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Short Article

GOLVEN Secretory Peptides Regulate Auxin Carrier Turnover during Plant Gravitropic Responses

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SUMMARY

Growth and development are coordinated by an array of intercellular communications. Known plant signaling molecules include phytohormones and hormone peptides. Although both classes can be implicated in the same developmental processes, little is known about the interplay between phytohormone action and peptide signaling within the cellular microenvironment. We show that genes coding for small secretory peptides, designated GOLVEN (GLV), modulate the distribution of the phytohormone auxin. The deregulation of the GLV function impairs the formation of auxin gradients and alters the reorientation of shoots and roots after a gravity stimulus. Specifically, the GLV signal modulates the trafficking dynamics of the auxin efflux carrier PIN-FORMED2 involved in root tropic responses and meristem organization. Our work links the local action of secretory peptides with phytohormone transport.

INTRODUCTION

Phytohormones can be synthesized and perceived in distinct tissues and, thus, relay information over a long range. Plant cells also deploy a repertoire of secretory peptides that carry short-range cell-to-cell signals (Butenko et al., 2009). These two classes of molecules are important for growth and development of multicellular plants and can be involved in the same processes, such as cell elongation (Santner and Estelle, 2009), cell proliferation control (Matsubayashi and Sakagami, 2006), organization of apical and cambial meristems (Hirakawa et al., 2008; Whitford et al., 2008; Benková and Hejátko, 2009; Jun et al., 2008; Matsuzaki et al., 2010), and organ abscission (Lewis et al., 2006). Nevertheless, little is known about the integration between hormonal actions and peptide signaling in the cellular microenvironment.

Plants respond to light and gravity by changing the organ growth direction and auxin is a key regulator during these tropic responses. Auxin gradients controlling tropic bending and root patterning (Vanneste and Friml, 2009) depend on intercellular polar auxin transport (PAT) mediated by influx carriers of the AUX1/LIKE-AUX1 family and efflux carriers of the PIN-FORMED (PIN) family (Vieten et al., 2007). Chemical inhibitors of PAT (Rashotte et al., 2000; Parry et al., 2001) and mutations in the *AUX1*, *PIN2*, and *PIN3* genes (Friml et al., 2002; Luschnig et al., 1998; Bennett et al., 1996; Chen et al., 1998; Swarup et al., 2005; Wiśniewska et al., 2006) abolish the lateral distribution of auxin upon gravistimulation and inhibit gravitropic responses.

Auxin gradients established and maintained by the PIN protein network also control the position of the stem cell niches, from embryogenesis on through the development of the adult plant. In addition, auxin influences cell division as well as cell expansion, and multiple *pin* mutants have a reduced root apical meristem (RAM) (Blilou et al., 2005). Plant peptides are also involved in stem cell homeostasis (Stahl and Simon, 2010). Recently, a family of secreted peptides, called root growth factors (RGFs), has been reported to be required for root meristem maintenance (Matsuzaki et al., 2010). The triple *rgf1 rgf2 rgf3* mutant has reduced root meristem size, and the phenotype was restored by the addition of a corresponding peptide. The structural characterization of the RGF1 mature peptide sequence revealed that it is posttranslationally modified by tyrosine sulfation (Matsuzaki et al., 2010). Posttranslational modifications have been described in several plant secretory peptide families and are important for their function. Specific posttranslational modifications considerably increase peptide bioactivity

