Transcriptional and Functional Classification of the GOLVEN/ROOT GROWTH FACTOR/CLE-Like Signaling Peptides Reveals Their Role in Lateral Root and Hair Formation

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The GOLVEN (GLV)/ROOT GROWTH FACTORS/CLE-Like small signaling peptide family is encoded by 11 genes in Arabidopsis (*Arabidopsis thaliana*). Some of them have already been shown to control root meristem maintenance, auxin fluxes, and gravitropic responses. As a basis for the detailed analysis of their function, we determined the expression domains for each of the 11 GLV genes with promoter-reporter lines. Although they are collectively active in all examined plant parts, GLV genes have highly specific transcription patterns, generally restricted to very few cells or cell types in the root and shoot and in vegetative and reproductive tissues. GLV functions were further investigated with the comparative analysis of root phenotypes induced by gain- and loss-of-function mutants or in treatments with GLV-derived synthetic peptides. We identified functional classes that relate to the gene expression domains in the primary root and suggest that different GLV signals trigger distinct downstream pathways. Interestingly, GLV genes transcribed at the early stages of lateral root development strongly inhibited root branching when overexpressed. Furthermore, transcription patterns together with mutant phenotypes pointed to the involvement of GLV4 and GLV8 in root hair formation. Overall, our data suggest that nine GLV genes form three subgroups according to their expression and function within the root and offer a comprehensive framework to study the role of the GLV signaling peptides in plant development.

In plants, intercellular signals are in part relayed by small-molecule phytohormones, such as auxin, cytokinin, ethylene, gibberellin, abscisic acid, and brassinosteroids. Recent biochemical and genetic studies have shown that plant cell-to-cell communication also involves peptide signaling pathways, similar to those previously identified in animals, regulating processes as diverse as plant-pathogen interaction, cell division and expansion, meristematic stem cell homeostasis, and pollen self-incompatibility (Butenko et al., 2009; Murphy et al., 2012). The recent analysis of the Arabidopsis (*Arabidopsis thaliana*) genome showed that plants code for hundreds of secreted small peptides, among which are numerous potential peptide hormones (Lease and Walker, 2006). Nevertheless, their characterization is not trivial, since most are encoded in multigene families, and the study of the corresponding loss-of-function (lof) mutants may not provide information on the function due to genetic redundancy. The expression patterns of all members of a given family, therefore, can serve as a guide for functional studies and suggest which genes are involved in identical or distinct developmental processes.

Alternatively, phenotypic analysis based on ectopic expression is another useful approach to gain insight into the molecular mechanisms controlled by peptide hormones, but it needs to be interpreted with caution as in any gain-of-function (gof) approach. In addition, synthetic peptides derived from the short conserved C-terminal domain of secreted peptide precursors (Matsubayashi, 2011) often mimic overexpression phenotypes, as is the case for the members of the large CLV3/ESR-RELATED (for CLAVATA3/
EMBRYO SURROUNDING REGION [CLE] family (Fiers et al., 2005, 2006; Strabala et al., 2006; Whitford et al., 2008; Jun et al., 2010).

In Arabidopsis, the GOLVEN (GLV)/ROOT GROWTH FACTORS (RGF) family coding for secretory peptides includes 11 genes that share the same structure (Matsuzaki et al., 2010; Whitford et al., 2012). They code for small precursor proteins of 79 to 163 amino acids containing a variable middle region that links two conserved domains: the N terminus that bears the signature of a signal peptide of 21 to 33 amino acids, with a cleavage site presumably processed when the GLV preproproteins enter the secretory pathway (Bendtsen et al., 2004), and a conserved motif of 13 to 16 amino acids at or near the C terminus that defines the GLV family, found in all higher plant genomes analyzed so far. The mature GLV1, GLV2, GLV3, and RGF1/GLV11 peptides were shown to be posttranslationally modified by sulfation of a conserved Tyr residue and hydroxylation of a Pro residue in the second half of the GLV motif (Matsuzaki et al., 2010; Whitford et al., 2012). The GLV/RFG peptides have also been associated with CLE18, a peculiar member of the CLV3/ESR family, and therefore named CLE-Like (CLEL; Meng et al., 2012). For clarity, related genes, proteins, and peptides are referred to hereafter following the GLV nomenclature. Table I lists the correspondence between Arabidopsis names reported in the three initial studies about the GLV/RFG/CLEL family.

Promoter-reporter line analysis showed that GLV1 and GLV2 are transcribed in the epidermis and cortex of the hypocotyl and are induced at its lower side upon reorientation, probably as part of the auxin transcriptional response induced by gravitimulation (Whitford et al., 2012). The promoters of GLV1 and GLV2 are also active in cotyledon, leaf, and flower tissues but not in the root. Instead, GLV3 expression was only detected in the root apical meristem (RAM), more precisely in precursor cells of the cortex, endodermis, and stele, close to the quiescent center (QC). In situ hybridization in RAM tissues showed that GLV11 is expressed in the QC and columella stem cells, while high levels of the GLV5 and GLV7 transcripts were detected mainly in the innermost layer of the central columella (Matsuzaki et al., 2010). Interestingly, the expression domains described so far provided clues about the GLV gene functions, because glv1, glv2, and glv3 lop mutants show impaired gravitropic response in the hypocotyl and the root, while the rfg1 rfg2 rfg3 triple mutant is defective in root stem cell maintenance (Matsuzaki et al., 2010; Whitford et al., 2012). Proteins identified as targets of the GLV/RFG signaling pathway(s) include the PLT1 and PLT2 transcription factors that are stabilized by the addition of the GLV11/RFG1 peptide (Matsuzaki et al., 2010) and the PIN2 auxin efflux carrier whose intracellular trafficking is regulated by the GLV3 peptide (Whitford et al., 2012).

As a first step toward a systematic analysis of GLV function, we mapped the transcription of the 11 Arabidopsis GLV genes in primary roots, young shoots, and inflorescences. In addition, we analyzed phenotypes in overexpression lines, focusing our analysis on organs and tissues highlighted by the GLV transcriptional patterns. Following this approach, we found that GLV genes are involved in multiple root development programs. This study offers a useful scaffold to guide the future genetic and biochemical studies necessary for the dissection of the signaling pathways triggered by the GLV peptides.

