a whole is composed of peptides that are (1) derived from nonfunctional precursors, (2) derived from functional precursors, or (3) not derived from a precursor protein (Figure 1). In the following sections, we will further discuss these groups and illustrate them and some key features with representative examples.

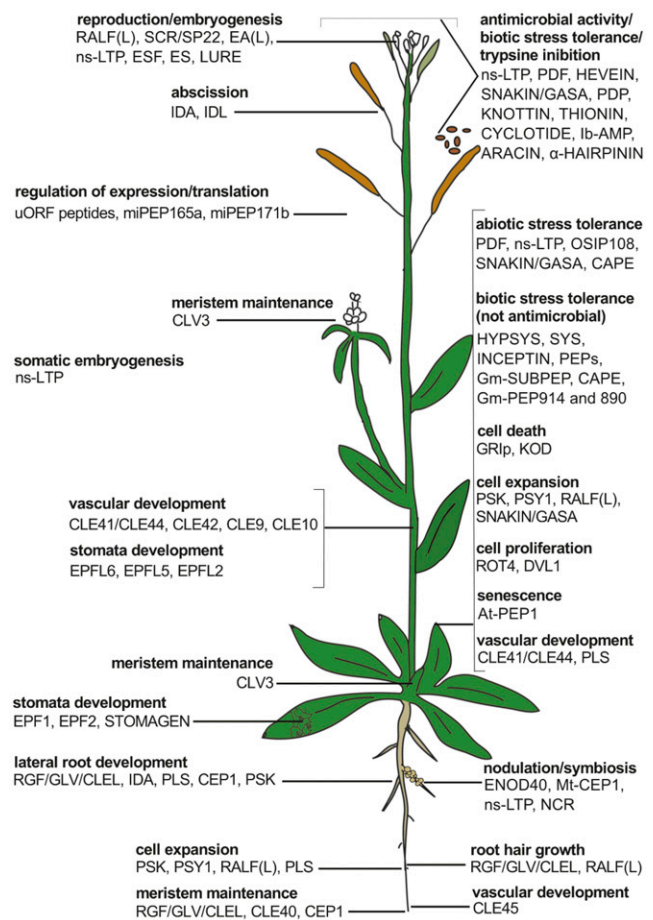
### PEPTIDES DERIVED FROM A NONFUNCTIONAL PRECURSOR

Most peptides identified up to now are derived from a longer precursor that has no known biological function as a preprotein, proprotein, or preproprotein. Based on specific characteristics, peptides within this group can be divided into three subgroups: (1) peptides characterized by specific posttranslational modifications, such as Pro hydroxylation, Pro glycosylation, and Tyr sulfation; (2) Cys-rich peptides; and (3) non-Cys-rich peptides without posttranslational modifications (Figure 1) (Matsubayashi, 2011a; Meng et al., 2012a; Murphy et al., 2012). Peptides within this group carry specific amino acid residues with dominant roles, such as Cys, Pro, Tyr, Gly, or Lys, because these are often essential for the peptide's activity.

#### Peptides with Specific Posttranslational Modifications

Posttranslationally modified (PTM) peptides typically consist of a maximum of 20 amino acids, have few or no Cys residues, contain modifications on their Pro and Tyr residues, and are released from preprotein precursors (Figure 1). Many peptide families involved in plant development, defense, cell identity, and cell-to-cell communication belong to this subgroup (Matsubayashi, 2011b). These peptides function as signaling molecules and are perceived by specific receptors. While progress has been made on molecular and physiological events downstream of PTM peptides, detailed modes of action are generally not known. In some cases, it has, for example, been demonstrated that PTM peptides can signal through a MITOGEN-ACTIVATED PROTEIN KINASE cascade, as such regulating transcription factor activity and/or other downstream events, that their activity ultimately leads to altered transcription factor expression patterns, that they affect apoplastic pH regulation, that they mediate the inhibition of proton transport, that they act as a chemo-attractant, etc. (Sparks et al., 2013; Haruta et al., 2014; Tabata et al., 2014; Butenko and Simon, 2015). To illustrate this subgroup of peptides, we selected some well-studied representatives (Figures 2 and 3) and refer to excellent, recent reviews for additional information (Fernandez et al., 2013b; Endo et al., 2014; Marmioli and Maestri, 2014; Matsubayashi, 2014; Grienerberger and Fletcher, 2015; Sauter, 2015).

In the context of this Review, we focus on specific post-translational modifications, such as Pro hydroxylation (Pro<sup>Hyp</sup>), Pro<sup>Hyp</sup> glycosylation (mainly arabinosylation), and Tyr sulfation (Matsubayashi, 2011a), which are often important for maturation of these peptides and play a role in peptide stability, activity, and



**Figure 2.** The Functional Diversity of Plant Peptides.

Plant peptides have a wide range of biological roles and act in different plant parts, as indicated in an illustration of a simplified plant (adapted from Czyzewicz et al. [2013]).

interactions with their receptor (Ogawa et al., 2008; Matsubayashi, 2011a; Okamoto et al., 2013; Tabata and Sawa, 2014). We do not consider other posttranslational modifications, such as proteolytic cleavage or folding, as listed more extensively by Wang (2012).

Pro hydroxylation is catalyzed by PROLYL-4-HYDROXYLASE, a type II membrane protein with an N-terminal transmembrane domain that is localized in the endoplasmic reticulum and Golgi apparatus (Gorres and Raines, 2010). Hydroxylation as such is not always important for the conformation and bioactivity of peptides, but can function as a necessary bridge for the posttranslational arabinosylation (Ito et al., 2006; Kondo et al., 2008). Pro<sup>Hyp</sup> arabinosylation is catalyzed by HYDROXYPROLINE O-ARABINOSYLTRANSFERASE, a Golgi-localized transmembrane protein. This enzyme transfers L-arabinose to the hydroxyl group of Pro<sup>Hyp</sup> residues (Amano et al., 2007; Ohyama et al., 2009; Ogawa-Ohnishi et al., 2013; Ogawa-Ohnishi and Matsubayashi, 2015). Pro<sup>Hyp</sup> arabinosylation is important for maintenance of proper peptide structure and as a consequence affects peptide activity (Amano et al., 2007; Ohyama et al., 2009; Shinohara and Matsubayashi, 2013; Mohd-Radzman et al., 2015; C. Xu et al., 2015). Tyr sulfation

