1. Introduction

1.1 Balance between plant growth and stress responses

Due to the phenomenon of climate change and thus altered environmental conditions for plants, the understanding of the plant's stress responses is moving into the focus of vast scientific studies. These responses are triggered by abiotic stresses such as salinity, drought, and heat (Zhang et al., 2022), or biotic stresses, for instance, caused by insects, fungi, or bacteria (Verma et al., 2016). The plant stress response involves a delicate balance between growth and stress defense/tolerance, ultimately influencing the plant's fitness and survival (Figure 1).

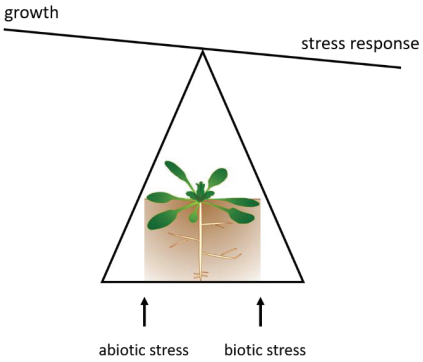

Figure 1. Balancing plant growth and stress signaling. Plants need to adapt upon abiotic and biotic stress. Thus, the plant needs to keep the balance between growth and stress response.

Morphologic changes observed in stressed plants include reduction of growth, senescence, early flowering, stomatal closure, and many mechanisms that enhance the stress tolerance (Zhang et al., 2022). Due to the importance for plant survival, plant stress response has to be regulated by many factors. Well-established regulators of plant stress response are the phytohormones, including abscisic acid (ABA), ethylene, cytokinin, auxin, gibberellin, jasmonate, salicylic acid, and strigolactones (Waadt et al., 2022). For instance, during osmotic stress triggered by salinity or drought, ABA adjusts growth and induces the closure of the stomata to reduce water deficit (Bharath et al., 2021; Verma et al., 2016). Auxin regulates hydropatterning, a mechanism that induces lateral root formation close to water, consequently increasing the water supply during low water availability (Orosa-

Puente et al., 2018). Root hydrotropism, the root growth towards moist soil, is regulated by asymmetric distribution of cytokinin in the root tip (Chang et al., 2019). Furthermore, auxin and brassinosteroids mediate the hyponastic response to elevated temperatures (Delker et al., 2021), and gibberellins regulate plant growth under cold stress (Lantzouni et al., 2020). During biotic stress, salicylic acid, ethylene, and jasmonate primarily mediate plant immunity (Bari et al., 2008). In addition to phytohormones, small posttranslational modified peptides, also known as peptide hormones, have emerged as regulators of plant stress responses (Kim et al., 2021, Hou et al., 2021).

1.2 Maturation of peptide hormones includes several steps of modifications

Plant peptides can be derived either from larger precursor proteins or transcribed directly from small open reading frames (Tavormina et al., 2015). Small posttranslational modified peptides (SPMPs) are classified into two main categories: cysteine-rich peptides and post-translationally modified peptides (Matsubayashi, 2014). Cysteine-rich peptides are characterized by a high number of cysteine residues, which enables the formation of disulfide bridges and thereby impacts on the folding and stability of the peptides. For instance, RAPID ALKALANIZATION FACTORS (RALFs) that regulate pollen tube growth and salt tolerance (Somoza et al., 2020; Zhao et al., 2018). LURE peptides, on the other hand, are identified as pollen tube attractants, thereby playing a crucial role in plant reproduction (Zhong et al., 2019).

Posttranslational modified peptides are synthesized as larger pre-pro-peptides that contain a N-terminal signal peptide. This signal peptide recruits the precursor into the secretory pathway and is cleaved upon entry. The biogenesis of posttranslationally modified peptides can involve several steps including proteolytic cleavage from the precursor, tyrosine sulfation, proline hydroxylation, and/or arabinosylation of hydroxyprolines (Figure 2). Many of these modifications are essential for the functional activity of the mature peptides (Stührwohldt and Schaller 2019). Once matured, the peptide is secreted into the apoplast and recognized by specific receptors, which initiate downstream signaling. Leucine-rich repeat receptor-like kinases (LRR-RLKs) are commonly involved as receptors in posttranslational modified peptide perception. Each stage of peptide maturation and perception will be described further in the subsequent sections.

