PEP7 acts as a peptide ligand for the receptor kinase SIRK1 to regulate aquaporin-mediated water influx and lateral root growth

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ABSTRACT

Plant receptors constitute a large protein family that regulates various aspects of development and responses to external cues. Functional characterization of this protein family and the identification of their ligands remain major challenges in plant biology. Previously, we identified plasma membrane-intrinsic sucrose-induced receptor kinase 1 (SIRK1) and Qian Shou kinase 1 (QSK1) as receptor/co-receptor pair involved in the regulation of aquaporins in response to osmotic conditions induced by sucrose. In this study, we identified a member of the elicitor peptide (PEP) family, namely PEP7, as the specific ligand of the receptor kinase SIRK1. PEP7 binds to the extracellular domain of SIRK1 with a binding constant of 1.44 ± 0.79 µM and is secreted to the apoplasm specifically in response to sucrose treatment. Stabilization of a signaling complex involving SIRK1, QSK1, and aquaporins as substrates is mediated by alterations in the external sucrose concentration or by PEP7 application. Moreover, the presence of PEP7 induces the phosphorylation of aquaporins in vivo and enhances water influx into protoplasts. Disturbed water influx, in turn, led to delayed lateral root development in the pep7 mutant. The loss-of-function mutant of SIRK1 is not responsive to external PEP7 treatment regarding kinase activity, aquaporin phosphorylation, water influx activity, and lateral root development. Taken together, our data indicate that the PEP7/SIRK1/QSK1 complex represents a crucial perception and response module that mediates sucrose-controlled water flux in plants and lateral root development.

Key words: receptor kinase, peptide signaling, receptor-ligand pair, sugar signaling, regulation of water influx

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INTRODUCTION

Plants as sessile organisms must be able to rapidly adapt to altering environmental conditions throughout the diurnal cycle and during their life span. This requires precise integration of extracellular information with intracellular (metabolic) signals. This integration of environmental and developmental signals in plants is often controlled by small peptides that then activate signaling cascades through receptor kinases. Receptor kinases constitute the biggest subclade within the plant kinome (Zulawski et al., 2014). The plant receptor kinases are involved in plant developmental processes as well as in responses to biotic and abiotic cues (Osakabe et al., 2013).

The first peptide with signaling function discovered in plants was systemin (Pearce et al., 1991), which at that time was postulated to be perceived by a leucine-rich repeat (LRR)-family receptor kinase (Scheer and Ryan, 2002), and for which in 2018 indeed an LRR receptor kinase was identified as its receptor (Wang et al., 2018). Since then, more and more signaling peptides were discovered, which are involved in cell-to-cell communication, developmental processes, and stress responses (Matsubayashi, 2014; Tavormina et al., 2015;
It is a characteristic of the small signaling peptides that they are mobile between cells. Thus, the site of their secretion may be different from the site of their perception. For example, peptide CLAVATA3 is recognized by an LRR-receptor kinase expressed in neighboring cell files (Clark et al., 1997). The peptides can even be translocated throughout the plant, as exemplified by the C-terminally encoded peptide family peptides, which are secreted by nitrogen-starved roots, but their receptors are located in the shoot (Tabata et al., 2014).

The biologically active peptides are defined as being smaller than 100 amino acids and usually undergo a process of maturation from larger precursor proteins (Tavormina et al., 2015; Olsson et al., 2019). Thus, the final active peptide is matured from its proprotein through proteolytic processing by specific peptidases (Schaller et al., 2018). In many cases, this involves two steps: firstly, the cleavage of an N-terminal signal sequence necessary for secretion of the protein to the apoplast, and secondly, release of the active peptide by cleavage of the prodomains. The peptides themselves are frequently subject to posttranslational modifications such as sulfatation, modification with sugar residues or hydroxyprolines (Matsubayashi, 2014). Formation of secondary structures through intramolecular disulfide bridges is a characteristic feature of cysteine-rich peptides, such as the rapid alkalinization factor (RALF) family (Moussu et al., 2020; Abarca et al., 2021). The process of peptide maturation has moved a variety of peptidase families into the focus of attention in the context of plant signaling pathways (Rautengarten et al., 2005). Subtilases were shown to be involved during maturation of the IDA peptide (Scharon et al., 2016), during biogenesis of TWS1 (Abarca et al., 2021), and also in phytosulfokine processing (Stühnwohldt et al., 2021). Although it is apparent that receptor kinases are primary candidates for the recognition of a variety of biologically active peptides with signaling functions, for most of the receptor kinases, the precise ligand remains unknown. In turn, also for many biologically active peptides, the receptors remain to be identified (Matsubayashi, 2003). Thus, for a more complete understanding of the functional implications of plant receptor kinases, it is of high interest to identify and characterize ligand–receptor pairs.

In the past, our group has studied sucrose-induced protein phosphorylation in a time course experiment resupplying sucrose to sucrose-starved Arabidopsis seedlings (Niittylä et al., 2007). Based on this time course information, sucrose-dependent regulation of an aquaporin and sucrose-induced phosphorylation of the sucrose exporter SWEET11 by a protein complex involving sucrose-induced receptor kinase SIRK1 was discovered (Wu et al., 2013). As a follow up to this work, we recently showed that the SIRK1 signaling complex is stabilized by the coreceptor QSK1 (Wu et al., 2019b). Together, SIRK1 and QSK1 regulate aquaporins through phosphorylation. Since aquaporin phosphorylation status was shown to correlate with the plant hydraulic status (Di Pietro et al., 2013), the SIRIK1 complex may affect water balance of root cells. However, the ligand of this SIRK1–QSK1 receptor complex remained unknown. We therefore conducted a series of biochemical and physiological experiments to identify PEP7 as the specific ligand of receptor kinase SIRK1.

### RESULTS

Sucrose-induced receptor kinase (SIRK1) belongs to the LRR-receptor kinases (Zulawski et al., 2014). SIRK1 was found to be activated by an external supply of sucrose (Wu et al., 2013). It interacts with co-receptor Qian Shou kinase (QSK1) and, in the active state, regulates the opening status of aquaporins (Wu et al., 2019b). The majority of the LRR-receptor kinases for which a ligand is known so far were found to bind small peptide ligands. Therefore, we followed the hypothesis that also SIRK1 could bind a small peptide ligand.