	Peptide family	Amino acid sequence	3D-structure		
NONFUNCTIONAL PRECURSOR	Representative peptide		Main characteristics		
	<b>CLE</b> CLV3	RLVPSGP*NPLHN (conserved motif: xRxcPsGpDPIHh)	arch-shape (helical arabinose chain)		
	<b>CEP</b> At-CEP1	DFRP <sup>*</sup> TNPGNSP <sup>*</sup> GVGH	β-turn-like conformation		
	specific PTM	<b>HYPSYS</b> Sl-HYPSYS-I	RT <sup>*</sup> P <sup>*</sup> YKTP <sup>*</sup> P <sup>*</sup> P <sup>*</sup> P <sup>*</sup> TSSSP <sup>*</sup> THQ	polyproline helix	
		<b>IDA/IDL</b> EPIP peptide	FGYLPKGVPIP <sup>*</sup> PSAP <sup>*</sup> SKRHN	nd	
		<b>GLV/RGF/CLEL</b> At-RGF1	DY <sup>*</sup> SNPGHHP <sup>*</sup> RHN	nd	
		<b>PSK</b> At-PSK1	<u>YIYTQ</u>	nd	
		<b>PSY1</b> At-PSY1	DYGDPSANPKHDPGV <sup>*</sup> P <sup>*</sup> PS	nd	
		Cys-rich	<b>nsLTP</b> Os-LTP2	AGCNAGQLTVCTGAAGGARPTAA <sup>*</sup> CCSSLR <sup>*</sup> AQQG <sup>*</sup> CF <sup>*</sup> QFAKDP <sup>*</sup> RYGRYVNSPNARKAVSSCGIALPTCH	four α-helices
			<b>PDF</b> At-PDF1.2	QKLC <sup>*</sup> EKPSGTW <sup>*</sup> SGV <sup>*</sup> CGNSNA <sup>*</sup> CKNQ <sup>*</sup> INLEGA <sup>*</sup> KHGS <sup>*</sup> CNYVFAHK <sup>*</sup> CIC <sup>*</sup> YVPC	α-helix, three β-sheets
			<b>PDFL</b> At-DEFL216	ILINESSDEQRIY <sup>*</sup> SFSP <sup>*</sup> TTSP <sup>*</sup> FDPRSLNQLKIGRIGY <sup>*</sup> CFD <sup>*</sup> CACRACMR <sup>*</sup> RGY <sup>*</sup> IRT <sup>*</sup> CSFERK <sup>*</sup> LCRCSISDIK	nd
			<b>NCR</b> Mt-NCR247	RNGCIVDPRCPYQQCRRPLYCRRR	nd
			<b>SNAKIN/GASA</b> Sl-SNAKIN1	GSSF <sup>*</sup> CDSK <sup>*</sup> CLR <sup>*</sup> CSKAGLADR <sup>*</sup> CLKY <sup>*</sup> CGIC <sup>*</sup> CEE <sup>*</sup> CK <sup>*</sup> CVPSGT <sup>*</sup> YGNKHEC <sup>*</sup> PCYRD <sup>*</sup> KKNSK <sup>*</sup> GKSK <sup>*</sup> CP	two predicted long α-helices
	<b>KNOTTIN</b> Mj-AMP1		QCIGNGGRCNENVGPPY <sup>*</sup> CCSG <sup>*</sup> F <sup>*</sup> LRQPGQGY <sup>*</sup> GCKNR	three β-sheets (cystine knot)	
	<b>SCR/SP11</b> SP11		NLRKT <sup>*</sup> CVHRLNSGGSC <sup>*</sup> CKSG <sup>*</sup> QHD <sup>*</sup> CEAFY <sup>*</sup> TNKT <sup>*</sup> NQAFY <sup>*</sup> CNCT <sup>*</sup> SPFR <sup>*</sup> TRYCDCAIK <sup>*</sup> CKVR	α-helix, three β-sheets	
	<b>LURE</b> Tf-LURE1		GEIPPEQLRYVEFCDLWSADFGSGCDLCKK <sup>*</sup> WGNFVGD <sup>*</sup> CDWYASTLWTSGDCV <sup>*</sup> SEK <sup>*</sup> KKK	predicted α-helix, three β-sheets	
	<b>ES</b> Zm-ES1		RDCLTQSTR <sup>*</sup> LPGL <sup>*</sup> CV <sup>*</sup> RSYD <sup>*</sup> CAIG <sup>*</sup> CRAE <sup>*</sup> GKGY <sup>*</sup> TGGR <sup>*</sup> CLIS <sup>*</sup> PIPL <sup>*</sup> DGIL <sup>*</sup> CY <sup>*</sup> CVK <sup>*</sup> CP <sup>*</sup> SNTTT	α-helix, three β-sheets	
	<b>ESF</b> At-ESF1		VRMKVKEIGRSNKIYIP <sup>*</sup> PCFRD <sup>*</sup> SCDH <sup>*</sup> VLK <sup>*</sup> DCY <sup>*</sup> CCG <sup>*</sup> SKPDL <sup>*</sup> CWED <sup>*</sup> QHYCN <sup>*</sup> THCP <sup>*</sup> PLK <sup>*</sup> PLIN	α-helix, three β-sheets	
	<b>THIONIN</b> At-THI2.1		KIC <sup>*</sup> CP <sup>*</sup> SNQARNGYSV <sup>*</sup> CRIR <sup>*</sup> FSKGR <sup>*</sup> CMQV <sup>*</sup> SGC <sup>*</sup> QNSD <sup>*</sup> T <sup>*</sup> CP <sup>*</sup> RGWVN	two antiparallel α-helices, two β-sheets	
	<b>HEVEIN</b> Hb-HEV1		EQCGRQAGGK <sup>*</sup> LC <sup>*</sup> PNNL <sup>*</sup> CC <sup>*</sup> SQWGW <sup>*</sup> CGST <sup>*</sup> DEY <sup>*</sup> CPD <sup>*</sup> HNC <sup>*</sup> QSNCKD	three antiparallel β-sheets, α-helix	
	<b>PDP</b> Ha-SFTI		GR <sup>*</sup> CTKSIP <sup>*</sup> IC <sup>*</sup> FPD	cyclic, two short antiparallel β-sheets	
	<b>CYCLOTIDE</b> Cyclotide phyb-A1		GIGCGES <sup>*</sup> CVWIP <sup>*</sup> CVSAAIG <sup>*</sup> SC <sup>*</sup> SNK <sup>*</sup> ICYRN	cyclic, three β-sheets (cystine knot)	
	<b>lb-AMP</b> lb-AMP1		QWGRRC <sup>*</sup> CGWGP <sup>*</sup> R <sup>*</sup> RY <sup>*</sup> CV <sup>*</sup> RWC	loop structure	
	<b>HAIRPININ</b> Sm-AMPX		VDPDVRAY <sup>*</sup> CKHQ <sup>*</sup> CMSTR <sup>*</sup> GDQARK <sup>*</sup> ICES <sup>*</sup> VCMR <sup>*</sup> QD	two α-helices	
	<b>ARACIN</b> At-ARACIN1		GSCGASIAEFNSSQILAKRAPP <sup>*</sup> CRRP <sup>*</sup> RLQNS <sup>*</sup> EDV <sup>*</sup> TH <sup>*</sup> TLP	nd	
	<b>RALF(L)</b> At-RALF23		ATRRYISY <sup>*</sup> GALRRNTIP <sup>*</sup> CSRRGAS <sup>*</sup> Y <sup>*</sup> NCRRGAQAN <sup>*</sup> PYSR <sup>*</sup> GC <sup>*</sup> SAIT <sup>*</sup> CRRS	nd	
	<b>EPF(L)/STOMAGEN</b> EPFL9=STOMAGEN		IGSTAPTCTYNE <sup>*</sup> CRG <sup>*</sup> RY <sup>*</sup> K <sup>*</sup> CR <sup>*</sup> AEQV <sup>*</sup> PE <sup>*</sup> GN <sup>*</sup> D <sup>*</sup> PINSAY <sup>*</sup> HYR <sup>*</sup> CV <sup>*</sup> CHR	two antiparallel β-sheets, loop	
	<b>EA(L)</b> Zm-EA1		GMMMKAPGAAGW <sup>*</sup> ICRAV <sup>*</sup> FEAN <sup>*</sup> PQ <sup>*</sup> LYFTILRTA <sup>*</sup> GAAAAA <sup>*</sup> FAAC <sup>*</sup> SIAS	nd	
	non-Cys-rich/ non-PTM	<b>SYS</b> Sl-SYS	AVQSKPPSKRD <sup>*</sup> PPKMQTD	polyproline helix	
		<b>PEP</b> At-PEP1	ATKVKAKQ <sup>*</sup> RGKEK <sup>*</sup> VSSGR <sup>*</sup> P <sup>*</sup> Q <sup>*</sup> HN	fully extended conformation	
		<b>GRip</b>	LLVSHYK <sup>*</sup> KIKK	nd	
		<b>Gm-PEP914</b>	D <sup>*</sup> HPRGGNY	nd	
	FUNCTIONAL PRECURSOR	<b>CAPE</b> Sl-CAPE-PR-1b	<u>PVGNWIGQRPY</u>	nd	
		<b>Gm-SUBPEP</b>	NTPPRRAKSRPH	nd	
		<b>INCEPTIN</b> Vu-INCEPTIN	<u>ICDINGVCVDA</u>	nd	
	NO PRECURSOR	<b>uORF peptides</b> SC-peptide (uORF2b)	MLNSTIRRRTHLVQSFVFLYWL <sup>*</sup> YVVS	nd	
		<b>miPEP171b</b>	MLLHRLSKFCKIERDIVIS	nd	
<b>PLS</b>		MKPRLCFNFRRRSISPCYISISYLLVAKLFLKFKIH	nd		
<b>ENOD40</b> Mt-ENOD40-I		MKLLCWEKSIHGS	nd		
<b>ROT4</b>		MAPEENGTCPECKTFGQKCSHVVKQRAKFIYLRRCIAMLCVWHDQNHDRKDS	nd		
<b>DVL1</b>		MEMKRVMMSSAERSKEKKRSIRRLGKYMKEQKGR <sup>*</sup> IIRRCMVMLLCSHD	nd		
<b>KOD</b>		MWWLVGLTPVELIHLCTFRERLCHL	α-helix		
<b>OSIP108</b>		MLCVLQGLRE	nd		

**Figure 3.** Key Features of All Peptide Types Based on Representative Examples.

Peptides derived from nonfunctional precursors can be classified in three subgroups: (1) PTM peptides, (2) Cys-rich peptides, and (3) non-Cys-rich/non-PTM peptides. PTM peptides are characterized by specific posttranslational modifications, such as Pro hydroxylation (orange), glycosylation (asterisk), and

is mediated by the Golgi-localized TYROSYLPROTEIN SULFO-TRANSFERASE, which catalyzes the transfer of a sulfonate moiety from 3'-phosphoadenosine-5'-phosphosulfate to the hydroxyl group of a protein-bound Tyr residue to form a Tyr O-sulfate ester and 3'-phosphoadenosine-5'-phosphate (Moore, 2003). Tyr sulfation possibly regulates biological activity, proteolytic processing of bioactive peptides, and/or extracellular protein-protein interactions (Kehoe and Bertozzi, 2000; Matsubayashi, 2011a).

A well-studied example of such PTM peptides is the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (CLE) family. Mature CLE consist of 12 to 13 amino acids with one to three highly conserved Pro residues, which are part of the conserved CLE motif located at or near the C terminus of the preproprotein (Cock and McCormick, 2001) (Figure 3). The fourth and seventh Pro residues of these CLE peptides are often hydroxylated and the seventh Pro<sup>HYP</sup> residue is also modified with three residues of L-arabinose (Ohyama et al., 2009; Matsubayashi, 2011b; Shinohara and Matsubayashi, 2013). Ala substitutions of CLV3 (Kondo et al., 2008) and experiments with *clv3* mutants (Ohyama et al., 2009; Shinohara and Matsubayashi, 2013) indicated that hydroxylation and arabinosylation, more specifically arabinose chain length and its influence on peptide conformation, are essential for the biological activity of CLV3. In *Arabidopsis thaliana*, the CLV3 peptide acts as a regulator of the shoot apical meristem by maintaining the stem cell population within the shoot apical meristem (Clark et al., 1995; Schoof et al., 2000). Since the discovery of CLV3 (Clark et al., 1995; Ito et al., 2006; Kondo et al., 2006; Ohyama et al., 2009), (putative) CLE genes have been identified in dicots, monocots, mosses, and algae (Jun et al., 2008; Oelkers et al., 2008; Miwa et al., 2009; Zhang et al., 2014; Strabala et al., 2014), and they were shown to act on diverse developmental processes, including shoot and root meristem development, nodulation, embryo and endosperm development, regulation of root architecture in response to nutrients, and vascular development (Hobe et al., 2003; Fiers et al., 2005; Hirakawa et al., 2008; Stahl et al., 2009; Jun et al., 2010; Mortier et al., 2010; Lim et al., 2011; Reid et al., 2011; Fiume and Fletcher, 2012; Okamoto et al., 2013; Araya et al., 2014; Richards et al., 2015; T.-T. Xu et al., 2015). Strikingly, plant-parasitic cyst nematodes also secrete CLE-like effector proteins that are required for successful nematode infection (Wang et al., 2010, 2011; Replogle et al., 2011; Miyawaki et al., 2013). 3D structure modeling demonstrated that CLV3, CLE1, CLE2, and CLE6 shared a similar arch-shaped molecular structure with conserved residues at the C terminus and middle region, suggesting that these regions could be involved in receptor selection and binding (Meng and

Feldman, 2010). Within the CLE family, the peptide-receptor relationships are diverse, since one receptor kinase can serve as the receptor for different peptides, but on the other hand, one peptide can be recognized by different receptors. To illustrate the former, CLV1 is a likely receptor for the CLV3 peptide during shoot meristem maintenance (Ogawa et al., 2008), for the CLE40 peptide during root meristem maintenance (Hobe et al., 2003) and for the CLE3 peptide during regulation of lateral root formation (Araya et al., 2014). To illustrate the latter, the CLE9 peptide interacts with the BARLEY ANY MERISTEM1 (BAM1) receptor, BAM2, BAM3, and CLV1, although with different affinity (Shinohara et al., 2012).

The C-TERMINALLY ENCODED PEPTIDE (CEP) family gives rise to peptides of 15 amino acids with two to four Pro residues that may be hydroxylated, thereby affecting biological activity (Ohyama et al., 2008; Imin et al., 2013; Roberts et al., 2013; Ogilvie et al., 2014) (Figure 3). In silico analysis showed that CEP-containing genes occur only in seed plants and are absent in mosses and algae (Ohyama et al., 2008; Delay et al., 2013; Imin et al., 2013; Roberts et al., 2013; Li et al., 2014; Ogilvie et al., 2014). Overexpression of *CEP1* in *Arabidopsis* results in the arrest of root growth (Ohyama et al., 2008), and, in *Medicago truncatula*, overexpression of *CEP1* or application of synthetic CEP1 results in inhibition of lateral root formation, enhancement of nodulation, and the induction of periodic circumferential root swellings (Imin et al., 2013; Mohd-Radzman et al., 2015). In addition, altering the levels of *CEP5* affects aboveground *Arabidopsis* growth and development (Roberts et al., 2013). Structural analysis showed that Mt-CEP1 contains a  $\beta$ -turn-like conformation and that Pro<sup>HYP</sup> increases conformational plasticity and alters surface area of the CEP peptide, which plays an important role in efficient physical interaction between peptide and receptor (Bobay et al., 2013). Remarkably, in an Mt-*CEP1* overexpression line, a vast majority of hydroxylated peptides was isolated, as well as only a minority of triarabinosylated peptides (Mohd-Radzman et al., 2015). Recently, two receptors for several members of the CEP family, CEP RECEPTOR1 (CEPR1) and CEPR2, were described in *Arabidopsis* (Tabata et al., 2014). Interestingly, plant-parasitic root-knot nematodes, such as *Meloidogyne hapla*, secrete peptides with high sequence similarity to CEPs (Bobay et al., 2013), and these nematode peptides possibly play a role in the formation of the novel plant cell types from which nematodes feed.

