Two phylogenetically unrelated peptide-receptor modules jointly regulate lateral root initiation via a partially shared signaling pathway in Arabidopsis thaliana

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Summary

- Peptide-receptor signaling is an important system for intercellular communication, regulating many developmental processes. A single process can be controlled by several distinct signaling peptides. However, since peptide-receptor modules are usually studied separately, their mechanistic interactions remain largely unexplored.
- Two phylogenetically unrelated peptide-receptor modules, GLV6/GLV10-RGI and TOLS2/PIP2-RLK7, independently described as inhibitors of lateral root initiation, show striking similarities between their expression patterns and gain- and loss-of-function phenotypes, suggesting a common function during lateral root spacing and initiation.
- The GLV6/GLV10-RGI and TOLS2/PIP2-RLK7 modules trigger similar transcriptional changes, likely in part via WRKY transcription factors. Their overlapping set of response genes includes PUCHI and PLT5, both required for the effect of GLV6/10, as well as TOLS2, on lateral root initiation. Furthermore, both modules require the activity of MPK6 and can independently trigger MPK3/MPK6 phosphorylation.
- The GLV6/10 and TOLS2/PIP2 signaling pathways seem to converge in the activation of MPK3/MPK6, leading to the induction of a similar transcriptional response in the same target cells, thereby regulating lateral root initiation through a (partially) common mechanism. Convergence of signaling pathways downstream of phylogenetically unrelated peptide-receptor modules adds an additional, and hitherto unrecognized, level of complexity to intercellular communication networks in plants.

Introduction

Coordination of growth and development, defense responses, and physiological processes in multicellular organisms relies on intricate networks of intercellular communication. One of the ways in which plant cells transmit signals to one another is via the exchange of signaling peptides that are secreted into the apoplast and perceived by target cells via transmembrane receptors, called receptor-like kinases (RLKs). The Arabidopsis genome encodes thousands of secreted peptides (Lease & Walker, 2006; Ghorbani et al., 2015; Hazarika et al., 2017) and over 600 RLKs (Shiu & Bleecker, 2001). These peptides and receptors are typically classified into phylogenetic groups or families based on sequence similarities. To date only a fraction of these peptides and receptors have been studied, but nonetheless, many peptide-receptor modules have been discovered and their involvement in a myriad of processes has been revealed. Multiple secreted signaling peptides and receptors, from a range of different families, are known to control several aspects of lateral root (LR) development, including initiation and spacing, primordium formation, spatial accommodation in the surrounding tissues, and elongation (Jourquin et al., 2020). Furthermore, some signaling peptides from different phylogenetic groups were found to control the same developmental steps in the LR formation process, but as these peptides are usually studied separately, it remains unclear whether they affect each other’s activity.

In Arabidopsis, LRs arise from subsets of xylem-pole pericycle cells, called LR founder cells (LRFCs) (Dubrovsky et al., 2000; Beeckman et al., 2001; Parizot et al., 2008). These cells are primed for LR formation in the elongation zone by periodic pulses of auxin signaling activity (De Smet et al., 2007; Moreno-Risueno et al., 2010; Xuan et al., 2015). Lateral root primordium formation is initiated in the maturation zone, where pairs of abutting LRFCs undergo another auxin response, resulting in nuclear migration towards the common cell wall, followed by an asymmetric anticlinal cell division, yielding two short central and two longer flanking cells, a configuration that is essential for further LR primordium morphogenesis (Malamy & Benfey, 1997;
De Smet et al., 2007; Dubrovsky et al., 2008; De Rybel et al., 2010; Goh et al., 2012). Among the signaling peptides currently known to regulate LR initiation are several members of two distinct phylogenetic groups, called the GOLVEN/ROOT MERISTEM GROWTH FACTOR/CLE-LIKE family (hereafter referred to as GLV), and the PAMP-INDUCED SECRETED PEPTIDE (PIP) family. Two auxin inducible after referred to as GLV), and the PAMP-INDUCED SECRETED PEPTIDE (PIP) family. Two auxin inducible peptides, GLV6 and GLV10, are expressed in LRFCs and throughout the shoot, and are known to regulate LR initiation are several members of two distinct phylogenetic groups, called the GOLVEN/ROOT MERISTEM GROWTH FACTOR/CLE-LIKE family (hereafter referred to as GLV), and the PAMP-INDUCED SECRETED PEPTIDE (PIP) family. Two auxin inducible peptides, GLV6 and GLV10, are expressed in LRFCs and throughout the shoot, and are known to regulate LR primordium development (Fernandez et al., 2015, 2020). During LR initiation, GLV6 and GLV10 peptides are perceived by ROOT GROWTH FACTOR INSENSITIVE (RGI) 1, RGI4 and RGI5 receptors (Ou et al., 2016; Shinohara et al., 2016; Song et al., 2016; Fernandez et al., 2020). RGI1 and RGI5 were also shown to be expressed in the pericycle and during LR formation (Fernandez et al., 2020). Double glv6glv10 mutants show increased total (nonemerged + emerged) LR primordium densities, which often appear clustered or paired, rather than regularly spaced (Fernandez et al., 2020). In contrast, overexpression of GLV6 (GLV6DE) or treatment with synthetic GLV6 peptides (GLV6p) disrupts the essential asymmetry of LRFC divisions, thereby inhibiting organogenesis, and resulting in excessive anticalinal pericycle cell divisions along the primary root (Fernandez et al., 2013, 2015). MITOGEN-ACTIVATED PROTEIN KINASE 6 (MPK6) was identified as a downstream component of the GLV6/10 signaling cascade. Accordingly, mpk6 mutants are largely resistant to the effects of GLV6DE and show increased densities and aberrant spacing of LR primordia, a phenotype that is similar to, but stronger than, that observed in glv6glv10 mutants (López-Bucio et al., 2014; Fernandez et al., 2020). The currently available data thus indicate that GLV6/10-RGI signaling serves to attenuate asymmetric LRFC divisions via a phosphorylation cascade involving MPK6, thereby preventing excessive LR initiation in the neighborhood of pre-existing initiation sites. GLV-RGI signaling was previously found to also regulate root apical meristem maintenance through induction of PLETHORA (PLT) 1 and PLT2 transcription factors (TFs) (Matsumaki et al., 2010; Zhou et al., 2010; Meng et al., 2012; Lu et al., 2020; Shao et al., 2020; Yamada et al., 2020). However, apart from the involvement of MPK6, it is not known how the GLV6/10-RGI module regulates LR spacing and initiation, so downstream targets of the pathway have yet to be discovered. Interestingly, many similarities exist between the activity of GLV6/10 and two members of the PIP peptide family, called TARGET OF LBD SIXTEEN 2/PIP-LIKE3 (TOLS2/PIPL3) and PIP2. As its name suggests, TOLS2 was identified as a direct transcriptional target of LATERAL ORGAN BOUNDARIES-DOMAIN 16 (LBD16) (Goh et al., 2019; Toyokura et al., 2019), downstream of the IAA14-ARF7/ARF19 auxin signaling module, which is of major importance for LR initiation (Fukaki et al., 2002, 2005; Okushima et al., 2005). TOLS2 and PIP2 are transcribed in LRFCs and throughout the course of LR organogenesis. The mature TOLS2 peptide is perceived by RECEPTOR-LIKE KINASE 7 (RLK7), which is preferentially expressed in the pericycle, LRFCs, and cells flanking developing primordia (Toyokura et al., 2019). Disruption of TOLS2-RLK7 signaling in tols2pip2 and rlk7 mutants, results in increased densities of auxin-responsive DR5: LUCIFERASE reporter expressing spots along the primary root, which are typically associated with LRFCs or LR primordia. Additionally, the DR5 spots in rlk7 or tols2pip2 mutants often appear in close proximity. These changes in DR5 spot density and distribution were not reported to result in changes in LR density, suggesting that additional mechanisms prevent ectopic LR initiation, but are nonetheless reminiscent of the increased LR primordium density and clustering observed in glv6glv10 mutants. Furthermore, treatment with synthetic TOLS2 peptide (TOLS2p), as well as TOLS2 overexpression, result in a strong reduction in total LR primordium density, indicating that LR initiation is also inhibited by this peptide. PUCHI, a known regulator of LR initiation and spacing, as well as primordium morphogenesis, was identified as a downstream transcriptional target of the TOLS2-RLK7 module (Hirot a et al., 2007; Kang et al., 2013; Goh et al., 2019; Trinh et al., 2019). As illustrated by the expression patterns and gain- and loss-of-function phenotypes described earlier, many parallels can be drawn between the roles of the GLV6/10-RGI and TOLS2-RLK7 signaling modules during LR initiation. Accordingly, both peptide-receptor modules were proposed to serve as inhibitors that prevent LR initiation events from occurring in close proximity, thereby ensuring proper LR spacing. These similarities prompted us to study GLV6/10 and TOLS2 signaling simultaneously, and investigate the functional relationship between these phylogenetically distinct peptide-receptor modules. This led us to identify new components of the GLV6/10 and TOLS2 induced signaling pathways and yielded new insights into the complexity of signaling peptide-mediated intercellular communication networks.

