Journal of Experimental Botany, Vol. 70, No. 16 pp. 4267–4277, 2019
doi:10.1093/jxb/erz292 Advance Access Publication 20 June, 2019
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REVIEW PAPER

Sulfated plant peptide hormones

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Received 26 March 2019; Editorial decision 10 June 2019; Accepted 11 June 2019

Editor: Stanislav Kopriva, University of Cologne, Germany

Abstract

Sulfated peptides are plant hormones that are active at nanomolar concentrations. The sulfation at one or more tyrosine residues is catalysed by tyrosylprotein sulfotransferase (TPST), which is encoded by a single-copy gene. The sulfate group is provided by the co-substrate 3´-phosphoadenosine 5´-phosphosulfate (PAPS), which links synthesis of sulfated signaling peptides to sulfur metabolism. The precursor proteins share a conserved DY-motif that is implicated in specifying tyrosine sulfation. Several sulfated peptides undergo additional modification such as hydroxylation of proline and glycosylation of hydroxyproline. The modifications render the secreted signaling molecules active and stable. Several sulfated signaling peptides have been shown to be perceived by leucine-rich repeat receptor-like kinases (LRR-RLKs) but have signaling pathways that, for the most part, are yet to be elucidated. Sulfated peptide hormones regulate growth and a wide variety of developmental processes, and intricately modulate immunity to pathogens. While basic research on sulfated peptides has made steady progress, their potential in agricultural and pharmaceutical applications has yet to be explored.

Keywords: Casparian strip integrity factor, LRR-RLK, phytosulfokine, plant peptide containing sulfated tyrosine, root meristem growth factor, sulfated peptide hormone, tyrosine sulfation, tyrosylprotein sulfotransferase.

Introduction

The identification of systemin, a peptide induced by wounding in Solanaceae (Pearce *et al.*, 1991) led to the discovery of a whole new class of phytohormones, the peptide hormones. Unlike the previously known classical hormones that are small metabolite molecules, peptide hormones have an amino acid backbone that is derived from a functional or non-functional precursor. The peptide hormones have been classified into 43 peptide types so far, but the actual number is expected to be higher due to their low native concentrations and technical difficulties regarding identification of peptides. Post-translationally modified peptides, which possess post-translational modifications (PTMs) such as proline hydroxylation or glycosylation, are classified in eight peptide hormone groups (Tavormina *et al.*, 2015). The isolation of the disulfated pentapeptide phytosulfokine (PSK) from cultured *Asparagus officinalis* cells (Matsubayashi and Sakagami, 1996) was the first evidence of an activating PTM of a plant hormone, tyrosine sulfation, that had previously only been reported for animal hormones (Moore, 2009). Unlike other PTMs, tyrosine sulfation is not present in metabolite hormones and it leads to activation of peptide hormones. By contrast, glycosylation, hydroxylation, or conjugation to amino acids of metabolite hormones such as indole-3-acetic acid, abscisic acid, and others by

Abbreviations: AHA, Arabidopsis H^+-ATPase; APK, APS kinase; APS, adenosine 5´-phosphosulfate; ATPS, ATP sulfurylase; CIF, Casparian strip integrity factor; CLE, CLAVATA3/EMBRYO SURROUNDING REGION-RELATED; CLEL, CLE-like; GLV, Golven; HPAT, hydroxyproline O-arabinosyltransferases; LRR-RLK, leucine rich repeat receptor-like kinase; P4H, prolyl-4-hydroxylases; PAPS, 3´-phosphoadenosine 5´-phosphosulfate; PAPST, PAPS transporter; PSK, phytosulfokine; PSKR, PSK receptor; PSY, peptide containing sulfated tyrosine; PSY1R, PSY1 receptor; PTM, post-translational modification; RGF, root meristem growth factor; RGFR, RGF receptor; SBT, subtilase; TPST, tyrosylprotein sulfotransferase.

a specific set of enzymes leads to reversible or irreversible inactivation (Nambara and Marion-Poll, 2005). Tyrosine sulfation links peptide hormone synthesis to sulfate metabolism via the common intermediate 3′-phosphoadenosine 5′-phosphosulfate (PAPS). The sulfate moiety provides stability of the peptide hormone that is secreted to the apoplast and specificity with regard to receptor recognition (Wang *et al.*, 2015; Song *et al.*, 2016). To achieve tyrosine sulfation, a unique sulfotransferase has evolved in plants, namely tyrosylprotein sulfotransferase. In this review, we summarize our current knowledge on the known groups of sulfated peptide hormones, their modifications, and their processing with a focus on sulfation, known peptide receptors, and physiological activities of peptide hormones. Long-distance transport of sulfated peptides has not been reported to date, but is easily conceivable. For example, the C-TERMINALLY ENCODED PEPTIDE involved in the regulation of nitrogen acquisition (Ohkubo *et al.*, 2017) and CLAVATA3 (CLV3)/ENDOSPERM SURROUNDING REGION-related (CLE) 12 from *Medicago trunculata* and MtCLE13 that regulates root nodule formation have been shown to be transported from root to shoot (Okamoto *et al.*, 2013; Kassaw *et al.*, 2017). The recent discovery of sulfated peptides in xylem sap fuels the idea of functions in long-distance organ-to-organ communication (Okamoto *et al.*, 2015; Patel *et al.*, 2018).

Tyrosine sulfation requires the co-substrate PAPS

Sulfur that is taken up by the plant as inorganic sulfate is activated by ATP sulfurylase (ATPS, encoded by *AtATPS1-4* in Arabidopsis, to adenosine 5′-phosphosulfate (APS) (Fig. 1A). APS kinases, located in plastids (AtAPK1/2/4) or in the cytosol (APK3), phosphorylate APS to 3′-phosphoadenosine 5′-phosphosulfate (PAPS). The fact that none of the *apk* single-mutants displays a phenotype whereas the *apk1 apk2* double-mutant has a dwarf phenotype (Mugford *et al.*, 2009) suggests that APK3 and APK4 only contribute to PAPS synthesis to a minor degree. In Arabidopsis, export of PAPS from the chloroplast occurs through PAPS transporter 1 (AtPAPST1) and probably also a second as yet unknown transporter (Gigolashvili *et al.*, 2012). The transporter(s) responsible for the import of PAPS into the Golgi, where peptide-precursor sulfation occurs, have not been identified.

