Dissecting Arabidopsis phospholipid signaling using reverse genetics
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Arabidopsis PLC3 is important for auxin mediated lateral root formation

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Abstract

Phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate and generates inositoltriphosphate and diacylglycerol, which are important second messengers in animal cells. In plants, the role of PLC remains enigmatic. Here we show that plc3 T-DNA insertion lines develop less lateral roots. Although the membrane permeable auxin analogue NAA could rescue this phenotype, addition of 2,4-D, which requires auxin influx carriers for its uptake, did not restore lateral root formation to wild-type levels. This suggests that PLC3 is involved in auxin influx. Promoter-reporter gene studies and database searches revealed that PLC3 is specifically expressed in the phloem and phloem companion cells, together with the auxin influx carrier AUX1 and its homolog LAX3. Red fluorescent protein-tagged PLC3 partially colocalized with the auxin influx carrier AUX1 at the plasmamembrane. We hypothesize that PLC3 plays a role in auxin transport.
Introduction

In mammals, phospholipase C (PLC) is at the heart of signal transduction processes by controlling the removal and formation of three distinct second messenger molecules. PLC hydrolyzes the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP₂), resulting in the formation of water-soluble inositoltrisphosphate (IP₃) and membrane localized diacylglycerol (DAG) [1]. PIP₂ binds to several proteins that regulate vesicular trafficking and cytoskeletal rearrangements [2]. In addition, PIP₂ has been shown to modulate ion channel activity [3, 4]. IP₃ triggers Ca²⁺ release from internal stores by activating Ca²⁺ channels [5], leading to a multitude of downstream effects [6]. DAG recruits protein kinase C (PKC) to the plasma membrane, resulting in phosphorylation of downstream signalling components [7, 8]. The DAG signal can be turned off by diacylglycerol kinase (DGK) that phosphorylates it to form phosphatidic acid (PA) [9, 10]. In addition to an attenuation product of DAG signalling, PA is involved in signal transduction and membrane trafficking [11], by binding and modulating the activity of several signaling proteins, including Raf-1 [12-14], Arf (ADP-ribosylation factor) [15] and mTOR [16].

Although PIP₂, IP₃ and DAG are present in plants, it is questioned whether similar functions are fulfilled, as no IP₃ receptors nor PKC homologues have ever been found. Instead of DAG, PA is thought to have signalling functions in plants [17]. This is based on the observation that PA is rapidly generated in response to various stress conditions and by the discovery of PA-binding proteins. These PA-binding proteins include the Raf-1 homologue CTR1 [18], ABI1 [19] and RCN1 [20], which are involved in responses to the phytohormones ethylene, abscisic acid and auxin respectively. Other Arabidopsis PA-binding proteins are AGD7 (ARF GAP (GTPase activating protein) Domain) [21] and heterodimeric capping protein [22]. The former stimulated Arf1 GTPase activity in a PA dependent manner whereas the

**PLC3 is important for lateral root formation**  •  129
actin-binding activity of the latter was inhibited by PA, suggesting that PA plays a role in membrane trafficking and cytoskeletal rearrangements.

The only PA-binding protein for which so far downstream targets have been identified is the protein kinase PDK1. In addition to PA, PDK1 binds to PIP$_2$ via its Pleckstrin Homology (PH) domain [23]. PDK1 phosphorylates many but not all members of the large AGC protein kinase family [24]. Among these is the PINOID (PID) protein kinase, which was also reported to bind PA [24, 25]. PID was shown to phosphorylate the auxin efflux carrier PIN1 [26, 27]. The phosphorylation state of PIN1 determines its subcellular localization, leading to directional auxin flux.

The model plant *Arabidopsis thaliana* contains 9 PLC genes [28]. These PLCs contain an X- and Y-domain, which are required for catalytic activity [29]. PLC1-5 have been shown to possess hydrolytic activity towards PIP$_2$ *in vitro* [28]. Here, we describe the characterization of PLC3 T-DNA mutant alleles that were found to develop less lateral roots. PLC3 mutants could be rescued by NAA, demonstrating that auxin perception is not affected. In contrast, 2,4-D could not restore lateral root formation to wild-type levels, indicating that auxin influx is compromised. *GUS/YFP*- reporter gene studies and database searches revealed that PLC3 is specifically expressed in the phloem, where the auxin influx carriers AUX1 and LAX3 are expressed as well. The putative role of PLC3 in regulating AUX1 and/or LAX3 is discussed.