RESULTS

GLV Expression in the Primary Root

GLV expression in the primary root was characterized with promoter-reporter lines. GLVpro::NLS-GFP or GLVpro::GUS-GFP Arabidopsis seedlings were grown for 4 to 5 d after germination (dag), and the GFP signal was analyzed with confocal microscopy (Fig. 1). The GLV root transcription profiles were surprisingly diverse yet always restricted to very few cell types, except for GLV1 and GLV2, which were not found to be expressed in any part of the primary root (Whitford et al., 2012; Fig. 1H). Three main GLV expression domains were defined as follows. I, GLV5, GLV7, GLV10, and GLV11 were transcribed in the QC and/or columella cells (CCs; Fig. 1, A–D). II, GLV3 and GLV9 patterns were positioned just above the QC, detectable in most cell layers, and restricted to the root meristem (Fig. 1, E and F). GLV6 was present in both domains I and II (Fig. 1G). III, GLV4 and GLV8 were transcribed only in the root portion above the meristem (Fig. 1, I–N).

GLV promoters within the same domain had somewhat distinct profiles. GLV5 expression was detected in the second, third, and fourth CC layers, at a lower level in undifferentiated CCs, but not, or only marginally, in the QC (Fig. 1A). GLV7 was also excluded from the QC but present only in the third and fourth CC layers (Fig. 1B). GLV10 and GLV11 patterns were largely overlapping: both genes were expressed in the QC and the CC initials (Fig. 1, C and D). Weak GLV10 transcriptional activity was also detected in additional underlying CCs and in the vascular initials above the QC (Fig. 1C).

GLV3 transcription was detected mainly in the endodermis, the cortex, and the vascular tissues (Fig. 1E). Expression was strongest within two to three cells in the QC vicinity. Limited expression was also observed in the epidermis. The GLV9 signal was strongest in the meristemical cortical cells but also detected in the epidermis and within the vasculature (Fig. 1F). Neither GLV3 nor GLV9 was transcriptionally active in the QC. GLV6 was transcribed within the meristem, mainly in the cortex but also in the epidermal and procambial cells closest to the QC (Fig. 1G). It was also present in the QC and the first and second CC layers. GLV6
Table I. Summary of GLV expression patterns and mutant phenotypes

Web sites for software programs are as follows: AtGenExpress Visualization Tool, http://www.weigelworld.org/resources/microarray/AtGenExpress; eFP browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Genevestigator, https://www.genevestigator.com/gv/plant.jsp. fl, Flowers; le, leaves; lr, lateral roots; n.a., not assayed; n.d., not detected; n.p., no probe found in the ATH1 GeneChip; ro, root; sh, shoot; st, stem.

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This report. Whitford et al. (2012). Meng et al. (2012). Matsuzaki et al. (2010). Observed for the glv5 glv7 glv11 triple mutant (Matsuzaki et al., 2010).
Figure 1. *GLV* transcription profiles in the primary root. A to D, *GLV* genes expressed in the QC and/or CCs (domain I): *GLV5pro:NLS-2xGFP* (A), *GLV7pro:NLS-2xGFP* (B), *GLV10pro:NLS-2xGFP* (C), and *GLV11pro:NLS-2xGFP* (D). E and F, *GLV* genes active in the RAM (domain II): *GLV3pro:GUS-GFP* (E) and *GLV9pro:GUS-GFP* (F). G, *GLV6pro:NLS-GFP-GUS*, GFP signal in the meristem, QC, and CCs (domains I and II). H, *GLV1* and *GLV2* not expressed in the primary root, *GLVpro:NLS-2xGFP*. I to N, *GLV* genes expressed above the meristem (domain III): *GLV4pro:NLS-2xGFP* (I–K) and *GLV8pro:NLS-GFP-GUS* (L–N). J, K, M, and N are higher magnification images, and the rectangle in I indicates the area enlarged in J and K. J and M represent median sections. K and N show epidermal cell files. Stars mark hair cells. Roots in A to H, M, and N were counterstained with PI (red). Bars = 20 μm (A–H), 100 μm (I and L), and 50 μm (J, K, M, and N).
expression was very strong in the initials surrounding the QC.

GLV4 transcription was only detected in the root elongation zone and the proximal region of the differentiation zone (Fig. 1I) and was restricted to all cells of the epidermis (Fig. 1, J and K; Supplemental Movie S1). The lower limit of the GLV8 expression domain coincided with the border of the maturation zone and extended shootward throughout the entire root, all the way up to the coleor (Fig. 1L). The reporter GFP signal was observed in the cortex and the epidermis (Fig. 1, M and N; Supplemental Movie S2). Unlike GLV4, GLV8 was restricted to the nonhair cell files in the epidermis, although it was present in all cortical cells regardless of their position.

To summarize, nine of the 11 GLV genes are transcribed with high tissue specificity in different domains of the Arabidopsis primary root.

Figure 2. Lateral root GLV expression and gof phenotype. A, First stage at which transcriptional activity is detected for each promoter. Corresponding developmental stages are indicated in the top right corner either as a number or a letter. E, Emerged LR; MLR, LR with established apical meristem. GLV1 expression was not detected in LRs (data not shown). B, LRs in wild-type (wt) and selected GLViOE plants at 12 dag. Bars = 20 μm (A) and 0.5 cm (B). C, Number of emerged LRs per 1 cm in GLViOE lines. GLV genes were organized according to their expression onset, from earliest to latest. Two categories are defined on top of the graph corresponding to GLV genes active before and after stage IV, respectively. Col, Columbia-0; n.e., not expressed in LRs. The chart shows data from two independent experiments ± se (n = 22–64). Stars indicate significant differences compared with the wild type (*P < 0.05, ***P < 0.001).
GLV Expression during Lateral Root Formation

GLV transcriptional activity was also analyzed in lateral roots (LRs). We found that all GLV genes, except GLV1, were expressed during LR formation. However, their respective spatial and temporal patterns were very different (Fig. 2A). According to the developmental stages defined by Malamy and Benfey (1997), GLV6 was the earliest gene and the only one active from stage I on, followed by GLV5 and GLV10 (stage II), GLV7 and GLV11 (stage IV), GLV3 (stage V), and finally GLV9 and GLV2 after emergence of the primordium. GLV4 and GLV8 expression was only detected in mature LRs in the same tissue as described for the primary root (Fig. 1). Surprisingly, GLV2 expression was also detected in LRs but was not found in the primary root.

GLV Expression in Shoot Tissues of Seedlings

GLV transcription in shoot tissues was analyzed in young seedlings transformed with a GLVpro::GUS-GFP or GLVpro::NLS-GFP-GUS construct and stained for GUS enzymatic activity at 5, 10, and 15 dag to image the cotyledons and the first two true leaves.