#### Identification of SIRK1 ligand candidates

To systematically screen for a putative peptide ligand to SIRK1, we prepared apoplasmic protein extracts derived from wild-type liquid-grown Arabidopsis seedlings. Apoplasmic proteins were ultrafiltered to exclude all protein components larger than 1 kDa and then separated into different fractions by reversed-phase chromatography (Figure 1A). Each fraction was tested for its ability to in vitro induce the kinase activity of SIRK1–GFP, which was enriched from root tissue of hydroponic cultures 2 days after sucrose starvation (Figure 1A). Kinase-active SIRK1–GFP (SIRK1–GFP<sub>suc</sub>), which also contains interacting co-receptor QSK1 (Wu et al., 2019b), was enriched from sucrose-stimulated hydroponic cultures (Wu et al., 2013) and used as a positive control (Figure 1B). Exposure of sucrose alone to SIRK1–GFP enriched from sucrose-starved plants (SIRK1–GFP<sub>starv</sub>) was not able to induce SIRK1 activity (Figure 1B). By contrast, SIRK1–GFP<sub>starv</sub> activity was highly induced after exposure to protein fraction F2 and, to a lesser extent, to protein fraction F1. Kinase activity was lowest upon exposure of SIRK1–GFP<sub>starv</sub> to protein fraction F3 (Figure 1B). An aliquot of each fraction was analyzed by mass spectrometry. We performed two runs in parallel—one with standard protocol including trypsin digestion, and one without prior trypsin digestion—in case the ligand candidates would not yield suitable tryptic peptides. Altogether, in fraction F1, we identified 1531 proteins, in fraction F2 4106 proteins, and in fraction F3 440 proteins. Among the identified proteins, we then selected those candidate proteins that were present with spectral counts higher than three in fractions F1 and F2 and that were not present (no spectra) in fraction F3. A total of 30 identified proteins were predicted to be secreted proteins, and 14 of these met the requirements of high abundance in fractions F1 and F2 but not in fraction F3 (Figure 1C). Two of these candidate proteins were also identified in the non-tryptic samples, namely RALF1 (AT1G02900) and PEP7 (AT5G09978). All tryptic and non-tryptic RALF1 and PEP7 peptides covered the C-terminal parts of the respective propeptides, which constitutes the biologically active RALF1 or PEP7 versions, respectively. Other RALF peptides such as RALF22, RALF23, and RALF33 were also identified in the fractions F1, F2, and F3 but did not strictly meet the criteria set for putative ligands as described above (supplemental Table 1).

We next used synthetic peptides of RALF1, RALF22, RALF32, and RALF33 at different concentrations to test for their ability to induce the kinase activity of SIRK1–GFP<sub>starv</sub> (supplemental Figure 1). Independently of the concentrations used, neither...
the RALF peptides nor the non-related reference peptides IDA and CLE were able to induce SIRK1–GFP\textsubscript{starv} kinase activity above background level (supplemental Figure 1). By contrast, with the exception of RALF1, the application of these peptides even reduced SIRK1–GFP\textsubscript{starv} kinase activity compared with the control conditions (supplemental Figure 1). Inhibition of complex formation between receptor and co-receptor by RALF23 has been described for the FLS2/EFR–BAK1 complex (Stegmann et al., 2017). In addition, recently it was shown that RALFs require internal disulfide bonding to acquire the required structure for receptor binding (Moussu et al., 2020). This may affect the performance of synthesized RALF peptides in this kinase assay.

PEP7 can bind to SIRK1 in biochemical and biophysical assays
The abundance of the signaling peptide PEP7 increased in the apoplasm of liquid-grown seedlings when resupplied with 30 mM sucrose after starvation (Figure 2A) but not when the sucrose-starved seedlings were supplied with related disaccharides such as sucrarose or trehalose or the monosaccharide glucose. Thus, the observed PEP7 accumulation was specific for sucrose-supplemented seedlings. To test, whether PEP7 influences SIRK1–GFP\textsubscript{starv} kinase activity, we performed an in vitro dose-response assay. Increasing concentrations of synthetic PEP7 enhanced SIRK1–GFP\textsubscript{starv} kinase activity, which reached saturation at PEP7 concentrations above 1 \textmu M (Figure 2B). We then tested whether PEP7 can directly bind to SIRK1–GFP. SIRK1–GFP, immobilized on anti-GFP magnetic beads (supplemental Figure 2A), was exposed to 1 \textmu M PEP7. After washing and elution, association of the putative ligand with SIRK1–GFP was detected by mass spectrometry. Indeed, SIKR1–GFP captured PEP7 from the solution (Figure 2C). Next, we performed a series of co-immunoprecipitation experiments to test for binding of PEP7 to the extracellular domain of SIRK1 (SIRK1–ECD). Firstly, StrepII-tagged SIRK1–ECD was purified after transient expression in Nicotiana benthamiana leaves.
and was then immobilized to streptavidin beads and exposed to PEP7 at a concentration of 1 μM. Again, PEP7 was detected by mass spectrometry among the eluted proteins that were bound to the SIRK1–ECD bait (Figure 2D). In a reverse co-immunoprecipitation experiment, synthetic (His)6-tagged PEP7 was immobilized to Ni-NTA magnetic beads (supplemental Figure 2C) and exposed either to 50 μg SIRK1–GFP, 20 μg SIRK1–ECD purified from N. benthamiana leaves after transient expression, or to root microsomal membrane preparations, which are expected to contain native full-length SIRK1 (Figure 2E). Indeed, in all three approaches, PEP7–(His)6 was found to capture SIRK1–GFP, StrepII-tagged SIRK1–ECD, or root-derived SIRK1 from the solutions. Next, we tested whether binding of SIRK1–ECD to immobilized PEP7–(His)6 can be competitively released by addition of free, non-tagged PEP7. We detected a release of SIRK1–ECD from the PEP7–(His)6 beads with increasing concentrations of free PEP7 (Figure 2F). Saturation was reached at PEP7 concentrations above 1 μM. When free PEP6 (1 μM) was used to elute SIRK1–ECD from the PEP7–(His)6 beads, no release of SIRK1–ECD was observed. These experiments suggest that indeed PEP7 associates with SIRK1 via its ECD.

We used microscale thermophoresis to obtain more quantitative data with respect to the binding of PEP7 to SIRK1–ECD. StreptII-tagged SIRK1–ECD was purified (supplemental Figure 3) and labeled with fluorescent dye RED-NHS. Incubation of different concentrations of PEP7 with SIRK1–ECD resulted in a sigmoidal binding curve, allowing the calculation of the binding constant (Kd value) of 1.4 ± 0.79 μM PEP7 (range: 1–1.8 μM) based on three assays from independent protein isolations (Figure 3). Adding an approximate amount of co-receptor QSK1–ECD as a third component to the binding assay reduced the binding constant to a Kd of 57.0 ± 22.2 nM (range: 33.9–80 nM). When PEP4 or PEP6 instead of PEP7 was used as a putative ligand in the microscale thermophoresis assay, no sigmoidal dose-response curve could be obtained, and the signal-to-noise ratio (around 1.4) was too small to calculate a Kd value. Thus, experimental evidence from the different association assays and from microscale thermophoresis point to PEP7 being able to physically bind to SIRK1–ECD.
PEP7 as the ligand for receptor kinase SIRK1

QSK1–ECD could increase the binding affinity of SIRK1–ECD and PEP7, as expected from QSK1 function as a co-receptor. Thus, PEP7 is a strong candidate to be the specific ligand of SIRK1.