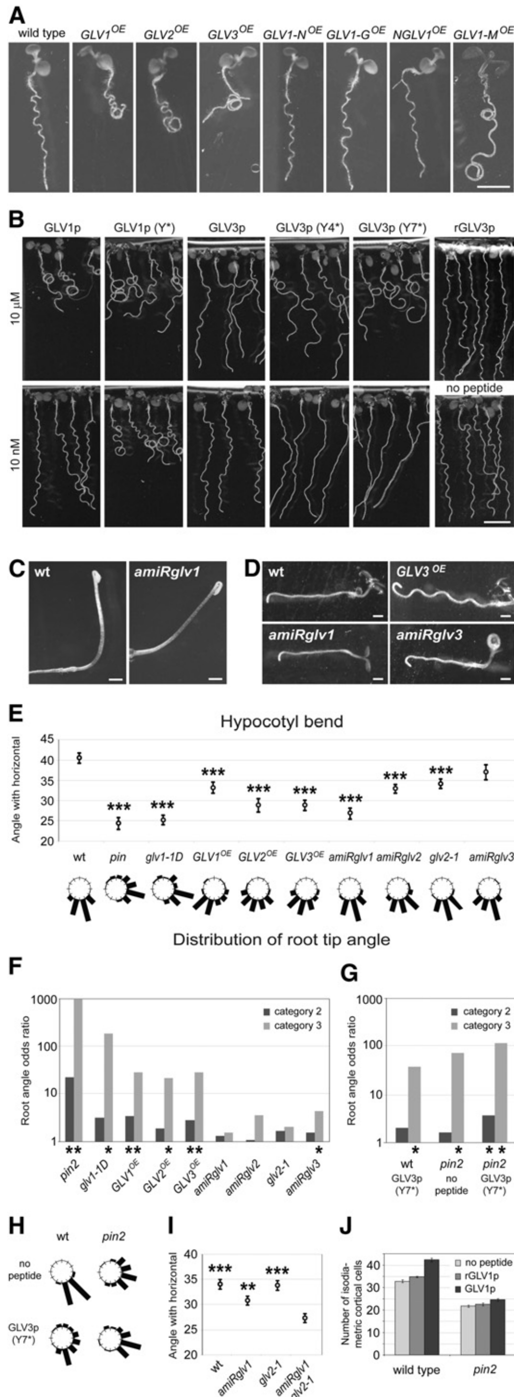

Figure 1. *GLV* Mutant Phenotypes

(A) Gain-of-function root growth. Seven day-after-germination (dag) seedlings were grown on 45° agar plates. OE denotes plants overproducing the GLV polypeptides or truncated variants. Overproduction of the full GLV1, GLV2, or GLV3 polypeptides, and of the truncated GLV1-M protein lacking the internal nonconserved region (amino acids [aa] 1–34 fused to 69–86), resulted in reduced root gravitropism. Other truncated forms of GLV1 did not: GLV1-N, GLV1 without the N-terminal leader (aa 35–86); GLV1-G, GLV1 without the GLV motif (aa 1–68); and NGLV1, N-terminal leader of GLV1 alone (aa 1–34).

(B) Dose-dependent wavy root phenotypes induced by GLV synthetic peptides. The asterisk and number indicate a sulfated tyrosine residue. rGLV3p peptide contains the same amino acids as its GLV3p counterpart, but in random sequence.

(C and D) Bending defects upon gravistimulation in etiolated hypocotyls (C) and roots (D).

(E and H–I) Quantitative gravitropic responses of hypocotyls (n = 57–145) and roots (circular distribution representation, n = 58–91) in *GLV* mutants (E and I) and seedlings grown on peptide-supplemented media (GLV3p, 100 nM) (H). *pin* denotes either *pin3-2* or *eir1-4* (*pin2* null) mutants as controls for mutants defective in hypocotyl and root bending, respectively. Asterisks in hypocotyl graphs mark significant differences: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Mean hypocotyl angle (±SEM) was compared in t tests to wild-type (E) and to the *amiRglv1 glv2-1* double mutant (I).

(F and G) A logistic regression model was applied to model the relationship between the nominal response variable angle and the different genotypes or treatments. The odds ratio (log scale) represents the odds of roots to fall in the angle categories 2 or 3 (further away from the vertical) rather than in the baseline category 1, either for a mutant line compared to the wild-type (F) or for the wild-type or the *pin2* mutant treated with a bioactive GLV peptide compared to the nontreated wild-type (G). For a detailed description, refer to the Supplemental Experimental Procedures. Asterisks in the root angle odds ratio graphs mark the mutant lines or treatments for which a root tip angle is more likely to deviate from the nontreated wild-type distribution.

(J) Increase of RAM size upon GLV1p treatment. Vertically grown 3-dag seedlings were transferred to solid media supplemented with GLV1p, rGLV1p, or without peptide, and grown vertically for a further 48 hr (n = 15). The number of cortical isodiametric cells were counted in the basal meristem (between quiescent center and elongation zone). Mean cell number (±SEM) was compared in two-sample t tests to control without peptide. Scale bars represent 2.5 mm (A), 5 mm (B), 1 mm (C), and 0.2 mm (D).

See also Figure S1, Tables S1 and S2, and Supplemental Experimental Procedures.

by enhancing the binding affinity to ligand receptors (Matsubayashi, 2011).

Here, we demonstrate that secretory peptides related to the RGF family are involved in the gravitropic response of plant organs and that they control the distribution of auxin by regulating the turnover of an auxin efflux carrier.

RESULTS

GOLVEN Genes Code for Small Secretory Proteins that Cause Root Agravitropism When in Excess

In a systematic reverse-genetics screen, we identified three *Arabidopsis thaliana* genes that altered root gravitropism when overexpressed: *GOLVEN1* (*GLV1*, At4g16515), *GLV2* (At5g64770), and *GLV3* (At3g30350). To visually score growth defects, *Arabidopsis* seedlings were placed on inclined impenetrable agar medium on which wild-type roots had an undulating regular pattern, called waving (Oliva and Dunand, 2007), whereas *GLV*-overexpressing roots formed irregular waves (“golven” in Dutch), including loops (Figure 1A). The 11 *GLV* genes identified in *Arabidopsis* based on sequence homology code for small proteins defining a family of plant-specific peptides, of which some have been recently described as RGFs (Matsuzaki et al., 2010) (see Figure S1A available online). In agreement with public gene expression compendia, promoter-reporter line analyses showed that *GLV1/RGF6* and *GLV2/RGF9* were expressed in leaves, hypocotyls, and flowers, and *GLV3/RGF4* specifically in the RAM (Figure 2). Because not all *GLV/RGF* genes are active in the root, they are referred to as *GOLVEN* (*GLV*) hereafter.