HYDROXYPROLINE-RICH GLYCOPEPTIDE SYSTEMINS (HYPYSYS) peptides are 15- to 20-amino acid glycopeptides with four to seven Pro residues possibly hydroxylated that are mainly found in members of the Solanaceae family (Pearce et al., 2001a, 2009; Pearce and Ryan, 2003; Chen et al., 2008; Bhattacharya

### Figure 3. (continued).

Tyr sulfation (blue). Cys-rich peptides carry at least two Cys residues to form stable disulfide bridges (square brackets). Non-Cys-rich/non-PTM peptides are not characterized by specific PTM or two or more Cys. Peptides can also be formed from functional precursors or directly translated from sORFs (<100 amino acids) in case no protein precursor is involved in the maturation process. To illustrate the features of all peptide types, the amino acid sequence of a representative mature peptide and the main characteristics of its 3D structure are shown. Additionally, dominant residues (bold), such as Pro, Gly, Lys, Cys, or Tyr, and conserved motifs (underlined) are indicated in the amino acid sequences of these representative peptides. *Arabidopsis thaliana* (At), *Glycine max* (Gm), *Helianthus annuus* (Ha), *Hevea brasiliensis* (Hb), *Impatiens balsamina* (Ib), *Mirabilis jalapa* (Mj), *Medicago truncatula* (Mt), *Oryza sativa* (Os); *Petunia hybrida* (phyb), SUCROSE CONTROL-peptide (SC-peptide); *Solanum lycopersicum* (Sl), *Stellaria media* (Sm), *Solanum tuberosum* (St), *Torenia fournieri* (Tf), *Vigna unguiculata* (Vu), *Zea mays* (Zm). nd, not determined.



et al., 2013) (Figure 3). Mature HypSys peptides are formed from large preproteins. Remarkably, one single protein precursor contains multiple mature HYPYSYS peptides. The latter contain either a characteristic Pro<sup>Hyp</sup>Pro<sup>Hyp</sup>XPro<sup>Hyp</sup>-motif (where X is Ala, Ser, Thr, or Pro<sup>Hyp</sup>) or a slightly different Pro<sup>Hyp</sup>XPro<sup>Hyp</sup>Pro<sup>Hyp</sup> motif (where X is Thr or Ser) in the case of potato (*Solanum tuberosum*) HYPYSYS I and black nightshade (*Solanum nigrum*) HYPYSYS I (Chen et al., 2008; Bhattacharya et al., 2013). Mass spectrometry analysis of HYPYSYS isolated from tomato (*Solanum lycopersicum*) leaf extracts revealed the presence of 3 to 17 pentose residues attached to the Pro<sup>Hyp</sup> residues. Chemically synthesized tobacco (*Nicotiana tabacum*) HYPYSYS I and II, both devoid of pentose residues, were found to be 10,000 times less active than native peptides in an alkalization assay (Pearce and Ryan, 2003). In general, HYPYSYS peptides adopt a polyproline helix stabilized by pentose appendages attached to Pro<sup>Hyp</sup> (Taylor et al., 2012). HYPYSYS has a role in defense against insect herbivores, activates protease inhibitors, and can, for example, enhance the resistance against *Helicoverpa armigera* larvae when overexpressed in tobacco (Ren and Lu, 2006; Pearce, 2011).

The mature peptide structure of INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and IDA-LIKE (IDL) peptides has not yet been determined, but they are believed to be derived from a preproprotein carrying a C-terminal conserved motif (EPIP) (Butenko et al., 2003). The EPIP motif (FGYLPKGVPI-PSAPSKRH) of Arabidopsis IDA and IDL1 can replace or partially substitute IDA function in vivo, and synthetic IDA and IDL1 EPIP peptides rescue *ida* mutants and induce early floral abscission in wild-type flowers (Stenvik et al., 2008). In addition to fulfilling a role in (floral) abscission, IDA also regulates cell separation during lateral root emergence (Butenko et al., 2003; Stenvik et al., 2006; Kumpf et al., 2013). Several IDL transcripts were predicted in dicots and monocots (Butenko et al., 2003) and the EPIP domain, which is well conserved between the IDL peptides of Arabidopsis, is also present in IDA and IDL sequences of bean (*Phaseolus vulgaris*), tomato, and soybean (*Glycine max*) (Tucker and Yang, 2012). IDA/IDL peptides act through the leucine-rich repeat receptor-like kinases HAESA (HAE) and HAE-LIKE2 (HSL2) (Stenvik et al., 2008; Kumpf et al., 2013; Butenko et al., 2014). It has been shown that the PIP motif (PIPPSAPSKRH) within the EPIP domain is significantly more effective for HSL2 than EPIP and induces an oxidative burst (used as a marker for receptor activation). However, no response to the PIP peptide was observed for the HAE receptor (Butenko et al., 2014). Substitution of Pro<sup>Hyp</sup> for Pro-1, Pro-3, Pro-7, but not Pro-4, revealed the importance of hydroxylation in the PIP peptide (Butenko et al., 2014) (Figure 3).

ROOT GROWTH FACTOR/CLE-LIKE/GOLVEN (RGF/CLEL/GLV) peptides are 13 to 18 amino acids long and contain Pro<sup>Hyp</sup> residues as well as sulfated Tyr residues (Matsuzaki et al., 2010; Meng et al., 2012b; Whitford et al., 2012) (Figure 3). The RGF/CLEL/GLV peptide family was discovered in Arabidopsis and is conserved throughout different plants, such as rice (*Oryza sativa*), quaking aspen (*Populus tremuloides*), and conifers (Whitford et al., 2012; Strabala et al., 2014). Mature RGF/CLEL/GLV peptides are produced by proteolytic cleavage of a preproprotein with a C-terminal conserved motif (Matsuzaki et al., 2010; Meng et al., 2012a; Whitford et al., 2012). To date, the RGF/CLEL/GLV family contains 12 members in Arabidopsis, including CLE18, previously

classified as a member of the CLE peptide family (Meng et al., 2012b). These peptides have a role in root gravitropism, root apical meristem maintenance, root hair development, and lateral root and shoot development (Matsuzaki et al., 2010; Whitford et al., 2012; Fernandez et al., 2013a). So far, receptors for this peptide family are unknown. Intriguingly, the putative Arabidopsis CLEL protein in the nucleus (CLELn) is possibly processed to a peptide that functions in intracellular signaling in plants, but this still requires further study (Meng et al., 2012a).

PHYTOSULFOKINE (PSK) is a 5-amino acid peptide containing two sulfated Tyr residues that has been detected in conditioned medium of plant cell cultures (Matsubayashi and Sakagami, 1996) (Figure 3). PSK is produced from a preprotein with a conserved characteristic motif in the C terminus, namely, YIYTQ in Arabidopsis and YIYSQ in rice (Lorbiecke and Sauter, 2002). PSK homologs are present in both monocots and dicots as small gene families (Lorbiecke and Sauter, 2002). In Arabidopsis, exogenous application of PSK caused enhanced root growth that was mainly a result of root cell elongation (Kutschmar et al., 2009). Post-translational Tyr sulfation of PSK is required for its activity (Matsubayashi and Sakagami, 1996). The sulfate group of Tyr-1 is more important for PSK activity than that of Tyr-3 (Matsubayashi et al., 1996). PSK acts through PHYTOSULFOKINE RECEPTORS (PSKRs) with kinase activity, and these were identified in Arabidopsis (At-PSKR2 and At-PSKR1) and in carrot (*Daucus carota*) (Dc-PSKR1) (Matsubayashi et al., 2006). Overexpression of Dc-PSKR1 caused enhanced callus growth in response to PSK (Matsubayashi et al., 2002).

PLANT PEPTIDE CONTAINING SULFATED TYROSINE1 (PSY1) is an 18-amino acid glycopeptide containing two Pro<sup>Hyp</sup> residues and one sulfated Tyr residue (Amano et al., 2007) (Figure 3). This peptide is glycosylated with three L-arabinose units attached to the Pro<sup>Hyp</sup>-16 residue (Amano et al., 2007). In addition, as for PSK, Tyr sulfation is required for PSY1 activity (Matsubayashi and Sakagami, 1996). PSY1, as well as PSK, significantly promotes cellular proliferation and expansion at nanomolar concentrations. The Arabidopsis genome contains two genes encoding PSY1 precursor homologs with significant similarity within the conserved PSY1 domain and flanked by basic amino acid residues possibly involved in proteolytic processing (Amano et al., 2007). PSY RECEPTOR1 (PSYR1) was identified as a receptor for PSY, and recent studies revealed that both PSKR1 and PSY1R are involved in the plant defense response by regulating salicylate- and jasmonate-dependent defense pathways (Matsubayashi et al., 2002; Mosher and Kemmerling, 2013).

### Cysteine-Rich Peptides

A second family of preprotein- or preproprotein-derived peptides are the Cys-rich peptides, characterized by a Cys-rich domain with 2 to 16 Cys residues, but considerably varying in both their length and primary sequences between peptide families and across plant species (Figures 1 and 3). Each Cys-rich peptide class has a characteristic number and linear arrangement of Cys residues. The latter form intramolecular disulfide bonds that are essential for proper class-specific secondary folding and activity (Marshall et al., 2011; Haag et al., 2012; Vriens et al., 2014). The majority of known Cys-rich plant peptides are thought to function

as AMPs during plant-microbe interactions, and they have been isolated from roots, leaves, stems, flowers, and seeds (Nawrot et al., 2014). Most AMPs are cationic, allowing them to interact with the negatively charged membranes of pathogens. However, anionic antimicrobial peptides, belonging to known AMP families (e.g., hevein and cyclotides) have been reported (Prabhu et al., 2013). Additionally, it has been shown that several Cys-rich peptides also have a role in plant development, pollen recognition and guidance, and seed development (Marshall et al., 2011; Qu et al., 2015) (Figure 2). In this section, we highlight some typical representatives of those Cys peptides, but refer to excellent recent reviews for a more complete overview on Cys-rich peptides (Marshall et al., 2011; Beale and Johnson, 2013; Richardson and Torii, 2013; van der Weerden et al., 2013; de Souza Cândido et al., 2014; Murphy and De Smet, 2014; Nawrot et al., 2014).