Materials and Methods

Plant material and growth conditions

Surface sterilized seeds were either sown directly on solid ½ Murashige & Skoog (MS) medium (2.154 g l⁻¹ MS (Duchefa Biochemie, Haarlem, the Netherlands), 1% sucrose, 0.1 g l⁻¹ Myo-inositol (Sigma-Aldrich, St Louis, MO, USA), 0.5 g l⁻¹ MES (Duchefa Biochemie), 0.8% Plant Tissue Culture Agar (Lab M, Heywood, UK)) or on nylon membranes with a 20 µm mesh size (Prosep, Zaventem, Belgium) placed on top of this medium. After 48 h of stratification at 4°C, plates were incubated at 21°C in continuous light. The following transgenic and mutant Arabidopsis lines were described elsewhere: GLV6:NLS-GFP-GUS/ Wave131Y (Geldner et al., 2009; Fernandez et al., 2015), GLV10:NLS-GFP-GUS (Fernandez et al., 2013), TOLS2:NLS-TdTomato and RLK7:NLS-TdTomato (Toyokura et al., 2019), RGI1:NLS-GFP, RGI5:NLS-GFP, iGLV6, rgi1rgi5/iGLV6, rgi1rgi4rgi5/iGLV6, mpk6-3AGLV6 and glv6glv10 (Fernandez et al., 2020), gLBD16:SRDX and lbd16lbd18lbd29 (Goh et al., 2012), mpk6-4 (López-Bucio et al., 2014), PLT5:GUS (Du & Scheres, 2017), LBD16:GUS (Okushima et al., 2007), PUCHI:GUS and puchi-1 (Hirot a et al., 2007), p35S:WRKY23-GR (Prasad et al., 2011), WRKY23:GUS and WRKY23:GFP (Grunewald et al., 2008), 35S:WRKY23-GR (Grunewald et al., 2012). rlk7-3 (SALK_120595) and rlk7-4 (SALK_083112) mutants were

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obtained for NASC. To generate the **WRKY23:**WRKY23-GFP line, a 2000 bp WRKY23 promoter fragment was cloned from genomic DNA using primers containing Golden Gate compatible A and G sequences and inserted into the pEN-L4-A-G-R1 plasmid (Houbarta et al., 2018), resulting in the pEN-L4-WRKY23pro-R1 entry clone. This plasmid was used in a multi-site Gateway reaction together with pEN-L1-gWRKY23-L2 (Grunewald et al., 2012), pEN-R2-GFP-L3 (Karimi et al., 2007), and pKm34GW (Karimi et al., 2005). The resulting expression vector was transferred to *Agrobacterium tumefaciens* strain C58C1, for floral dip transformation of *Arabidopsis thaliana* of the Columbia (Col-0) ecotype. Synthetic GLV10p (DY(SO3)) PKPSTRPPRH) and TOLS2p (ASGP(OH)SRGGAGH) were obtained from GenScript (Piscataway, NJ, USA) (> 70% purity).

**Reverse transcription quantitative polymerase chain reaction experiments**

To compare auxin inducibility of *GLV6* and *GLV10* expression between Col-0 and gLBD16-SRDX or lbd16lbd18lbd33 lines, 7-d old seedlings grown on nylon membranes were transferred to medium containing 10 µM 1-naphthalene acetic acid (NAA; Duchefa Biochemie) or an equivalent volume of dimethylsulfoxide (DMSO). Whole roots were sampled at the indicated time points after NAA or mock treatment. Roots of estradiol inducible *iGLV6*, *rgi1rgi4rgi5/iGLV6* and *mpk6-3/iGLV6* seedlings were sampled 12 d after germination (DAG) on solid ½MS medium supplemented with 2 µM estradiol (Sigma-Aldrich) or DMSO. Peptide treated roots were sampled from 7 DAG seedlings that were incubated for 24 h in liquid ½MS, supplemented with 1 µM GLV10p or TOLS2p. Total RNA was isolated using the ReliaPrep™ RNA Miniprep System (Promega, Madison, WI, USA). Complementary DNA (cDNA) was synthesized with the qScript® cDNA SuperMix (Quantabio, Beverly, MA, USA). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments were performed using SYBR® Green Mix (Roche, Basel, Switzerland) in a LightCycler® 480 (Roche). *CKA2* and *CDKAI* were included as housekeeping genes (all primer sequences; Supporting Information Table S1).