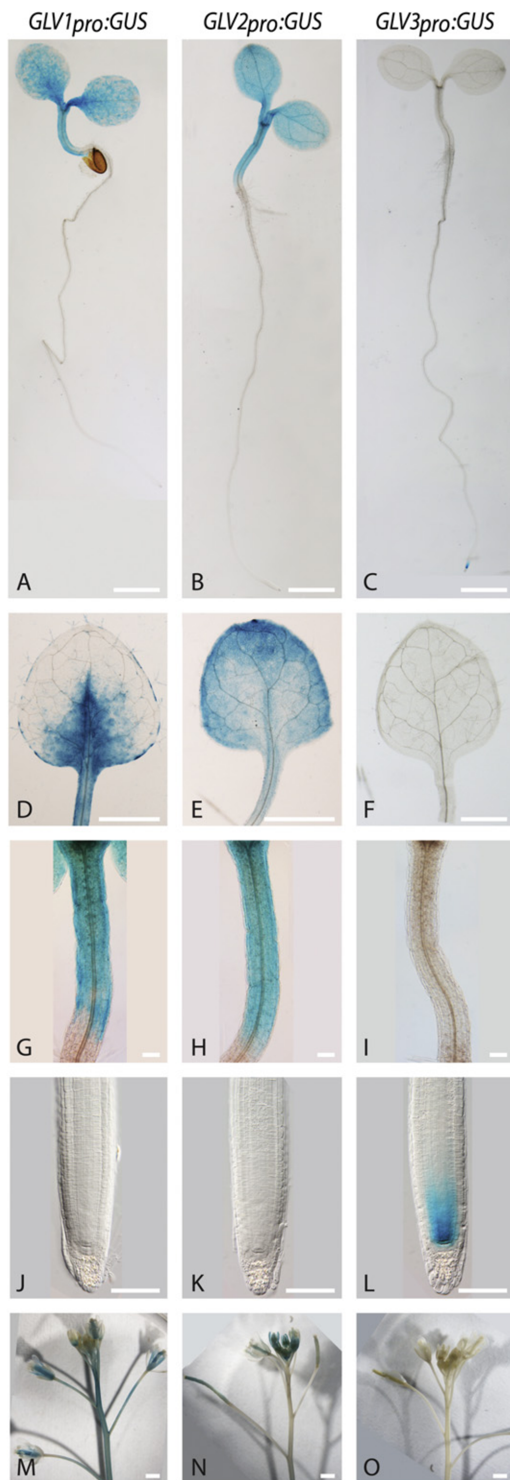

Figure 2. *GLV* Transcription Profiles

All lines stained for GUS activity carried a homozygous single-locus *GLV* promoter:*GUS* transcriptional fusion transgene as indicated.

(A–C) Five-day-old whole seedlings.

(D–F) First or second leaf, 2 weeks after germination.

(G–L) Detail of hypocotyls (G–I) and root meristem (J–L) of 5-day-old seedlings.

(M–O) Inflorescences.

Scale bars represent 1 mm (A–F) and (M–O) and 100 μm (G–L).

The GLV proteins are composed of a nonconserved region linking an N-terminal sequence predicted to be cleaved off upon secretion of the remaining polypeptide and a C-terminal conserved motif, dubbed the GLV motif (Figure S1A). This tripartite structure is shared by other plant signaling peptide families, including CLV3/CLE, RALF, PSK, and IDA (Butenko et al., 2009). Overexpression of truncated versions of *GLV1* in transgenic *Arabidopsis* lines revealed that together the N-terminal leader sequence and the GLV motif were necessary and sufficient for the *GLV* gain-of-function agravitropic root phenotype (Figure 1A). Furthermore, peptides containing the GLV motif were identified by mass spectrometry in liquid medium conditioned with plants overexpressing either the *GLV1*, *GLV2* or *GLV3* genes. Mature GLV peptides likely resulted from the proteolytic cleavage of the corresponding precursor protein, and carried sulfated tyrosine and hydroxylated proline residues as determined by fragmentation mass spectra (Figure S1B, Table S1, and data not shown), in agreement with the structure of the RGF1 mature peptide (Matsuzaki et al., 2010).

To investigate the role of the GLV motif, we examined the phenotype of *Arabidopsis* plants grown in the presence of the corresponding synthetic peptides (see Supplemental Experimental Procedures for amino acid sequences). At micromolar concentrations, treatment with the GLV1p, GLV2p, and GLV3p peptides resulted in a dose-dependent abnormal gravitropic root phenotype, consistent with the genetic gain-of-function results. The synthetic peptides carrying the GLV1 and GLV3 conserved motif, in which a tyrosine residue was sulfated caused similar phenotypes at nanomolar concentrations, exhibiting two to three orders of magnitude higher bioactivity than their unmodified counterparts (Figure 1B and Figure S1C).