Probably the best studied Cys-rich peptides are PLANT DEFENSINs (PDFs) (Terras et al., 1995). This family of ~5-kD peptides is characterized by an  $\alpha$ -helix and a triple-stranded  $\beta$ -sheet, stabilized by four disulfide bonds (Figure 3). Though PDFs are derived from a preprotein, there are some reports on PDFs derived from preproteins containing a C-terminal prodomain necessary for vacuolar targeting and preventing phytotoxicity (De Coninck et al., 2013b; Lay et al., 2014). PDFs are widely distributed over monocots and dicots and belong to the pathogenesis-related protein family 12 (PR12 family) (van Loon et al., 2006). They mainly have antifungal activity, although some of them have reported antibacterial activity. Heterologous overexpression of various PDFs leads to increased resistance of both model plants and crops against different fungi and bacteria (Carvalho Ade and Gomes, 2011; De Coninck et al., 2013b; Gaspar et al., 2014). The modes of action of several PDFs, including radish (*Raphanus sativus*) AFP2, *Nicotiana glauca* D1, alfalfa (*Medicago sativa*) Def1, *M. truncatula* Def4, and pea (*Pisum sativum*) d1, have been well studied and point toward specific interactions with various fungal sphingolipids and phospholipids (Thevissen et al., 2004, 2012; Aerts et al., 2007; Lobo et al., 2007; Ramamoorthy et al., 2007; van der Weerden et al., 2008, 2010; Sagaram et al., 2011, 2013; Muñoz et al., 2014). Upon interaction, PDFs are either internalized by the fungal cell and interact with intracellular targets, or they stay outside the cell and induce cell death through induction of a signaling cascade (Vriens et al., 2014). As such, they represent a typical example of host defense peptides acting in a more targeted way than through the aspecific lipid bilayer perturbation classically proposed for AMPs (Wilmes et al., 2011). Other activities have also been reported for PDFs, including trypsin,  $\alpha$ -amylase, and protein synthesis inhibition and blocking of sodium channels. Additionally, some PDFs appear to play a role in pollen recognition and abiotic stress tolerance and inhibit root growth (van der Weerden and Anderson, 2013).

Initially, 15 PDFs were identified in *Arabidopsis* (Thomma et al., 2002), but motif model-based searches uncovered 317 defensin-like genes (DEFLs) in the *Arabidopsis* genome (Silverstein et al., 2007), 70% for which evidence for transcription has been provided (Tesfaye et al., 2013). Most *Arabidopsis* DEFLs have eight Cys but some contain only four to six Cys (Figure 3). This is also the case for a special group of DEFLs, i.e., the nodule-specific Cys-rich (NCR) peptides abundantly formed during the interaction between rhizobia soil bacteria and legumes. In *M. truncatula*, for example,

~500 genes encode putative NCRs that are targeted to the nitrogen-fixing bacteroids and via a recent proteomics study, 138 NCR peptides could be detected (Figure 3). Interestingly, in contrast to most AMP family members, a majority of these peptides are anionic or neutral (Durgo et al., 2015), and they were suggested to neutralize the toxic activity of cationic NCRs. However, although cationic NCRs display *in vitro* antimicrobial activity, it is doubtful that they exert this activity *in vivo*, as peptide concentrations in nodules are lower than those used in *in vitro* assays (Van de Velde et al., 2010; Tiricz et al., 2013; Maróti and Kondorosi, 2014). Instead, they are essential for bacteroid differentiation and for maintenance of their bacterial endosymbionts (Van de Velde et al., 2010; Farkas et al., 2014; Penterman et al., 2014). Intriguingly, it seems that these NCRs interact with bacterial proteins (Farkas et al., 2014) and target intracellular regulatory pathways of the bacteroids (Penterman et al., 2014). These data, together with their specific expression in nodules and their lack of induced expression during pathogenic interactions, suggest that NCRs do not have a role as AMPs (Guefrachi et al., 2014).

Nonspecific LIPID TRANSFER PROTEINs (nsLTPs) (Kader, 1996) are grouped into two subfamilies, based on their molecular masses of ~10 kD (LTP1) and ~7 kD (LTP2) (Douliez et al., 2000). The LTP mature peptide, derived from a preprotein, shows a structure of four  $\alpha$ -helices, stabilized by four disulfide bridges (Figure 3). This structure allows the formation of a hydrophobic cavity accommodating binding to lipids, hence, their reported *in vitro* ability to bind and transport various lipids (Carvalho Ade and Gomes, 2007; Tousheh et al., 2013). Almost 600 nsLTPs from dicots, monocots, and gymnosperms have been identified (Wang et al., 2012), but their mode of action is still poorly understood. Emphasis lies on their role in plant-microorganism interactions, although they have reported roles in cutin synthesis, somatic embryogenesis, reproduction, as well as responses to abiotic stress. nsLTPs, belonging to the PR14 family (van Loon et al., 2006), can have direct antimicrobial activity or play a role as signaling molecules during both pathogenic as well as symbiotic interactions (Terras et al., 1992; Cammue et al., 1995; Blein et al., 2002; Pii et al., 2012; Champigny et al., 2013; Nawrot et al., 2014).

THIONINs are peptides of ~5 kD, with a common structure of two antiparallel  $\alpha$ -helices and a double-stranded  $\beta$ -sheet, stabilized by three to four disulfide bonds (Figure 3). It is generally accepted that THIONINs are derived from a preprotein containing an acidic C-terminal prodomain, hypothesized to neutralize the basic mature peptide (Stec, 2006). Recently, however, an unprocessed 15-kD THIONIN has also been detected in *Arabidopsis* (Asano et al., 2013). THIONINs are found in both monocots and dicots and are grouped within the PR13 family (van Loon et al., 2006). They display a broad range of toxicity against bacteria, fungi, yeast, insect larvae, and mammalian cells (Stec, 2006), and this is attributed to their interaction with anionic phospholipids leading to membrane permeabilization (Stec et al., 2004; Majewski and Stec, 2010). Moreover, overexpression of THIONIN genes in several plants led to decreased susceptibility against various bacteria, fungi as well as nematodes (Epple et al., 1997; Shirasawa-Seo et al., 2002; Muramoto et al., 2012; Ji et al., 2015).

KNOTTIN-type peptides of 3 to 4 kD are characterized by a triple-stranded  $\beta$ -sheet and three disulfide bonds, one of which crosses the macrocycle formed by the two other disulfide bridges

(Figure 3). This knotted structure, also referred to as cystine knot, provides an extraordinary stability to peptides and has also been described for CYCLOTIDES (see below). KNOTTIN-type peptides, found in *Mirabilis jalapa* (Mj-AMP1 and Mj-AMP2) and *Phytolacca americana* (PAFP-S), are derived from a preprotein and display mainly antifungal activity (Cammue et al., 1992; Gao et al., 2001; de Souza Cândido et al., 2014). Interestingly, PA1b, a KNOTTIN from pea, is embedded in albumin PA1. Upon processing, this preproprotein yields two peptides, including PA1a, an uncharacterized peptide, and PA1b, displaying insecticidal activity (Chouabe et al., 2011).

A special type of knotted peptides is represented by the CYCLOTIDES, characterized by ~30-amino acid peptides with a head-to-tail cyclized backbone resulting in ultrastable peptides (Figure 3). They have been detected in plants of the Rubiaceae, Violaceae, Fabaceae, Solanaceae, and Cucurbitaceae. CYCLOTIDES are embedded in preproteins with a C-terminal prodomain and often containing several CYCLOTIDE peptides (Craig and Malik, 2013). A wide range of activities has been reported for CYCLOTIDES, including inhibitory activity on fungi, bacteria, viruses, insects, molluscs, barnacles, and nematodes, as well as inhibition of trypsin (Daly et al., 2009; Craig et al., 2010). Within the CYCLOTIDES, a special subclass is formed by the PawS-derived peptide (PDP) family. These cyclic Asteraceae peptides consist of 12 to 18 amino acids and two Cys residues (Elliott et al., 2014). PDPs are embedded in precursors for albumin seed storage protein named PawS (Mylne et al., 2011). The latter encodes a preproprotein comprising a central PDP domain and two C-terminal regions for the small and large subunit of albumin. Sunflower (*Helianthus annuus*) Trypsin Inhibitor 1 (SFTI-1) and Trypsin inhibitor 1-Like (SFT-L1) were the first members of this family to be isolated from sunflower seeds (Mylne et al., 2011). So far, only SFT-1 was shown to inhibit trypsin (Elliott et al., 2014).

SNAKIN/GIBBERELLIC ACID STIMULATED-LIKE (GASA) peptides are 6-kD peptides with 12 Cys residues, detected in monocots and dicots (Figure 3). The structure of the SNAKIN/GASA peptides has not yet been determined (Harris et al., 2014) but through a combination of ab initio and comparative molecular modeling and disulfide bond prediction, it was suggested that they consist of two long  $\alpha$ -helices (Porto and Franco, 2013). The mature peptide is derived from a preprotein, in the case of *S. tuberosum* St-GSL1 (previously known as St-SN1), or a preproprotein, in the case of St-GSL2 (St-SN2) harboring a 15-amino acid region between the NSS and the mature St-GSL2. Both St-GSL peptides, as well as the alfalfa SNAKIN Ms-SN1, display in vitro broad antimicrobial activity (Segura et al., 1999; Berrocal-Lobo et al., 2002; García et al., 2014) and overexpression of their corresponding genes in several plants resulted in increased resistance against fungal and bacterial pathogens (Almasia et al., 2008; Rong et al., 2013; García et al., 2014). Additionally, SNAKIN/GASA peptides have been suggested to have an important role in plant development. For example, silencing of St-GSL1 resulted in plants with smaller leaves and affected cell division, metabolism, and cell wall composition of leaves (Nahiriřak et al., 2012).

HEVEIN-like peptides are chitin binding peptides of ~4 kD with 6 to 10 Cys residues and are derived from a preproprotein (Andreev et al., 2012; R Shukurov et al., 2012). The structure is represented by an  $\alpha$ -helix and an antiparallel three- or four-

stranded  $\beta$ -sheet (Andersen et al., 1993; Xiang et al., 2004; Dubovskii et al., 2011) (Figure 3). Up to now, only a few HEVEIN-like peptides have been detected in both monocots and dicots (Porto et al., 2012). Based on their chitin binding capacity, they were proposed to play a role in plant defense. Indeed, HEVEIN-like peptides display antimicrobial activity against various fungi (Broekaert et al., 1992; de Souza Cândido et al., 2014), although the exact mode of action has not yet been elucidated. Strikingly, some HEVEIN-like peptides also affect microorganisms without chitin in their cell wall, such as oomycetes and bacteria (de Souza Cândido et al., 2014). Overexpression of HEVEIN-like peptides resulted in increased plant resistance against various fungal and oomycete pathogens (Lee et al., 2003; R Shukurov et al., 2012).

*Impatiens balsamina* ANTIMICROBIAL PEPTIDES (Ib-AMPs), isolated from seeds (Tailor et al., 1997), represent four closely related basic peptides of 20 amino acid, each defined by two disulfide bonds which form a distinctive loop structure (Patel et al., 1998) (Figure 3). They display antifungal and antibacterial activity. Ib-AMP1 was shown to bind the cell surface and penetrate into the cell membrane of fungi (Lee et al., 1999), albeit not through interaction with proteinaceous receptors nor with glucosylceramides reported to act as specific binding sites for PDFs (Thevissen et al., 2005). The Ib-AMPs appear unique as no homologs proteins could be identified in protein databases. Moreover, Ib-AMPs are all embedded in a single preproprotein and are processed from a multi-peptide precursor.

Recently, a novel class of ~4-kD Cys-rich peptides, referred to as  $\alpha$ -HAIRPININS, has been identified in seeds of several monocots and dicots (Oparin et al., 2012; Utkina et al., 2013; Slavokhotova et al., 2014).  $\alpha$ -HAIRPININS are defined by four cysteines and two  $\alpha$ -helices (Figure 3). They are derived from a preproprotein containing 5 to 12  $\alpha$ -HAIRPININS (Utkina et al., 2013; Slavokhotova et al., 2014). Various peptides display broad antimicrobial and/or trypsin-inhibitory activity (Oparin et al., 2012; Utkina et al., 2013; de Souza Cândido et al., 2014; Slavokhotova et al., 2014).