Figure 3. GLV expression in shoot tissues. Images show GLV1pro::GUS-GFP, GLV2pro::GUS-GFP, GLV6pro::NLS-GFP-GUS, and GLV8pro::NLS-GFP-GUS. The expression of other GLV genes was not detected in these tissues (data not shown). A, Cotyledons of 5-dag seedlings. B, Shoots of 10-dag seedlings. C, Differential interference contrast images showing GLV expression in the SAM (GLV1, GLV2, GLV6) and the stipules (GLV8). Arrowheads point to the SAM, and arrows point to GLV6 expression at the border between the meristem and the leaves and to GLV8 expression in stipules. D, GLV expression in first and second leaves (10 dag). Bars = 1 mm (A and B), 50 μm (C), and 0.5 mm (D).
corresponding to developmental stages 0.7, 1.02, and 1.04, respectively (Boyce et al., 2001; Fig. 3). We previously reported that GLV1 and GLV2 were transcribed in cotyledons, leaves, and hypocotyls of 5-dag seedlings (Whitford et al., 2012). The two genes could already be distinguished at that stage based on the GUS staining of cotyledons: GLV1 was transcribed irregularly throughout the cotyledon and at a higher level at the base of the organ; GLV2 was transcribed homogeneously across the cotyledon (Fig. 3A). In the true leaves of older plants, GLV1 and GLV2 transcription patterns were partly complementary: GLV1 was expressed at a high level in the basal part of the leaf, close to the main veins, irregularly in the lamina joining these veins, and in separate segments of the leaf margin; GLV2 was expressed in the whole leaf lamina, with a stronger signal in the outer part of the leaf (Fig. 3, B and D; Whitford et al., 2012). GLV2 transcriptional activity was higher in younger leaves and decreased as leaves expanded (Fig. 3B). GLV1 transcription level was similar in all leaves regardless of age. Neither of the two genes was found to be expressed in the shoot apical meristem (SAM; Fig. 3C).

Two additional GLV genes were transcribed in the aerial part of the Arabidopsis plants. GLV6 promoter activity was detected in the vasculature of cotyledons and leaves (Fig. 3, A, B, and D) but also in the SAM, more precisely at the border between the meristem and the leaf primordia (Fig. 3C). The GLV8 pattern was irregular. The restricted GUS staining of cotyledons and leaves marked patches that could not be associated with lamina substructures (Fig. 3, A and D). Expression was also observed at the base of the cotyledon petioles and in stipules, but not in the SAM (Fig. 3, A–C). Furthermore, no promoter activity was detected in the rosette for GLV3 (Whitford et al., 2012), GLV4, GLV5, GLV7, GLV9, GLV10, and GLV11 (data not shown).

**GLV Expression in the Inflorescence**

GLV1 promoter activity was detected in the stem, the rachis of the inflorescence, and the lower portion of the flower pedicel. Noticeably, GUS staining was asymmetric in the pedicel and only detected at its upper side (Fig. 4A), a pattern reminiscent of the asymmetric expression of the gene in gravistimulated hypocotyls (Whitford et al., 2012). Within the flower, GLV1 transcription was detected throughout flower development at the base of the sepals, in the petals, the filament of the stamens, and the gynoecium (Fig. 4E). GLV2 transcription was observed in the stem and sepals and in the gynoecium of the developing flowers (Fig. 4, B and F). Both genes were also expressed later in the silique (Fig. 4, A and B). GLV6 transcription was only detected in the flower, the vasculature of sepals, stamens, and petals (Fig. 4, C and G) as well as at the tip of the gynoecium and later in the siliques. GLV8 was expressed irregularly in the sepals, similar to its leaf pattern, as well as in pollen grains (Fig. 4, D and H). Although GLV7 activity was not detected in other shoot tissues, it was transcribed in the pollen (Fig. 4I). None of the other GLV promoters was found to be active in the inflorescence (data not shown).

**GLV Promoter-Reporter Profiles Agree with Quantitative Reverse Transcription-PCR and Other Transcriptome Data**

To determine whether the results obtained with GLV promoter-reporter lines correspond to endogenous gene transcription, we extracted RNA from wild-type plants and measured GLV transcript levels by quantitative reverse transcription (qRT)-PCR in entire seedlings (5 dag), shoots (5 dag), roots (5 dag), true leaves (14 dag), and inflorescences (30 dag; Fig. 5). All GLV transcripts were detected in young seedlings, except...
GLV3, whose level was close to the detection limit. In agreement with reporter line analysis, the GLV3 to GLV11 transcripts were all detected in the main root, while GLV1 and GLV2 were absent from this organ. Only the GLV1, GLV2, and GLV10 transcripts were detected in aerial tissues (5-dag seedlings and true leaves), reflecting reporter data for GLV1 and GLV2 but not GLV10. GLV1, GLV5, GLV6, and GLV10 were highly expressed in the inflorescence, while the other genes had low or undetectable transcript levels. Reporter lines and qRT-PCR data matched in most cases. However, GLV10 transcripts were detected in the root, as determined with reporter lines, but also in other shoot tissues, suggesting that the 5' promoter region analyzed in this study may not contain all the cis-regulatory sequences controlling GLV10 tissue specificity (Lee et al., 2006).

In a previous report, in situ hybridization analysis showed that the RGF1/GLV11 transcript is present in the QC and CCs, while RGF2/GLV5 and RGF3/GLV7 transcripts were mainly detected in the innermost layer of central CCs (Matsuzaki et al., 2010). These results are in accordance with the data obtained with the corresponding GLVpro::NLS-2xGFP lines (Fig. 1, A, B, and D). Finally, transcript-level data are available in public ATH1 microarray compendia for seven GLV genes: GLV1, GLV2, GLV3, GLV4, GLV6, GLV7, and GLV9 (Schmid et al., 2005; Winter et al., 2007; Hruz et al., 2008). As summarized in Table I and Figure 6, the expression profiles deduced from microarray data agreed in almost all cases with the patterns described above (Figs. 1–4). To conclude, taking the available data into consideration, our promoter-reporter lines reliably defined the expression domain of all but one GLV gene.