**Expression pattern analyses**

To assess the effects of GLV10p and TOLS2p treatments on *PLT5*, *LBD16* and *PUCHI* expression patterns, 3 DAG seedlings expressing a GUS (β-glucuronidase) marker were transferred to untreated medium or medium containing 200 nM GLV10p or TOLS2p and collected for GUS staining at 9 DAG. GUS stainings were performed as previously described after fixation in 90% acetone at 4°C (Beeckman & Engler, 1994). Images were taken with a BX53 DIC microscope (Olympus, Tokyo, Japan) or a VXH-7000 digital microscope (Keyence, Mechenlen, Belgium). Confocal images were taken using an LSM710 (Zeiss, Jena, Germany) or SP8 (Leica, Wetzlar, Germany) microscope. Green fluorescent protein (GFP) was excited at 488 nm and acquired at 495–540 nm or at 495–516 nm when combined with yellow fluorescent protein (YFP). tdTomato was excited at 561 nm and acquired at 570–690 nm. YFP was excited at 514 nm and acquired at 519–554 nm. Propidium iodide was excited at 561 nm and acquired at 570–700 nm.

**Phenotypic analyses**

Emerged LRs were counted using a stereo microscope on 12 DAG seedlings. Total LR (nonemerged + emerged) and clustered LR (within 500 µm) numbers were quantified in 9 DAG seedlings using an Olympus BX53 DIC microscope after clearing the roots as described previously (Malamy & Benfey, 1997). Scans of roots were used to measure primary root lengths with Fiji (Schindelin et al., 2012; Schneider et al., 2012) and densities were calculated by dividing emerged or total LR numbers by primary root lengths.

Gravistimulation induced LR primordia were analyzed in 4 DAG seedlings grown on nylon membranes that were turned 90° counterclockwise. The effects of estradiol-induced *GLV6* in wild-type and mutant backgrounds was assessed by transferring 8 h gravistimulated seedlings to plates containing 2 µM estradiol or DMSO. LR primordia were analyzed at various time points after gravistimulation by mounting seedlings on slides in a chloral hydrate solution for 30 min and analyzing the root bends with an Olympus BX53 DIC microscope.

The effect of peptide treatments on the pericycle of Col-0 and gLBD16-SRDX roots was assessed using 9 DAG seedlings grown on untreated or 100 nM GLV10p or TOLS2p containing medium. Similarly, 9 DAG 35S:WRKY23-GR seedlings grown on 10 µM DEX or DMSO containing medium were used. Seedlings were fixed and cleared using the ClearSee protocol (Kurihara et al., 2015; Ursache et al., 2018) and cell walls were stained using Calcofluor White (Sigma-Aldrich), excited at 405 nm and acquired at 430–470 nm using a Zeiss LSM710 microscope.

**Protein extraction and western blotting**

For MPK3/6 phosphorylation assays, 6 DAG seedlings were transferred to liquid ½MS medium and left to acclimatize for 1 h. Next, 1 µM of GLV10p or TOLS2p was added to the medium and seedlings were collected in liquid nitrogen at the indicated time points after treatment. Plant material was ground and 2 µl of extraction buffer (50 mM Tris–HCl with pH 7.5, 100 mM NaCl, 10% (v/v) glycerol, 0.5% (v/v) Triton x-100, 1 mM diethiothreitol, 1 mM ethylene-diamine-tetraacetic acid, 1 mM phenylmethylsulfonylfluoride, 1× cOmplete™ Ultra Tablet (Roche) per 50 ml and 1% (v/v) Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich)) was added per milligram of tissue. Samples were centrifuged at 13 000 g for 30 min. Protein concentrations in the supernatant were quantified using the Qubit protein assay kit (ThermoFisher, Waltham, MA, USA). Equal amounts of protein (20 µg) were separated in 7.5% Mini-Protein® TGXTM precast gels (Bio-Rad, Hercules, CA, USA) and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 3% bovine serum albumin (BSA), membranes were incubated overnight with anti-phospho-p44/42 antibodies (Cell Signaling Technology, Danvers, MA, USA; 1:2500). Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (GE Healthcare, Chicago, IL, USA;
1 : 10 000) were visualized with SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher). Antibodies were stripped in a 1 : 1 (v/v) 10% SDS and 100 mM glycine-HCl (pH 2.5) solution and incubated overnight with primary anti-MPK6 (Sigma-Aldrich; 1 : 8000) and anti-MPK3 (Sigma-Aldrich; 1 : 2500) antibodies. HRP-conjugated anti-rabbit secondary antibodies were visualized using Western Lightning Plus ECL (PerkinElmer, Waltham, MA, USA). Blots were imaged using a ChemiDoc XRS+ imaging system (Bio-Rad).

RNA-sequencing and bioinformatic analysis

Seedlings were grown on nylon membranes for 4 d, after which they were gravistimulated by turning plates 90° counterclockwise. Eight hours after gravistimulation, seedlings were transferred to ½MS, containing 2 μM estradiol or an equivalent volume of DMSO. Then, 3 and 6 h after transfer, c. 400 root bends were dissected and collected per sample. Four biological replicates were obtained. Total RNA was extracted using the RNaseasy Plant Mini Kit (Qiagen, Hilden, Germany). RNA concentration and purity were determined using the Nanodrop™ 1000 (Nanodrop Technologies, Wilmington, DE, USA) and RNA integrity was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Sequencing-libraries were prepared using the TruSeq® Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA, USA), pooled, and sequenced on the NextSeq 500 system (Illumina; High Output, 75 bp, Single Reads). Raw sequencing data was analyzed using the GALAXY platform (Afgan et al., 2018). Quality control and trimming were performed using FASTQC (Babraham Bioinformatics, Cambridge, UK) and TRIMMOMATIC (Bolger et al., 2014), respectively. Reads were mapped to the Arabidopsis exome as annotated in the TAIR10 genome assembly and transcript abundances were quantified using the Salmon method (Patro et al., 2017). Differential gene expression analysis between corresponding treated and untreated samples was performed in R (R Core Team, 2020) using DESeq2 (Love et al., 2014) with batch-effect correction. Gene ontology (GO) terms for biological processes, as defined by the PANTHER classification system (Mi et al., 2019), were performed via a Fisher's Exact test with Bonferroni correction using the PANTHER GO tool (www.pantherdb.org). TF binding site (TFBS) enrichment analyses were performed on the 1000 bp upstream regions of GLV6 and TOLS2 response genes using both the PScan tool (Zambelli et al., 2009) against the JASPAR2020 TFBs database (Fornes et al., 2020) and the TF2Network tool (Kulkarni et al., 2018).

Results

GLV6/10 and TOLS2 peptides have similar effects on pericycle cells and are transcriptionally regulated via LBD16

Although GLV6/10 and TOLS2/PIP2 peptides, as well as their receptors, have no apparent phylogenetic relationship, their expression patterns and mutant phenotypes show many similarities, suggesting their activities during LR initiation might be related. In agreement with previous reports, roots treated with synthetic GLV6p, GLV10p or TOLS2p showed reduced emerged- and total LR densities, consistent with their proposed function as inhibitors of LR initiation (Fig. 1a) (Fernandez et al., 2015, 2020; Toyokura et al., 2019). Interestingly, microscopic analysis of GLV10p or TOLS2p-treated roots revealed similar aberrant anticlinal cell divisions in the pericycle along the primary root (Fig. 1b), reminiscent of the ectopic cell divisions previously described upon GLV6OE (Fernandez et al., 2015).