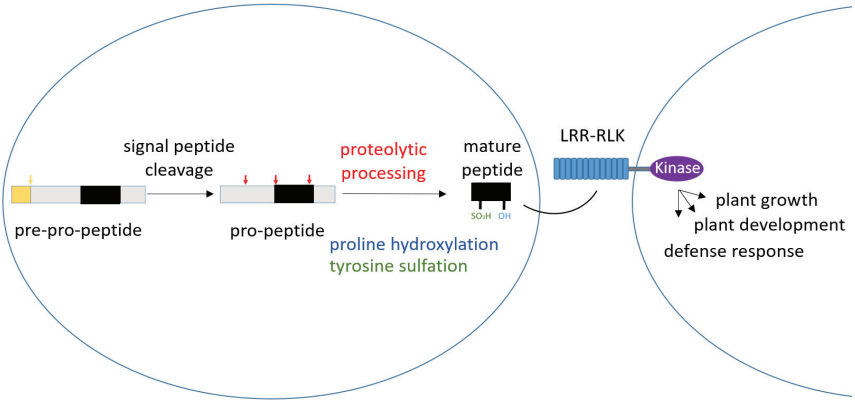

Figure 2. Simplified scheme of the posttranslational modification of peptides. The larger pre-pro-peptide is cleaved to release the signal peptide (yellow) upon entering the secretory pathway. Proteolytic processing events (red) done by subtilases cleave the peptide to its mature length. Tyrosine sulfation (green) is catalysed by the TPST enzyme, while proline hydroxylation (blue) is catalysed by P4Hs. The mature peptide is released into the apoplast and is perceived by a receptor (LRR-RLK) which can trigger effects on plant growth, plant development or defence responses.

1.3 Modification and perception of peptide hormones

1.3.1 Proteolytic processing by subtilases

The proteolytic cleavage of posttranslationally modified peptide precursors is mediated by enzymes called subtilases, which are encoded by 56 genes in Arabidopsis (Rautengarten et al., 2005). Due to redundancy, the specific functions of many subtilases have yet to be discovered. The term "subtilases" originates from subtilisin, a protease found in *Bacillus subtilis*, and their catalytic activity is defined by the arrangement of Aspartate, Histidine, and Serine residues, known as the catalytic triad (Smith et al., 1966; Meyer et al., 2016). Plant subtilases are synthesized as pre-proenzymes and undergo several activation steps, similar to their ligands, the SPMPs (Meyer et al., 2016). The structure of subtilases comprises a signal peptide, prodomain, subtilase domain, PA domain, and FnIII-like domain (Schaller et al., 2018). The signal peptide guides the subtilase into the secretory pathway and is cleaved upon entry (Schaller et al., 2018). The prodomain is necessary for proper folding and inhibits subtilase activity until it reaches its destination. The prodomain is subsequently cleaved autocatalytically in a pH-

dependent manner upon entering the acidic trans-Golgi (Meyer et al., 2016). The subtilase domain contains the catalytic triad and is responsible for cleaving the subtilase's substrate. The PA domain serves as a protein-protein interaction domain (Mahon et al., 2000). Subtilase processing can either involve pre-processing of the precursor to facilitate correct folding or cutting the peptide into its final length. For example, the SPMP CLAVATA3/ESR-RELATED (CLE40), which regulates stem cell differentiation, is processed by SBT1.4, SBT1.7, and SBT4.13 (Stührwohldt et al., 2020a). The INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) peptide, involved in floral organ abscission, is processed by SBT4.12, SBT4.13, and SBT5.2 (Schardon et al., 2016). In tomato, the wound response-regulating peptide systemin is cleaved by phytaspase1 and phytaspase2 (Beloshistov et al., 2018). To address the redundancy of subtilases, the expression of subtilase-specific inhibitor proteins from *Phytophthora infestans*, such as EPI1 (extracellular proteinase inhibitor 1) and EPI10, under the control of tissue-specific promoters, has become an important tool for analysing peptide functions. This approach has facilitated the identification of subtilases involved in IDA maturation (Schardon et al., 2016).