In summary, the structure of the GLV proteins, their secretion, and the biological activity of peptides resembling mature GLV proteolytic cleavage products suggest that GLV functions are mediated by extracellularly secreted peptides derived from the GLV motif, posttranslationally modified, and possibly recognized by membrane receptors (Butenko et al., 2009; Ohyama et al., 2009).

GLV Gain- and Loss-of-Function Mutants Have Altered Gravitropic Responses

To determine whether the *GLV* genes are required for gravitropic responses, we measured hypocotyl and root bending after reorientation of *Arabidopsis* seedlings in which *GLV1*, *GLV2*, or *GLV3* had either been knocked down via RNA interference (*amiRglv*) or overexpressed (GLV^{OE}). We also analyzed two insertional mutants carrying a T-DNA locus either in the promoter (*glv1-1D*) or in the coding sequence (*glv2-1*) of the gene of interest. The corresponding transcripts were up- and downregulated, respectively (Table S2). Hypocotyl gravicurvature was reduced in *amiRglv1*, *amiRglv2*, *glv2-1*, $GLV1^{OE}$, $GLV2^{OE}$, $GLV3^{OE}$, and *glv1-1D*, but was not affected in *amiRglv3* (Figures 1C and 1E). The *amiRglv1 glv2-1* double mutant showed a stronger defect than either of the single mutants (Figure 1I). Root gravicurvature was altered in $GLV1^{OE}$, $GLV2^{OE}$, and $GLV3^{OE}$, as well as in *glv1-1D* and *amiRglv3*, but not in *amiRglv1* or *amiRglv2* (Figures 1D–1F). The phenotype of the *GLV* overexpression lines was similar to the altered gravitropic response of wild-type roots treated with GLV3p (Figures 1G and 1H). The

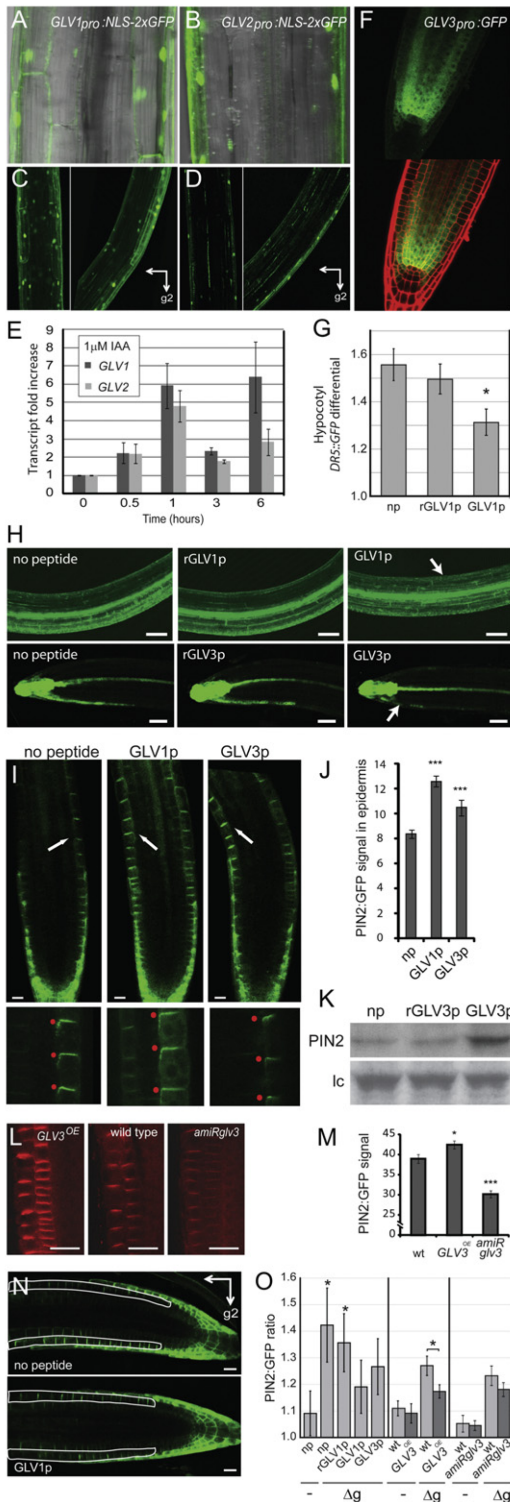

Figure 3. *GLV* Expression, GLV Control of Auxin Gradients, and PIN2 Protein Level

(A and B) Expression of *GLV1* and *GLV2*. Promoter-reporter lines carry a double GFP fusion protein with a nuclear localization signal.