We then comparatively analyzed expression patterns of GLV6, GLV10 and TOLS2 during LR formation. Confocal imaging of crosses between TOLS2-NLS-TdTomato and GLV6-NLS-GFP-GUS/Wave131Y or GLV10-NLS-GFP-GUS reporter lines showed that all three peptide-encoding genes start to be expressed in LRFCs before their first anticlinal cell division, and continue to be expressed throughout LR development, mainly in the primordium center (Fig. 1c). Expression can occasionally also be observed in endodermis and cortex cells overlying a developing primordium, in which GLV6/10 and TOLS2 transcription also coincide. Expression of TOLS2 was previously shown to be regulated by the auxin-IAA14-ARF7/ARF19-LBD16 pathway (Toyokura et al., 2019). Similarly, transcription of GLV6 and GLV10 was shown to be auxin inducible in an ARF7/ARF19 dependent manner (Fernandez et al., 2020). Additionally, published micro-array datasets showed an ARF7/ARF19 and IAA14 quasipoisson model in case of over- or under-dispersion) was fitted to the LR or cluster counts with, where applicable, treatment and/or genotype and/or log_{10}(concentration) and their interactions as fixed effects. A log-link function was applied, and log-transformed primary root lengths were used as an offset variable. Contrasts and post hoc interaction analyses were set up using the EMMEANS or the PHIA package (De Rosario-Martinez, 2015; Lenth, 2021) and Dunnett, Tukey or Bonferroni corrections were implemented where applicable. For the statistical analysis of RT-qPCR data, log2 fold changes (FCs) in expression levels between treatment and mock conditions were calculated for each genotype, and two-tailed Student’s t-tests were performed to compare treatment effects between genotypes. To determine statistical differences between the proportions of mock- or estradiol-treated seedlings containing a LR primordium of certain developmental stages in gravistimulation induced root bends, Chi-square tests were performed. For pairwise comparisons of transcriptomic datasets, the probability of finding the observed number of overlapping genes (within the total number of genes for which reads/hits were found in both experiments) was determined using hypergeometric tests.

Gene ontology and transcription factor binding site enrichment analyses

Overrepresented gene ontology (GO) terms for biological processes, as defined by the PANTHER classification system (Mi et al., 2019), were performed via a Fisher’s Exact test with Bonferroni correction using the PANTHER GO tool (www.pantherdb.org). TF binding site (TFBS) enrichment analyses were performed on the 1000 bp upstream regions of GLV6 and TOLS2 response genes using both the PScan tool (Zambelli et al., 2009) against the JASPAR2020 TFBs database (Fornes et al., 2020) and the TF2Network tool (Kulkarni et al., 2018).

Statistical analyses

Statistical analyses were performed in R (R Core Team, 2020). For LR- and clustered LR density data, a Poisson model (or a
dependent regulation of GLV6 (Fig. S1) (Okushima et al., 2005; Vanneste et al., 2005). Given their matching expression patterns with TOLS2, we investigated whether GLV6 and GLV10 are also transcriptionally regulated via LBD16. Indeed we found that the NAA induced upregulation of GLV6 and GLV10 is attenuated in the roots of dominant negative gLBD16-SRDX seedlings, as well as in lbd16lbd18lbd33 triple mutants (Figs 1d, S2).

Analysis of RGI1:NLS-GFPxRLK7:NLS-TdTomato and RGI5: NLS-GFPxRLK7:NLS-TdTomato crosses showed that expression patterns of the GLV6/10 and TOLS2/PIP2 receptors during LR

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GLV6/10 and TOLS2 peptides have similar effects on pericycle cells and are transcriptionally regulated via LBD16. (a) Nonemerged, Emerged and Total lateral root (LR) densities of 12-d old wild-type Arabidopsis seedlings treated or not with 100 nM GLV10p or TOLS2p. Individual datapoints and mean ± SD are shown. n = 15. Statistical significance was determined via Poisson regression with Dunnett’s correction. (b) Confocal images of longitudinally sections through the maturation zone of 9-d old wild-type Arabidopsis roots treated or not with 100 nM of GLV10p or TOLS2p. Anticlinal divisions within a single pericycle cell file are indicated with yellow arrowheads. The different tissue layers are indicated; v, vasculature; p, pericycle; e, endodermis; c, cortex; ep, epidermis. Cell walls were stained with Calcofluor White. Bar, 30 μm. (c) Confocal images of different LR developmental stages in GLV6-NLS-GFP-GUS/Wave131Y X TOLS2:NLS-TdTomato (upper row), and GLV10-NLS-GFP-GUS X TOLS2:NLS-TdTomato (bottom row) transcriptional reporter lines in Arabidopsis thaliana. Green fluorescent protein (GFP) and TdTomato signals are shown in green and magenta respectively. LRFcs, lateral root founder cells. White arrowheads indicate instances of overlapping signals observed in cortex or endodermis cells overlying a developing primordium. Yellow fluorescent protein (YFP) marked plasma membranes are shown in gray. Bar, 30 μm. (d) Log2 fold changes in GLV6 and GLV10 expression levels at different time points after 10 μM NAA (1-naphthaleneacetic acid) vs mock treatment in wild-type and gLBD16-SRDX Arabidopsis roots. Individual datapoints and mean ± SD are shown. n = 3. Statistical significance was determined using Student’s t-tests. (e) Confocal images of different LR developmental stages in RGI1-NLS-GFP X RLK7:NLS-TdTomato (upper row) and RGI5-NLS-GFP X RLK7-NLS-TdTomato (bottom row) reporter lines in Arabidopsis thaliana. GFP and TdTomato signals are shown in green and magenta respectively. White arrowheads indicate instances of overlapping signals observed in cortex or endodermis cells overlying a developing primordium. Bar, 30 μm.

initiation and organogenesis also overlap (Fig. 1e). RLK7 is transcription more broadly throughout the stele, but both RGI1 and RLK7 are expressed in xylem-pole pericycle cells and all three receptors are transcribed in LRFcs, as well as in stage I primordia after the first anticlinal cell division. During later developmental stages, expression of both RGI1 and RLK7 is reduced in the central part of the primordium but clearly maintained in primordium margins and flanking pericycle cells, while transcription of RGI5 is strongly reduced throughout the whole primordium and pericycle. Thus, there seems to be a large overlap between the expression patterns of these receptors, especially in LRFcs and young LR primordia. Additionally, RLK7 is occasionally transcribed in cells overlying a developing primordium, which also often coincides with expression of RGI1 or RGI5. However, in contrast to RLK7, RGI1 and RGI5 are also expressed in the tips of a newly formed LR when they are about to emerge, in agreement with their role in RAM maintenance.