1.3.2 Proline hydroxylation by P4Hs

Proline hydroxylation is a common PTM and catalyzed by proline-4-hydroxylases (P4H), which are encoded by 13 genes in Arabidopsis (Hieta et al., 2001; Velasquez et al., 2015). Besides proline, the catalyzed reaction includes oxygen (O_2) and 2-oxoglutarate as co-substrates, Fe^{2+} as a co-factor and ascorbate for full activity (Kivirikko et al., 1989; Annunen et al., 1998). The Fe^{2+} ions are bound by a conserved histidine-x-aspartate motif in the P4Hs (Yamaguchi et al., 1998; Yuasa et al., 2004). Proline hydroxylation plays a crucial role in the function of peptides. For instance, CLE40 proline hydroxylation prevents further cleavage events in the mature peptide sequence during the proteolytic processing of the CLE40 precursor, thus ensuring correct maturation (Stührwohldt et al., 2020a). Proline hydroxylation of IDA was found to be essential for receptor binding (Santiago et al., 2016). In contrast to the proteolytic processing by subtilases, proline hydroxylation is not always essential for the function of the peptides. The ROOT GROWTH FACTOR6 (RGF6) or RGF4 peptides are also active in the absence of their proline hydroxylation (Whitford et al., 2012; Ghorbani et al., 2016). Furthermore, proline hydroxylation serves as a basis for modifications like O-linked glycosylation, which

is crucial for receptor binding (Amano et al., 2007). Golgi-located HPAT, encoded by three genes in Arabidopsis, catalyze the O-linked glycosylation (Ogawa-Ohnishi et al., 2013). O-linked glycosylation was identified as a critical factor for the functional activity of the sulfated peptide PLANT PEPTIDES CONTAINING SULFATED TYROSINE (PSY) (Amano et al., 2007).

1.3.3 Tyrosine sulfation by TPST

Tyrosine sulfation is a crucial modification that is catalyzed by the enzyme tyrosylsulfoprotein transferase (TPST). A gene encoding for the TPST enzyme is observed in both plant, and animal genomes (Moore et al., 2009; Komori et al., 2009). TPST utilizes the co-substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to transfer a sulfate group to the hydroxyl group of tyrosine (Moore et al., 2009). The animal genome encodes for two *TPST* genes. Both are classified as type II transmembrane proteins and are located in the trans-Golgi. Tyrosine sulfation is widespread in animals and has been associated with important roles in cellular processes. Notably, a *tpst1*/*tpst2* double mutant in mice leads to severely impaired postnatal survival (Huttner et al., 1982; Westmuckett et al., 2008). In contrast, the Arabidopsis genome encodes a single-copy gene for the TPST enzyme, which is classified as a type I transmembrane protein (Komori et al., 2009). *AtTPST* is widely expressed throughout the plant (Komori et al., 2009; Matsubayashi 2011). In plants, tyrosine sulfation mainly occurs in SPMPs and has been shown to be essential for the function of many peptides, particularly in receptor binding and proteolytic processing by subtilases (Amano et al., 2007; Meng et al., 2012; Ou et al., 2016; Doll et al., 2020; Royek et al., 2022; Stintzi and Schaller, 2022).