Finally, two novel cationic and hydrophobic peptides, named ARACINs, were recently identified in Arabidopsis and appear specific for the Brassicaceae family. The mature peptides of 39 to 40 amino acids are derived from a preproprotein and contain two Cys residues (Figure 3). Chemically synthesized ARACINs displayed in vitro antifungal activity while overexpression of the ARACIN1 precursor in Arabidopsis resulted in increased resistance against *Botrytis cinerea* and *Alternaria brassicicola* (Neukermans et al., 2015).

All above-mentioned Cys-rich peptides have been mainly studied for their antimicrobial activity, but based on the examples given, it seems that several of those peptides acquired novel functions in plant development. This phenomenon, called protein promiscuity, has been reported for several peptides in the plant defense response and postulated as being essential for peptide evolution (Franco, 2011). Although less abundantly documented, some Cys-rich peptide families lack reported antimicrobial activity, and those will be exemplified below.

RAPID ALKALINIZATION FACTOR (RALF) peptides of ~5 kD, derived from the C-terminal end of a preproprotein, have been detected using mass spectrometry techniques on tobacco leaves (Pearce et al., 2001b). RALF and RALF-like peptides were found in Arabidopsis (Pearce et al., 2001b), rice (Cao and Shi, 2012), poplar



(*Populus tremula*) (Haruta and Constabel, 2003), tomato (Pearce et al., 2001b), sugarcane (*Saccharum officinarum*) (Mingossi et al., 2010), *M. truncatula* (Combiere et al., 2008), and maize (*Zea mays*) (Cao and Shi, 2012). RALF and RALF-like peptides mainly contain four Cys residues forming two disulfide bridges (Pearce et al., 2001b) (Figure 3). They are reportedly involved in plant development through arresting primary root growth, inhibiting root hair growth and cell elongation in hypocotyl and root cells, decreasing nodulation, and regulating pollen tube growth (Pearce et al., 2001b; Wu et al., 2007; Combiere et al., 2008; Covey et al., 2010). Using a phosphoproteomics approach, FERONIA (FER), a member of the *Catharanthus roseus* RLK1-LIKE KINASE (Cr-RLK1L) family of receptors, could be identified as a RALF receptor (Haruta et al., 2014); subsequently, a model for the signaling transduction pathway was presented (Wolf and Höfte, 2014). For more information on the RALF peptide family, we refer to a recent review (Murphy and De Smet, 2014).

EPIDERMAL PATTERNING FACTOR (EPF) and EPF-LIKE (EPFL) peptides contain six or eight conserved Cys residues in the C-terminal mature peptide region (Figure 3). They are 45 to 76 amino acids long and are derived from a preproprotein. In Arabidopsis, the EPF family consists of two EPF peptides (EPF1 and EPF2) and nine EPF-like peptides (EPFL1 to EPFL9). *EPF/EPFL* genes are present in mosses, monocots, and dicots (Takata et al., 2013). Overexpression of *EPF1*, *EPF2*, *EPFL4*, and *EPFL5* inhibits stomatal development when ectopically overexpressed (Hara et al., 2009). In contrast, overexpression of *EPFL9* (*STOMAGEN*) results in increased stomatal density and clustering (Hunt et al., 2010). EPFL9 was recently shown to be involved in the light response of the stomatal pathway (Hronková et al., 2015). NMR-derived structural information showed that STOMAGEN represents two antiparallel  $\beta$ -strands connected by a loop (Ohki et al., 2011) (Figure 3). Additional 3D structure modeling revealed that EPF1/EPF2-LIKE (EPFL) peptides form a disulfide bond in the loop region absent in EPFL9/STOMAGEN-LIKE peptides (Takata et al., 2013). Recent studies demonstrated direct binding of EPF peptides to ERECTA family receptor kinases (Lee et al., 2012).

Based on their Cys arrangement and predicted Cys stabilized structure, several peptides, including S-LOCUS CYSTEINE-RICH PROTEIN/S-LOCUS PROTEIN11 (SCR/SP11), LURE, and maize EMBRYO SAC4 (ES4), have been classified as DEFLs. SCR/SP11 peptides, identified in Brassicaceae, consist of ~50 amino acids with eight Cys residues linked by disulfide bridges and are derived from a preprotein. SCR/SP11 functions as the male determinant in the pollen coat and is perceived by the female determinant, the S-locus receptor kinase. Recognition leads to inhibition of self-pollen germination and pollen tube growth (Takayama et al., 2000, 2001; Higashiyama, 2010). LURE peptides are 60- to 70-amino acid peptides abundantly expressed in synergid cells (an egg-accompanying haploid cell) of the ornamental plant *Torenia fournieri* and Arabidopsis. They have six conserved Cys residues and are derived from a preprotein (Figure 3). Tf-LUREs were shown to attract pollen tubes in vitro, while silencing of *At-LURE1* resulted in impaired pollen tube guidance (Okuda et al., 2009; Takeuchi and Higashiyama, 2012). *ES4* is abundantly expressed in the synergid cell and is involved in pollen tube growth arrest, burst, and explosive sperm release in maize (Amien et al., 2010). ES1-4 peptides have antifungal activity, since they inhibit

the germination and growth of the maize pathogens *Fusarium graminearum* and *Ustilago maydis* (Woriedh et al., 2015). The ES family of maize encodes 61- to 68-amino acid peptides with eight Cys residues, and they are highly conserved with little variation at the C terminus. Secondary structure analysis of mature ES peptides predicts an  $\alpha$ -helix and a triple-stranded antiparallel  $\beta$ -sheet (Cordts et al., 2001; Woriedh et al., 2015) (Figure 3).

EMBRYO SURROUNDING FACTOR1 (ESF1) peptides were recently discovered in Arabidopsis as regulators of suspensor development and auxin distribution in early developing embryos (Costa et al., 2014). Mature ESF1 peptide, containing 68 amino acids, is characterized by an  $\alpha$ -helix and three  $\beta$ -sheets supported by four disulfide bonds. It was shown that ESF1 peptides protein structure was essential for its biological activity, as ESF1 peptide application induced suspensor cell elongation at nanomolar concentrations only when it was folded correctly (Costa et al., 2014). In addition, removal of any of the disulfide bonds or both tryptophan residues also resulted in the loss of peptide activity (Costa et al., 2014).

Maize EGG APPARATUS1 (EA1) is a 49-amino acid peptide with two Cys residues and is released from an Ala-rich precursor containing an internal signal sequence (Figure 3). This peptide is secreted from the egg apparatus to the micropylar region of the ovule integument, required for micropylar pollen tube guidance in maize, the last step of the pollen tube journey during the double fertilization process in flowering plants. EA1 attracts maize pollen tubes in vitro and arrests their growth at higher concentrations (Márton et al., 2012). In-depth in silico analysis of peptide sequence revealed that all peptides in this family shared a highly conserved C-terminal domain and the EA box (Zm-EA1-like motif) found in both monocot and dicot species (Gray-Mitsumune and Matton, 2006). The EA box is 27 to 29 amino acids in length with the conserved residues near the C terminus (Gray-Mitsumune and Matton, 2006). *EAL* transcripts were found in both reproductive and vegetative tissues (Gray-Mitsumune and Matton, 2006).

### Non-Cysteine-Rich Peptides without Specific Posttranslational Modifications

A diverse group of plant peptides is not characterized by specific PTMs, such as Pro hydroxylation and glycosylation and/or Tyr sulfation, or the presence of two or more Cys, though we do not exclude the possibility that any of these PTMs could be detected in the future (Pearce et al., 1991; Huffaker et al., 2006; Yamaguchi et al., 2011; Wrzaczek et al., 2015). These “non-Cys-rich/non-PTM peptides” can contain functionally important amino acids in their primary structure such as Pro, Gly, and Lys and are mainly released from a proprotein (Figures 1 and 3) (Constabel et al., 1995; Huffaker et al., 2006; Yamaguchi et al., 2011; Wrzaczek et al., 2015). They are 8 to 36 amino acids in length and have reported roles mainly in the defense response of plants (Figure 2).

SYSTEMINS (SYS) are 18-amino acid peptides characterized by four to six Pro residues and a conserved C terminus (PPKMQTD) (Figure 3). They are specifically found in the Solanoideae subfamily of Solanaceae, including tomato, bell pepper (*Capsicum annuum*), potato, and black nightshade (*S. nigrum*) and are not posttranslationally modified in contrast to HYPSSYS (Pearce et al., 1991; Constabel et al., 1998). SYS is a primary,

mobile signal that is released into the vascular system of tomato plants at sites of herbivore attacks and further transported to distal tissues. SYS also induces the production of volatiles to attract natural enemies of insect herbivores and the synthesis of two wound-inducible proteinase inhibitor proteins (Pearce et al., 1991; Pearce, 2011). Ala-scan studies revealed that Pro-13 or Thr-17 of SYS are essential residues for the latter activity (Pearce et al., 1993). In addition to a role in defense against herbivores, it was demonstrated that SYS mediates the jasmonate signaling pathway required for resistance against the necrotrophic fungus *B. cinerea* in tomato (El Oirdi et al., 2011). Although a SYS receptor was not detected yet, SYS was found to adopt a poly(L-Pro) II type helix known to be important for receptor recognition events in animals (Toumadje and Johnson, 1995).

PLANT ELICITOR PEPTIDES (PEPs) are 23- to 36-amino acid peptides carrying Gly as dominant residue in their motif (S/G)(S)Gxx(G/P)xx(N) and are conserved across monocots and dicots (Figure 3). All PEPs reveal a strict conservation of Gly-17, and this residue as well as Ser-15 was proven to be important for the alkalinizing activity of At-PEPs (Pearce et al., 2008). PEPs are likely to function as enhancers of immunity, damage-signaling peptides, and elicitors of systemic defense responses against pathogens and/or herbivores (Huffaker et al., 2011, 2013; Albert, 2013; Bartels et al., 2013; Klausner et al., 2015). Recently, it was shown that At-PEPs also have a role in dark/starvation-induced senescence via an early induction of chlorophyll degradation and autophagy (Gully et al., 2015). Although PEPs are released from precursors without NSS, PEPs must be exported from the cells to interact with cell surface leucine-rich repeat receptor kinases PEP RECEPTOR1 (PEPR1) and PEPR2 (Krol et al., 2010; Yamaguchi et al., 2010). Recently, analysis of the crystal structure of the At-PEP1-PEPR1 complex revealed that At-PEP1 adopts a fully extended conformation and binds to the inner surface of the superhelical extracellular LRR domain of PEPR1 (Tang et al., 2015). The LRR domains of PEPRs coevolved with the PEPs, leading to distinct PEP motifs in each plant family and interfamily incompatibility of recognition, in contrast to the highly conserved downstream signaling (Lori et al., 2015). In general, the PEP immune signaling pathway is associated with an early cytosolic Ca<sup>2+</sup> influx that activates protein kinases, reactive oxygen species, and nitrogen oxide (Ma et al., 2013). Moreover, this pathway coactivates the jasmonate- and salicylate-mediated immune branches to link local and systemic immunity (Ross et al., 2014).