PEP7 induces the formation of SIRK1 signaling complex and phosphorylation of SIRK1 substrates

SIRK1–GFP fusion expressed in the sirk1 and pep7 background was used to determine the interactome of SIRK1 after addition of sucrose, PEP7, or mock treatment following an established affinity purification protocols (Wu et al., 2019b) (supplemental Table 2). Protein abundances were normalized to the protein abundance ratios were calculated as log2 ratios of treatment SIRK1/QSK1 signaling complex (Wu et al., 2013, 2019b).

Corresponding to the known pore-gating phosphorylation sites from SoPIP2A (Figure 5C), suggesting that, indeed, PEP7 induced pore-opening phosphorylations of aquaporins, similar to the treatment with external sucrose (Figure 5D).

PEP7 may stabilize in vivo association of SIRK1 and QSK1 for forming active complexes

To investigate potential effect of PEP7 on SIRK1 and QSK1 associations, we measured their spatial proximity in vivo by Förster resonance energy transfer by fluorescence lifetime (FLT) imaging (FRET–FLIM). In transiently transformed N. benthamiana epidermal leaf cells, QSK1–GFP and SIRK1–mCherry co-localize in the plasma membrane with or without the supply of PEP7 (supplemental Figures 4A and 4B). To determine the background FRET–FLIM values, the FRET donor QSK1–GFP was expressed alone, and the FLT was measured. In all cases, where QSK1–GFP and SIRK1–mCherry (FRET acceptor) were co-expressed, a reduction in FLT was observed, indicating a close spatial proximity (less than 10 nm; Glocker et al., 2019) of the two proteins. There was no significant further reduction in FLT when PEP7 was applied compared with mock treatment.

In those experiments, hydroponic cultures of Arabidopsis plants where starved for sucrose and resupplied with 30 mM sucrose solution for 5 min prior to harvesting of tissue and analysis of protein phosphorylation (Wu et al., 2013, 2019b).

Here, we show that in the wild type, an external supply of PEP7 (instead of sucrose) also resulted in increased phosphorylation of QSK1/QSK2 and aquaporins (Figure 5B). The sucrose-induced phosphorylation of QSK1/QSK2 and aquaporins was reduced in the previously described sirk1 loss-of-function mutant (Figure 5B). The external supply of PEP7 instead of sucrose to sirk1 seedlings resulted in an even lower phosphorylation of QSK1/QSK2 and aquaporins compared with wild type. Moreover, the phosphorylation of aquaporins and QSK1/QSK2 was not observed in the pep7 mutant upon sucrose supply. However, the increased phosphorylation of QSK1/QSK2 and aquaporins in pep7 was restored by external supply of PEP7. In the sirk1;pep7 double mutant, the phosphorylation of QSK1/QSK2 and aquaporins was neither induced by sucrose nor rescued by an external supply of PEP7.

Importantly, the quantified phosphopeptides of aquaporins (supplemental Table 3) corresponded to the known pore-gating phosphorylation sites from SoPIP2A (Figure 5C), suggesting that, indeed, PEP7 induced pore-opening phosphorylations of aquaporins, similar to the treatment with external sucrose (Figure 5D).
**Figure 4.** Interactome of SIRK1 induced by PEP7 and sucrose.

(A) Protein abundance distribution normalized to bait (SIRK1) abundance. LFQ values were taken as measure of protein abundance.

(B) Distribution of treatment/control comparisons centered on median values. The two median values within each comparison are shown in the violin plot, respectively.

(C) Venn diagram of responding proteins upon sucrose or PEP7 treatment.

(D) Correlation analysis of all protein comparisons under two treatments. Proteins with values greater than the median were considered to be recruited by SIRK1 under both treatments.

(E) Correlation analysis of only receptor kinases.

(F) Correlation analysis of only PIPs under two treatments.
To exclude the possibility that endogenous PEP7 may mask the effects of externally applied PEP7 on QSK1–GFP/SIRK1–mCherry association, we performed the FRET–FLIM experiments in the presence of the protease inhibitor leupeptin. Leupeptin inhibits the metacaspase 4 (MC4) (Vercammen et al., 2004), which releases PEP7 from its propeptide (Moussu et al., 2020). After treatment with leupeptin, the FLT of QSK1–GFP in the donor-only control was reduced in response to PEP7 addition (supplemental Figure 4B). However, the FLT of QSK1–GFP in presence of SIRK1–mCherry was not significantly altered in response to PEP7 application even after treatment with leupeptin (supplemental Figure 4B). Likely, SIRK1 and QSK1 are associated in preformed nano-structured membrane domains in the absence of PEP7 as was described for several other LRR–RK complexes (Bucherl et al., 2017; Glöckner et al., 2019; Gronnier et al., 2022). Therefore, PEP7 may act as a stabilizing factor of the QSK1/SIRK1 association, thereby transforming the preformed inactive complexes into active ones. Indeed, when the SIRK1–QSK1 complex was activated by sucrose or PEP7 treatment, SIRK1 was reliably detected in pull-downs of QSK1–GFP (supplemental Figure 4C). Moreover, when the QSK1 complex was fixed in the active state (e.g., by mutation of the phosphorylated serines of QSK1 to aspartates), SIRK1 was also detected in pull-downs under non-stimulated conditions (supplemental Figure 4C).

**PEP7 affects water influx to protoplasts via receptor kinase SIRK1**

Sucrose as an osmotic agent was previously shown to induce water influx into protoplasts, and this sucrose-induced water influx was significantly reduced in the sirk1 mutant (Wu et al., 2013). We performed protoplast swelling assays with wild type and sirk1 and pep7 single mutants as well as the sirk1;pep7 double mutant to test if supply of PEP7 was also able to induce water influx and whether this was dependent on the presence of SIRK1 receptor kinase. In wild type, mannitol and sucrose induced water influx density (Figures 6A and 6B). An external

### Figure 5. PEP7-induced changes of phosphorylation levels of SIRK1 substrates.

(A) SIRK1 signaling scheme highlighting substrates that presented previously.

(B) Heatmap of means of Z-scored phosphosite intensities of different phosphopeptides identified for known members of the SIRK1 signaling pathway. Each row represents one phosphopeptide.