A unique tyrosylprotein sulfotransferase catalyses tyrosine sulfation of peptides in the *cis*-Golgi

The transfer of sulfate to the hydroxyl group of a substrate is catalysed by sulfotransferases (SOTs). In Arabidopsis, the SOT family (EC 2.8.2.-) consists of 22 members, most of which catalyse sulfation of glucosinolates, flavonols, and brassinosteroids (Hirschmann *et al.*, 2014). The identification of PSK was the first evidence for tyrosine sulfation in plants (Matsubayashi and Sakagami, 1996; Moore, 2009). It was reported earlier in animals and is catalysed by tyrosylprotein sulfotransferases (TPSTs; EC 2.8.2.20). The Arabidopsis TPST, also termed ACTIVE QUIESCENT CENTER 1 (AQC1) (Zhou *et al.*, 2010), HYPERSENSITIVE TO Pi STARVATION 7 (Kang *et al.*, 2014), or SCHENGEN2 (SGN2) (Doblas *et al.*, 2017), transfers sulfate from the co-substrate PAPS to the hydroxyl group of one or more defined tyrosine residue(s) of a peptide precursor to produce a tyrosine O^4-sulfate ester (Moore, 2003) (Fig. 1B). Plant-specific TPSTs were first identified in rice (*Oryza sativa*), carrot (*Daucus carota*), *Asparagus officinalis*, and subsequently in Arabidopsis (Hanai *et al.*, 2000; Komori *et al.*, 2009). Aside from their shared catalytic activity, plant TPSTs are distinct from other plant SOTs or animal TPSTs in that they have an overall low sequence identity and differ in their domain structure. Human TPSTs, with 370 and 377 residues, are type II membrane proteins with a short N-terminal domain in the cytoplasm and a putative stem region between the transmembrane and catalytic domains, whereas the Arabidopsis TPST has 500 residues and is a type I membrane protein with a short C-terminus in the cytoplasm, an N-terminal signal peptide, and a heparan sulfate 6-*O*-sulfotransferase 2 homology domain (Moore, 2009). TPST is located in the *cis*-Golgi where sulfation occurs, as shown by co-localization with the *cis*-Golgi marker SYP31 (Komori *et al.*, 2009). The gene is ubiquitously expressed, with highest transcript levels in the root apical meristem, lateral root primordia, and the vasculature (Komori *et al.*, 2009). To our current knowledge, *TPST* is a unique gene in plants and the TPST enzyme is responsible for sulfation. TPST not only sulfates PSK but also other sulfated peptide hormones including PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1 (PSY1) and ROOT GROWTH MERISTEM FACTOR 1 (RGF1), as shown by incubation of TPST-containing microsomal protein fractions with the radiolabeled TPST-substrate [^{35}S]PAPS and affinity purification of TPST with a PSY precursor column (Amano *et al.*, 2007; Komori *et al.*, 2009; Matsuzaki *et al.*, 2010). Knockout of TPST in Arabidopsis *tpst-1*, *aqc1-1*, and *sgn2-1* causes a pleiotropic phenotype with shorter roots, a reduced root apical meristem size, early senescence, a reduced number of reproductive organs (Komori *et al.*, 2009; Zhou *et al.*, 2010), a defective Casparian strip (Doblas *et al.*, 2017), increased metaxylem cell numbers (Holzwart *et al.*, 2018), and an altered immunity to specific pathogens (Mosher *et al.*, 2013). An altered immune response has also been observed in TPST-silenced *Solanum lycopersicum* (tomato) plants (Zhang *et al.*, 2018). Some *tpst* phenotypes can be restored by specific sulfated peptides, for example root growth is partially restored in *tpst-1* by exogenous application of PSK and PSY1, which promote cell elongation, and RGF1, which stimulates cell division in the root apical meristem, and is fully restored by applying all three peptides (Matsuzaki *et al.*, 2010). Application of CASPARIAN STRIP INTEGRITY FACTOR (CIF) rescues the defect in the Casparian strip diffusion barrier. The altered immunity responses of *tpst-1* (partially) rely on the loss of PSK and PSY signaling (Mosher and Kemmerling, 2013). While a recognition motif for TPST has not been identified so far, it has been shown that adjacent and distant acidic amino acids in the flanking sequence of the proPSK sequence are important for recognition and that an aspartate residue N-terminal of the targeted tyrosine is required (Hanai *et al.*, 2000). Indeed, all sulfated peptides known to date (Fig. 2) contain a DY-motif except GLV9 (Golven), whose