Recently, GRIM REAPER PEPTIDE (GRIP) was identified in *Arabidopsis* as a novel, basic Lys-rich 11-amino acid peptide with homologs in crucifers (Wrzaczek et al., 2015) (Figure 3). The formation of GRIP from the preproprotein GRI is unique because this is the first report in plants of a specific type of protease, METACASPASE-9, involved in the release of a mature functional peptide. Since METACASPASE-9 also cleaves GRI at other sites, it cannot be excluded that other peptides are formed. GRIP is released in the extracellular space and binds to the extracellular domain of the plasma membrane-localized atypical leucine-rich repeat receptor-like kinase POLLEN-SPECIFIC RECEPTOR-LIKE KINASE5 (PRK5). Upon binding with its receptor, GRIP triggers oxidative stress and reactive oxygen species-dependent cell death in *Arabidopsis* (Wrzaczek et al., 2015).

In soybean, the PLANT ELICITOR PEPTIDES 914 and 890 (PEP914 and PEP890) and homologs in Fabales and Cucurbitales are 8-amino acid peptides that share a highly similar sequence [DxPRG(G/H)NY] (Yamaguchi et al., 2011) (Figure 3). Although no single, dominant residue occurs in Gm-PEP914, the C-terminal Asp-7 or Tyr-8 residues are important for its alkalinizing activity. Moreover, synthetic Gm-PEP914 and Gm-PEP890 induce the expression of defense related genes in soybean cell cultures (Yamaguchi and Huffaker, 2011).

## PEPTIDES DERIVED FROM A FUNCTIONAL PRECURSOR

Although most known peptides are derived from nonfunctional precursors (see above), some studies reported the existence of unique peptides derived from functional precursor proteins (Schmelz et al., 2006; Pearce et al., 2010a; Chen et al., 2014) (Figure 1). In some cases, these functional precursors have a different primary function from that of the peptides buried within them. Such peptides, released from the precursor by proteolytic enzyme activity, are called cryptides (Samir and Link, 2011). In humans, ~35 cryptides have been reported (Pimenta and Lebrun, 2007), but in plants, only three have been reportedly identified (Figures 2 and 3) (Schmelz et al., 2006; Pearce et al., 2010a; Chen et al., 2014).

The soybean SUBTILASE PEPTIDE (Gm-SUBPEP) is a unique 12-amino acid peptide abundant in Pro and Arg residues (Pearce et al., 2010a) (Figure 3). An Ala-scan revealed that Arg-10 and His-12 are important for Gm-SUBPEP alkalinizing activity and putative receptor interaction (Pearce et al., 2010b). Gm-SUBPEP has a high basic net charge and was purified from soybean leaves as a non-posttranslationally modified peptide. Gm-SUBPEP is embedded in the C-terminal end of a preproprotein with an independent metabolic role as subtilase, an extracellular protease, and member of the subtilisin-like protease family. Gm-SUBPEP induces the same defense-related genes as Gm-PEP914 and Gm-PEP890, without sharing sequence homology (Pearce et al., 2010a; Yamaguchi and Huffaker, 2011).

Recently, a targeted and quantitative peptidomics strategy was employed to discover novel defense signaling peptides in tomato (Chen et al., 2014). CYSTEINE-RICH SECRETORY PROTEINS, ANTIGEN 5, AND PATHOGENESIS-RELATED 1 PROTEINS derived peptide 1 (CAPE1) was successfully identified as an 11-amino acid peptide derived from the preproprotein PATHOGENESIS-RELATED PROTEIN 1b (PR1b), a marker gene for the salicylic acid signaling pathway and systemic acquired resistance. For many years it was thought that tomato *PR-1b* encodes a 14-kD protein with antifungal activity (Niderman et al., 1995). Now it appears that it also encodes the CAPE1 peptide with antibacterial activity and antiherbivory activity and a role in the regulation of salt stress responses (Chen et al., 2014; Chien et al., 2015). At least 30 novel putative CAPE1-like peptides carrying the characteristic Pro-rich PxGNxxxxPY motif were found in Solanoideae, Nicotianoideae, Vitaceae, Brassicaceae, Fabaceae, and Poaceae (Chen et al., 2014) (Figure 3). This could mean that CAPE1 and its homologs form a new, highly conserved peptide family functioning in the defense response of plants. Tomato plants presprayed with CAPE1 exhibited increased resistance to the bacterial pathogen

*Pseudomonas syringae* pv tomato DC3000 and reduced *Spo-doptera litura* larval growth and weight. CAPE1 was significantly upregulated after wounding and/or methyl jasmonate treatment, similar to systemin (Chen et al., 2014). It is suggested that CAPE1 may be a novel DAMP (damage-associated molecular pattern) for induction of immunity, similar to HYP SYS, RALF, and At-Pep1 (Pearce et al., 2001a, 2001b; Huffaker et al., 2006; Albert, 2013; Trivilin et al., 2014). Recently, it was shown that At-CAPE1 functions as a negative regulator of salt stress (Chien et al., 2015).

INCEPTINS are 11- to 13-amino acid long peptides embedded in a functional plant protein, i.e., chloroplastic ATP synthase. Remarkably, after uptake by herbivorous insects, the latter undergoes nonspecific proteolysis and releases the INCEPTIN back into the plant where it functions as an elicitor of herbivore defense (Schmelz et al., 2006; Schmelz et al., 2012). These acidic INCEPTINS carry a core motif consisting of one disulfide bridge (Figure 3). However, the latter is nonessential for INCEPTIN's reported antiherbivore activity, in contrast to Asp-3, Asp-10, Cys-8, and the C-terminal Ala (Schmelz et al., 2007). INCEPTINS are conserved in cowpea (*Vigna unguiculata*), bean, Arabidopsis, maize, and rice (Schmelz et al., 2006). This example shows that diversity in plant peptide synthesis is not only created by the plant processing machinery, but also by that of species interacting with plants, thereby increasing even more the potential of the plant proteome.

Evidence is emerging on bioactive peptides formed from functional protein precursors that are suggested to serve (themselves) as important functional components of plant metabolism (Fesenko et al., 2015). For example, in gametophore, protonema, and protoplast cells of the moss *Physcomitrella patens*, more than 20,000 unique endogenous peptides ranging from 5 to 78 amino acids were detected with mass spectrometry analysis (Fesenko et al., 2015). These specific peptide pools are hydrolysis products of functional proteins, although the exact mechanisms of endogenous peptide formation have not been elucidated yet. In both the protonema and protoplast states of the moss, plastid proteins served as the main source of peptides. In gametophores, stress-related proteins were among the most productive precursors (Fesenko et al., 2015). The functional characterization of endogenous peptide pools is ongoing, though 117 peptides already were predicted to have antimicrobial potential (Torrent et al., 2012; Fesenko et al., 2015). It is hypothesized that the formation of these specific endogenous peptide pools or "peptide burst" is a form of biotic stress response based on the production of AMPs from existing functional proteins (Fesenko et al., 2015). Nevertheless, experimental evidence on the bioactivity of these endogenous peptides is essential to consider them as functional components of plant metabolism and not as degradation products of the proteome.

## NONPRECURSOR-DERIVED PEPTIDES

So far, the majority of characterized plant peptides are derived from precursor proteins (Tabata and Sawa, 2014). Recently, some sORFs (usually <100 codons) were proposed to represent a potential additional source of functional peptides, called "short peptides encoded by sORFs" or sPEPs (Andrews and Rothnagel,

2014). This type of peptide is directly translated from an sORF. The involvement of an intermediate precursor or further processing is not required for the synthesis of these peptides (Figure 1). Therefore, we define this type of peptides as "nonprecursor-derived peptides." In contrast to previous literature, we do not employ the terminology "micropeptides" for sPEPs for two reasons (Crappé et al., 2013). First, posttranslationally modified peptides are 20 amino acids or less and thus are smaller than micropeptides. Second, the prefix "micro" was used to point to a regulatory function, as in microproteins (Staudt and Wenkel, 2011) rather than commonly implemented as a small-size indicator. sPEPs have been demonstrated to fulfill various biological roles ranging from plant development to regulation of gene expression, and we will illustrate this novel group with representative examples (Figures 2 and 3).

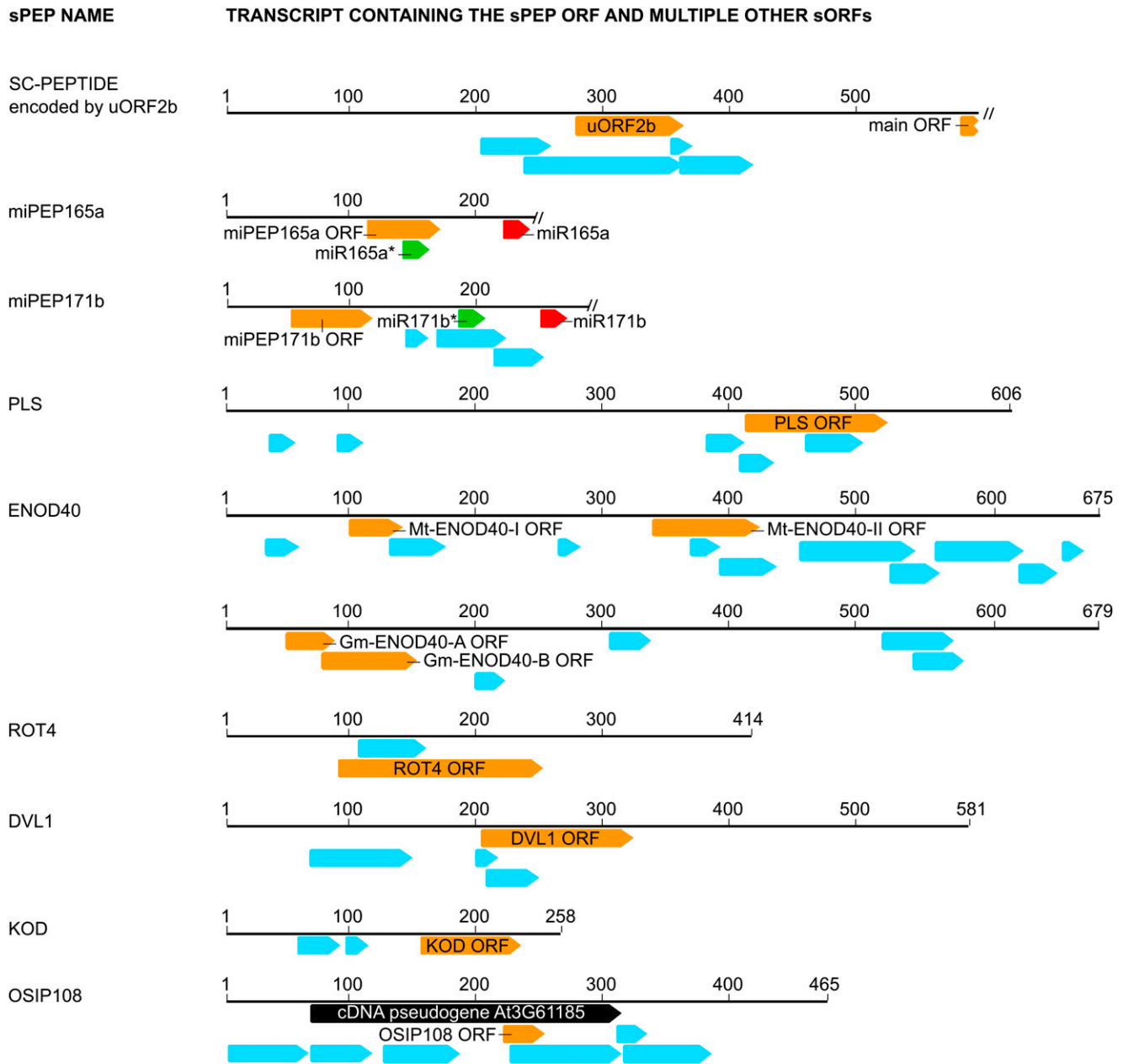
Up to now, nonprecursor-derived peptides from plants could be subdivided in three subgroups, depending on the genomic location of the sORF(s) encoding the peptide(s). These sORFs can be located (1) upstream of the main ORF in the 5' leader sequence of a gene, (2) in primary transcripts of miRNA, or (3) in other transcripts not encoding longer (>100 amino acid) proteins. The latter class includes annotated protein-encoding transcripts with one or more ORFs encoding peptides <100 amino acids, transcripts from intergenic regions, and some transcripts previously annotated as long noncoding RNA (lncRNA) (Figure 1).