(C) Sequence alignment of C-terminal phosphorylated plant aquaporins. Gray area indicates the conserved residues that can be phosphorylated.

(D) Z-scored phosphorylation levels of conserved residues of PIPs that correspond to the pore gating that induced by sucrose and PEP7. Small letters indicate significant differences (p < 0.05; pairwise t-test) for each phosphopeptide between genotypes.
Figure 6. Water influx density of protoplasts induced by osmotic changes through mannitol, sucrose, or by the supply of PEP7 in the wild type (Col-0), sirk1, pep7, the double mutant sirk1pep7, and mc4.

(A) Boxplots show the water flux density relative to wild type. The average of Col-0 under mannitol treatment was used as the control and set to 1. Vertical lines in the boxes indicate the median and upper/lower borders represent the 25th percentile. White dots represent individual data points.

(legend continued on next page)
supply of PEP7 in wild type significantly further increased water influx density compared with mannotil or as observed in presence of sucrose. In the sirk1 mutant, mannotil induced water influx density, but sucrose treatment did not. Supply of PEP7 to sirk1 mutant did not increase water influx density (Figures 6A and 6B), which remained low, similar to water flux density observed in sirk1 with sucrose treatment. In the pep7 mutant, mannotil induced water influx, but sucrose treatment resulted in low water influx density, similar as in the sirk1 mutant. Strikingly, external supply of PEP7 to the pep7 mutant restored water influx densities to higher values statistically not different from wild type under mannotil treatment (Figures 6A and 6B). In the sirk1:pep7 double mutant, neither sucrose nor PEP7 treatment resulted in enhanced water influx densities, suggesting that SIRK1 is required for the protoplast swelling response.

Recently, the MC4 was identified to be involved in PEP7 maturation (Moussu et al., 2020). Interestingly, water influx rates of the mc4 loss-of function mutant resembled the water influx rates of the pep7 mutant. In mc4, as in pep7, sucrose did not induce water influx, but PEP7, also in mc4, was able to restore water influx to the protoplasts (Figures 6A and 6B). This further supports our conclusion of PEP7, as being produced by MC4 activity, to be involved in regulation of water influx.

We then tested whether other members of the PEP family were able to affect water influx to protoplasts in a similar way. PEP4 and PEP6 were used since they were also found to be expressed in root tissue (Shulse et al., 2019). Water influx densities in wild type were not significantly affected by the presence of PEP4 or PEP6. When the receptor kinase SIRK1 was absent (sirk1 mutant), water influx densities in the presence of PEP4 or PEP6 were not different from wild type, suggesting that PEP4 and PEP6 did not require the presence of receptor kinase SIRK1 (supplemental Figure 5A). In the pep7 mutant, no difference in water influx densities compared with wild type was observed when PEP4 or PEP6 was supplied externally (supplemental Figure 5A). To differentiate the effects of SIRK1 from other known PEP-family receptors, we performed protoplast swelling assays using pep1 and pep2 mutants. PEPR1 and PEPR2 are known receptors to PEP1 (Bartels et al., 2013). Although pep1 and pep2 showed an increased water influx density in response to mannotil compared with wild type, PEP7 was still able to increase water influx density similarly as in wild type, suggesting that PEPR1 and PEPR2 were not involved in the PEP7 pathway (supplemental Figure 5B).

**Lateral root growth mediated by PEP7 requires SIRK1**

Next, we explored the role of the PEP7/SIRK1 signaling pathway on the organ level of roots. We hypothesize that particularly lateral root growth, which relies on sucrose supply as carbon source and water transport for turgor buildup (Péret et al., 2012), may—in addition to auxin—be affected by the SIRK1/ QSK1/PEP7 signaling module. In wild type, PEP7 treatment resulted in lateral root primordia (LRP) being more frequently found at later developmental stages (stages VI + VII, VIII) (Figure 7) compared with untreated plants. In the sirk1 mutant, LRP were equally distributed across stages, and no effect of PEP7 treatment was observed, suggesting a generally delayed lateral root development in the sirk1 mutant, and this was not affected by PEP7 treatment. In the pep7 mutant under control conditions, most LRP were observed at early stages II + III. PEP7 supply significantly decreased LRP at early stages, and we found a significant increase of LRP frequency at stages VI + VII and VIII (Figure 7). This may indicate that PEP7 contributes to progressing LRP from early stages (I to III) to later stages (VI + VII and VIII). In the sirk1;pep7 mutants, LRP distribution under control conditions was similar to the distribution observed in the pep7 mutant, with low frequency of LRP at stages IV + V. PEP7 treatment resulted in a similar distribution of LRP stages as in the sirk1 mutant, lacking a significant progression of LRP stages to later developmental stages.

In general, it must be kept in mind that root growth and lateral root development are complex traits affected by multiple signaling pathways over a more long-term time scale. In previous work, LRP emergence was shown to be affected by aquaporin activity, which was in turn affected by auxin (Péret et al., 2012). Thus, the observed low numbers at stages IV and V in in pep7 and sirk1;pep7 may be attributed to complex interactions of multiple signaling pathways (e.g., auxin signaling) affecting aquaporin activities, and these may still be active even in the absence of SIRK1. Also, the more long-term time scale compared with other biochemical process, such as protein phosphorylation and protoplast swelling assays, must be considered. However, our data clearly show a contribution of PEP7 and SIRK1 to the complex trait of LRP emergence.

**DISCUSSION**

PEP7 (AT5G09978) belongs to a family of “danger signaling peptides” consisting of eight family members (Zhang et al., 2016a). Out of this family, PEP1 was shown to be recognized by receptors PEPR1 (AT1G73080) and PEPR2 (AT1G17750) (Krol et al., 2010; Yamaguchi et al., 2010), but very little is known about the other PEP-family members. Here, we point to a signaling pathway for PEP7. Based on the sequences of the active peptides, PEP6 shows strongest similarity to PEP7 and therefore was used as a control. PEP4, although its propeptide shares expression with PEP7 in root tissue, shows the strongest dissimilarity to PEP7 and was used as a second negative control (Zhang et al., 2016a).

PROPEP7 was found by single-cell sequencing of root cells, clustering with marker genes of mature root hairs (Ryu et al., 2019). In the same study, PROPEP6 was found in a gene cluster with marker genes of the endocortex, while other members of the
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The PEP family or the respective PEPR receptors were not found. Most interestingly, in another single-cell sequencing study of root cells (Shulse et al., 2019), expression of PROPEP7 was induced by external sucrose supply, while expression of PROPEP6 and PROPEP4 were not induced by sucrose (Shulse et al., 2019). Thus, members of the PEP family are involved in other signaling pathways besides biotic stress responses and were shown to be released also upon changes in plant central carbon metabolism (e.g., sucrose). Our research points to an involvement of PEP7 in regulatory processes induced by osmotic imbalances in sucrose availability.