In combination with previous reports, our data show that loss-and gain-of-function of both GLV6/10-RGI and TOLS2-RLK7 modules affect LR density, LR spacing and pericycle cell divisions in a similar manner, and that these peptides and receptors have overlapping spatiotemporal expression patterns. The considerable overlap in the cells in which these peptides are produced and perceived, their common transcriptional regulation via LBD16, and their matching phenotypic effects, strengthen the hypothesis that GLV6/10 and TOLS2 signaling modules have a similar function during LR initiation. Formation of LRs can be triggered by gravity-induced root bending, so we used gravistimulation to synchronize LR initiation in large numbers of seedlings (Lucas et al., 2008; Péret et al., 2012; Voß et al., 2015). Under our experimental conditions, LR initiation events (i.e. nuclear migration and the first anticlinal cell division of LRFcs) were strongly enriched in root bends of seedlings between 11 and 15 h after gravistimulation (Fig. S3). To induce the GLV pathway, we used the recently reported estradiol-inducible GLV6OE line (iGLV6) (Fernandez et al., 2020). Exposing iGLV6 seedlings to estradiol, 8 h after gravistimulation, resulted in the inhibition of LR organogenesis in over 80% of seedlings, an effect that was almost completely lost in rgi1rgi5/iGLV6 and mpk6/iGLV6 lines (Figs 2a, S4) (Fernandez et al., 2020). Based on these data, an experiment was designed in which 8 h long gravistimulated iGLV6 and rgi1rgi5/iGLV6 seedlings received an estradiol or mock treatment for 3 or 6 h (corresponding to 11 and 14 h after gravistimulation, respectively), after which root bends were dissected for RNA extraction and sequencing (Fig. 2b).

Differential gene expression analysis yielded 828 genes that showed a significant change in expression level in the iGLV6 line after 3 h and/or 6 h of estradiol treatment, relative to the corresponding mock conditions. After 3 and 6 h, 404 and 590 genes were differentially regulated, respectively, with an overlap of 166 genes between the two time points (Fig. 2c; Table S2). As expected, the large majority of these genes did not show a significant response, or responded to a lesser extent, to GLV6OE in the rgi1rgi5 mutant. This is consistent with the partial suppression of the GLV6OE phenotype in the rgi1rgi5/iGLV6 line and confirms that the RNA-sequencing captured an RGI-dependent response. GO-enrichment analysis revealed an overall enrichment of genes associated to, among others, auxin transport and responses, LR formation, and root system development, in agreement with the proposed function of GLV6/10 signaling during LR initiation (Table S3).

In view of the observed similarities in expression patterns and phenotypes related to the GLV6/10-RGI and TOLS2-RLK7 modules, we compared our RNA-sequencing dataset with recently published micro-array data that captured the transcriptional response to 3 h of TOLS2p treatment in roots of wild-type and rlk7 mutants (Toyokura et al., 2019). Interestingly, 90 of the 257 TOLS2 response genes also showed a significant response to...
which constitutes a highly significant overlap between both datasets, and all of these genes reacted in a similar way to both peptides (Fig. 2d). Moreover, a less stringent analysis of all genes responding to either TOLS2p treatment (adj. $P < 0.1$; absolute FC $> 1.3$), and/or $GLV6^{OE}$ (adj. $P < 0.05$; absolute FC $> 1.3$), revealed an even bigger overlap between these datasets, showing that the majority of genes followed the same trend in each experiment, albeit not consistently statistically significant in both (642 of the 808 genes that fulfill these criteria show a similar response in the $GLV6^{OE}$ and TOLS2p dataset) (Table S4;
Fig. 2 Comparative analyses of GLV6, TOLS2 and WRKY23 induced transcriptional responses and lateral root (LR)-related WRKY23 expression patterns and gain-of-function phenotypes. (a) Percentage of iGLV6 and rgi1rgi5/iGLV6 Arabidopsis seedlings with a LR primordium at a certain developmental stage 50 h after gravitropism, when grown on mock or estradiol-containing (2 µM) medium. The observed developmental stages are indicated, ranging from 0 (no primordium) to VIII (right before emergence). E, emerged. n = 30. Statistical significance was determined via Chi-square tests. (b) Schematic representation of the RNA-sequencing setup used to identify genes that are differentially regulated upon overexpression of GLV6 (GLV6OE) during LR initiation in Arabidopsis thaliana. (c) Heatmap representing fold changes (FCs) of the 828 differentially regulated genes (adj.P-value < 0.05) after 3 h and/or 6 h of estradiol induced GLV6OE in the wild-type background. Genes were grouped according to the timing at which they showed a significant FC (i.e. after 3 h, 6 h or both). (d) Venn diagram displaying the overlap between the significantly regulated genes in the GLV6OE and TOLS2p transcriptomic datasets (P < 0.05, abs. FC > 1.3) and a heatmap representing the FCs of the 90 overlapping genes in both datasets. Statistical significance of the overlap was determined using a hypergeometric test. (e) Enrichment score (calculated as log(P-value)) of the transcription factor binding sites (TFBS) of all 530 Arabidopsis TFs (JASPAR2020 database) in the promoter regions (~1000 bp) of genes that are upregulated 3 h after GLV6OE or TOLS2p treatment. Each dot represents a single TF. Yellow dots represent all WRKY TFs in the database. The dotted line indicates the cut-off above which enrichment is statistically significant (P < 0.05). Statistical significance was determined using a z-test with Bonferroni correction. (f) Venn diagram displaying the overlapping response genes between WRKY23-GR (P < 0.1) and WRKY23-SRDX (P < 0.05) with GLV6OE (P < 0.05) and TOLS2p (P < 0.05) transcriptomic datasets in a way that conform with a positive regulation of GLV6 and TOLS2 response genes by WRKY23 (i.e. a similar response in the WRKY23-GR datasets or the opposite response in the WRKY23-SRDX datasets) and a heatmap representing FCs of the 406 overlapping genes in each of these datasets. Statistical significance of pairwise overlaps between datasets was determined using hypergeometric tests. (g) DIC images of the WRKY23::GUS expression pattern in lateral root founder cells (LRFCs) and later stages of LR development in Arabidopsis. Bar, 30 µm. (h) Confocal images comparing the WRKY23::GFP and RGI1-NLS-GFP expression patterns in LR primordia of Arabidopsis thaliana, right before emergence. Green fluorescent protein (GFP) signals are shown in green. Cell walls were stained with propidium iodide (red) or Calcofluor White (white) in the left and right panels, respectively. Bar, 30 µm. (i) Representative images of 10-d-old Arabidopsis seedlings of a relatively weak 35S:WRKY23-GR line grown on mock or dexamethasone (DEX)-containing (10 µM) medium. Note the absence of LRs while primary root growth is hardly affected in the presence of DEX. The dots in the image highlight the location of emerged LRs and the number of emerged LRs along each primary root is indicated on top. Bar, 0.5 cm. (j) Emerged LR densities of 12-d-old seedlings grown on mock or DEX-containing (10 µM) medium. Individual datapoints and mean ± SD are shown. n = 16. Statistical significance was determined via Poisson regression. (k) Confocal images of longitudinal sections through the maturation zone of 9-d-old 35S: WRKY23-GR Arabidopsis roots grown on mock or DEX-containing (10 µM) medium. Anticlinical divisions within a single pericycle cell file are indicated with yellow arrowheads. The different tissue layers are indicated: v, vasculature; p, pericycle; e, endodermis; c, cortex; ep, epidermis. Cell walls were stained with Calcofluor White. Bar, 50 µm.