**gof** Root Phenotypes Point to the Involvement of GLV Genes in Diverse Developmental Processes

Because genes coding for plant signaling peptides are in most cases part of multigene families, single *lof* mutants do not often display developmental defects due to redundancy. In such cases, *gof* mutants are useful to initiate the functional classification of these genes, for example in developmental processes. For this purpose, transgenic lines were created in which each of the GLV genes was overexpressed under the control of the Cauliflower mosaic virus 35S promoter. At least five independent single-locus lines were obtained per gene (referred to as GLV<sup>OE</sup>). We confirmed that the corresponding GLV transcript was up-regulated in most lines (Supplemental Table S2). To score defects in GLV<sup>OE</sup> mutants, seedlings were grown on inclined plates (1% agarose). We have previously reported *gof* phenotypes for the GLV1, GLV2, and GLV3 genes (Whitford et al., 2012). Transgenic Arabidopsis seedlings in which one of these genes was overexpressed showed a wavy-root phenotype when

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**Figure 5.** Relative GLV transcript levels in different parts of the plant. GLV transcript levels were measured by qRT-PCR. The data are shown for two independent biological replicates ± s.e.

**Figure 6.** Schematic representation of the GLV gene expression domains and functions.
Figure 7. GLV gof and lof root phenotypes. A, Representative GLV<sup>OE</sup> lines grown on inclined plates showing increasing defects in root gravitropic growth. B, Gravitropic response of GLV<sup>OE</sup> lines following gravistimulation. Stars indicate significant...
grown on inclined plates, which can be explained by a
defective gravitropic response (Oliva and Dunand,
2007; Whitford et al., 2012). With this study, we
continued the detailed analysis of root growth and de-
velopment in Arabidopsis lines overexpressing one of
the GLV1 to GLV11 genes (Table I).

Overexpression of all GLV genes, except GLV4 and
GLV8, markedly affected the growth of the primary
root on the agar surface of inclined plates, resulting in
dramatically larger and more irregular waves than in the
wild type, and leading in many cases to root curling (Fig. 7A; Table I; data not shown). Root growth
was quantified with the gravitropic index (GI), defined as
the ratio of the linear distance connecting the cotot and
the root tip over the root length (Grabov et al.,
2005). Two independent T3 homozygous lines were
measured for each GLV gene (Fig. 7C). GI analysis
showed that all GLV overexpression lines, except GLV4OE
and GLV9OE (corresponding to domain III genes), have
significant gravitropic growth defects and can be ranked
according to phenotype severity. Overexpression of genes
normally active in the meristematic zone (domain II) in-
duced the strongest alteration: first GLV6, followed
by GLV3 and GLV9. The phenotype of GLV5OE,
GLV7OE, GLV10OE, and GLV11OE lines (domain I) was
moderate. GLV1 and GLV2 overexpression also
resulted in lower GI, although neither gene is expressed
in the main root.

In addition, we studied the gravitropic response of
selected GLVOE lines in experiments where vertical
plates were reoriented by a 90° angle. The root cur-
vature was measured 6 h after gravistimulation. The
chosen GLVOE lines represented the different pheno-
typic classes as quantified with the GI (Fig. 7B)
and included genes expressed in different parts of
the root (Fig. 1). The results confirmed that some GLVOE
lines did not respond properly to gravity, as reported
previously for GLV3OE (Fig. 7B). In agreement with GI
measurements, GLV5OE and GLV6OE had the stron-
gest agravitropic phenotype, GLV5OE showed an
intermediate defect, whereas GLV4 overexpression had
little effect.

In summary, the distribution of the GLV genes in
three gof phenotypic classes matched their correspond-
ing domains of expression (compare Figs. 1 and
7C) and suggests that GLV genes active in domain II
(GLV3, GLV6, and GLV9) are involved in gravitropic
responses. To further test this hypothesis, we mea-
sured the GI of available GLV lof mutants grown under
the same conditions and representing each defined
expression domain: a double glv5 glv7 transfer DNA
(T-DNA) insertion mutant (domain I); lines silenced
for GLV3 (amiRglv3; domain II), GLV4 (amiRglv4; do-
main III), and GLV1 (amiRglv1; not expressed in the
root); and the glv2-1 T-DNA insertion mutant (not
expressed in the main root). Only amiRglv3 plants had
a reduced GI (Fig. 7F), confirming the role of domain II
GLV genes in gravitropism.

Previous reports showed that the overexpression of
certain GLV genes also resulted in a larger RAM (Table
I and refs. therein). We expanded this analysis to all
the lines overexpressing GLV genes. The size of the
RAM in the GLVOE lines was larger than that in the
wild type, although GLV6OE displayed only mild de-
fects (Fig. 7, D and E). The ranking and classification
based on either GI or RAM size were not strictly
equivalent (e.g. see GLV4OE phenotypes), suggesting
that the GLV peptides may be recognized by distinct
receptors and trigger different cellular responses.

Finally, we investigated root-branching defects in
GLVOE lines, since 10 of the GLV genes were found to
be expressed during LR development (Fig. 2A). In
wild-type plants, several emerged LRs are already
visible at 12 dag. In some GLVOE lines, however, only a
few or no LRs were observed at that time, while root
length was not significantly different between lines
(Fig. 2, B and C; data not shown). Interestingly, the
timing of GLV expression onset during LR initiation
and emergence correlated in most cases with the de-
fects observed in gof lines (i.e. overexpression of GLV
genes transcribed at earlier stages resulted in a smaller
number of LRs [Fig. 2, A and C], except for GLV2).

Treatments with GLV Peptides Mimic
Overexpression Phenotypes

Treatment of roots with synthetic peptides encom-
passing the GLV conserved motif at the C terminus
of GLV1, GLV2, and GLV3 preproteins caused grav-
itropic defects (Meng et al., 2012; Whitford et al., 2012).
On the other hand, roots grown on all GLV/GRF-
derived peptides, except GLV6/GRF8p, resulted in
the enlargement of the RAM (Matsuzaki et al., 2010),


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GOLVEN Signaling Peptides Control Diverse Processes