Processing of PEP7

Recently, type II metacaspases were found to be involved in processing of the plant elicitor peptides (Moussu et al., 2020), specifically MC4 (AT1G79340) was able to process also PROPEP7 by releasing the active peptide from the C terminus of the propeptide by cleavage after arginine or lysine. The processing of PEPs was induced by calcium signals (Hander et al., 2019) and required intramolecular proteolysis even of the metacaspase. The mRNA of MC4 was shown to be cell-to-cell mobile (Thieme et al., 2015). Little is known about further requirements for processing of the PEP precursors. Our work supports that MC4 may indeed be involved in PEP7 maturation as concluded from the protoplast swelling assays of the mc4 mutant with supply of external PEP7 and/or sucrose (Figure 6).

The signaling pathway of PEP7

The expression pattern of PROPEP7 does not show overlaps with expression of the known PEP receptors PEPR1 and PEPR2 (Bartels et al., 2013), suggesting a different perception pathway for PEP7. Also, SIRK1 shows unique expression patterns compared with PEPR1 and PEPR2, especially in sink tissues of shoot and root (Winter et al., 2007). Distinct signaling pathways for SIRK1 and PEPR1/2 were also concluded here, since pep1 and pepr2 mutants remained responsive to PEP7 treatment in protoplast swelling assays (supplemental Figure 5B). However, the SIRK1 signaling pathway is linked with components of PEPR1 and PEPR2 signaling through common interaction partners. For instance, BAK1, two HERK2 paralogs, and QSK1—all classified as co-receptors (Xi et al., 2019)—are suggested as common interaction partners for all three receptors (PEPR1, PEPR2, SIRK1) (Arabidopsis Interactome Mapping Consortium, 2011; Jones et al., 2014).

One important function of co-receptors is in the stabilization of the interaction of the receptor with the ligand. The co-receptor contributes as a shape-complementary component and, by interaction with the receptor, holds the ligand in place (Sun et al., 2013a, 2013b; Santiago et al., 2013; Zhang et al., 2016b, 2016c). Our biochemical assays and the proteomics study of protein complexes and their phosphorylation, as well as the cellular and whole-plant responses, support PEP7 as a ligand to receptor kinase SIRK1. QSK1 was previously proposed to have a role in stabilization of the activated SIRK1 complex (Wu et al., 2019b; supplemental Figure 6C). It may even be speculated that QSK1 could also function as co-receptor with other receptor kinases, possibly also involving other PEPs as ligands. A ligand-stabilizing function was clearly also demonstrated for co-receptor BAK1, in which different regions of the ECD are involved in interaction with different ligands (Sun et al., 2013a, 2013b; Santiago et al., 2013).

Receptor-ligand binding and preformed complexes of SIRK1 and QSK1

Receptor kinases are known to have strong binding affinities for their ligands. Brassinolide binds to the BRI1 receptor with a Kd of 15 nM as determined by immunoprecipitation assays (Wang et al., 2001). Scatchard plots suggested the Kd of Clavata3 binding to its receptor Clavata1 at 17 nM (Ogawa et al., 2008). The binding of CLE41/44 to receptor PYX with a Kd of 33 nM was detected by using isothermal titration calorimetry (Zhang et al., 2016b). The binding of RALF to its receptor Feronia with a Kd of around 1 μM was determined by microscale thermophoresis. Interestingly, far higher binding constants were found for binding of the peptide IDA to its receptor Hasea with a Kd of 20 μM using isothermal titration calorimetry (Santiago et al., 2016). The higher Kd values, as in the case of IDA, were obtained under conditions in which the co-receptor (SERK1) was not present. In that regard, the Kd values obtained here for the binding of PEP7 to receptor SIRK1 are within this range at around 1 μM. Strikingly, by adding the co-receptor QSK1 to the binding assay, a significantly higher binding affinity for PEP7 was observed (Kd = 57.01 ± 22.3 nM). We use the approximated same amount of QSK1–ECD as the SIRK1–ECD in the binding assays, but it is not yet known if different ratios of receptor and co-receptor could result in different Kd of SIRK1 binding PEP7.

Apparently, PEP7 is produced under conditions of changed sucrose supply (Shulse et al., 2019). According to our biochemical data, the ligand PEP7 would induce the formation of the SIRK1 signaling complex. However, our attempts to visualize the recruitment of co-receptor QSK1 to ligand-activated SIRK1 using FLIM/FRET experiments revealed no clear differences between treatments with PEP7 and controls. This supports the hypothesis that receptor and co-receptor are arranged in preformed complexes within nano-domains (Bucherl et al., 2017; Gronnier et al., 2022) and that these complexes are likely stabilized or rearranged by the ligand to reveal their full activity. This was already observed for other receptor/co-receptor pairs (Caesar et al., 2011; Ladwig et al., 2015).

We found biochemically stable interactions between SIRK1 and QSK1 only after treatment with PEP7 (Figure 4). This supports the view that the complex is stabilized so that it can be detected after biochemical purification. The phosphorylation of scaffold proteins may be important in formation and rearrangement of such preformed signaling domains (Penaki et al., 2018). In any case, we here could show that binding of PEP7 to the SIRK1–QSK1 protein complex leads to the activation of a downstream signaling cascade involving activation of aquaporins and subsequent changes in water influx to the cell.

PEP7 and the response to sucrose

Our findings strongly point to a receptor/co-receptor signaling pathway of SIRK1/QSK1 that is activated by the elicitor peptide PEP7 and results in the opening of aquaporins by
Figure 7. Lateral root development in the wild type (Col-0), sirk1, pep7, and sirk1;pep7.

(A) Experimental setup and definition of stages as described (Péret et al., 2012).

(B) Percentage of the plants on different lateral root developmental stage sections, without PEP7 (gray) and with 1 μM PEP7 supply (green) in wild type and mutants. Data are shown as mean values ± SEM (n = 27–30). Small letters indicate significant differences of lateral root primordia frequencies within one genotype without PEP7 supply. Capital letters indicate significant differences of lateral root primordia frequencies within one genotype under 1 μM PEP7 supply. Asterisks mark the statistical comparisons between no PEP7 and with 1 μM PEP7 supply at each stage section (Student’s t-test, *p < 0.05 and **p < 0.01).
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SIRK1-dependent phosphorylation. The opening status of aquaporins was shown to also have effects on lateral root emergence (Péret et al., 2012) via auxin signaling, and root hydraulic conductivity is affected by aquaporin abundance, even in response to signals from the shoot (Vandeleur et al., 2014). Thus, effects of the SIRK1/QSK1/PEP7 signaling pathway on lateral root development may also be indirect through reduced cell expansion in mutants where aquaporin activation is impaired. In general, the SIRK1/QSK1/PEP7 signaling pathway could have biological functions during sucrose allocation within the plant and/or during response to osmotic stresses and drought.