Fig. S5). This is especially clear at 3 h after induction of GLV6OE, which likely corresponds best with the 3 h peptide treatment assayed in the TOLS2 micro-array dataset. It should be noted that, due to technical differences between both transcriptional experiments (i.e. root bends vs full roots), the comparison between these datasets has its limitations, potentially leading to an underestimation of the similarities. Lack of a complete overlap could also be due to genes showing a specific response to either GLV6 or TOLS2. The set of genes that only show a significant response to GLV6OE contains some potentially important factors for LR initiation (Table S4), including ARF19, some auxin transporters like PIN1 and ABCB19, auxin conjugating GH3 enzymes, and a number of cyclins and microtubule-related genes involved in the cell cycle. These response genes might be part of a GLV specific mechanism. However, since many of the potentially GLV6 specific response genes are only induced after 6 h of GLV6 overexpression, their response to TOLS2 might simply be missed at the assayed 3 h time point after treatment. Overall, the extensive overlap between the GLV6OE- and TOLS2p-induced response genes suggests that the matching GLV6/10- and TOLS2-related LR phenotypes are likely underpinned by the same, or at least a very similar, transcriptional response.

If GLV6/10 and TOLS2 peptides indeed trigger a similar transcriptional response during LR initiation, they likely act via the post-translational regulation of the same TF(s). TFBS enrichment analyses were performed on the promoters of genes that were differentially regulated shortly (3 h) after induction of GLV6OE or TOLS2p treatment. These analyses revealed that binding sites for TFs of the WRKY family were strongly enriched in the promoter sequences of GLV6 and TOLS2 inducible genes (Fig. 2e; Table S5), suggesting that their expression might be positively regulated by WRKYs (Rushston et al., 2010). The role of these TFs during LR development has not been thoroughly investigated, but some WRKYs, including WRKY23, WRKY46 and WRKY75, were previously suggested to be involved (Devaiah et al., 2007; Grunewald et al., 2012; Ding et al., 2015; Prát et al., 2018). Transcriptomic data on roots after dexamethasone (DEX)-induced WRKY23-GR activation, as well as after NAA treatment on dominant negative 35S:WRKY23-SRDX roots, were recently reported (Hajný et al., 2020). Comparative analyses of these datasets with the GLV6OE and TOLS2 transcriptomic data revealed sets of GLV6 and/or TOLS2 upregulated or downregulated genes that show a similar response upon DEX-induced WRKY23-GR activation and/or an opposing response in the 35S:WRKY23-SRDX line (Fig. 2f; Table S6), consistent with a possible role for WRKY23 as a positive regulator in the GLV6/10-RGI1 and TOLS2-RLK7 pathways. However, these overlaps are not as extensive as the overlap between the GLV6OE and TOLS2p transcriptomic datasets, suggesting that apart from WRKY23, additional TFs are responsible for the regulation of GLV and TOLS2 response genes. Like GLV6/10 and TOLS2, WRKY23 was shown to be auxin inducible via the IAA14-ARF7/19 auxin signaling module and is expressed during LR development (Grunewald et al., 2008, 2012). Closer inspection of transcriptional and translational WRKY23 reporter lines showed expression in LRFCs and throughout LR development (Figs 2g, S6). From stage III onwards, WRKY23 is preferentially expressed in the flanks of LR primordia, and around stage V/VI expression resumes at the tip of the primordium. This expression pattern is reminiscent of RGI1 and RLK7, consistent with a possible role for WRKY23 downstream of these receptors (Fig. 2h). Although WRKY23 overactivity usually results in a dramatic decrease in
primary root length (Grunewald et al., 2012) (Fig. S7), a weaker overexpression effect can be achieved in some DEX-inducible 35S:WRKY23-GR lines, resulting in only minor decreases in primary root length, allowing for the analysis of LR densities (Fig. 2i). Interestingly, DEX-induced WRKY23-GR activation resulted in a strong decrease in emerged LR density (Fig. 2i,j). Furthermore, microscopic analysis revealed the occurrence of ectopic anticlinal divisions throughout the pericycle, akin to the aberrant pericycle divisions observed upon GLV6OE, as well as GLV10p and TOLS2p treatment (Fig. 2k). In combination with the enrichment of WRKY binding sites in the promoters of GLV6 and TOLS2 induced genes, and the partial overlaps between the WRKY23, GLV6 and TOLS2 transcriptional responses, these data suggest that WRKY23 might be an important component of the GLV6/10 and TOLS2 signaling pathways, potentially serving as one of the TFs that induce their common transcriptional response.

GLV6/10 and TOLS2 peptide signaling affect lateral root initiation through PLETHORA and PUCHI

The TFs PLT5, PUCHI, and LBD16 were among the strongest upregulated genes in the GLV6OE RNA-sequencing dataset (Table S2). Their induction was also confirmed via RT-qPCR experiments on roots after GLV6OE and GLV10p treatment, and was clearly dependent on RGI1, RGI4 and RGI5 (Figs 3a, S8). Since these TFs are recognized regulators of LR development, they might indeed serve as important downstream targets of the GLV6/10-RGI module.

Interestingly, TOLS2 was previously shown to affect LR initiation via the induction of PUCHI, and PLT5 and LBD16 were also upregulated in the TOLS2p micro-array dataset (Toyokura et al., 2019). We confirmed the RLK7-dependent induction of LBD16, PLT5, and PUCHI transcription in TOLS2p-treated roots via RT-qPCR (Fig. 3a), and studied the effect of GLV10p and TOLS2p treatments on LBD16:GUS, PLT5:GUS and PUCHI:GUS lines. Whereas the expression of each of these TFs is usually strictly associated with LR primordia, GLV10p and TOLS2p treatments trigger their expression throughout large portions of the pericycle from the young maturation zone onwards (Fig. 3b). To investigate whether these TFs could be responsible for the inhibitory effect of GLV10p and TOLS2p on LR initiation, we analyzed the LR phenotypes of peptide-treated mutant lines. Since PLT5 is known to act redundantly with PLT3 and PLT7, a plt3plt5plt7 triple mutant was used (Hofhuis et al., 2013; Du & Scheres, 2017). In the absence of peptides, plt3plt5plt7 mutants hardly produce any emergent LRs and primordium development typically halts around stage IV. However, quantification of all LR primordia in the presence of GLV10p or TOLS2p revealed that the decrease in total LR primordium density is suppressed in the plt3plt5plt7 mutant compared to Col-0 (Fig. 3c). Similarly, treatment of the puchi-1 mutant with GLV10p or TOLS2p revealed a reduced response to both peptides (Fig. 3c). These data suggest that the GLV6/10- and TOLS2-induced inhibition of LR initiation is at least partially dependent on the activity of PLTs and PUCHI.