**Results**

*PLC3 is important for lateral root development*

In our ongoing efforts to study PLC function in plants, we discovered that a PLC3 T-DNA insertion line, plc3-2, develops less lateral roots than wild-type (Fig. 1a).
A second insertion line, *plc3-3*, also exhibited this phenotype, which was similar to that of the auxin influx carrier mutant *aux1-7* [30, 31] (Fig. 1b). Primary root elongation of *plc3* lines was similar to wild-type, although a small difference for *plc3-2* was observed (Fig. 1c). When corrected for these differences, the lateral root density of *plc3-2* and *plc3-3* was found to be ~30% lower than wild-type and comparable to *aux1-7* (Fig. 1d). Lateral roots of *plc3* mutants were morphologically normal, suggesting that *plc3* is not affected in lateral root growth *per se* but rather in their initiation. No other developmental phenotypes were observed.

To confirm that *plc3-2* and *plc3-3* are loss of function mutants, *PLC3* expression was determined by RT-PCR. Using primers on both sites of the insertion (Fig. 2a), no expression was found in either *plc3-2* or *plc3-3* (Fig. 2b). To obtain additional evidence that loss of *PLC3* causes the lateral root phenotype, *PLC3* cDNA under control of a 2.4 kb promoter fragment was introduced in the *plc3-2* background. Lateral root formation in T3 seedlings, that are homozygous for this construct, was
Fig. 2. (a) Gene structure of PLC3. Filled boxes represent exons, grey boxes represent the X- and Y-domains, lines represent introns, open boxes represent untranslated regions and triangles represent T-DNA insertions. Primers used for RT-PCR are indicated by arrows. Drawing is approximately to scale. (b) PLC3 expression in plc3 alleles. RNA was extracted from wild-type and plc3 alleles and cDNA was made, which was subsequently PCR amplified for 40 cycles with PLC3 specific primers (indicated in d) and for 30 cycles with primers specific for TUBULIN4 (TUB) as a loading control. (c) Complementation of plc3-2 with PLC3 cDNA. Indicated genotypes were grown on plates for eight days, lateral roots were counted under a dissecting microscope and the primary root length was measured. Shown is the number of lateral roots per mm primary root. Statistical significant differences are indicated by letters (Tukey, α = 0.05).

restored to wild-type level, strongly supporting the conclusion that PLC3 is required for a normal lateral root density (Fig. 2c)

*Exogenous NAA, but not 2,4-D, can rescue the plc3 phenotype*

The phytohormone auxin is well known to induce lateral root development. Lateral root formation in the plc3 mutant alleles was determined in response to the exogenously applied auxin analogues 2,4-D and NAA. 2,4-D depends on auxin
Fig. 3. Lateral root formation in *plc3* is rescued by NAA but not by 2,4-D. Indicated genotypes were grown with or without 20 nM 2,4-D or 30 nM NAA, for 8 days. Lateral roots were counted under a dissecting microscope and the primary root length was measured. Shown is the number of lateral roots per mm primary root.

Influx carriers to enter the cell while NAA is membrane permeable and bypasses this requirement. Therefore, *aux1* mutants are rescued by NAA but not by 2,4-D [32]. As shown in Fig. 3, treatment with NAA rescued lateral root formation in *plc3* mutants and *aux1-7*, demonstrating that *plc3* is not affected in auxin perception. Treatment with 2,4-D enhanced lateral root formation in wild-type but not in *plc3* mutants and *aux1-7*. The difference in responses elicited by NAA and 2,4-D in *plc3* may reflect a defect in auxin influx in this mutant.