(C and D) Asymmetric activity of *GLV1* and *GLV2* promoters in gravistimulated (right) compared to nongravistimulated (left) hypocotyls. Arrows mark the gravity vector before and after (g_2) reorientation.

(E) qRT-PCR analysis of *GLV1* and *GLV2* transcript levels after auxin treatment. Fold increase (±SEM) was calculated relative to mock-treated plants and averaged from three independent biological repeats.

(F) Expression of *GLV3* in the RAM.

(G) GLV peptide effect on auxin gradient formation after gravistimulation. *$DR5_{pro}$:GFP* plantlets were gravistimulated on solid medium with either no peptide (np), a random control peptide (rGLVp), or a bioactive peptide (GLVp). Hypocotyls (top) and roots (bottom) were pretreated for 6 hr and 12 hr, and gravistimulated for 16 hr and 6 hr, respectively. Means of differentials (±SEM) were compared using a two-sample t test.

(H) GFP signal ratio between the outer and inner side of the gravistimulated hypocotyls shown in (G) (n = 24). Arrow highlights upper and lower side of hypocotyl and root, respectively.

(I) Increase of PIN2 in the PM of *eir1-4 35Spro:PIN2:GFP* root tips exposed to GLVp (arrows). 5-dag plants were incubated overnight with the indicated peptides. The apical polarity of the PIN2 protein in epidermal cells (red dots) was identical in all samples.

(J) Quantification of the PIN2-GFP signal (arbitrary units; ±SEM) in epidermal cells from seedlings as shown in (I) (n = 14–21).

(K) Immunoblot analysis of PIN2 levels in membrane extracts. Roots were harvested 8 hr after mock treatment (np) or application of the indicated peptides. PIN2 level increased in the presence of GLV3p compared to rGLV3p and np samples. lc, loading controls stained with Coomassie Brilliant Blue.

(L) PIN2 immunolocalization in *GLV3* mutants. Protein levels in the PM of epidermis and cortex coincided with *GLV3* expression levels.

(M) Quantification of the PIN2 signal (arbitrary units; ±SEM) in the PM of epidermal cells in mutant seedlings (n = 22–26).

(N) PIN2 gradient across top and bottom epidermis in gravistimulated roots. 5-dag *eir1-4 $35S_{pro}$:PIN2:GFP* seedlings were transferred to medium with or without GLV1p, incubated vertically (3 hr), and gravistimulated (4 hr). White contours highlight epidermal cell files where PIN2:GFP was quantified.

(O) Quantification of PIN2 root asymmetry after gravistimulation. Ratio of PIN2-GFP signal between top and bottom epidermis (as shown in N; ±SEM) was calculated after peptide treatment (left, n = 12–18), or in F1 *$PIN2_{pro}$:PIN2:GFP* plants crossed either to wild-type, *$GLV3^{OE}$*, or *amiRglv3* plants (center and right, n = 24–36). The mean signal ratio was compared in two-sample t tests to controls without peptide (np) before reorientation (left) or to the wild-type (center and right) before (−) and after gravistimulation (Δg; after 4 hr for the *$GLV3^{OE}$* and 2 hr for the *amiRglv3* plants).

Scale bars represent 50 μm (A–G) and 20 μm (I–N). See also Figures S1 and S3 and Table S3.

lack of loss-of-function phenotypes in the root for *GLV1* and *GLV2* and in the hypocotyl for *GLV3* is in agreement with the absence of expression of the respective genes in these organs (Figure 2). These data suggest that GLV peptides act locally to regulate gravitropic growth responses.

GLV Genes Are Expressed in Specific Cell Types, and Some Are Induced by Auxin

In plants, tropic curvature requires the asymmetric distribution of the hormone auxin (Went, 1974) that triggers transcriptional responses followed by differential cell elongation at the opposite sides of the bending organs (Esmon et al., 2006). Upon gravistimulation, auxin accumulates at the lower side of shoots and roots where it stimulates and inhibits cell elongation, respectively (Friml et al., 2002). The analysis of promoter:reporter *Arabidopsis* lines indicates that the transcription of *GLV1* and *GLV2* was restricted to the epidermis and cortex and enhanced at the lower side of the reoriented hypocotyl (Figures 3A–3D). Transcription of both genes was also rapidly induced by auxin (Figure 3E). In addition, *GLV1* correlated with genes known to be upregulated by auxin and associated with gravitropism and phototropism

(Table S3). *GLV3* transcription followed a very different pattern and was restricted to the RAM, primarily in cells of the cortex, endodermis, and stele, close to the quiescent center (Figure 3F). No transverse gradient in *GLV3* promoter activity could be detected within reoriented root tips.