In addition to PLT5 and PUCHI, transcription of LBD16 is also induced by GLV6/10 and TOLS2 peptides. Since LBD16 has now been established as an upstream activator of GLV6, GLV10 and TOLS2 transcription, this suggests that these peptides are part of a positive feedback loop via which LBD16 enhances its own transcription. However, GLV10p treatment still results in a strong reduction in emerged LR density of higher order ldb16ldb18ldb33 mutants (Fig. 3d), and induces ectopic anticlinal divisions in the pericycle of gLBD16-SRDX roots (Fig. 3e), indicating that LBD16 is not required for these effects of GLV peptides. Interestingly, TOLS2p treatment was previously shown to reduce the density of DR5-marked spots in wild-type as well as gLBD16-SRDX seedlings, suggesting that LBD16 is also not needed for the effects of TOLS2p on the pericycle (Toyokura et al., 2019). Hence, neither GLV6/10, nor TOLS2 signaling, seem to require LBD16 for their phenotypic effects. In contrast, the changes in PLT5 and PUCHI expression levels and patterns, in combination with the reduced effects of GLV10p and TOLS2p treatments on the total LR density of plt3plt5plt7 and puchi mutants, indicate that PLTs and PUCHI serve as important and common downstream targets of the GLV6/10-RGI and TOLS2-RLK7 modules during LR initiation.

GLV6/10-RGI and TOLS2-RLK7 modules do not require each others activity

The strikingly similar phenotypic effects and transcriptional responses triggered by GLV6/10-RGI and TOLS2-RLK7 signaling, as well as their overlapping expression patterns during LR initiation, might indicate that both peptide-receptor modules are part
of the same genetic pathway. For example, one module might be activated downstream of the other, or RGIs and RLK7 may act together in the same receptor complex to perceive their peptide ligands. However, quantification of total LR densities upon GLV10p treatment on \textit{rlk7} mutants and TOLS2p treatment on \textit{rgi1rgi4rgi5} mutants showed that \textit{rlk7} and \textit{rgi1rgi4rgi5} mutants are still completely sensitive to GLV10p and TOLS2p, respectively (Fig. 4a). These data exclude the possibility that these peptide-receptor modules require each other’s activity to affect LR initiation, but rather function independently to perform a similar
function. Additionally, the effect of combined GLV10 and TOLS2 peptide treatments on emerged LR density was assessed over a range of different concentrations (Fig. 4b). The combination of both peptides consistently resulted in a stronger effect compared to equimolar concentrations of the individual peptides. This suggests that the simultaneous stimulation of both peptide-receptor pathways activates their shared downstream response more efficiently, resulting in a synergistic effect on LR initiation.

GLV6/10-RGI and TOLS2-RLK7 signaling pathways converge in MPK6 phosphorylation

Previous studies uncovered that MPK6 is part of the GLV6/10-RGI signaling cascade during LR initiation, downstream of the RGI receptors (Fernandez et al., 2020; Lu et al., 2020; Shao et al., 2020). In agreement with this, mpk6 mutants showed an increased total LR density and aberrant LR spacing, reminiscent of glv6glv10 double mutants (López-Bucio et al., 2014; Fernandez et al., 2020). Nevertheless the mpk6 phenotype is stronger than the glv6glv10 phenotype, indicating that MPK6 activity during LR initiation is controlled by additional factors. Considering all previously discussed data, we therefore tested whether TOLS2p treatment can induce MPK6 phosphorylation. Indeed, both GLV10p and TOLS2p treatment triggered quick and transient phosphorylation of MPK6, as well as MPK3, and this response was largely suppressed in the rgi1rgi4rgi5 and rlk7 mutants, respectively (Fig. 5a). However, TOLS2p treatments on rgi1rgi4rgi5 mutants, and GLV10p treatments on rlk7 mutants, still resulted in a clear induction of MPK3/6 phosphorylation, again confirming that these peptide-receptor modules do not require each other’s activity.

TOLS2p-induced MPK6 phosphorylation suggests that MPK6 acts downstream of TOLS2-RLK7 signaling. Indeed, as previously described for GLV6OE, we observed a less pronounced reduction in total LR density upon GLV10p or TOLS2p treatment of mpk6 mutants (Fig. 5b), suggesting that these mutants are less responsive to these peptides during LR initiation. This convergence of GLV6/10 and TOLS2 peptide signaling into the same phosphorylation cascade might explain why they trigger such a similar transcriptional and phenotypic output. Accordingly, RT-qPCR experiments on mpk6 mutant roots confirmed that the upregulation of PLT5, PUCHI and LBD16 upon GLV6OE, as well as TOLS2p treatment, partially depends on MPK6 (Fig. 5c). However, the attenuation of the GLV6OE and TOLS2p-induced transcriptional response in mpk6 mutants is not as strong as observed in rgi1rgi4rgi5 and rlk7 mutants for their respective peptide ligands, indicating that knocking out MPK6 alone is not sufficient to completely block the response to either of these peptides. This is likely due to the redundant activity of MPK3, as it is also phosphorylated upon treatment with GLV10p or TOLS2p (Fig. 5a) and known to act redundantly with MPK6 in diverse biological processes, including RGF1/GLV11 regulated RAM maintenance (Wang et al., 2007; Cho et al., 2008; Meng et al., 2013; Lee et al., 2019; Fernandez & Beeckman, 2020; Lu et al., 2020; Shao et al., 2020; Shen et al., 2020).

If GLV6/10-RGI and TOLS2/PIP2-RLK7 signaling indeed converge on the same pathway, the glv6glv10 mutant phenotype is expected to be complemented not only by GLV10p, but also TOLS2p treatments. Indeed, we found that treatment with very low doses (10 nM) of GLV10p, as well as TOLS2p, can reduce the total LR density of glv6glv10 mutants back to wild-type-like

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**Fig. 4** GLV6/10-RGI and TOLS2-RLK7 modules do not require each others activity. (a) Total (nonemerged (NE) + emerged) lateral root (LR) primordium densities of 9-d old wild-type, rgi1rgi4rgi5 and rlk7 mutant Arabidopsis seedlings, treated or not with 100 nM GLV10p or TOLS2p. Individual datapoints and mean ± SD are shown. n = 15. Statistical significance was determined via quasi-poisson regression with Dunnett’s correction. (b) Emerged LR densities of 12-d old wild-type Arabidopsis seedlings, treated with a range of different GLV10p and TOLS2p concentrations, as well as equimolar 50 : 50 combinations of both peptides. The mean ± SE are shown for each condition. n = 15. Statistically significant differences between treatments were determined via Poisson regression with Tukey correction.
levels (Fig. 6a). Additionally, the increased density of clustered LR primordia in glv6glv10 double mutants was also reduced by both GLV10p and TOLS2p treatment, while wild-type seedlings were unaffected at these concentrations (Fig. 6b). These results confirm that GLV6/10 and TOLS2 likely regulate LR initiation via a (partially) shared signaling pathway (Fig. 6c).