We here show that PEP7 is secreted in response to external sucrose supply. Thus, activation of SIRK1 by sucrose, as studied in previous work (Wu et al., 2013, 2019a), requires sensing of sucrose status and maturation of PEP7 before the receptor SIRK1 is activated. A challenging question remains for further study as to whether external sucrose triggers the PEP7 maturation or whether sucrose acts after uptake into the cells.

MATERIALS AND METHODS

Plant materials

Arabidopsis seeds of wild type (col-0), sirk1 (SALK_125543), qsk1 (SALK_019840), pep7 (SALK_025824), pep6 (SALK_141703), and mc4 (SAIL_856_D05) were used. Double mutants sirk1;pep7 and sirk1;qsk1 were obtained by crosses of the respective single mutants. Furthermore, we overexpressed of SIRK1 (35 S::SIRK1-GFP (SAIL_856_D05) were used. Double mutants sirk1;pep7 and sirk1;qsk1 were obtained by crosses of the respective single mutants. Furthermore, we overexpressed SIRK1 in the background of sirk1 and pep7. Homozygous T-DNA insertional mutants sirk1 and double mutant sirk1;qsk1 were confirmed via PCR amplification as reported previously (Wu et al., 2019b). Mutants of pep7 and sirk1;pep7 were confirmed via PCR amplification using T-DNA border primer LbB1.3 (5’-ATTTCGGCG GATTTCGGGAAC-3’) and gene-specific primers (PEP7-RP: 5’- GGA AGGTGGCCTAGTTGGTACCAAT TCGG-3’; SIRK1-RP: 5’-TTTCCAGCATTTCCACACT-3’; SIRK1-LP: 5’-CACTAGCTTTGAGGTGTCG-3’) (supplemental Figure S6).

Hydroponic cultures, treatments, and phenotyping

Plants were germinated and grown under 16/8 day/night (22°C, 120 μE/s/m²) in ½MS medium plus 0.5% sucrose in a hydroponic culture system (Schlesier et al., 2003). Whole seedling cultures were grown in 50 ml ½MS medium with 0.5% sucrose as described (Niittyla et al., 2007). Sucrose starvation–resupply experiments were performed as described (Niittyla et al., 2007). Plant material was collected after sucrose starvation (no resupply), after sucrose treatment (1%), after PEP7 treatment (1 μM PEP7 for 5 min). Addition of glucose (33 mM), sucralose (30 mM), and trehalose (40 mM) with the same osmolarity (Vapro 5600, Wescor Biomedical Systems) as the 30 mM sucrose solution were used as control treatments in some experiments. For lateral root growth phenotypical analysis, lateral root induction was performed on 5-day-old seedlings grown on ½MS plates with or without 1 μM PEP7 by rotating the plates at 90° (Péret et al., 2012).

Protein extraction and ultrafiltration

At harvesting, tissue was flash frozen in liquid nitrogen. After breaking 300 g of frozen tissue to coarse pieces, the frozen tissue was transferred to a glass grinder (GLASS/PTFE Potter Elvehjem Tissue Grinder 30 ml), and 0.1% trifluoroacetic acid (TFA) was added to a volume of about 30 ml per sample. After tissue homogenization, the solution was filtered through four layers of gauze (Miracloth, Merck Millipore). Remaining cell debris was pelleted at 4°C and 10 000 x g for 15 min. The supernatant was used for further ultrafiltration and fractionation. The protein extract was firstly filtered through a 0.45 μm filter to get rid of any insolubilized material. Afterward, the protein extract was concentrated over a tangential flow filtration (Minimate Tangential Flow Filtration Systems, Pall Corporation) using a molecular weight cutoff of 1 kDa (i.e., retaining anything larger than 1 kDa).

Fractionation by size-exclusion chromatography

The ultrafiltrate was fractioned by fast protein liquid chromatography (NGC chromatography systems, BioRad) reverse-phase chromatography using a Bio-scale MT column (2 ml, BioRad). The column was packed with Macro-Prep t-Butyl and Methyl Hydrophobic Interaction Chromatography Media. Before fractionation, the fast protein liquid chromatography system was washed with 20% ethanol using a flow of 10 ml min⁻¹ (50% pump A/50% pump B). The system was then washed with 2.5 ml H₂O at a speed of 0.5 ml min⁻¹ for 5 min. The column was equilibrated with 6 ml 2% acetonitrile and 0.1% TFA (8.57 min × 0.7 ml min⁻¹), and a 500 μl sample was loaded to the column. A linear elution gradient was run from 100% pump A to 100% pump B. Pump A was supplied with 2% acetonitrile with 0.1% TFA and pump B with 50% acetonitrile with 0.1% TFA. The gradient was run for 28 min, of which the last 3 min were 100% pump B. The fractioned solution was collected with a fraction collector (BioFrac Fraction Collector, BioRad) in 10 2 ml fractions.

Transient expression of recombinant SIRK1 in N. benthamiana

Agrobacterium tumefaciens GV3101 strain harboring the relevant constructs and strain CS8C1 (pBinG1 vector with P19 gene) were grown in liquid LB medium (10 g⁻¹ Tryptone, 5 g⁻¹ yeast extract, and 10 g⁻¹ NaCl) at 28°C with appropriate antibiotics. For infiltration, Agrobacterium culture was adjusted to final OD 600 0.5 for GV3101 and 0.25 for strain CS8C1 in a mixture using infiltration buffer 10 mM MES-KOH (pH 5.6), 200 μM Acetylsyringone, and 10 mM MgCl₂. Three- to four-week-old N. benthamiana leaves were syringe infiltrated and harvested after 48 h. The harvested leaves were flash frozen in liquid nitrogen before protein extraction and purification. SIRK1–ECD and QSK1–ECD were expressed as C-terminal fusion with an HA and Strepl tag using the pXCS vector series in GV3101::pMP90RK strain (Witte et al., 2004).