GLV Peptide Treatment Interferes with Auxin Fluxes during Gravitropic Responses

We hypothesized that the GLV peptides themselves participate in the formation of gravity-induced gradients, in which case their exogenous application should impair the differential accumulation of auxin as visualized with the synthetic transcriptional reporter $DR5_{pro}$:*GFP* (Friml et al., 2003). Accordingly, *DR5* asymmetry was reduced in reoriented hypocotyls and roots pretreated with GLV1p and GLV3p, respectively (Figures 3G and 3H). Collectively, *GLV* gain- and loss-of-function phenotypes suggest a functional link between *GLV* tissue-specific expression and the regulation of gravitropic growth responses that involve the establishment of auxin gradients both in shoot and root tissues (Vanneste and Friml, 2009).

The Auxin Efflux Carrier PIN2 Is a Target of the GLV Signaling Pathway

The GLV activity enlarged the meristem as seen by the increased number of isodiametric cells in GLV1p-treated seedlings and as previously reported for similar RGF peptides (Matsuzaki et al., 2010). This enlargement was partially suppressed in the *eir1-4* line, a null mutant hereafter referred to as *pin2*, suggesting that *PIN2* is a target of the GLV signaling pathway (Figure 1J). Furthermore, *pin2* was also resistant to GLV peptide treatment with regard to gravitropism because the response of the mutant to gravistimulation was similar when grown on medium containing GLV3p (Figures 1G and 1H).

We studied whether GLV activity affected expression or distribution of the AUX1, PIN1, PIN2, and PIN3 auxin carriers in *Arabidopsis* root tips. Transcription of some of these genes was slightly altered by the application of GLV synthetic peptides, but transcriptional regulation did not appear to be the main mode of action by which the GLV pathway regulated gravitropism (Figure S2). In contrast, we found that the PIN2 protein level had increased in the membrane protein fraction of roots treated with GLV peptides, although PIN2 cellular polarity did not change (Figures 3I–3K). Accordingly, in $GLV3^{OE}$ and *amiRglv3* roots, the PIN2 level was higher and lower, respectively, than that of the wild-type at the plasma membrane (PM) of epidermal and cortical cells (Figures 3L and 3M). GLV-induced PIN2 accumulation was specific because the abundance of the other membrane-bound proteins AUX1, PIN1, PIN3, PIP2, and BRI1 was not altered by GLV peptides (Figures S3A–S3E).

Peptide treatments and mutant analysis suggested that GLV activity controlled PIN2 abundance in the root meristem by a posttranscriptional mechanism. During gravistimulation, asymmetric auxin distribution across the root meristem requires the differential turnover of PIN2 between the upper and lower epidermis, resulting in the accumulation of the protein at the lower side of the reoriented root tip (Abas et al., 2006; Kleine-Vehn et al., 2008). The treatment with bioactive GLV peptides and *GLV3* overexpression both significantly reduced this differential PIN2 turnover (Figures 3N and 3O), probably decreasing auxin asymmetry and causing root agravitropism. The differential turnover was also reduced in all assays with the *amiRglv3* plants, but not significantly (Figure 3O).

GLV Peptides Modulate PIN2 Trafficking Dynamics

The level of PIN2 in the PM is highly dependent on posttranslational control, such as protein turnover, polar targeting, and endocytosis (Kleine-Vehn and Friml, 2008). Hence, GLV peptide application was used to investigate the mechanism of the apparent GLV-dependent regulation of PIN2 targeting. Within minutes, the GLV3 peptide treatment increased the localization of PIN2 at the PM (Figures 4A–4C) and the occurrence of PIN2-GFP-labeled endomembrane vesicles in root epidermal cells (Figures 4A, 4D, and 4E and Movie S1). GLV1 peptide treatment had the same effects (data not shown). The PIN2 level at the PM and the number of PIN2-labeled vesicles also increased in roots treated with the translation inhibitor cycloheximide (Figures 4C and 4E). In contrast, the uptake of the endocytic tracer FM4-64 was not altered after GLVp application or in $GLV1^{OE}$ plants (Figures S3F–S3H). The transient intracellular PIN2 accumulation and increased PIN2 stability at the PM collectively suggest that GLV signaling controls vesicular sorting events.

GLV Peptides and Auxin Act Together on PIN2 Trafficking

Auxin has been shown to transiently increase the PIN2 pool at the PM presumably through the inhibition of an endocytic step involved in constitutive protein cycling (Paciorek et al., 2005). Intriguingly, large PIN2-labeled vesicles rapidly accumulated after the combined treatment of root tips with GLV3p and auxin, at levels higher than with either of the two compounds separately (Figures 4F and 4G and Movie S1).

DISCUSSION

We have shown that GLV secretory peptides are involved in the control of tropic growth. Furthermore, our data indicate that the apoplastic GLV signal specifically regulates the PIN2 level in different compartments of root epidermal cells, causing the transient accumulation of the protein in endosomes or prevacuolar compartments, and increasing the PIN2 pool at the PM, suggesting that GLV activity might regulate specific aspect(s) of PIN2 trafficking.