## Peptides Encoded by Upstream ORFs

Upstream ORFs (uORFs) are putative protein-coding regions located in the 5' leader sequence of a main protein-coding region or overlapping with such a main ORF (Andrews and Rothnagel, 2014; von Arnim et al., 2014) (Figure 4). Currently, more than 20,000 uORFs are annotated in Arabidopsis (von Arnim et al., 2014). Only a minor fraction of these uORFs show conservation of their primary sequences (conserved peptide uORFs or CPuORFs), whereas the majority does not (nonCPuORFs) (Jorgensen and Dorantes-Acosta, 2012; von Arnim et al., 2014). The length of uORFs with a reported function varies from 1 to 92 codons (von Arnim et al., 2014). For an overview of all plant uORFs, we refer to uORFdb, a database on eukaryotic uORF biology (<http://cbdm.mdc-berlin.de/tools/uorfdb>; Wethmar et al., 2014).

In several case studies, uORFs function as regulators of metabolism or mediators of developmental gene regulation by influencing the expression and/or translation of the main ORF. Often, this posttranscriptional regulation occurs in response to compounds such as sucrose, polyamines, phosphocholine, and ascorbate (Jorgensen and Dorantes-Acosta, 2012; von Arnim et al., 2014; Ebina et al., 2015; Laing et al., 2015). A genome-wide ribosome footprinting study in Arabidopsis revealed that uORFs capture ribosomes and thereby limit the translation of the downstream main ORF, meaning that uORFs can act as a barrier for translation (Juntawong et al., 2014).

Translational evidence for uORFs is supported by in vitro translation, mutational analysis in planta, and ribosome footprinting data, though not all uORFs are necessarily translated (Andrews and Rothnagel, 2014; Juntawong et al., 2014; von Arnim et al., 2014). In contrast to human cell lines, uORF peptides have not yet been detected by mass spectrometry in plants



**Figure 4.** A Wide Variety of sPEP Transcripts.

Transcripts of sPEPs harbor many sORFs (<100 amino acids; blue arrows), though only a few sORFs have been experimentally characterized (orange arrows). All sORFs starting with AUG and consisting of at least six codons in sense direction are displayed. Interior ORFs located within the same frame of a longer ORF are omitted, except for the SC-PEPTIDE. Only the 5' part of the SC-PEPTIDE transcript and the miPEP transcripts is displayed (indicated by a double slash). A red arrow represents mature miRNA found from one arm of a hairpin; a green arrow represents mature miRNA (with asterisk) formed from the opposite arm of a hairpin.

(Kim et al., 2014). One possible explanation could be that uORF peptides do not accumulate because short peptides may typically be turned over rapidly in the cell (von Arnim et al., 2014). Remarkably, in a recent report, a functional uORF initiated by a noncanonical codon (a non-AUG initiation codon) has been described, meaning that noncanonically translated uORFs too

should be taken into account in future research (Laing et al., 2015). Leaky scanning and reinitiation are two major mechanisms via which translation of uORFs and the main ORF are coordinated. For more information on the latter, we refer to excellent recent reviews (Andrews and Rothnagel, 2014; von Arnim et al., 2014).

### Peptides Encoded by sORFs in Primary Transcripts of miRNAs

Recently, it was shown that primary transcripts of microRNAs (pri-miRNAs) contain sORFs encoding regulatory peptides termed miRNA-ENCODED PEPTIDES (miPEPs) (Lauressergues et al., 2015). For each of the 50 pri-miRNAs in Arabidopsis, a putative peptide with a canonical initiation codon was predicted. These 50 miPEPs are 3 to 59 amino acids long and share no common signature, suggesting that each miPEP is specific for its corresponding miRNA. The *M. truncatula* miPEP171b and Arabidopsis miPEP165a were studied in detail and function as transcriptional activators. Overproduction of miPEP171b and miPEP165a in roots of *M. truncatula* and Arabidopsis, respectively, enhanced the accumulation of their respective endogenous miRNAs, resulting in downregulation of target genes involved in root development. miPEPs were not yet detected by mass spectrometry analysis in planta, though translational evidence for miPEP171b and miPEP165a was supported by a combination of in vivo translational GUS fusions, overexpression experiments, immunolocalization studies, and ribosome footprinting (Juntawong et al., 2014; Lauressergues et al., 2015). Altogether, these findings strongly indicate that the first ORF in pri-miR171b and pri-miR165a functions as a peptide rather than as RNA (Lauressergues et al., 2015) (Figure 4). As such, these peptides derived from pri-miRNAs form a novel subclass with important regulatory features in plants and probably in other eukaryotes as well.

### Peptides Encoded by sORFs in Transcripts Not Encoding Longer (>100 Amino Acids) Proteins

sORFs (<100 codons) are not only found in the 5' leader sequence of a main protein-coding region or in primary transcripts of miRNA, but also in other transcripts not encoding longer (>100 amino acid) proteins. A striking overlap exists between features of some of the latter coding transcripts and lncRNAs, since some lncRNAs also contain one or more ORFs <100 codons (Mercer et al., 2009). An unambiguous definition of sPEPs encoded by these sORFs and a strict distinction with lncRNA is impeded at the moment because functional studies and translational evidence for these peptides are limited (Andrews and Rothnagel, 2014). Therefore, we carefully discuss the indications for functionality and translatability of these sPEPs to discriminate between functional peptides and functional transcripts.

Currently, six functionally characterized plant sPEPs encoded by transcripts not encoding longer proteins have been reported in the literature, namely, POLARIS (PLS; 36 amino acids), EARLY NODULIN GENE 40 (ENOD40; 12, 13, 24, or 27 amino acids), ROTUNDIFOLIA FOUR (ROT4; 53 amino acids), KISS OF DEATH (KOD; 25 amino acids), BRICK1 (BRK1; 84 amino acids), Zm-908p11 (97 amino acids), and Zm-401p10 (89 amino acids) (Ma et al., 2008; Dong et al., 2013; Crappé et al., 2013; Andrews and Rothnagel, 2014). However, based on the TAIR10 annotation of the Arabidopsis genome, 131 functionally annotated peptides (<100 amino acids) without an NSS can be identified. This group of peptides, previously not regarded as sPEPs, includes 18 DEVIL (DVL)/ROT peptides, 6 methallothioneins, 2 NUCLEAR RNA POLYMERASE (NRPD) peptides, 10 members of the SMALL

AUXIN-UP RNA (SAUR)-like auxin-responsive protein family, and many other Arabidopsis peptides encoded by sORFs in transcripts not encoding longer (>100 amino acids) proteins (Wen et al., 2004; Ream et al., 2009; Grennan, 2011; Spartz et al., 2012) (Supplemental Data Set 1). Therefore, we emphasize that many more peptides than previously thought are covered by the definition of this type of sPEPs. Here, we discuss the functionality and translatability of the five best-characterized sPEPs (PLS, ENOD40, ROT4, DVL1, and KOD) in plants and of novel, potential sPEPs in Arabidopsis.

The 36-amino acid peptide PLS is required for correct root growth and leaf vascular patterning (Casson et al., 2002; Chilly et al., 2006). This peptide is encoded by the longest and fifth of six sORFs in the *PLS* transcript (Figure 4). Overexpression of the *PLS* transcript in a *pls* knockout mutant resulted in partial rescue of the short root mutant phenotype, whereas overexpression of a modified *PLS* transcript (with mutated initiation codon) did not. These complementation assays demonstrate that the PLS initiation codon is required for activity, supporting a functionality as sPEP (Casson et al., 2002). Nevertheless, it remains unclear how the PLS peptide is translated, since the PLS-ORF is preceded by three other sORFs. It was hypothesized that these sORFs are uORFs and the long PLS-ORF is the main ORF.

*M. truncatula* ENOD40 is involved in symbiotic nodule development, and its activity can be mediated by the peptides ENOD40-I (13 amino acids) and ENOD40-II (27 amino acids), as well as by the ENOD40 transcript (Sousa et al., 2001; Campalans et al., 2004). This transcript contains 12 sORFs, though only ENOD40-I and -II span two nucleotide sequences that are conserved in all legume ENOD40 genes (Wan et al., 2007). Local overexpression of ENOD40 and derived variants into *M. truncatula* roots demonstrated the involvement of ENOD40-I and -II peptides as well as of a structured RNA signal in cortical cell division (Sousa et al., 2001). The translatability of ENOD40-I (second sORF) and -II (fifth sORF) has been demonstrated by in vivo translational GUS fusions in *M. truncatula* roots, even if they were preceded by other sORFs in the transcript (Sousa et al., 2001) (Figure 4). These results suggest that sORFs do not arrest 5'-to-3' ribosome scanning, since reinitiation can occur along the ENOD40-1 transcript and the initiation codons of ENOD40-I and -II can be recognized by ribosomes for translation (Sousa et al., 2001). However, in vitro translation studies of all ENOD40 sORFs in wheat germ extract could not detect the corresponding peptides, possibly due to instability of such small peptides (<39 amino acids) under artificial conditions (Sousa et al., 2001). Strikingly, the ENOD40 transcript as well as a modified variant (with mutated initiation codons of ENOD40-I and -II) relocalized an RNA BINDING PROTEIN1 (RBP1) from the cytoplasm to the nucleus, proving that the ENOD40 transcript itself, and not the ENOD40 encoded peptides, is responsible for this specific activity (Campalans et al., 2004). Overall, it can be hypothesized that ENOD40 encodes a bifunctional or dual RNA, since both the ENOD40 transcript and peptides are functional in *M. truncatula* and exert different roles (Bardou et al., 2011).