To substantiate the role of PLC3 in auxin transport, dose-response experiments were performed. Primary root elongation remained essentially unaffected by the addition of NAA (Fig. 4a) while lateral root density increased in a dose-dependent manner (Fig. 4b). Lateral root development was completely rescued in *plc3-2*,

PLC3 is important for lateral root formation
Fig. 4. Dose-dependent induction of lateral root formation in *plc3* by auxins
Indicated genotypes were grown at increasing concentrations of NAA for 8 days (a, b) or at increasing concentrations of 2,4-D for 9 days (c, d). Lateral roots were counted and the primary root length measured. Shown are the primary root length (a, c) and the lateral root density (b, d).

confirming that auxin perception is not affected. Addition of 2,4-D had an inhibitory effect on the root elongation of wild-type and *plc3*-2 but not on *aux1*-7 (Fig. 4c). Lateral root development in wild-type seedlings was dose-dependently induced by 2,4-D (Fig. 4d). Although lateral root development in *plc3*-2 was still responsive to 2,4-D, it was lower than wild-type at every concentration tested, shifting the dose-response curve to the right, suggesting that *plc3* mutants have a
reduced influx capacity. For comparison, aux1-7 was completely unresponsive to 2,4-D.

**DR5 expression**

The synthetic auxin responsive element *DR5* has been widely used to monitor auxin responsive-gene expression [33]. In auxin response mutants such as *axr1* and *axr3*, *DR5* expression is reduced. Altered *DR5* expression patterns are found in auxin efflux mutants *pin1* and *pin2* but not in the auxin influx mutant aux1-7 [34]. *DR5rev::GFP* was crossed to plc3-2. Subsequently, plc3 and wild-type plants homozygous for *DR5rev::GFP* were identified in the F2. In root tips of plc3, no difference in *DR5* expression was observed compared to wild-type (Fig. 5).
PLC3 is co-expressed with members of the LAX family of auxin influx carriers in the phloem

In order to study the expression of PLC3, a 2.4 kb fragment of the PLC3 promoter, which was sufficient to drive PLC3 cDNA to rescue the plc3-2 phenotype (Fig. 2c), was used to drive expression of a GUS:YFP fusion. GUS staining revealed ubiquitous PLC3 expression in young seedlings, mainly associated with the vasculature, in four independent transgenic lines (Fig. 6a). Diffuse staining was observed in the elongation zone but was absent in the root tip (Fig. 6b). In 9 days-old seedlings, expression was predominantly found in the elongation zone and less intense in the more mature parts of the root. Strong staining was observed at the base of outgrowing lateral roots (Fig. 6c). Confocal imaging revealed that PLC3 expression was confined to two separate cell layers in the stele (Fig. 6d,e). When imaged in a transverse section, PLC3 expression could be observed in two vascular bundles (Fig. 6f). Brady et al. have published a comprehensive expression map of the Arabidopsis root by isolating specific cell types followed by micro-array experiments. Using this database, PLC3 expression was found to be confined to the phloem and the phloem companion cells (Supplemental table 1) [35, 36]. Using an independent marker line, these data have recently been confirmed [37]. Since our data suggests that plc3 mutants have a lower auxin influx capacity and auxin influx is mediated by the LAX family of auxin influx carriers [38], the expression of AUX1 and LAX1-3, was analyzed in the cells that express PLC3. This analysis
revealed that \textit{PLC3} is co-expressed with \textit{AUX1} and \textit{LAX3} in the phloem and phloem companion cells (Fig. 6g).

\textit{RFP-tagged PLC3 colocalizes with AUX1 at the plasmamembrane}

To study the subcellular localization of \textit{PLC3}, it was translationally fused to monomeric red (cherry) fluorescent protein (RFP). This construct, driven by its native promoter, was transformed into \textit{plc3-2} or, under control of the 35S promoter, into wild-type. \textit{pPLC3::RFP:PLC3} rescued the lateral root phenotype of \textit{plc3-2} (supplemental figure 1), indicating that RFP:PLC3 is functional. Unfortunately, the fluorescence was too low to study the subcellular localization of \textit{PLC3} by confocal microscopy (data not shown). Analysis of \textit{35S::RFP:PLC3} however, revealed strong fluorescence in root tissue, which localized to both the cytoplasm and plasma membrane (Fig. 7a). Plasma membrane localized fluorescence was also observed when RFP and PLC3 were swapped (data not shown). RFP alone localizes to the cytosol only. The plasma membrane localization of \textit{PLC3} is very likely to reflect the true localization of \textit{PLC3} while the fluorescence in the cytosol is probably due to the strong overexpression. In support of this, plasma membrane localization of \textit{PLC} is consistent with subcellular fractionation studies, followed by activity assays [39].