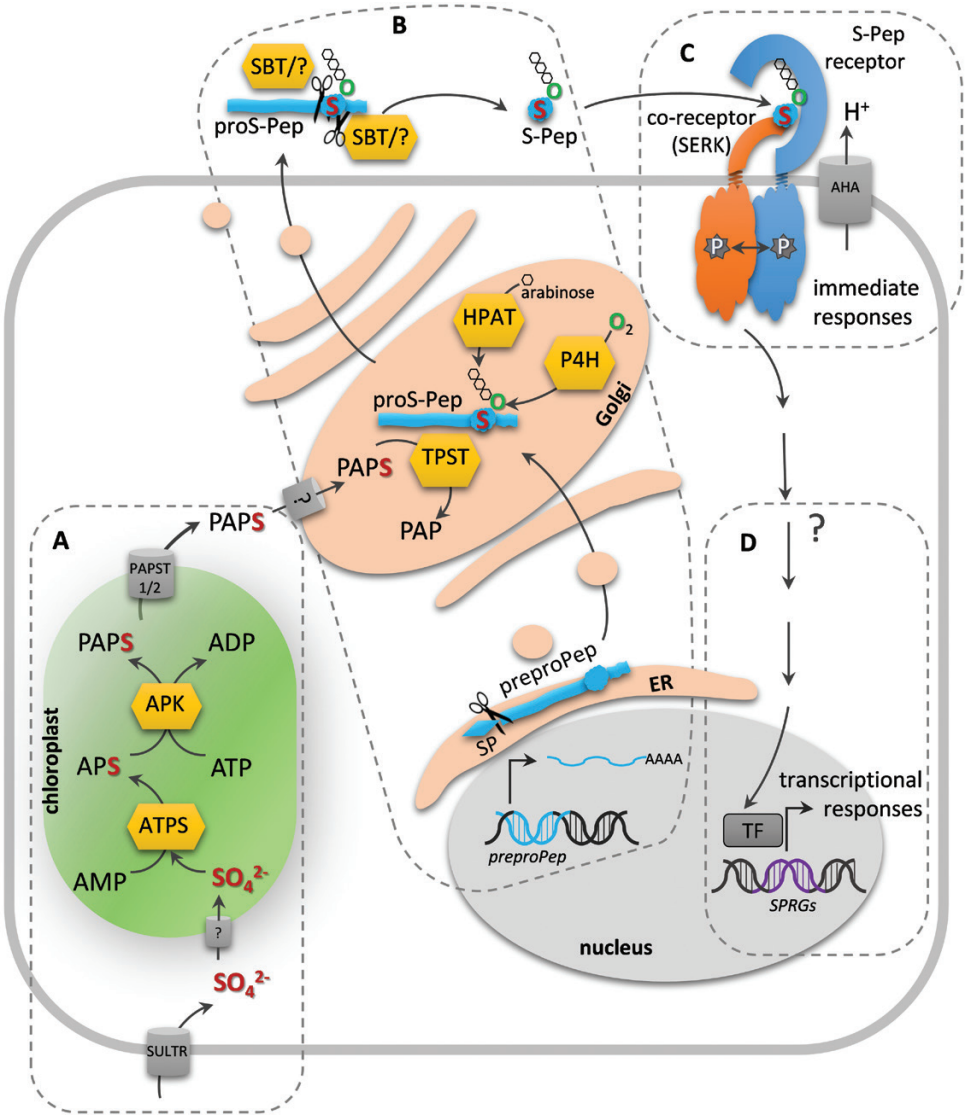

Fig. 1. Activation of sulfate, maturation of sulfated peptides, and peptide perception. (A) Sulfate uptake occurs via sulfate transporters (SULTR) into the cytoplasm, but the transporter into the chloroplast is unknown. ATP sulfurylase (ATPS) and APS kinase (APK) produce the tyrosylprotein sulfotransferase (TPST) co-substrate 3′-phosphoadenosine 5′-phosphosulfate (PAPS) (Mugford *et al.*, 2009). The PAPS transporter (PAPST) exports it into the cytoplasm, but how PAPS enters the Golgi is not known (Gigolashvili *et al.*, 2012). (B) Maturation of sulfated peptides includes cleavage of the signal peptide (SP) in the ER during preproprotein synthesis, tyrosine sulfation by TPST in the Golgi (Komori *et al.*, 2009), proline hydroxylation by prolyl-4-hydroxylase (P4H) and triarabinosylation by hydroxyproline O-arabinosyltransferase (HPAT) in the case of PSYs in the Golgi (Amano *et al.*, 2007), and cleavage of the N- and C-terminals by subtilases (SBT) and/or other unknown proteases probably in the apoplast to release the mature sulfated peptide (S-Pep) (Srivastava *et al.*, 2008; Ghorbani *et al.*, 2016). (C) PSKR, PSY1R, and RGFRs are perceived at the plasma membrane by a receptor/co-receptor pair that mutually transphosphorylate each other and activate the receptor (Hohmann *et al.*, 2017). The proton-pumping H^+-ATPase (AHA) has been identified as a direct target of the S-Pep receptors PSKR1 and PSY1R (Fuglsang *et al.*, 2014; Ladwig *et al.*, 2015) (D) S-Peps are predicted to regulate S-Pep-responsive genes (SPRGs) via unknown signaling intermediates and transcription factors (TF).

tyrosine is preceded by the acidic amino acid glutamate (Fig. 2B). Whether or not GLV9 is actually sulfated has not been determined but its biological activity in meristem maintenance that is required for root elongated growth is massively reduced compared to RGF1-6 (Matsuzaki *et al.*, 2010).

Why tyrosine sulfation?

The transfer of a sulfate group from PAPS to the tyrosine side-chain alters the chemical properties of the peptide by increasing hydrophilic binding. This alters the affinity to proteins that bind sulfated peptides and act as peptide receptors. Binding of PSK to its receptor PSKR1 (PSK RECEPTOR1) occurs mainly by hydrogen-bond formation whereby the sulfate moieties of PSK interact with specific residues in the perception domain of PSKR1 (Wang *et al.*, 2015). It is therefore not surprising that both the amino acid backbone (Bahyrycz *et al.*, 2004, 2005) and sulfation of both tyrosine residues are required for full biological activity of PSK (Matsubayashi *et al.*, 1996; Matsubayashi and Sakagami, 1996; Kobayashi *et al.*, 1999; Kwezi *et al.*, 2011; Stührwohldt *et al.*, 2011; Igarashi *et al.*, 2012; Mosher *et al.*, 2013). Similarly, unsulfated RGF1 has a 185-fold reduced affinity to RGF RECPTOR1 (RGFR1), one of its three receptors (Shinohara *et al.*, 2016). Song *et al.*, (2016)