Figure 7. (Continued.)
differences compared with the wild type (wt; for odds ratio estimates of root tip angle distribution, see Supplemental Table S5). C. Quantification of
the GLVOE root growth phenotype according to the GI ± s.e. (n = 17–45). For clarity, the results for only one of two analyzed independent lines
are shown (for details, see “Materials and Methods”). D. Representative GLVOE root tips. Arrowheads show the boundary between the root meristem
and the elongation zone. E. Quantification of root meristem size (μm) in GLV5OE lines ± s.e (n = 14–84). For clarity, only one of two analyzed independent
lines is shown. F. GI quantification of GLV lof mutants roots ± s.e (n = 25–30). G. GI quantification of wild-type roots treated with GLV-derived
synthetic peptides ± s.e (n = 6–8), as shown in H and I. H and I. Root growth defects induced by GLV synthetic peptides. Wild-type roots were treated
with either unmodified peptides at 10 μM (H) or 100 nM (I, bottom panel) or Tyr-sulfated peptides (Y*) at 100 nM (I, top panel). As indicated at the top
of C, E, and F, GLV genes or peptides were grouped according to their expression domains within the root: I, colliumella/QC; II, meristem; III,
mature zone. n.e., Not expressed in the main root; np, no-peptide treatment; rGLVp, random GLVp. Stars indicate significant differences compared with the wild type (*P < 0.05, **P < 0.01, ***P < 0.001). Bars = 0.5 cm (A, H, and I) and 50 μm (D).
reminiscent of the corresponding overexpression phenotypes. Building on these previous studies, we wished to confirm that the functional domain in the other GLV precursors was also encoded in their C-terminal region by comparing peptide-induced and gof phenotypes. We analyzed the root growth pattern of wild-type roots transferred onto solid medium containing synthetic peptides (GLVp) corresponding to all members of the GLV family expressed in roots, except for GLV3p, whose effect on root growth was previously reported (Whitford et al., 2012). As a control, roots were also treated with randomized peptides (rGLVp), with identical amino acid content but a scrambled sequence.

For this assay, 3-dag wild-type seedlings were transferred on the surface of inclined plates in which the solid medium contained peptides at a concentration of 10 μM (for amino acid sequences, see Supplemental Table S4). Five days after transfer, GLVp-treated roots had wavy or agravitropic phenotypes, whereas roots grown without peptide or with randomized peptides did not (Fig. 7H). However, the severity of the peptide-induced growth pattern varied. From weakest to strongest: GLV8p, GLV6p, and GLV4p treatments resulted in a partial loss of wave formation; GLV9 and GLV10p induced exaggerated wavying; and GLV11p, GLV5p, and GLV7p induced the formation of loops, indicative of a poor gravitropic response (Fig. 7, G and H). Most peptides induced the formation of waves and loops similar to those observed in GLV6OE roots. Addition of GLV4p or GLV8p altered only slightly the root growth direction, in accordance with the phenotype of the corresponding overexpression lines. However, the addition of GLV6p had a similar effect, contradicting in this case the phenotype of the GLV6OE lines that showed the highest GI alteration. Also, opposite to the overexpression lines, peptides corresponding to genes in the meristematic region (GLV6 and GLV9) did not induce the strongest phenotype (Fig. 7, G and H).

Four endogenous mature GLV peptides have been shown to carry posttranslational modifications (GLV1, GLV2, GLV3, and GLV11/RGF1), and synthetic Tyr-sulfated versions of these peptides have a higher bioactivity than their unmodified counterparts (Matsuzaki et al., 2010; Whitford et al., 2012). Therefore, we tested whether additional GLV peptides carrying a sulfated Tyr, noted GLVp(Y*), may induce root growth defects at lower concentrations (100 nm) than the unmodified ones. The growth of roots treated with GLV4p(Y*) was not significantly altered. However, GLV5p(Y*) and GLV11p(Y*) induced a wavy phenotype similar to the unmodified peptides at higher concentrations (Fig. 7, G–I). Interestingly, GLV6p(Y*) treatment resulted in markedly slanted roots, in contrast to its unmodified counterpart, which did not significantly alter root growth at any of the assayed concentrations (Fig. 7, G and I).

We have noticed that peptide addition may affect root growth somewhat differently than overexpression of the corresponding gene (i.e. gene overexpression sometimes resulted in altered root waving, while peptide treatment instead induced root slanting). Such a discrepancy might be explained in several ways. The level of signaling peptides accumulating in the cellular microenvironment under the transcriptional control of the 35S cauliflower mosaic virus promoter can be higher than the local concentration of the synthetic peptides added exogenously. Alternatively, the maturation process might differ between GLV peptides, and some of them may require unknown posttranslational modifications to be active, for example, to bind to their cognate receptor protein with high affinity.

In summary, root growth phenotypes induced by Tyr-sulfated and unmodified GLV-derived peptides resemble in most cases those resulting from overexpression of the corresponding gene, indicating that the functional domain of the GLV protein is contained within the C-terminal portion of the precursor proteins. Furthermore, as reported in previous studies, synthetic peptides are more active when carrying a sulfated Tyr, confirming the importance of such posttranslational modifications for GLV functions (Fig. 7, G and I).

Effect of GLV Peptides on PIN2 Distribution in RAM Epidermal Cells

We previously reported that the GLV1 and GLV3 peptides induced a rapid increase in the level of PIN2 auxin efflux carrier in intracellular vesicles as well as in the plasma membrane (PM) of epidermal cells in the RAM (Whitford et al., 2012), thereby explaining how GLV gain of function prevents the formation of an asymmetric auxin gradient required for normal gravitropic responses. To study whether other GLV peptides have similar effects on PIN2 distribution, we analyzed the dynamics of the PIN2-GFP fusion protein immediately after peptide addition. PIN2pro:PIN2-GFP roots were treated with GLV peptides carrying a sulfated Tyr and derived from the GLV4, GLV5, and GLV6 precursor proteins (Supplemental Table S4). These were selected as representatives of each of the three main root expression domains (Table I). We also included RGF1/GLV11p as a reference to the work of Matsuzaki et al. (2010) and a random GLV6 peptide (rGLV6p) as a control.

The GLV3p-induced changes in PIN2-GFP signals associated with the PM and vesicles already reached a plateau 10 min after peptide treatment (Whitford et al., 2012). Therefore, the effect of the other GLV peptides was analyzed at the same time following peptide addition. Similar to the effect of GLV3p(Y*), GLV6p(Y*), GLV5p(Y*), and GLV11p(Y*), treatments resulted in the accumulation of PIN2-GFP in the PM (Fig. 8). Like GLV3p(Y*), GLV5p(Y*) and GLV11p(Y*) strongly induced PIN2 accumulation in intracellular vesicles, while GLV6p resulted in more subtle differences. GLV4p(Y*) and the rGLV6p control did not show any change in the PIN2-GFP signal. As summarized in
Table I, the effect of synthetic GLV peptides on PIN2 distribution is consistent with their respective effect on root growth direction.