Thus far, four groups of sulfated peptides have been identified in Arabidopsis: phytosulfokine (PSK), PSYs, RGFs and CASPARIAN STRIP INTEGRITY FACTORs (CIFs) (Matsubayashi et al., 2014; Tavormina et al., 2014; Sauter et al., 2019). A knock out of the *AtTPST* gene results in a loss of function mutant for all sulfated peptides in Arabidopsis. The *tpst-1* mutant serves as a valuable tool for studying the function of these peptides. Initially, the *tpst-1* phenotype was described as dwarf plants accompanied with stunted roots, pale green leaves, reduced higher order veins, early senescence, and a reduced number of flowers and siliques (Komori et al., 2009). Further studies have revealed additional phenotypic characteristics of *tpst-1*, including hypersensitivity to fructose (Zhong et al., 2020),

defects in embryonic cuticle formation (Doll et al., 2020; Giorgi et al., 2021), hypersensitivity to phosphate deficiency (Kang et al., 2014), defective maintenance of the root stem cell niche, and decreased meristematic activity (Zhou et al., 2010). Additionally, sulfotyrosine has been identified as an extracellular pH sensor (Liu et al., 2022). Thus far all sulfated peptides are perceived by receptors from the LRR-RLK family.

1.3.4 Peptide perception by LRR-RLKs

Leucine-rich repeat receptor-like kinases (LRR-RLKs) are receptors located in the plasma membrane that regulate various aspects of plant growth, development, and responses to abiotic and biotic stresses (Xu et al., 2016; Xi et al., 2019; Soltabayeva et al., 2022). In Arabidopsis, the LRR-RLK family consists of approximately 230 members (Xu et al., 2016). Structurally, they comprise an extracellular domain, a transmembrane domain, and a cytoplasmic kinase domain. Regarding the length of the extracellular domain, LRR-RLKs are divided into main or co-receptors (Xi et al., 2019). The large helical ectodomain of the main LRR-RLK binds its ligand by forming a heterodimer with a smaller ectodomain containing the co-receptor (Figure 3). In contrast, the human LRR-RLKs, known as Toll-like receptors (TLRs), homodimerize (Hothorn et al., 2016; Sameer et al., 2022). In plants, the co-receptor stabilizes the complex of the main LRR-RLK with the ligand and is required for kinase activation (Zhang et al., 2016). This triple complex is ligand-induced and ligand-dependent. Thus, in the absence of the ligand, the main- and co-receptor do not interact (Hohmann et al., 2017). Upon formation of the triple complex (ligand/receptor/co-receptor), the kinase domain of the main receptor, which determines downstream signaling, is activated by autophosphorylation and activates a cytoplasmic signaling cascade (Hohmann et al., 2017). The ligands of the LRR-RLK can include hormones, polypeptides or SPMPs (Zhang et al., 2016).

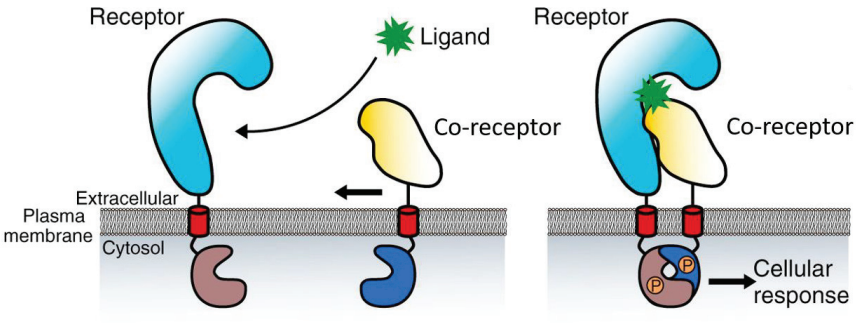

Figure 3. Main LRR-RLK forms a heterodimer with the ligand and co-receptor. Adapted from Brandt et al., 2016.