Protein extraction and strep-tag purification

N. benthamiana leaf material harboring SIRK1–ECD or QSK1–ECD was ground in liquid nitrogen (Witte et al., 2004) and thawed in 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2% v/v PVPP, 0.5% Triton X-100, 1 mM PMSF, and 5 mM DTT. The mixture was incubated at 4°C for 2 h under constant shaking. After centrifugation (14 000 RPM, 10 min, 4°C), the supernatant was subjected into an equilibrated Strep-Tactin column (2-4013-001, IBA GmbH) according to the manufacturer’s tutorial. The Streptagged protein was eluted with 50 mM biotin, 100 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM EDTA. Eluates were passed through a Macrosep Advance Centrifugal Devices with Omega Membrane 10K (MAP010C36, Pall Corporation) to get rid of the biotin contamination in the eluate. The target protein (SIRK1–ECD and QSK1–ECD) was collected from the sample reservoir.

Microsomal membrane preparation

Microsomal membranes were enriched by differential centrifugation as described (Perl et al., 2001; Wu et al., 2013, 2019a). 1.5 g fresh tissue was homogenized in 10 ml ice-cold 330 mM mannitol, 100 mM KCl, 1 mM EDTA, 50 mM Tris-MES (pH 7.5), 5 mM DTT, 1 mM PMSF, and 0.5% v/v Protease inhibitor cocktail (P9599, Sigma-Aldrich) and phosphatase inhibitors (25 mM NaF, 1 mM Na3VO4, 1 mM benzamidin, 3 μM leupeptin). The homogenate was centrifuged for 15 min at 7500 × g at 4°C. The supernatant was centrifuged again for 75 min at 48 000 × g at 4°C, resulting in the microsomal membrane pellet.
PEP7 as the ligand for receptor kinase SIRK1

Pull-downs of GFP-tagged SIRK1
Microsomal proteins (100 μg) resuspended in 330 mM mannitol, 25 mM Tris-MES (pH 7.5), and 0.5 mM DTT was incubated with 25 μl anti-GFP agarose beads (Chromotek) (Wu et al., 2013). After incubation, beads were collected and washed twice with 500 μl 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, and 0.01% IGEPAL. For protein–protein interaction assays, the proteins were eluted from the beads with 100 μl buffer (10 mM Tris-HCl [pH 8.0], 6 μg urea, 2 M thiourea). For kinase activity assays, three more washing steps were carried out, once with 10 mM Tris-HCl (pH 7.5), 300 mM NaCl, and 0.5 mM EDTA and twice with 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.1% BSA, and 2 mM DTT.

Kinase activity assay
SIRK1–GFP fusion proteins were affinity purified over anti-GFP beads (see above). A luciferase-based kinase activity assay was performed as described (Wu et al., 2013). The agarose beads with GFP-tagged proteins were resuspended in 30 μl kinase reaction buffer with ATP and the generic kinase substrate myelin basic protein (40 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 0.1% BSA, 2 mM DTT, 10 μM ATP, 0.4 μg μl⁻¹ myelin basic protein). After incubation, 30 μl ADP-GLO Reagents (Promega) was added. Then, kinase detection reagents were added and incubated for another hour. Luminescence as a measure of the amount of ATP conversion from ADP was recorded with a luminometer (TecanM200Pro).

Binding assays of PEP7–HIS to immobilized SIRK1
The C-terminal HIS-tagged PEP7 (Pepmic) was used as bait. 20 μl HisPur Ni-NTA Magnetic Beads (Thermo Fisher Scientific) were equilibrated with 100 mM NaH₂PO₄/Na₂HPO₄, 600 mM NaCl, 0.05% Tween-20, and 30 mM imidazole (pH 8.0), and the beads were collected by a magnet. The mixture of 100 μl equilibration buffer containing final concentration 1 μM PEP7–HIS was incubated with beads for 1 h at 4°C. After incubation, the beads were washed three times with 100 mM NaH₂PO₄/Na₂HPO₄, 600 mM NaCl, 0.05% Tween-20 Detergent, and 50 mM imidazole (pH 8.0). SIRK1 full-length GFP protein extract from N. benthamiana (50 μg), SIRK1 full-length GFP from microsomal fraction of SIRK1–GFP overexpression line (50 μg), or purified SIRK1ECD–HA–StrepII (20 μg) was added as prey protein. Formed complexes were enriched on a magnet and eluted with 40 μl 100 mM NaH₂PO₄/Na₂HPO₄, 600 mM NaCl, and 250 mM imidazole (pH 8.0). Ten μg of the eluate were vacuum dried and stored for further use.

Binding assays of SIRK1 to immobilized PEP7–HIS
SIRK1 full-length GFP protein obtained from membrane fraction and SIRK1–ECD–HA–StrepII protein obtained from transient expression in N. benthamiana were used as bait to capture PEP7. For binding assay of SIRK1 full-length GFP with PEP7, 100 μg SIRK1–GFP protein extract was prebound to GFP-Traps-MA beads (Chromotek) for 2 h under rotation at 4°C. The slurry was separated with a magnet, the supernatant was discarded, and the beads were washed three times with 500 μl 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.5 mM EDTA. Then, prey PEP7 (1 μM) was added to the beads for 1 h at 4°C. Beads were washed three more times, and proteins were eluted with 60 μl 6 M urea and 2 M thiourea. For the binding assay of SIRK1–ECD to PEP7, 200 μg SIRK1–ECD protein extracted from N. benthamiana was prebound to Strep-tagII beads (Thermo Fisher Scientific) for 2 h under rotation at 4°C. The beads were collected by centrifugation and washed three times in 100 mM HEPES (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 0.05% Triton X-100, and 2 mM DTT. PEP7 was added, and bead slurry was eluted with 100 μl 100 mM HEPES (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 0.05% Triton X-100, 2 mM DTT, and 10 mM biotin (Witte et al., 2004). Ten μg of the eluates were vacuum dried and stored for further use.

Competitive binding assay
All buffers used were identical to those described for the PEP7–HIS pull-down assay described above. PEP7–HIS (final concentration 1 μM) was firstly immobilized on 20 μl HisPur Ni-NTA Magnetic Beads, and subsequently 100 μg purified protein SIRK1ECD–HA–StrepII was bound to the immobilized PEP7–HIS. Different concentrations of untagged PEP7 (100 μM ddH₂O containing 0, 0.2, 0.5, 1, 1.5, 2, or 3 μM PEP7) was added to elute the SIRK1–ECD from the immobilized PEP7–HIS. The remaining SIRK1–ECD that was not eluted by PEP7 was then eluted with HIS–beads elution buffer (100 mM NaH₂PO₄/Na₂HPO₄, 600 mM NaCl, 250 mM imidazole).