**Discussion**

As increasingly more peptide-receptor modules are being characterized, it becomes apparent that distinct modules can be involved in the control of the same process. However, being studied separately, functional interactions between peptide signaling pathways remain largely unexplored.

Previous studies have independently shown that GLV6/10 and TOLS2/PIP2 regulate LR spacing and initiation (Fernandez et al., 2015, 2020; Toyokura et al., 2019). By studying these signaling peptides simultaneously, we identified new downstream targets of the GLV6/10-RGI and TOLS2-RLK7 modules, and show that they inhibit LR initiation via a common mechanism. We found that TOLS2-RLK7 signaling triggers the phosphorylation of MPK3/MPK6, and that MPK6 activity is required for a full effect of TOLS2 peptides on LR initiation, as described previously for GLV6/10. Furthermore, comparative transcriptome analyses revealed that GLV6/10 and TOLS2 induce a very similar transcriptional response downstream of MPK6. This common transcriptional response might partially result from the activation of WRKY TFs, including WRKY23. In agreement with this, several other WRKYs are known to be phosphorylated by MPK6, and MPK6 is able to interact with a number of WRKYs, including WRKY23 (Popescu et al., 2009; Mao et al., 2011; Guan et al., 2014). Although further investigation is required, the matching LR and pericycle phenotypes observed upon GLV6/10 and TOLS2 overabundance or WRKY23 overactivation, in combination with the overlapping expression patterns of RGI1, RLK7 and WRKY23, suggest that this TF could indeed be a component of the GLV6/10 and TOLS2 induced signaling cascade.

In addition to MPK6 as a common component of the GLV6/10-RGI and TOLS2-RLK7 induced phosphorylation cascade, we identified PLT5 and PUCHI as common transcriptional targets.
Furthermore, we show that GLV6/10 and TOLS2 both require the activity of PUCHI as well as PLTs for their effect on LR initiation. Interestingly, while these TFs promote proper primordium morphogenesis, they also act as inhibitors of LR initiation (Hirota et al., 2007; Hofhuis et al., 2013; Du & Scheres, 2017; Trinh et al., 2019). Consistently, higher order plt, puchi and mp-k6 mutants, were reported to show increased total LR primordium densities and clustering of LR primordia, reminiscent of the phenotypes described for glv6glv10 and tols2pip2 mutants.

The inhibitory effect of GLV6/10 and TOLS2 on LR initiation thus arises via a (partially) shared downstream mechanism. Moreover, the common upstream regulation of GLV6, GLV10 and TOLS2 transcription through the IAA14-ARF7/ARF19-LBD16 mediated auxin response, and the overlapping expression patterns of these peptides and their receptors, suggest that they simultaneously induce this mechanism in the same target cells. Nevertheless, the undiminished sensitivity of rkl7 and rgi1rgi4rgi5 mutants to GLV10p and TOLS2p treatments respectively, indicates that both modules trigger this shared mechanism independently. Combined peptide treatments suggest that GLV6/10 and TOLS2 even have a synergistic effect. This may be the result of a lack of competition between these peptides for receptor binding, as they are independently recognized by their specific receptors, thereby jointly stimulating the pathway more efficiently. Finally, we found that small doses of TOLS2p can complement the glv6glv10 mutant phenotypes. Defects in one peptide-receptor module can thus be complemented by stimulation of the other, suggesting that GLV6/10 and TOLS2 peptides indeed function at least partially redundantly during LR initiation. Altogether, our data indicate that, despite the lack of strong phylogenetic relationships, the GLV6/10-RGI and TOLS2-RLK7 modules independently trigger MPK3/MPK6 phosphorylation in the same target cells (i.e. LRFCs and pericycle cells surrounding LR primordia), leading to the induction of a
shared transcriptional response that underlies their matching effects on LR initiation (Fig. 6c).

For now, we can only speculate about the reason why these two distinct peptide-receptor modules regulate the same pathway during LR development. Apart from the enrichment in root development-related genes, GO-enrichment analyses also indicated an enrichment for genes involved in water and ion transport among the GLV6 response genes, as well as slight enrichments for genes related to defense responses and immunity in both the GLV6 and TOLS2 transcriptome datasets. This raises the possibility that the expression of GLV6, GLV10, TOLS2 and PIP2 could be modulated in response to different abiotic and/or biotic stressors, integrating these inputs into the same signaling pathway to modify root system architecture accordingly. Since GLVs and TOLS2, as well as their receptors, were recently found to be implicated in plant immunity, an involvement of these peptides in the interplay between defense responses and LR initiation seems particularly likely (Hou et al., 2014; Najafi et al., 2020; Stegmann et al., 2021; Wang et al., 2021).

Redundant regulation of the same or related processes by multiple peptides from the same family have often been recorded. Furthermore, peptides from the same family can also regulate different processes via quite different mechanisms, and even opposing effects of peptides from the same family have been described (Hara et al., 2007, 2009; Hunt & Gray, 2009; Sugano et al., 2010; Schlegel et al., 2021). In addition, we propose here that phylogenetically unrelated peptides can perform a common or redundant function via a common mechanism in the same cells. To our knowledge, this is the first report on the convergence of signaling pathways downstream of peptide-receptor modules from different families. Interestingly, MPK3 and MPK6 are known to be involved in many peptide signaling pathways from several different phylogenetic groups (Jewaria et al., 2013; Hou et al., 2014; Zheng et al., 2018; Zhang et al., 2019; Zhu et al., 2019; Fernandez et al., 2020; Lu et al., 2020; Shao et al., 2020), but despite this common central hub, these peptides typically induce a different response. How specificity of downstream responses is maintained when different input signals pass through the same phosphorylation cascade is currently not well understood, but seems to rely largely on the separation of these signals in space and time (Lampard et al., 2008; Wengier et al., 2018). Conversely, GLV6/10 and TOLS2 peptides are perceived in the same target cells at the same time, and trigger a similar response downstream of MPK3/6. This suggests that other unrelated peptide signals might also converge into a similar response through these MPKs, especially when they simultaneously affect the same cells or tissues. The integration of inputs from multiple distinct peptide-receptor modules adds another layer of complexity to intercellular communication networks in plants.

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Author contributions

JJ, AIF and TB conceptualized the study. JJ, AIF and TB contributed to methodology. JJ and BP performed formal analysis. JJ, KX, WG and AM investigated the study. HF and TB provided resources. JJ wrote the original draft. JJ, AIF, HF and TB wrote, reviewed and edited the manuscript. JJ contributed to visualization. JJ, AIF and TB supervised the study. JJ, AIF, HF and TB performed funding acquisition.

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Data availability

The GLV6OE RNA-sequencing data were deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) and are accessible through accession number E-MTAB-10891.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 IAA14 and ARF7/ARF19 dependent inducibility of GLV6, TOLS2 and PIP2 by auxin.

Fig. S2 LBD dependent inducibility of GLV6 and GLV10 transcription by auxin.

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