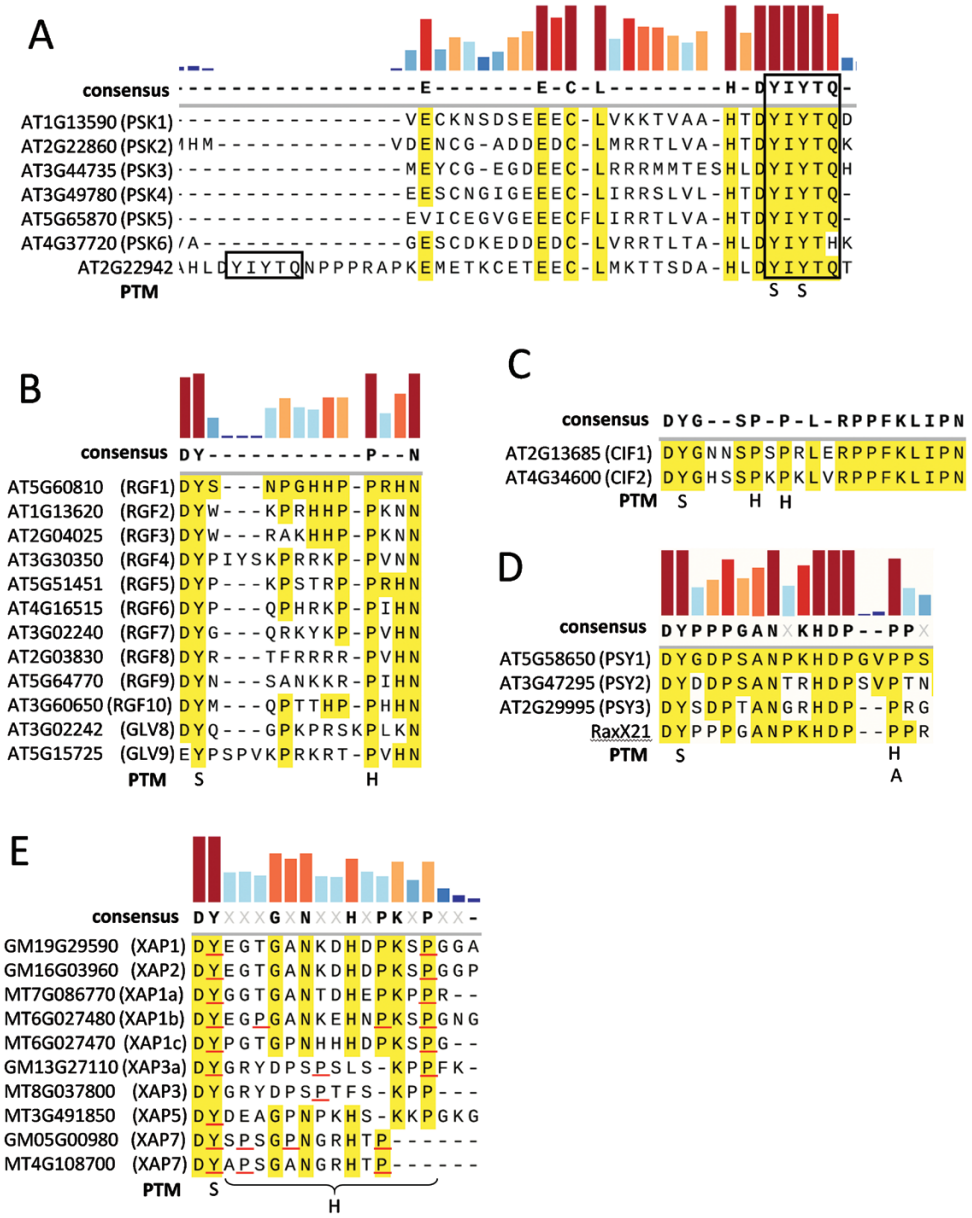

Fig. 2. Protein sequence alignments (MUSCLE) of sulfated peptide families of the mature peptide motif regions. All sulfated peptides, except GLV9, share a DY-motif. Hydroxylated and glycosylated amino acids are present in the majority of sequences. (A) The PSK sequences are N-terminally extended to align a functionally undescribed ORF with two PSK motifs (black boxes). In (A–E) coloured bars indicate sequence conservation between all sequences with a gradient (in 10 % steps) between dark-blue (low, <10%) and dark-red (high, 90–100%). Yellow highlighting indicates consensus to the identified peptides: (B) RGF1, (C) CIF1, and (D) PSY1. (E) Uncharacterized sulfated peptides (XAPs) were identified from the xylem sap of *Glycine max* and *Medicago truncatula* (Okamoto *et al.*, 2015; Patel *et al.*, 2018). Experimentally verified post-translational modifications (PTMs) are indicated at the bottom of each figure (if underlined in red in the case of XAPs): S = tyrosine sulfation; H = hydroxylation of proline residues; A = triple L-arabinosylation of hydroxyproline.

identified an RxGG motif in RGFRs that is responsible for specific recognition of the sulfate group. Some studies have reported activity of the unmodified peptide, which raises the question as to how crucial this PTM actually is (Kutschmar *et al.*, 2009; Meng *et al.*, 2012; Doblas *et al.*, 2017). A requirement of sulfation for bioactivity has controversially been considered for the RGF family member RGF1/CLEL8/GLV1. Meng *et al.* (2012) showed that CLEL8 (CLE-like) peptide without sulfation and hydroxylation alters root growth direction and lateral root development whilst the sulfated RGF1 peptide but not the unmodified peptide backbone is able to rescue the defective root apical meristem of *tpst-1* (Matsuzaki *et al.*, 2010). In some studies it is possible that the activity of unmodified peptide has been due to the high concentration applied (Kutschmar *et al.*, 2009; Meng *et al.*, 2012; Whitford *et al.*, 2012). When investigating modified peptides, a dose–response analysis and titration curves to determine the physiological activity and binding affinity of the non-/modified peptide are advisable, as has been shown for RGF1 (Matsuzaki *et al.*, 2010), PSK (Kutschmar *et al.*, 2009; Stührwohldt *et al.*, 2011), PSY1 (Amano *et al.*, 2007), and CIFs (Doblas *et al.*, 2017). Unsulfated peptides are active in the micromolar range

whereas binding affinity assays with the ectodomains of the corresponding peptide receptors and growth assays have shown a K_D or half-maximal biological activity of the sulfated peptides in the nanomolar range (Matsubayashi *et al.*, 2002, 2006; Stührwohldt *et al.*, 2011; Whitford *et al.*, 2012; Fernandez *et al.*, 2013; Doblas *et al.*, 2017). Aside from increasing the specificity and affinity of peptides to their receptors, tyrosine sulfation as well as proline hydroxylation are very stable and irreversible modifications (Moore, 2003; Gorres and Raines, 2010). To date, desulfation by enzymes has not been reported for either plant-derived or for plant pathogen-derived sulfatases, demonstrating that sulfated peptides are stable signaling molecules even in a highly biochemically active environment. In mammals, arylsulfatases, present in lysosomes, may be involved in tyrosine-sulfated protein degradation (Parenti *et al.*, 1997). By contrast, phosphorylation and acetylation are reversible PTMs catalysed by phosphatases and deacetylases, which allows for dynamic regulation of signaling pathways. Aside from enzymatic inactivation, tyrosine-sulfated peptides are fairly stable even in non-physiological, harsh acidic conditions of pH 1–3, as shown for the model peptides gastrin-17, caerulein, and drosulfokinin (Balsved *et al.*, 2007), which suggests that sulfated peptides are stable in the acidic apoplast. The same holds true for neutral and alkaline conditions (Huttner, 1984).