One of the most extensively studied LRR-RLKs is BRASSINOSTEROID INSENSITIVE 1 (BR1), which binds brassinosteroids using SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3) as a co-receptor, thereby regulating numerous growth-related processes in plants (He et al., 2000; Santiago et al., 2013). SERK3 (also known as BAK1) also functions as a co-receptor for the FLAGELLIN SENSITIVE 2 (FLS2) receptor, which recognizes flagellin and triggers plant immunity (Chinchilla et al., 2007), highlighting the fact that the main LRR-RLK determines downstream signaling specificity. The SERK family comprises five members and has been shown to be essential for many processes related to plant growth, development, and immunity (Brandt et al., 2016). Furthermore, LRR-RLKs have been identified as receptors for SPMPs, as demonstrated e.g. by CLAVATA1 (CLV1), which binds the CLV3 peptide involved in shoot and floral meristem development, and HAESA, which binds the IDA peptide (Rojo et al., 2002; Cho et al., 2008). Recently, LRR-RLKs, including SERKs, have also been established as receptors for many sulfated peptides (Table 1).

Table 1. Perception of sulfated peptides by LRR-RLK.

Sulfated peptide	Receptor	Co-receptor	Publication
PSK	PSKR1, PSKR2	SERK3	Wang et al., 2015; Ladwig et al., 2015; Hartmann et al., 2014; Kaufmann et al., 2021
RGF1	RGI1, (RGI2, RGI3)	SERK	Ou et al., 2022
RGF5, RGF8	RGI	-	Fernandez et al., 2020
RGF7	RGI4, RGI5	SERK4, SERK5	Wang et al., 2021
RGF9	RGI3	SERK3	Stegmann et al., 2022
PSY1	PSY1R	-	Amano et al., 2007
PSY1-9	PSYR1, PSYR2, PSYR3	-	Ogawa-Ohnishi et al., 2022
CIF1, CIF2	GSO1, GSO2	-	Nakayama et al., 2017; Doublas et al., 2017
CIF3, CIF4	GSO1, GSO2		Truskina et al., 2022
TWS1	GSO1, GSO2	SERK1-3	Zhang et al., 2021

1.4 Sulfated peptides function as regulators of growth and development

1.4.1 PSK as growth and development regulator in various species

Sulfated peptides have gained increasing importance in plant stress responses. Among them, PSK has been extensively studied. Originally, PSK was discovered as a cell division-promoting factor in *Asparagus officinalis* cells (Matsubayashi et al., 1996). In Arabidopsis, there are seven genes encoding the same YIYTQ penta-peptide, with the exception of PSK6, which differs in the last amino acid (Figure 4).

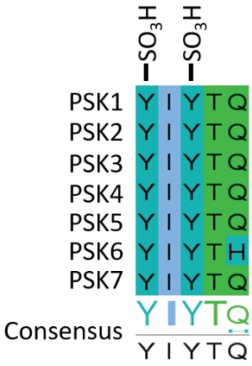

Figure 4. Alignment of the mature PSK peptides in Arabidopsis. Protein sequence alignment of the mature PSK peptides. Posttranslational modifications are indicated by SO_3H (tyrosine sulfation). Protein sequence alignment was done using MUSCLE (Multiple Sequence comparison by Log- expectation). The default Clustal X color scheme was used to assign colors to conserved amino acids based on their amino acid properties. PSK sequences were obtained from Kaufmann et al., 2019.

The maturation of PSK involves several steps. It may require pre-processing by SBT1.1, followed by proteolytic cleavage by unknown subtilases, and undergoes tyrosine sulfation by TPST (Srivastava et al., 2008). The proteolytic processing of PSK is unique because it involves the removal of the aspartate residue from the conserved DY motif at the N-terminus of sulfated peptides. The specific subtilase responsible for cleaving PSK to its final length in Arabidopsis is still unknown, while in tomato, it is catalyzed by phytaspase2 (Reichardt et al., 2020). In tomato, stress-induced PSK formation leads to flower drop (Reichardt et al., 2020). Further studies have revealed that PSK optimizes plant growth and defense by triggering the phosphorylation of the glutamine synthetase GS2 in tomato (Ding et al., 2022). In Arabidopsis, PSK has been attributed with various growth-promoting functions, including regulation of root growth, hypocotyl length, cell expansion, and pollen tube growth (Kutschmar et al., 2009; Stührwohldt et al., 2011; Hartmann et al., 2014; Stührwohldt et al., 2015). It also plays a role in plant immunity against diverse pathogens (Loivamäki et al., 2010; Igarashi et al., 2012; Mosher et al., 2012; Rodiuc et al., 2015). PSK functions through the LRR-RLKs PSKR1 and PSKR2, involving the co-receptor SERK3 (Ladwig et al., 2015; Hartmann et al., 2014; Kaufmann et al., 2021). Downstream signaling of the PSK/PSKR module involves CYCLIC NUCLEOTIDE-GATED CHANNEL17 and H^+-ATPase (Ladwig et al., 2015). Moreover, studies have revealed a potential application for PSK in the floral industry. Exogenous PSK treatment has been shown to delay the senescence of cut rose flowers and strawberry fruits (Aghdam et al., 2021a; Aghdam et al., 2021b). In summary, PSK has emerged as an important regulator of plant growth and development, as well as a mediator of stress responses in diverse plant species.