Microscale thermophoresis
A buffer exchange column was firstly applied to purified SIRK1–ECD protein to remove any unfavorable reagents. Concentration of purified ECDs was measured by BCA assay. The RED-NHS 2nd generation amine reactive dye (MO-L011, NanoTemper Technologies) was used to label the purified SIRK1–ECD (2 μM) with a 5:1 dye:protein ratio for 30 min at room temperature in the dark. Column B was employed to remove the excess of dye and produced the labeled protein in PBST buffer (20 mM NaH₂PO₄/Na₂HPO₄, 100 mM NaCl [pH 7.5], 0.05% (v/v) Tween 20). Labeled SIRK1–ECD was adjusted to final concentration of about 20 nM in PBST and then titrated with serial (1:1) dilutions of PEP7 (starting at 0.5 mM), PEP6, or PEP4 (all peptides obtained from Pepmic). The complex was established to allow for 10 min at room temperature before the samples were loaded into the capillaries (MO-K025, NanoTemper Technologies). Binding was detected by a Monolith NT.115 instrument (NanoTemper Technologies) at 24°C with 80% excitation power and 40% MST power, and a dose–response curve was generated. QSK1–ECD with a final concentration 20 nM was applied for triple binding affinity estimation. All experiments were repeated at least three times. Raw data were analyzed by MO Affinity Analysis software (v.2.2.4) and OriginPro software.

Protoplast swelling assays
Surface-sterilized seeds after vernalization for 48 h were germinated and grown vertically on sucrose-starved medium (½MS solid medium with 0.02% sucrose). Approximately 30 seedlings were cut into small pieces, and cell walls were digested in 300 mM mannitol, 10 mM MES-KOH (pH 5.8), 10 mM CaCl₂, 10 mM KCl containing 1% (w/v) cellulase Onozuka R10 (Duchefa), and 1% (w/v) macerozyme R10 (Duchefa). After 3 h of gentle shaking in the dark at room temperature, the protoplasts were prepared via a 50 μm nylon mesh filter. Protoplasts were then enriched by centrifugation at 80 x g at 4°C for 10 min and washed three times with ice-cold wash buffer (300 mM mannitol, 10 mM MES-KOH [pH 5.8], 10 mM CaCl₂, 10 mM KCl). The protoplasts were finally resuspended in 150 μl wash buffer and stored in the dark on ice for at least 30 min before an experiment was started. In principle, protoplast swelling experiments were performed as described earlier (Sommer et al., 2007; Wu et al., 2013). Approximately 20 μl of the protoplast suspension were pipetted to 200 μl high osmolarity solution (supplemental Table 4) in a perfusion chamber mounted on the stage of an inverted microscope (DMi8, Leica). Protoplasts were allowed to settle down for 5 min. The chamber was perfused with 3 ml of high osmolarity solution to select protoplasts sticking well to the glass bottom of the chamber, then the chamber was perfused with low osmolarity solution. Solution change in the chamber took about 15 s. A video was recorded for 5 min to capture the dynamic change of the protoplasts with the time interval of 3 s. Buffers with 30 mM sucrose and/or 1 μM PEP7 are described in supplemental Table 4. The diameter of the protoplasts was measured directly by using the Leica software LAS X Core, followed by the calculation of volume and surface area at each time point. A regression curve was fitted to the volume change, and the maximal slope was obtained from the first derivative of the curve. The maximal water flux density that corresponds to aquaporin activity thus was determined by the maximal slope divided by the protoplast surface area at the corresponding time point.
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Curve fitting and derivative calculation were performed with OriginPro software.

**FLIM–FRET analysis**

The coding sequences of SIRK1 and QSK1 were expressed as C-terminal fluorophore fusions in 2in1 vectors, namely pFRETcg-2in1-CC (Hecker et al., 2015). To obtain GFF-donor-only controls, a coding sequence of gentamycin fused to mCherry was used. The binary vectors and p19 as gene silencing suppressor were introduced into GV3101 and infiltrated into 3- to 4-week-old N. benthamiana leaves. After infiltration, the plants were put in darkness for 2 days. Then, 1 μM PEP7 or water was added to the system by infiltration into the leaves right before the measurement. In some experiments, the protease inhibitor leupeptin was infiltrated directly with the constructs at a concentration of 10 μM. The measurements were performed for a maximum time of 10 min based on a modified protocol (Glockner et al., 2015) with a SP8 confocal laser scanning microscope (Leica Microsystems) equipped with Leica Microsystems Application Suite software and a FastFLIM upgrade from PicoQuant consisting of Sepia Multichannel Picosecond Diode Laser, PicoQuant Timeharp 260, TCSPC Module, and Picosecond Event Timer (Picoquant). Imaging was done by using a 63x/1.20 water-immersion objective and focusing on the plasma membrane of the abaxial epidermal cells. The presence of the fluorophores was detected by excitation with 488 or 561 nm and 500–500 or 600–650 nm detection range for GFP or mCherry, respectively. Co-localization was demonstrated by reading out signal intensities over the plasma membrane. GFP FLT in nanoseconds of either donor-only-expressing cells or cells expressing the indicated combinations was measured with a pulsed laser as an excitation light source of 470 nm and a repetition rate of 40 MHz. The acquisition was performed until 500 photons in the brightest pixel were reached at a resolution of 256 × 256 pixels. For data processing, a region of interest at the plasma membrane was defined in the SymPhoTime software, and bi-exponential curve fitting as well as correction for the instrument response function was applied. A total range of 23 ns was evaluated. Statistical analysis was carried out with JMP 14 and OriginPro software.

**Tryptsin digestion and phosphopeptide enrichment**

Protein pellets were solubilized in 10 mM Tris–HCl (pH 8.0), 6 M urea, and 2 M thiourea and by subjecting the protein solution to ultrasonic bath for 10 min. In-solution trypsin digestion was carried out as described (Wu et al., 2017). Digested peptides were resuspended in 1 M glycolic acid, 80% v/v Acetonitrile, and 8% v/v TFA 80%. Phosphopeptides were enriched by TiO2 beads (Titansphere, 5 mg beads) at 80% v/v Acetonitrile, and 6% v/v TFA 80%. Phosphopeptides were eluted from a LC-MS system and Sandra Herold for her assistance during the revision process. Research in our laboratories was supported by the German Research Foundation (DFG) with grants to W.X.S. (SCHU1533/10-1 and SCHU1533/11-1) and to K.H. (CRC 1101-D02, HA 2146/22-2, and HA 2146/23-1) and a grant for scientific equipment (INST 37/819-1 FUGG).

**SUPPLEMENTAL INFORMATION**

Supplemental information is available at Molecular Plant Online.

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**AUTHOR CONTRIBUTIONS**

J.W. and L.X., performed experiments, analyzed data, and wrote manuscript; X.N.W., S.K., L.R., T.N., and J.W., performed experiments and analyzed data; K.H. and W.X.S., analyzed data and wrote manuscript.

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