Glycosylation and hydroxylation of sulfated peptides

Sulfated peptides can undergo proline hydroxylation and glycosylation (Fig. 1B). Proline hydroxylation is catalysed by prolyl-4-hydroxylases (P4Hs), which are encoded by 13 genes in Arabidopsis. The specific P4Hs involved in maturation of sulfated peptides have not been identified (Myllyharju, 2003; Matsubayashi, 2012). PSY1 is a glycosylated sulfated peptide. L-arabinosylation (L-Ara_3) at the hydroxylated proline of PSY1 is catalysed by hydroxyproline O-arabinosyltransferases (HPATs) (Amano *et al.*, 2007; Ogawa-Ohnishi *et al.*, 2013). Triarabinosylation has been shown to be important for the biological activity of AtCLV3 (Ohyama *et al.*, 2009; Shinohara and Matsubayashi, 2013), MtCLE12 and 13 (Kassaw *et al.*, 2017; Imin *et al.*, 2018), as well as CLE ROOT SIGNAL 1 and 2 in *Lotus japonicus* (Okamoto *et al.*, 2013).

Proteolytic cleavage of sulfated peptide precursors

Sulfated peptides are encoded as non-functional precursors. The modified precursors are proteolytically cleaved by endo- and probably exopeptidasen to release the mature and active peptides, most likely after secretion from the Golgi into the apoplast, as suggested for example for peptide hormones from *M. trunculata* and AtIDA (Djordjevic *et al.*, 2011; Schardon *et al.*, 2016; Patel *et al.*, 2018) (Fig. 1B). In animals, proteolytic processing has been reported for precursor molecules of peptide hormones and neuropeptides such as insulin and melanocyte-stimulating hormones (Seidah and Prat, 2012). Precursor processing catalysed by prohormone convertases is essential to form the bioactive entities. The enzymes are considered as therapeutic targets, which highlights the importance of this activating step. In plants, less is known about proteolytic enzymes of secreted hormone peptides. Partial cleavage of the N-terminus of Arabidopsis proPSK4 has been demonstrated *in vivo* by SUBTILASE (SBT) 1.1, a prohormone convertase of the subtilisin-like serine protease family, with the highest specificity observed *in vitro* for proPSK4 over other PSK precursors (Srivastava *et al.*, 2008). Since the cleavage product still has additional N- and C-terminal residues, further trimming must be predicted. PSK derivatives with additional amino acids attached to the N- or C-terminus of the disulfated YIYTQ-sequence have a 1000-fold lower bioactivity compared to PSK (Matsubayashi *et al.*, 1996). For the RGF/GLV/CLEL peptide family, SBT 6.1 and 6.2 have been shown to cleave the RGF6/CLEL6/GLV1 and RGF9/CLEL9/GLV2 precursors and to be required for peptide activation (Ghorbani *et al.*, 2016), For the majority of the 56 AtSBTs, targeting to the secretory pathway is predicted by bioinformatics tools (Rautengarten *et al.*, 2005) and has been shown experimentally as well for SBT1.1, 4.12, 4.13, and 5.2 (Srivastava *et al.*, 2008; Schardon *et al.*, 2016). Interestingly, the target predictions for S1P/SBT6.1 and SBT6.2 localization are mitochondria and chloroplasts (Rautengarten *et al.*, 2005), but S1P/SBT6.1 co-localizes with the Golgi-marker BODIPY TR ceramide (Liu *et al.*, 2007) and interacts with the protease inhibitor Serpin1 in the apoplast, as shown by BiFC analysis (Boruc *et al.*, 2010; Ghorbani *et al.*, 2016). SBT6.1 targets not only RGF precursors but also the precursor of RAPID ALKALINIZATION FACTOR 23 (Srivastava *et al.*, 2009; Schaller *et al.*, 2018). It is as yet unclear whether the tyrosine sulfation in the *cis*-Golgi is required for recognition by the protease. For SBTs it has been shown that these proteases are kept inactive by an auto-inhibiting prodomain until they reach the acidic *trans*-Golgi where the prodomain is released in a pH-dependent manner (Meyer *et al.*, 2016; Schaller *et al.*, 2018), further indicating that proteolytic cleavage occurs in the extracellular space, or at least upstream of the *trans*-Golgi.

PSK

Phytosulfokine-α (PSK) is a Tyr-disulfated pentapeptide that displays hormone-like activities at nanomolar concentrations (Stührwohldt *et al.*, 2011). PSK is encoded as a 75–123-amino-acid preproprotein by nuclear genes (Lorbiecke and Sauter, 2002). The conserved PSK protein family PF06404 (http://pfam.xfam.org/family/PF06404) consists of 352 homologous sequences found in 53 species from both mono- and dicotyledonous plant species, including *Arabidopsis thaliana*, *Glycine soja*, *Brassica napus*, *Zea mays*, and *Oryza sativa*. Sequence conservation and the presence of multiple genes encoding PSK in each species examined to date and an *in silico* conservation mapping for ligand-binding sites for PSKR1 orthologs suggest a conserved ligand–receptor interaction (Orr and Aalen, 2017). In Arabidopsis, seven loci have been annotated as putative PSK precursor genes. *AtPSK1-5* contains the canonical PSK domain YIYTQ (Fig. 2A). Transcriptome analysis by RNA-seq

and reporter gene expression analysis has revealed PSK expression at varying levels throughout all tissues and developmental stages, resulting in a ubiquitous supply of the PSK precursor (Stührwohldt *et al.*, 2011; Cheng *et al.*, 2017). The pseudogene *AtPSK6* (At4g37720) encodes the PSK-related sequence YIYTH. As the result of a new Arabidopsis genome annotation (Araport 11; Cheng *et al.*, 2017), the locus *At2g22942* has been identified as a previously unrecognized putative phytosulfokine precursor gene. Interestingly, the encoded protein sequence contains two canonical PSK sequences (Fig. 2A). For *AtPSK6*, no expressed sequence tags have been reported and RNA-seq data show very low expression levels for *AtPSK6* in pollen and for *At2g22942* in the receptacle (Cheng *et al.*, 2017), possibly suggesting minor or highly specific biological functions of these genes that have yet to be described.