The GLV signal might thus potentiate PIN2-mediated PAT, thereby explaining why the perturbation in the GLV function results in abnormal gravitropic responses. According to this model, if the GLV signal is increased ectopically (GLV peptide treatment or GLV^{OE}), the fine regulation of the PIN2 turnover would be lost, resulting in its preferential accumulation in the lower epidermis of the gravistimulated root because of the PIN2 stabilization on both sides of the organ, hence preventing the formation or maintenance of the auxin gradient; however, if the GLV signal is reduced (*GLV* knockout or silencing), the PIN2 level would decrease and a reduced auxin flux would prevent the formation of the gradient. We speculate that the GLV regulatory mechanism enhances the robustness of auxin gradient responses, also considering that positive feedback

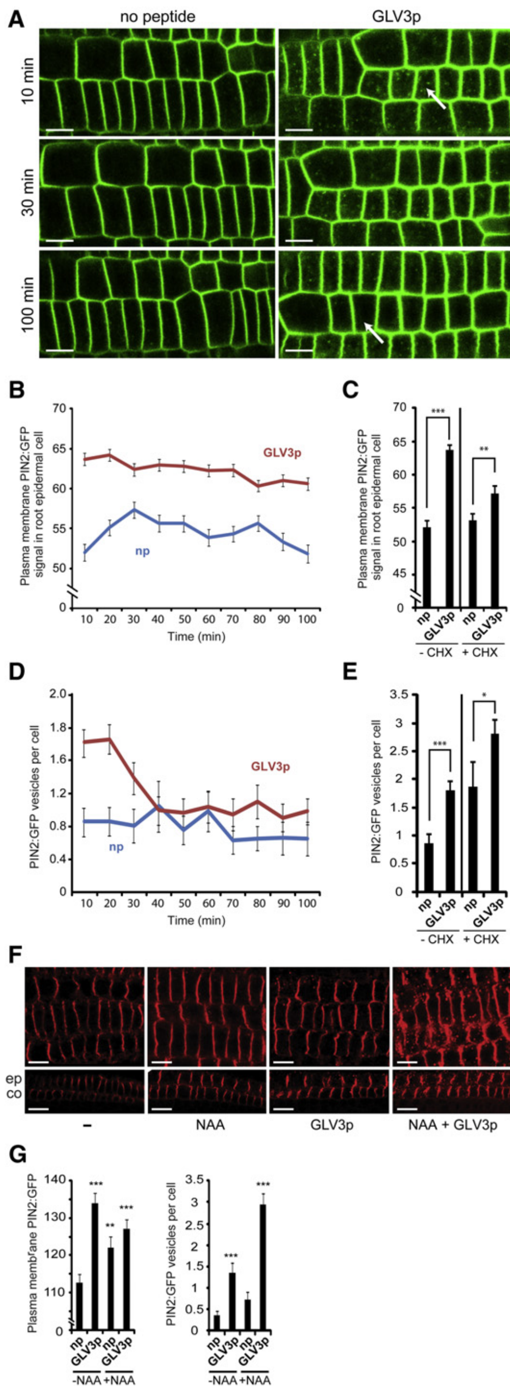

Figure 4. PIN2 Trafficking upon GLV Peptide Treatment

(A) PIN2 subcellular localization in epidermal cells. 4-dag *eir1-4 $PIN2_{pro}$:PIN2:GFP* roots were transferred to the indicated treatment for 10–100 min. Arrows mark vesicular signals.

(B–E) Relative to the control without peptide, the sulfated GLV3p initially increased the PM-localized PIN2-GFP fluorescence expressed in arbitrary units (B and C) and the number of PIN2:GFP-labeled vesicles (D and E). These rapid GLV-induced changes were also observed when plants were treated with the translation inhibitor cycloheximide (CHX), indicating that they do not result from transcriptional changes (C and E). All means are presented ± SEM.

(F) PIN2 immunolocalization after treatment with GLV peptide and auxin. From left to right, epidermal cells (top) mock-treated for 30 min (–), treated with auxin alone (10 μM α-naphthalene acetic acid [NAA]), with GLV3p alone, or with both. Longitudinal medial sections (bottom) show corresponding epidermis (ep) and cortex (co) cell files.

(G) Quantification of the PIN2 signal in the PM (left) and in endosomes (right) from seedlings as shown in (F). In each data point resulting from the analysis of 12–16 roots, the signal was quantified for on average 12–23 cells per treatment.

Means are presented ± SEM. Scale bars represent 10 μm. See also Movie S1.

loops might be created in which specific *GLV* genes are induced by auxin, for instance in the hypocotyl.

Future research will be necessary to unravel additional steps in the GLV pathway. Other secretory peptides are recognized by receptor kinases in the PM (Butenko et al., 2009), and the peptide signal perception triggers the posttranslational modification of membrane-associated proteins, possibly regulating their stability or their trafficking between different membrane systems. The rapidity of the changes in the PIN2 distribution in response to the GLV peptide application argues for such a mechanism. In addition, the receptor-ligand recognition might also initiate a signaling cascade, resulting in transcriptional reprogramming.