Root Hair Development Is Impaired in GLV gof and lof Plants

GLV8OE lines had a weak root meristem phenotype, and GLV8 transcription was restricted to the cortical and nonhair epidermal cells of the root maturation zone. Therefore, we examined whether GLV8 is involved in the developmental programs specific to these tissues, including the formation and growth of the root hairs (Tominaga-Wada et al., 2011). While some wild-type hairs branch during cell differentiation, they rarely form more than two prongs. In contrast, GLV8OE roots carried a large number of bifurcated and higher order branched root hairs (Fig. 9A). The GLV3OE, GLV4OE, and GLV5OE lines had also more branched hairs than the wild type, but always fewer than GLV8OE. The shape of GLV8OE root hairs was also sometimes aberrant, including bent tips (Fig. 9B).

DISCUSSION

Definition of the GLV Expression Domains

Transcription pattern analysis with promoter-reporter lines provides high-resolution expression maps, including for genes not represented in the ATH1 Gene-Chip. In the case of the GLV/RGB/CLEL family, ATH1 transcript profile data are available for only seven of the 11 genes (GLV1, GLV2, GLV3, GLV4, GLV6, GLV7, and GLV9). This study showed that nine of them are transcribed in the Arabidopsis primary root with specific patterns, grouped in three distinct domains: I, the QC and CCs; II, the root meristem; and III, the maturation zone (Fig. 6). Furthermore, transcription was also detected in the course of LR development for 10 GLV genes, again each with specific patterns. Finally, localized GLV expression was detected in shoot tissues and in the inflorescence for five GLV members (Fig. 6).

To confirm the role of GLV8 in root hair development, we analyzed hair morphology in lof mutant plants. The SALK_054452 insertional line carries a T-DNA in the GLV8 promoter region approximately 80 bp before the start codon. The GLV8 transcript could not be detected by qRT-PCR in this line, hereafter renamed glv8-1 (Supplemental Table S2). In glv8-1 mutant roots, the hairs were shorter and the number of branched hairs was reduced compared with the wild type (Fig. 9, C and D). The other tested lof mutants, comprising lines silenced for GLV3 (amiRglv3) or GLV4 (amiRglv4), and a double glv5 glv7 T-DNA insertion mutant had a normal number of branched hairs (Fig. 9C). While root hair length was not affected in the amiRglv3 and glv5 glv7 mutants, amiRglv4 plants had shorter root hairs than the wild type, but this phenotype was weaker than in glv8-1 (Fig. 9D). Because GLV4 is expressed in the epidermal cells of the elongation and proximal maturation zones, it may act redundantly with GLV8 in that region of the root.

In summary, both the peculiar expression patterns of GLV4 and GLV8 and the morphological analysis of related mutants indicate that GLV signaling is also involved in root hair development.
GLV Functions Are Difficult to Dissect Genetically But May Be Classified According to Expression Domains

The diversity of GLV expression patterns suggests that GLV peptides relay cell-to-cell signals in diverse developmental processes. However, several members of the family are transcribed in close or overlapping domains, implying that single lof mutants may not yield altered phenotypes because of genetic redundancy. The combination of multiple GLV lof mutations is not trivial, because signaling peptides are encoded by small-size genes and T-DNA insertion lines are lacking for some of them. Furthermore, silencing multiple GLV genes with a single artificial mRNA (Schwab et al., 2006) is not possible, because they share little DNA sequence homology and only in short stretches. Therefore, the comparative analysis of gof mutant lines is a useful approach to gain the first insights into GLV gene function.

Our phenotypic classification focused on root gof defects detected in young seedlings. Although older soil-grown GLVOE plants were not exhaustively characterized, we did not observe any obvious phenotypes in their aerial parts. However, expression data suggest that a closer inspection is needed to investigate the
involvement of GLV signaling in developmental processes that take place in the shoot.

The overexpression of most GLV genes resulted in root gravitropic defects as well as increased meristem size, probably because the corresponding mature peptides bind identical receptor(s), as reported for other peptide families (Ogawa et al., 2008). Thus, multiple GLV genes are likely to carry partly redundant signals during gravitropic responses (Whitford et al., 2012; this report) and in meristem maintenance mechanisms (Matsuzaki et al., 2010; this report), according to their expression pattern in the RAM. The severity of the phenotypes varied, possibly reflecting ligand-receptor affinity and sequence similarity between mature GLV peptides. Based on GI measurements, GLV genes could be classified into functional groups corresponding to their expression domains, but this was not strictly the case when RAM sizes were compared. These results suggest that two different receptors might relay GLV signals involved in gravitropic responses and RAM maintenance and that one may be more specific or saturable than the other. Furthermore, because they do not act cell autonomously, secreted peptide signals may consist of integrated gradients resulting from the addition of redundant molecules encoded by genes with overlapping but distinct, expression profiles.

Although gof studies, via overexpression or peptide application, cannot determine the precise contribution of each GLV family member to specific processes, our results indicate that a larger number than initially reported may trigger the same pathway. For example, RGF1/GLV11, RGF2/GLV5, and RGF3/GLV7 were shown to control meristem maintenance (Matsuzaki et al., 2010). But GLV6 and GLV10 are also expressed in the QC and CCs, and the corresponding gof lines also displayed increased RAM size, suggesting redundancy.

Similarly, GLV3, GLV6, and GLV9 may all be involved in root gravitropism because they have similar expression domains and gof phenotype as indicated by their GI. An important site for the response to a gravistimulus is the RAM epidermal cell layer, in which the GLV signal controls the level of PIN2 in the PM (Abas et al., 2006; Whitford et al., 2012). In that framework, the closest cells expressing GLV genes are those found in the inner root tissues defining domain II within the RAM, where GLV3, GLV6, and GLV9 are transcribed. Interestingly, the overexpression of these genes led to the strongest defects in GI, possibly reflecting a high affinity of the encoded signaling peptides for their cognate receptor(s). Furthermore, only the down-regulation of GLV3 resulted in a lower GI than the wild type, while the loss of function of GLV genes expressed in other domains of the root did not. These results support a model in which the signal encoded by the GLV3, GLV6, and GLV9 peptides, secreted from inner cell layers, is perceived in the epidermis, where it regulates the differential root tip growth resulting in root gravibending and waving.

Interestingly, GLV1 and GLV2 are involved in the regulation of the gravitropic curvature in the hypocotyl (Whitford et al., 2012), and the molecular pathways downstream of the GLV signals in the two organs may be related.