As with metabolite plant hormones, PSK has been shown to have multiple physiological functions. It stimulates somatic embryogenesis (Kobayashi *et al.*, 1999; Igasaki *et al.*, 2003) and promotes adventitious root formation in cucumber hypocotyls (Yamakawa *et al.*, 1998). Furthermore, PSK promotes the differentiation of mesophyll cells of *Zinnia elegans* to tracheary elements in cell cultures (Matsubayashi *et al.*, 1999) by down-regulating stress-response genes at the onset of the trans-differentiation process (Motose *et al.*, 2009). In *tpst-1* knockout mutants and in the PSK receptor null mutant the metaxylem cell number is increased, which suggests a role for PSK in the control of xylem cell fate (Holzwart *et al.*, 2018). Interestingly, the modified peptide hormone TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR acts in an opposite manner (Etchells *et al.*, 2016). In plant reproduction, PSK promotes pollen germination (Chen *et al.*, 2000) and pollen-tube elongation (Stührwohldt *et al.*, 2015), and it acts as a short-distance signal that helps to guide the pollen tube from the transmission tract along the funiculus to the embryo sac. Plants that lack PSK receptors produce fewer seeds (Stührwohldt *et al.*, 2015).

With the use of synthetic PSK and genetic knockout of PSK receptors, PSK signaling has been shown to promote hypocotyl elongation (Stührwohldt *et al.*, 2011), leaf (Hartmann *et al.*, 2014) and root growth (Kutschmar *et al.*, 2009), and cotton fiber-cell elongation (Han *et al.*, 2014). The growth-promoting activity is mainly driven by increased cell expansion rather than cell division, as is evident from increased leaf epidermal cell size, longer hypocotyl cells, and longer differentiated root cells.

Finally, PSK signaling differentially affects plant immunity depending on the type of the invading pathogen. The contribution of PSK signaling to plant immunity seems to be dependent on the lifestyle rather than the phylogenetic origin of the pathogen (Rodiuc *et al.*, 2016). Arabidopsis plants deficient in PSK perception are more susceptible to pathogens with a necrotrophic lifestyle such as the fungi *Alternaria brassicicola* and *Sclerotinia sclerotiorum* and the bacterium *Ralstonia solanacearum*, whereas their resistance against hemi-/biotrophs such as the bacterium *Pseudomonas syringae* and the oomycete *Hyaloperonospora arabidopsidis* is increased (Loivamäki *et al.*, 2010; Igarashi *et al.*, 2012; Mosher *et al.*, 2013; Rodiuc *et al.*, 2016). PSK signaling reduces the expression of microbe-associated molecular pattern-inducible genes elicited by elf18 or flg22, as determined by quantitative PCR after 8 h (Igarashi *et al.*, 2012). The expression of PSK precursor genes and of PSK receptor 1 (*PSKR1*) is induced by wounding, the fungal elicitor E-Fol, elf18, and following infection with necrotrophic fungi, whereas infection with the hemibiotrophic bacterium *P. syringae* does not change the expression (Loivamäki *et al.*, 2010; Igarashi *et al.*, 2012), which is suggestive of a role for PSK signaling in specific host–pathogen interactions. Recently, Zhang *et al.* (2018) revealed that PSK signaling induces auxin-dependent immune responses in tomato when it is infected with the necrotrophic fungus *Botrytis cinerea*. Besides growth promotion through cell expansion, PSK contributes to the regulation of quiescent center cell division and the differentiation of distal stem cells (Heyman *et al.*, 2013; Kong *et al.*, 2018).

RGFs/GLVs/CLELs

The sulfated and hydroxylated peptide ROOT GROWTH MERISTEM FACTOR 1 (RGF1) was identified as the first member of the RGF group in a search for sulfated peptides that can rescue the stunted root growth phenotype of the *tpst-1* mutant (Matsuzaki *et al.*, 2010). Like all sulfated peptides, the RGF members contain the typical DY-motif, except for GLV9, and a highly conserved hydroxylated proline residue. Unlike the mature PSK peptide that is fully conserved within and across species, RGFs have conserved and non-conserved amino acids (Fig. 2B). PSK and PSY have been shown to partially restore root growth in *tpst-1* (Matsuzaki *et al.*, 2010). RGF1 affects root development by increasing the abundance of the transcription factor PLETHORA, which leads to maintenance of the root stem-cell niche. Application of synthetic RGF1 restores meristematic activity in *tpst-1*. RGF1, PSK, and PSY together fully restore root growth (Matsuzaki *et al.*, 2010). RGF1 is a member of the RGF/GOLVEN(GLV)/CLE-LIKE(CLEL) peptide family (Fig. 2B) (Matsuzaki *et al.*, 2010; Meng *et al.*, 2012) with partially redundant functions. RGF7–9 have reduced or no biological activity in root elongation growth (Matsuzaki *et al.*, 2010). The expression patterns differ but, taking all members together, RGF peptides seem to be present throughout the plant (Fernandez *et al.*, 2013). Aside from their activity in the root apical meristem, RGFs control root gravitropism (Meng *et al.*, 2012; Whitford *et al.*, 2012), lateral root development (Meng *et al.*, 2012; Fernandez *et al.*, 2015), and root hair development (Fernandez *et al.*, 2013).