In agreement with our observations, several peptides of the GLV/RGF family have recently been shown to be required for the maintenance of the root stem cell niche and the proliferation of transit-amplifying cells that determine the RAM size. RGF peptides act through the positive regulation of the PLETHORA1 (PLT1) and PLT2 transcription factors (Matsuzaki et al., 2010; Zhou et al., 2010). The GLV/RGF signals might control in parallel RAM size and gravitropic response via the posttranslational regulation of PIN2 trafficking. Alternatively, the GLV/RGF-dependent PLT activity might be controlled by the PIN2 auxin efflux carrier, because *PLT* expression is positively regulated by auxin (Aida et al., 2004) and because peptide treatments alter the PIN2 trafficking within minutes, thus hours before the reported changes in PLT transcription or translation triggered by the GLV11/RGF1 peptide (Matsuzaki et al., 2010).

In conclusion, we have identified a mechanism in which environmental cues guide plant growth and the auxin distribution is regulated by an extracellular peptide signal. We speculate that the action of auxin and other phytohormones is intertwined with that of multiple secretory peptide families.

EXPERIMENTAL PROCEDURES

Growth Conditions

Seeds were surface sterilized and germinated on half-strength Murashige and Skoog medium (MS; Duchefa Biochemie B.V.), 1%–1.5% (w/v) sucrose, and 1.2% (w/v) agarose, at pH 5.8, in climate-controlled growth chambers at 22°C under continuous light (100 μmol × $m^{-2}s^{-1}$). For a detailed description of mutants, transgenic lines, DNA constructs, and quantitative RT-PCR experiments, see Supplemental Experimental Procedures.

Peptide Treatments

Seedlings were transferred to solid media supplemented with synthetic peptides and incubated on plates vertically or at a 45° angle. All peptides (~70% purity or higher) were dissolved in sterile sodium phosphate buffer (50 mM, pH 6.0). Peptides were either purchased from commercial vendors (Pepscan Systems or GeneScript) or synthesized in house. Peptides were applied at 100 μM unless otherwise specified. In some cases, peptide

treatment was combined with the protein synthesis inhibitor cycloheximide (CHX, 50 μM) to avoid possible transcriptional effects caused by GLV treatment.

Quantitative Analysis of Gravitropic Responses

For hypocotyls, vertically grown etiolated seedlings (4 days after germination [dag]) were rotated 90° in the dark. For roots, light-grown seedlings (4 dag) were aligned and rotated 135° in the dark. The bending angle was measured with ImageJ (http://rsbweb.nih.gov/ij/) 20 hr and 6 hr after gravistimulation for hypocotyl and root, respectively (n = 20–40). Gravicurvature was quantified consistently in at least three independent experiments and with at least two T3 single-locus homozygous *GLV* overexpression and amiR lines (Table S2).

Histochemical and Microscopic Analysis

All fluorescence images were collected and analyzed on a confocal microscope 100 M with the software package LSM 510 (version 3.2; Zeiss) equipped with a 25 mW argon and 2 mW HeNe laser. PIN2 was immunolocalized with a rabbit PIN2 antibody (1:1,000 dilution) and a Cy3-labeled anti-rabbit antibody (1:600 dilution). Movies were collected and analyzed on a Zeiss LSM 710 confocal microscope with the ZEN 2009 software. For live cell imaging, whole seedlings were mounted in water or MS media with dyes or drugs added at the appropriate concentrations. For imaging of GFP, the 488 nm line of the argon laser was used for excitation and emission was detected at 520 nm. For imaging of the *N*-(4-triethylammoniumpropyl)-4-(*p*-dietheylaminophenylhexatrienyl) pyridium dibromide (FM4-64), the 543 nm line of the HeNe laser was used for excitation and emission was detected at 590–620 nm. The laser power, pinhole, gain and offset settings for the confocal microscope were kept identical among different treatments. DIC images were captured with the transmission light detector of the confocal microscope. Fluorescence intensity from confocal sections was quantified with ImageJ.

Immunoblot Analysis of PIN2 Levels in Root Membrane Extracts

Wild-type roots (7 dag) were grown on solid medium containing no peptide, rGLV3p, or GLV3p for 8 hr. Membrane fractions were prepared and processed as described Abas and Luschnig (2010). Membrane extracts were stored in sample buffer at −70°C. For each separate treatment, membrane extracts prepared from equal amounts of starting root material (7 mg) were analyzed by SDS-PAGE. Immunoblots were carried out with a purified rabbit antibody raised against PIN2 (1:2,000 dilution) and a horseradish peroxidase-conjugated secondary antibody. PIN2 levels were quantified with ImageJ.

Statistical Tests

Unless otherwise specified, means between samples were compared by a two-sample Student's t test, taking into account the equality between population variances (assessed with an F test). Data pooled from independent biological replicates were analyzed statistically.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, three tables, and one movie and can be found with this article online at doi:10.1016/j.devcel.2012.02.002.

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