1.4.2 RGFs regulate responses beyond those specific to roots

The family of RGF (ROOT MERISTEM GROWTH FACTOR) peptides was initially identified by three independent research groups, resulting in three different names: CLAVATA 3/EMBRYO SURROUNDING REGION-LIKE (CLEL), ROOT MERISTEM GROWTH FACTOR (RGF), and GOLVEN (GLV) (Whitford et al., 2012; Meng et al., 2012; Matsuzaki et al., 2010). In Arabidopsis, the number of CLEL/RGF/GLV peptides varies depending on the study, with approximately 11 to 12 members. These peptides have a final length of 13-16 amino acids and contain a conserved

DY motif at the N-terminus, a conserved P (proline) fourth to the last amino acid, and a conserved N (asparagine) at the C-terminus (Figure 5).

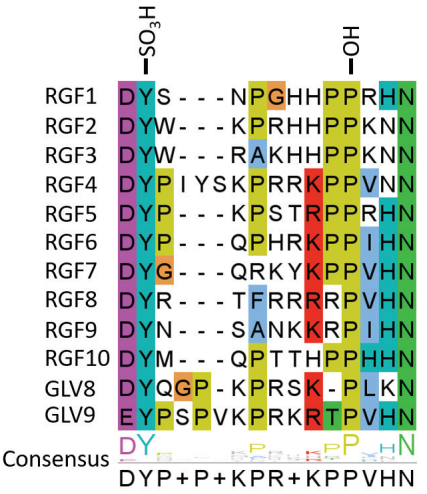

Figure 5. Alignment of the mature RGF peptides in Arabidopsis. Protein sequence alignment of the mature RGF/GLV peptides. Posttranslational modifications are indicated by SO_3H (tyrosine sulfation) and proline hydroxylation (OH). Protein sequence alignment was done using MUSCLE (Multiple Sequence comparison by Log- expectation). The default Clustal X color scheme was used to assign colors to conserved amino acids based on their amino acid properties. RGF/GLV sequences were obtained from Kaufmann et al., 2019.

The maturation process of RGF peptides involves tyrosine sulfation by TPST, proline hydroxylation at the conserved P residue, and proteolytic cleavage by subtilases. The aspartate residue at the N-terminus was found to be important for subtilase cleavage by SBT3.8 in the case of RGF1, RGF6, and RGF9, occurring in a post-Golgi compartment (Stührwohldt et al., 2020b; Stührwohldt et al., 2021). However, mutation of *sbt3.8* did not result in any specific phenotype related to RGF peptides, suggesting redundancy in N-terminal cleavage (Stührwohldt et al., 2020b). For RGF6 and RGF9, a pre-processing event by SBT6.1 at the RRLR/RRAL motifs of the precursor was shown to be essential for the function of these peptides (Ghorbani et al., 2016; Stührwohldt et al., 2020b). Most of the RGF peptides are primarily expressed in the roots, while RGF6 and RGF9 are primarily expressed in the aerial parts of the plant (Figure 6).