CIFs

Recently, a new group of tyrosine-sulfated peptides, the CASPARIAN STRIP INTEGRITY FACTORs 1 and 2 (CIF1 and CIF2), have been identified (Doblas *et al.*, 2017; Nakayama *et al.*, 2017). The peptides consist of 21 amino acids with one sulfated tyrosine in the typical DY-motif and two hydroxylated proline residues as post-translational modifications (Nakayama *et al.*, 2017), whilst the often-accompanying glycosylation of hydroxyprolines has not been reported (Fig. 2C). In contrast to other tyrosine-sulfated peptides, CIFs appear to have one highly

specific function: CIF peptide signaling is required for the formation of the Casparian strip diffusion barrier of endodermal cells (Doblas *et al.*, 2017; Nakayama *et al.*, 2017). CIFs are predominately expressed in the stele of primary (CIF1 and CIF2) and lateral roots (CIF2) (Nakayama *et al.*, 2017). They act locally, as only tyrosine-sulfation activity in the stele is able to rescue the diffusion-barrier defective phenotype. This conclusion was based on an extensive analysis of tissue-specific TPST-expression mutants and propidium iodide permeability (Doblas *et al.*, 2017). Mutants lacking CIF signaling due to mutations in the genes encoding for the peptide precursors *cif1-1* and *cif2-1*, the tyrosylprotein-sulfotransferase (*TPST*) *sgn2-1*, or the CIF receptors *gso1/sgn3-3* and *gso2-1* display mislocalized CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN 1 and lignin in the endodermis cell layer, resulting in a discontinuous casparian strip (Doblas *et al.*, 2017; Nakayama *et al.*, 2017).

PSYs

PLANT PEPTIDE CONTAINING SULFATED TYROSINE 1 (PSY1) is a sulfated and triarabinosylated 18-amino-acid peptide (Fig. 2D) that promotes cell expansion and cell division. It originates from a 75-amino-acid precursor, encoded in Arabidopsis by three nuclear genes (Amano *et al.*, 2007). PSY1 is expressed in a wide range of tissues but high expression is found in the root elongation zone and the shoot apical meristem. Wounding enhances PSY1 expression, as shown by northern blot analysis (Amano *et al.*, 2007), which points to a role in biotic stress (Matsuzaki *et al.*, 2010). Indeed, PSY signaling promotes susceptibility to the hemi-biotrophic fungus *Fusarium oxysporum* (Shen and Diener, 2013) and increases resistance to the necrotrophic fungus *Alternaria brassicicola* (Mosher *et al.*, 2013). PSY1 and PSK are therefore also classified as damage-associated molecular pattern factors (Wang *et al.*, 2014; Tang *et al.*, 2017) that modulate immunity responses.

Perception and downstream signaling of tyrosine-sulfated peptides

A common factor of all sulfated peptides is that they are perceived by plasma membrane-localized leucine-rich repeat receptor-like kinases (LRR-RLKs) from classes X and XI (Diévart and Clark, 2003) (Fig. 1C and Table 1), in most cases by more than one receptor per peptide, for example PSK by PSKR1 and PSKR2 (Matsubayashi *et al.*, 2002, 2006; Amano *et al.*, 2007), RGFs by RGFR1–5 (Ou *et al.*, 2016; Shinohara *et al.*, 2016; Song *et al.*, 2016), and CIFs by GSO1 and GSO2 (Doblas *et al.*, 2017; Nakayama *et al.*, 2017), which leaves PSY1R (PSY RECEPTOR1) for PSYs as the only single-copy receptor known so far (Amano *et al.*, 2007). Several peptide receptors heterodimerize with co-receptors of the SOMATIC EMBRYOGENESIS RECEPTOR KINASE family, as shown for the sulfated peptide receptors PSKR1, PSY1R, and RGFRs (Table 1), resulting in subsequent mutual transphosphorylation of the cytoplasmic kinase domains within the activation loops. Kinase activity of the receptor is essential for peptide signaling (Hartmann *et al.*, 2014, 2015; Ladwig *et al.*, 2015; Wang *et al.*, 2015; Ma *et al.*, 2016; Song *et al.*, 2016, 2017; Hohmann *et al.*, 2017; Kaufmann *et al.*, 2017; Oehlenschlæger *et al.*, 2017). In the case of CIF signaling, it has not yet been reported that SGN3 forms a dimer with a co-receptor; however, based on the extremely high ligand affinity to SGN3 as measured by isothermal titration calorimetry in the absence of a possible co-receptor, Doblas *et al.* (2017) speculated that the paradigm of peptide receptor–co-receptor formation might not apply for SGN3 activation. Only few downstream signaling components of peptide receptors have been identified (Table 1), most of which are localized to the plasma membrane, such as the cytoplasmic receptor-like kinase SGN1 that acts in CIF signaling (Doblas *et al.*, 2017) and RECEPTOR-LIKE PROTEIN 44 that participates in PSK signaling (Holzwart *et al.*, 2018). RECEPTOR-LIKE PROTEIN 44 controls procambial cell identity and connects the PSK and brassinosteroid signaling pathways through interactions with PSKR1 and the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE 1 (Holzwart *et al.*, 2018). For PSKR1, formation of a nanocluster at the plasma membrane has been shown with the ARABIDOPSIS H^+-ATPases 1 and 2 (AHA1 and AHA2) and the CYCLIC NUCLEOTIDE GATED CHANNEL 17 (Ladwig *et al.*, 2015) (Fig. 1C). Aside from being an active kinase, PSKR1 is a calmodulin-binding protein and has been described to possess guanylyl cyclase activity, making the PSK receptor a versatile protein both with respect to regulation of its activity and to downstream signaling through phosphorylation, Ca^{2+}-calmodulin signaling, and cGMP synthesis (Kwezi *et al.*, 2011; Hartmann *et al.*, 2014; Kaufmann *et al.*, 2017). Regulation of root elongation by PSY1 depends on the interaction with PSY1R and subsequent activation of AHA2 through transphosphorylation (Fuglsang *et al.*, 2014). A similar mechanism may apply for PSK, which induces protoplast swelling within minutes (Stührwohldt *et al.*, 2011) (Fig. 1C). Evidence for crosstalk between sulfated peptides and hormone signaling has also been reported. Auxin increases TPST expression, which probably promotes the maturation of sulfated peptides (Zhou *et al.*, 2010). Furthermore, PSK promotes auxin synthesis (Zhang *et al.*, 2018) whereas RGFs change auxin flux by altering the trafficking dynamics of the auxin efflux carrier PIN2 (Whitford *et al.*, 2012) (Table 1). Transcriptional regulation by sulfated peptides for a few S-Pep responsive genes (SPRGs) has been shown (Fig. 1D). PSK signaling inhibits ethylene production by regulation of ACC SYNTHASEs (ACSs) and ACC OXIDASEs (ACOs) (Wu *et al.*, 2015; Zhang *et al.*, 2018), and CIFs regulate CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN LIKE (CASPL) and MYB DOMAIN PROTEIN 15 (MYB15), resulting in lignification (Drapek *et al.*, 2018).