GLV Signals Are Involved in LR Formation

As recently reported for two members of the gene family (GLV1/CLEL6 and GLV10/ CLEL7; Meng et al., 2012), overexpression resulted in impaired LR development, suggesting that GLV signals take part in this process. Our transcriptional and mutant analysis confirms that this indeed is the case. Interestingly, with the exception of GLV2, genes transcribed at early stages of LR initiation and yielding the strongest gof LR inhibition phenotype are also active at the core of the RAM, including the CCs, the QC, and the surrounding initials (domain I). GLV genes with lower or no inhibitory activity are either transcribed in tissues farther up in the primary root or absent from it. Remarkably, GLV5, GLV6, GLV7, GLV10, and GLV11 expressed in domain I in the primary root also showed increased RAM size when overexpressed (Matsuzaki et al., 2010; Meng et al., 2012; this report), suggesting that they may control meristematic activity in both the pericycle and the RAM. Because the expression of GLV genes is turned on at successive stages of LR formation, the corresponding peptides may be involved in different steps of the developmental program or may have an additive effect. By analogy with other signaling ligands, GLV peptides may be perceived by membrane receptor kinases involved in LR initiation. In that context, ARABIDOPSIS CRINKLY4 is an interesting candidate because it is expressed at the core of the LR primordium and controls cell division in the pericycle cell layer at the early stages of LR development (De Smet et al., 2008).

The GLV8 Signal Regulates Root Hair Development

GLV8 gain of function resulted in no or minor primary root growth defects. Furthermore, GLV8 is transcribed in the maturation zone, specifically in cortical and nonhair cells, outside the root meristem. Thus, this gene may have evolved to fulfill specific functions different from its paralogous counterparts active in the RAM and in the course of LR initiation. In Arabidopsis, the differentiation of root epidermal cells into root hair or nonhair cells depends on their position relative to the underlying cortical cells. Root hair cells are in contact with two cortical cells, whereas epidermal cells in contact with only one cortical cell remain hairless (Dolan et al., 1994). The peculiar transcription pattern of GLV8 prompted us to search for root hair phenotypes in related mutant lines. The GLV8 gof and loc phenotypes are partly opposite: GLV8gof lines produced hairs with more complex and irregular
GLV Functions and Auxin Signaling

The molecular mechanisms downstream of GLV perception in the LR meristem and the epidermal cells of the root maturation zone remain to be analyzed. We speculate that similar processes may be at play as deduced previously (Whitford et al., 2012). The GLV promoter was either a double GFP fused to a nuclear localization signal (NLS-2xGFP) or a translational fusion between GUS and GFP (GUS-GFP). In GLVpro::NLS-2xGFP lines, transcriptionally active cells were marked with a bright fluorescent nucleus, yielding high spatial resolution and high sensitivity in confocal microscopy analysis. However, GLVpro::GUS-GFP lines were preferred to study expression patterns in shoot tissues to avoid chlorophyll autofluorescence. In some cases, the reporter gene consisted of an open reading frame (ORF) coding for a nuclear GFP-GUS fusion protein (GLVpro::NLS-GFP-GUS lines; for additional information, see Supplemental Table S1). Absence of a reporter line in Figures 2 to 4, documenting GLV expression, indicates that transcriptional activity was not detected for the corresponding gene in the studied plant organ.

The GLVpro::GUS-GFP, GLVpro::NLS-GFP-GUS, GLVpro::GUS, GLVpro::NLS-GFP-GFP, and GLVpro::GUS-GFP lines were described previously (Whitford et al., 2012). The GLV4 to GLV11 promoter sequences (700–1,700 bp upstream of the ORF initiation codon) were PCR amplified with Arabidopsis genomic DNA as template. Primers were designed so that the encoded peptides act as a paracrine signal carrier, thereby affecting the formation of auxin gradients in the root tip (Whitford et al., 2012). Likewise, changes of auxin concentration at the core of the LR primordium (De Rybel et al., 2010) or in root hair cells (Pitts et al., 1998; Rahman et al., 2002; Jones et al., 2009) depending on GLV activity may control their respective development.

CONCLUSION

We have conducted an exhaustive analysis of the domains of expression for each member of the GLV family coding for signaling peptides in Arabidopsis. We have also studied related glof and lof phenotypes focusing on root development. The combined data sets suggest that nine GLV genes form three subgroups according to their expression and function within the root (Fig. 6). Our study provides useful tools to predict the functions of specific GLV genes in particular developmental processes, as demonstrated in the case of LR and root hair formation.

MATERIALS AND METHODS

Growth Conditions

Arabidopsis (Arabidopsis thaliana) seeds were sown on half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie) supplemented with 1.5% (w/v) Suc and 1% (w/v) agarose, pH 5.8, and stratified for 2 d at 4°C before germination. Seedlings were germinated in climate-controlled growth chambers at 22°C under long-day conditions (100 μmol m⁻² s⁻¹). To score root growth defects and GI measurements, seedlings were grown on plates inclined at an angle of approximately 45°. For gravistimulation experiments, 4-d auxin-light seedlings germinated on vertical plates were placed in the dark and reoriented by a 90° angle.

Recombinant DNA Constructs and Arabidopsis Lines

In most cases, the reporter protein driven by a GLV promoter was either a double GFP fused to a nuclear localization signal (NLS-2xGFP) or a translational fusion between GUS and GFP (GUS-GFP). In GLVpro::NLS-2xGFP lines, transcriptionally active cells were marked with a bright fluorescent nucleus, yielding high spatial resolution and high sensitivity in confocal microscopy analysis. However, GLVpro::GUS-GFP lines were preferred to study expression patterns in shoot tissues to avoid chlorophyll autofluorescence. In some cases, the reporter gene consisted of an open reading frame (ORF) coding for a nuclear GFP-GUS fusion protein (GLVpro::NLS-GFP-GUS lines; for additional information, see Supplemental Table S1). Absence of a reporter line in Figures 2 to 4, documenting GLV expression, indicates that transcriptional activity was not detected for the corresponding gene in the studied plant organ.

The GLVpro::GUS-GFP, GLVpro::NLS-GFP-GUS, GLVpro::GUS, GLVpro::NLS-GFP-GFP, and GLVpro::GUS-GFP lines were described previously (Whitford et al., 2012). The GLV4 to GLV11 promoter sequences (700–1,700 bp upstream of the ORF initiation codon) were PCR amplified with Arabidopsis genomic DNA as template. Primers were designed so that the encoded peptides act as a paracrine signal carrier, thereby affecting the formation of auxin gradients in the root tip (Whitford et al., 2012). Likewise, changes of auxin concentration at the core of the LR primordium (De Rybel et al., 2010) or in root hair cells (Pitts et al., 1998; Rahman et al., 2002; Jones et al., 2009) depending on GLV activity may control their respective development.