The soybean ENOD40-A and -B peptides, 12 and 24 amino acids long, respectively, are involved in the regulation of sucrose use in nitrogen-fixing nodules and specifically bind to NODULIN 100, a subunit of sucrose synthase (Röhrig et al., 2002). The sORFs

encoding these peptides are embedded in a transcript containing six sORFs (Figure 4). However, only Gm-ENOD40-A and -B, similar to Mt-ENOD40-I, span a nucleotide sequence that is conserved in all legume *ENOD40* genes. The translatability of Gm-ENOD40-A and -B is supported by *in vitro* translation studies and mutational assays (Röhrig et al., 2002). Moreover, the Gm-ENOD40-B peptide was detected in nodules via protein gel blotting (Röhrig et al., 2002). Since Gm-ENOD40-A and -B are located out of frame, it is remarkable that both sORFs are translated and function as peptides in the same biological conditions. The translation mechanism of Gm-ENOD40-A and -B could be explained by leaky scanning and ribosome reinitiation, as was also reported in the context of uORFs (Andrews and Rothnagel, 2014).

The 53-amino acid ROT4/DVL16 peptide regulates polar cell proliferation in lateral organs and leaf morphogenesis in Arabidopsis (Narita et al., 2004). The *ROT4* transcript contains a longer sORF encoding ROT4 and a shorter sORF (Figure 4). A dominant mutant, *rot4-1D*, overexpressing *ROT4* possesses shortened, rounded leaves and short floral organs. Moreover, overexpression of only the conserved ROT4-LIKE (RTFL) domain (RKCWVKEQ-RARLYIIRRCVLMMLLCWHD) embedded in ROT4 and its homologs was sufficient to confer the *rot4-1D* phenotype (Figure 3). Loss-of-function mutations in *ROT4* and several ROT4-LIKE genes were aphenotypic, suggesting that there may be some functional redundancy between family members (Narita et al., 2004; Guo et al., 2015). Translatability of ROT4 is supported by *in vivo* translational GFP fusion experiments (Narita et al., 2004). In contrast to ENOD40 and PLS, ROT4 could be translated via a classical translation mechanism as this peptide is encoded by the first and longest sORF in the transcript.

ROT18 (or DVL1) is a 51-peptide member of the RTFL or DEVIL (DVL) family in Arabidopsis, which consists of many proteins that are widely conserved among land plants and function mainly in plant organogenesis (Wen et al., 2004; Valdivia et al., 2012; Guo et al., 2015). ROT18/DVL1 is encoded by the longest ORF in the *ROT18/DVL1* transcript, which contains four sORFs in total (Figure 4). Downregulation and overexpression of the *ROT18/DVL1* ORF resulted in phenotypic changes similar to corresponding mutants of ROT4 (Wen et al., 2004). Overexpression of modified *ROT18/DVL1* (with frame-shift or point mutations inhibiting ROT18/DVL1 translation) and complementation assays suggest a role for the ROT18/DVL1 peptide in plant development (Wen et al., 2004). However, it remains unclear how this peptide is translated, since the ROT18/DVL1-ORF is preceded by two other sORFs, similar to PLS.

KOD is a 25-amino acid peptide unique in Arabidopsis and involved in programmed cell death regulation (Blanvillain et al., 2011). More specifically, it modulates suspensor elimination during embryogenesis and root hair programmed cell death. KOD is encoded by the longest and last ORF in a short *KOD*-transcript containing three sORFs in sense. Experiments using knockout mutants, carrying a T-DNA insertion and a point mutation (Pro-9 to Ser), respectively, and transient assays in onion (*Allium cepa*) using mutated and truncated variants of *KOD* indicate that *KOD* functions as a peptide rather than as RNA (Blanvillain et al., 2011). *In vivo* translational GFP fusions showed that the initiation codon of KOD can be recognized for translation in planta, though it

remains unclear how the KOD peptide is translated, similar as for PLS (Figure 4). Nevertheless, NMR studies on synthetic KOD peptide pointed out that it adopts an  $\alpha$ -helix (from Pro-9 to Arg-21), whereas the N-terminal region is disordered (Blanvillain et al., 2011).

Based on a recent transcriptome study of Arabidopsis leaves upon oxidative stress and subsequent functional analysis in the eukaryotic model yeast *Saccharomyces cerevisiae*, different putative oxidative stress-induced peptides (OSIPs) could be identified (De Coninck et al., 2013a). The sORF encoding the decapeptide OSIP108 is contained within a pseudogene harboring seven sORFs and induced by oxidative stress. Both overexpression and infiltration of synthetic OSIP108 in Arabidopsis leaves resulted in increased oxidative stress tolerance. Moreover, treatment of other eukaryotes with OSIP108 was demonstrated to prevent apoptosis triggered by different oxidative stress inducers (Spincemaille et al., 2014a, 2014b). Although these findings support a function for OSIP108 as a peptide, the latter could not yet be identified as such in planta and its translation can be questioned.

Recently, 7901 sORFs potentially encoding sPEPs of 30 to 100 amino acids were identified in intergenic regions of the Arabidopsis genome (Hanada et al., 2007, 2013). A subset of these sORFs (473), showing high homology with sORFs in other plants and unbiased expression evidence, was overexpressed in Arabidopsis. Altered morphogenesis of Arabidopsis plants was shown for ~10% of these sORFs, a proportion that is approximately 7 times higher than that of randomly chosen known genes (Hanada et al., 2013). Translational evidence for the latter 49 functional sORFs could not be found in reported data sets of ribosome footprinting experiments and mass spectrometry analysis (Castellana et al., 2008; Juntawong et al., 2014). On the other hand, other sORFs predicted in the initial pool but without reported phenotypes are associated with ribosomes and possibly translated (Juntawong et al., 2014) or detected by mass spectrometry (Castellana et al., 2008).

In general, although various approaches have been employed for sPEPs to discriminate between a functional peptide and a functional transcript, direct evidence demonstrating the presence of sPEPs in planta is lacking as well as an in-depth explanation of the translation mechanism for certain sORFs. Generally applied to assess potential translation of an ORF are ribosome footprinting techniques that determine whether ribosomes are associated with a certain region in the transcript. While these could support the translatability of, e.g., miPEPs, no such conclusions can yet be made for PLS, ROT4, DVL1, KOD, and OSIP108 ORFs due to insufficient reads present in recent ribosome footprinting data from Arabidopsis (Juntawong et al., 2014). However, this approach still provides indirect evidence for the presence of a specific sPEP, since the observed interaction of an ORF with ribosomes does not necessarily imply translation of that ORF, but it can also function in regulating the translation of other ORFs. Cutting-edge techniques such as puromycin-associated nascent chain proteomics (PUNCH-P) allowing intact ribosome-nascent polypeptide chains to be analyzed by mass spectrometry (Aviner et al., 2014) could overcome this problem, but are not yet been optimized for plants. The most direct evidence for the presence of peptides in planta can be obtained from mass spectrometry



analyses. The latter allowed successful detection of several sPEPs in other higher eukaryotes, but many of these were noncanonical sPEPs (Slavoff et al., 2013; Prabakaran et al., 2014). Moreover, since sPEPs often occur at low abundance (Hanada et al., 2013; Andrews and Rothnagel, 2014) and classical proteomic analyses involving data-dependent acquisition mass spectrometry have a bias for highly abundant peptides, they have limited potential to detect sPEPs. Targeted proteomic techniques as data-independent acquisition mass spectrometry and selected/multiple reaction monitoring could be promising in this regard for detection of specific sPEPs though less suitable for discovery-based applications (Samir and Link, 2011; Chen et al., 2014; Doerr, 2014; Taylor et al., 2014). Finally, it should be noted that in planta identification of a peptide does not necessarily imply that it fulfills any biological role but the peptide can instead be part of suggested translational noise (Guttman and Rinn, 2012).

### Concluding Remarks

Peptides (<100 amino acids) form a subset of the plant proteome that has been receiving a lot of attention over the past several years. In particular, the different types and the number of peptides identified in different plant species have been expanding. In addition to peptides derived from a nonfunctional precursor, such as well-known AMPs and signaling peptides, evidence is emerging on peptides derived from functional protein precursors as well as peptides directly translated from sORFs (<100 codons). These sORFs can be located in the 5' leader sequence of protein-encoding genes, in primary transcripts of miRNA, or in other transcripts not encoding longer (>100 amino acids) proteins. Moreover, plant peptides display diverse functions. This functional diversity ranges from roles in plant development, growth, fertilization, senescence, cell death, cell-to-cell communication, nodulation, and defense response to regulation of expression and translation. Peptides exert these functions via direct interactions with pathogens or through receptor binding and downstream signaling. In addition to this diversity in synthesis and function, a peptide varies in length, 3D structure, amino acid composition, and possible posttranslational modifications. Thus, it can be concluded that diversity is key in the peptide world.

Nevertheless, this diversity still remains largely unexplored, mainly because of technical limitations. First, identification of ligand-receptor pairs remains challenging and even less is known about the downstream signaling. Various approaches are available to study physical ligand-receptor interactions, including photoaffinity labeling of peptide-receptor interactions (Tabata et al., 2014), phosphoproteomics revealing the phosphorylation status of plasma membrane proteins in planta upon treatment with synthetic peptides (Haruta et al., 2014), and a cellular bioassay using the oxidative burst as readout for receptor activation by synthetic peptides (Butenko et al., 2014). For more details, we refer to a recent discussion on these approaches (Stes et al., 2015). Second, it is still controversial and unclear whether the plant peptidome harbors a wide array of functional peptides encoded by sORFs. Future experiments should focus on cutting-edge ribosome footprinting, mass spectrometry techniques and in-depth functional analyses of nonprecursor-derived peptides in planta to further explore this dark matter of the peptidome. Expanding the

peptidome brings us close to the field of noncoding RNA, since it was recently hypothesized that lncRNA transcripts too could form a repository for new peptides (Ruiz-Orera et al., 2014). Furthermore, transcripts could be bifunctional, meaning that the transcript as well as one or more peptides from one transcript play a role, as was shown for Mt-*ENOD40* (Bardou et al., 2011). Therefore, we want to put forward the notion that it is difficult to define an exact border, since there is rather a continuum between coding and noncoding sequences. In conclusion, bringing together biological questions and emerging trends as well as cutting-edge techniques in the field of peptide research will deepen our understanding of the complex peptidome as a fascinating and essential part of the plant proteome.

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL libraries under the following accession numbers: *CEP1* (AT1G47485), *CEP5* (AT5G66815), *GSL1* (FJ195646), *EPF1* (AT2G20875), *EPF2* (AT1G34245), *EPFL4* (AT4G14723), *EPFL5* (AT3G22820), *EPFL9* (AT4G12970), *LURE1* (AT5G43285), *ES4* (NM\_001112150.1), *PR-1b* (AT2G14610), *PLS* (AT4G39403), Mt-*ENOD40* (X80264.1), Gm-*ENOD40* (X69154), *ROT4* (AT2G36985), *DVL1* (AT5G16023), and *KOD* (JF411843).

### Supplemental Data

**Supplemental Data Set 1.** List of potential Arabidopsis peptides encoded by short ORFs (<100 amino acids) in transcripts not encoding longer (>100 amino acids) proteins.

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### AUTHOR CONTRIBUTIONS

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## The Plant Peptidome: An Expanding Repertoire of Structural Features and Biological Functions

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