Sulfated peptide hormones produced by microbes

Tyrosine sulfation was first reported for plants and animals (Moore, 2009). Interestingly, RaxST, a tyrosylprotein sulfotransferase, was recently identified from the biotrophic plant pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae*

Table 1. *Overview of sulfated peptides, peptide receptors, molecular signaling components, and physiological responses*

Peptide	PTM	Receptor	LRR-RLK class*	Co-receptor	Downstream signaling	Physiological responses
RGFs/GLVs/CLELs (Matsuzaki *et al.*, 2010)	Tyr-sulfation Pro-hydroxylation	RGFR1/RGI1 RGFR2/RGI2 RGFR3/RGI3 RGI4 RGI5 (Ou *et al.*, 2016; Shinohara *et al.*, 2016)	XI	SERKs (Ou *et al.*, 2016; Song *et al.*, 2016)	PIN2 (Whitford *et al.*, 2012) *PLT2* (Matsuzaki *et al.*, 2010; Ou *et al.*, 2016; Shinohara *et al.*, 2016)	Root apical meristem homeostasis (Matsuzaki *et al.*, 2010) Root hair formation (Fernandez *et al.*, 2013) Lateral root formation (Fernandez et al., 2013, 2015) Gravitropic responses of root and shoot (Whitford *et al.*, 2012)
PSK (Matsubayashi and Sakagami, 1996)	Tyr-sulfation	PSKR1 PSKR2 (Matsubayashi et al., 2002, 2006; Amano *et al.*, 2007)	X	SERKs (Ladwig *et al.*, 2015; Wang *et al.*, 2015)	AHA1/2 and CNGC17 (Ladwig *et al.*, 2015) RLP44 (Holzwart *et al.*, 2018) CaMs (Hartmann *et al.*, 2014; Kaufmann *et al.*, 2017) cGMP (Kwezi *et al.*, 2011) YUCs → auxin ↑ (Zhang *et al.*, 2018) Ca^{2+} influx (Zhang *et al.*, 2018) *ACS*s, *ACO*s → ethylene ↓ (Wu *et al.*, 2015; Zhang *et al.*, 2018)	Cell expansion in leaves, hypocotyls and primary root (Kutschmar *et al.*, 2009; Stührwohldt *et al.*, 2011; Han *et al.*, 2014; Hartmann *et al.*, 2014) Quiescent center cell division (Heyman *et al.*, 2013) Reproduction: pollen germination (Chen *et al.*, 2000), pollen-tube growth (Stührwohldt *et al.*, 2015), funicular pollen tube guidance (Stührwohldt *et al.*, 2015) Immunity response to necrotrophic fungi (Zhang *et al.*, 2018) Xylem formation (Holzwart *et al.*, 2018) Adventitious root formation (Yamakawa *et al.*, 1998) Production of secondary metabolite taxol (Kim *et al.*, 2006)
PSYs (Amano *et al.*, 2007)	Tyr-sulfation Pro-hydroxylation Hyp-triarabinosylation	PSY1R (Amano *et al.*, 2007)	XI	SERKs (Oehlenschlæger *et al.*, 2017)	AHA2 (Fuglsang *et al.*, 2014)	Cell expansion and cell division (Amano *et al.*, 2007) Immunity response to necrotrophic fungi (Mosher *et al.*, 2013)
CIFs (Doblas *et al.*, 2017; Nakayama *et al.*, 2017)	Tyr-sulfation Pro-hydroxylation	GSO1/SGN3 (Nakayama *et al.*, 2017)	XI	–	SGN1 (Doblas *et al.*, 2017) *CASPL, MYB15* → CASP+lignin ↑ (Drapek *et al.*, 2018)	Casparian strip formation (Doblas *et al.*, 2017; Nakayama *et al.*, 2017)
XAPs (Okamoto *et al.*, 2015; Patel *et al.*, 2018)	Tyr-sulfation Pro-hydroxylation	–	–	–	–	Lateral root formation (Patel *et al.*, 2018)

* LRR-RLK, leucine rich repeat receptor-like kinase class according to Diévart and Clark (2003).
PTM, post-translational modification.

(Han *et al.*, 2012; Ronald, 2014). This bacterium produces the sulfated peptide RaxX, which mimics PSY1 (Fig. 2D) and triggers PSY1 responses, suggesting that RaxX can hijack the PSY1 signaling pathway. RaxX is perceived by the Xa21 immune receptor and modulates host defense (Pruitt *et al.*, 2017). As mentioned above, tyrosine sulfation is a non-reversible modification that results in the formation of highly stable signaling molecules that are targets in the arms race between plant hosts and pathogens.

Future perspectives

Research on sulfated peptides has so far mainly focused on the model plant Arabidopsis, whereas genes encoding for sulfated peptides and their signaling components are highly conserved throughout the higher plant kingdom (Lorbiecke and Sauter, 2002; Hartmann *et al.*, 2015). Beneficial effects of sulfated peptides on crop plant yields is an emerging topic that has great potential. Examples for agricultural exploitation of sulfated peptides have been described for an RGF in peach fruit ripening (Busatto *et al.*, 2017) and for PSK in the development of longer cotton fibers (Han *et al.*, 2014). Sulfated peptides seem to also affect secondary metabolism, as shown for PSK that synergistically acts with methyljasmonate to promote production of taxol (Kim *et al.*, 2006), a diterpenoid used as a therapeutical drug against cancer, thus revealing a great potential in pharmaceutical applications. Recently, a group of sulfated peptides were discovered from the xylem sap of *G. max* and *M. trunculata*, namely the XYLEM ASSOCIATED PEPTIDES (XAPs) (Fig. 2E) (Okamoto *et al.*, 2015; Patel *et al.*, 2018). Their functions are largely unknown and their characterization and elucidation as potential long-distance signals will be an exciting topic in the coming years.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft through grant SA495/13-2. The authors declare no conflict of interest.

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