CONCLUSION

We have conducted an exhaustive analysis of the domains of expression for each member of the GLV family coding for signaling peptides in Arabidopsis. We have also studied related glof and lof phenotypes focusing on root development. The combined data sets suggest that nine GLV genes form three subgroups according to their expression and function within the root (Fig. 6). Our study provides useful tools to predict the functions of specific GLV genes in particular developmental processes, as demonstrated in the case of LR and root hair formation.
GOLVEN Signaling Peptides Control Diverse Processes

Columbia-0 accession by Agrobacterium tumefaciens (strain C58C1) flower dip (Clough and Bent, 1998). In all experiments, the wild-type accession was Columbia-0.

The mutant lines glv8-1 (SALK_054452), glv9-1 (same as rgf2-1; Matsuzaki et al., 2010; SALK_145834), and glv10-1 (same as rgf1-1; Matsuzaki et al., 2010; SALK_053439) were obtained from the European Arabidopsis Stock Center. The presence of the relevant T-DNA was confirmed by PCR with a primer corresponding to the T-DNA left border, GLV gene-specific primers, and genomic DNA as template. The relevant T-DNA is in the promoter of glv8-1 and in an intron in glv9-1 and glv10-1. The glv8 and glv10 lines were crossed to obtain the glv8 glv10 double mutant.

**qRT-PCR Analysis**

To determine GLV expression levels in different plant organs, 5-dag seedlings were split in two: the shoot part (above the cotyledon) and the whole main root (below the cotyledon). Whole 5-dag seedlings were also included in the analysis. The first/second leaves were harvested in 14-dag seedlings, and inflorescences (approximately 30 dag) were cut approximately 3 cm below the apex. Total RNA was isolated with Trizol (Invitrogen). Residual DNA contaminants were removed by treating the RNA samples with RNase-free DNase (Roche). One microgram of RNA from each sample was used as a template to synthesize the first cDNA strand with the Invitrogen cDNA Synthesis Kit (Bio-Rad). Expression levels were analyzed by qRT-PCR. Reactions were performed on 384-well plates with a LightCycler real-time thermocycler (Roche) and SYBR Green to monitor double-stranded DNA synthesis. Each quantitative PCR was performed in three technical replicates. Data were analyzed with the “delta-delta method” (Pfafl, 2001), taking primer efficiency into consideration, and normalized with UBQUITIN, CKIIa2, and CDKA as reference transcripts. The sample with the maximum value for each gene was chosen as the calibrator (set to 1), and the results of two biological replicates were averaged.

Expression levels of GLV genes in gof and l0f Arabidopsis lines were quantified in a similar way. Transcript fold change was calculated with respect to the wild type. At least five independent T3 homozygous lines were obtained for each construct. Supplemental Table S2 indicates the level of GLV transcripts in the glv T-DNA insertional mutants and independent gof and l0f T3 homozygous transgenic lines that were characterized phenotypically.

**Peptide Treatment**

GLV peptides derived from the C-terminal conserved region of the GLV preprotein were either obtained from a commercial provider (GenScript; http://www.genscript.com/) or synthesized in house as described previously (Whitford et al., 2012). To study root phenotypes induced by peptide treatment, seedlings were germinated in solid MS medium and treated for 10 min in liquid MS medium containing no peptide or supplemented with 100 nM Tyr-sulfated GLV peptides. Immediately after treatment, seedlings were mounted in the same liquid medium on a microscopic glass slide and imaged on a Zeiss Axiovert 100M confocal laser scanning microscope with the software package LSM 510 (version 3.2; Zeiss) equipped with 25-mW argon and 2-mW helium-neon lasers. Other images were taken with an Olympus Fluoview FV1000 microscope. GFP signals were detected with a 488-nm filter for excitation and a 520 nm for detection. Cell membranes were counterstained with propidium iodide (PI) and imaged with a 543-nm filter for excitation and 590 to 620 nm for detection. For the three-dimensional reconstruction of root tissues, longitudinal sections of root segments (approximately 300 μm long) were imaged with a 40× objective across one-half of the root cylinder (from epidermis to vasculature). The Z-stack step size was 1 to 1.5 μm. Movies were generated with the Velocity software (version 6.2; Perkin-Elmer) to show successive transverse planes and to visualize the nuclear GFP marker in the different root cell files.

Tissues were stained for GUS activity as described previously (Beeckman and Engler, 1994), and stained plants were analyzed and imaged with either a binocular Leica microscope or an Olympus microscope (DIC-BX51). Photographs were taken with a CAMEDIA C-3040 zoom digital camera (Olympus).

**Morphological Analysis**

Imaged root features were measured with the ImageJ software (http://rsweb.nih.gov/ij/). Two independent T3 homozygous lines were analyzed for each GLV transgenic line in all quantification experiments. The phenotype was very similar in the two lines analyzed, indicating that the GLV transcript levels are saturating in both of them. Therefore, for clarity in Figure 7, only one is shown. Metamer size was measured in GLV-NC peptides germinated on solid medium. For this purpose, 5-dag roots were stained with PI for 2 min, mounted in water, and directly imaged with the confocal microscope. For GI calculation, the root length and the distance between the cotyledon and the root tip were measured in 7-dag seedlings grown on inclined plates. Root hairs of 4- to 5-dag seedlings were imaged with a binocular Leica microscope. Root hair length was quantified in a 1-mm segment, approximately at the same distance from the root tip, where hairs are fully elongated.

**Statistical Analysis**

Statistical differences between mutant lines and the wild type were assessed with Student’s t test. Results were obtained by pooling data from two independent experiments. Error bars show se. In gravitostimulation experiments, statistical differences were analyzed using a regression model as described previously (Whitford et al., 2012; Supplemental Table S5): *P < 0.05, **P < 0.01, ***P < 0.001.

**Supplemental Data**

The following materials are available in the online version of this article.

Supplemental Table S1. GLV primers for amplification of promoter sequences.

Supplemental Table S2. Transcript levels in GLV mutant lines.

Supplemental Table S3. GLV primers for amplification of ORF sequences.

Supplemental Table S4. Synthetic peptides.

Supplemental Table S5. Odds ratio estimates of root tip angle distribution.

Supplemental Movie S1. Expression of GLV4 in the root epidermal cells.

Supplemental Movie S2. Expression of GLV8 in the root cortical and non-epidermal cells.

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**LITERATURE CITED**