Next, the extent of colocalization between RFP:PLC3 and AUX1:YFP was studied. AUX1 localizes to the plasma membrane, endosomes and the Golgi apparatus. This localization is sensitive to the ARF-GEF inhibitor Brefeldin A, which causes AUX1 to accumulate in large multivesicular bodies, called BFA compartements [40]. As expected, RFP:PLC3 and AUX1:YFP co-localized at the plasmamembrane (Fig. 7b,c). However, when the plants were treated with BFA for 30 min, AUX1:YFP accumulated in BFA compartments (Fig. 7e), whereas RFP:PLC3 remained at the plasma membrane (Fig. 7d, f).
Fig. 7. Subcellular localization of RFP:PLC3 and AUX1:YFP 
5 days-old F₁ seedling from a cross between 35S::RFP:PLC3 and 
pAUX1::AUX1:YFP, before (a-c) or after (d-f) treatment with 50 µM Brefeldin A. 
(a,d) RFP:PLC3, (b,e) AUX1:YFP, (c,f) overlay.
Discussion

The role of the Arabidopsis PLC3 gene was studied using T-DNA loss-of-function mutants. Two independent plc3 alleles were found to develop less lateral roots than wild-type, which could be restored by expression of PLC3 cDNA behind its own promoter, demonstrating that PLC3 is involved in lateral root development.

Lateral root formation in plc3 could be restored to wild-type level by the addition of the membrane permeable auxin analogue NAA, suggesting that the level of auxin was the limiting factor. Lateral root formation could not be completely restored with 2,4-D, which depends on auxin-influx carriers to enter the cell. Accordingly, 2,4-D cannot rescue aux1, whereas NAA can [32]. At higher 2,4-D concentrations, plc3 became responsive, suggesting that auxin influx capacity is reduced in plc3.

GUS/YFP-reporter gene analysis, combined with results from gene expression databases revealed that PLC3 is expressed in phloem cells and phloem companion cells. Auxin is known to move basipetally via the phloem. The pericycle, from which lateral roots emerge, surrounds the vasculature. It is tempting to speculate that auxin is taken up from the phloem and moves laterally toward the pericycle cells, which are stimulated by auxin to divide into a different direction, leading to a new lateral root and that this process is disturbed in plc3. DR5 expression is a function of the auxin concentration and the auxin responsiveness of a cell and this was not altered in plc3. As PLC3 expression was not found in the root tip, this experiment did not address the auxin responsiveness of plc3 but it does, however, show that the auxin maximum is not disturbed, which is often observed with auxin efflux but not with auxin influx mutants [34]. These results are therefore consistent with a role for PLC3 in auxin influx. In contrast to aux1, plc3 is not agravitropic and (data not shown), which can be explained by the different expression patterns of
AUX1 and PLC3. AUX1 is expressed in the graviresponding lateral root cap and epidermal cells [41] while PLC3 is not.

Reciprocally, cells that express PLC3, also express genes encoding for auxin influx carriers AUX1 and LAX3. In protophloem cells, AUX1 is asymmetrically localized at the apical membrane and cycles between the plasmamembrane and endosomes [40]. Perhaps, PLC3 contributes to auxin influx by regulating the function of AUX1 and/or LAX3. PLC hydrolyses PIP2 into DAG which can be quickly phosphorylated to PA. Several connections between PIP2 and AUX1/LAX3 function can be thought of. PIP2 functions in vesicular trafficking in yeast and mammals [2]. A yeast mutant with elevated PIP2 levels was impaired in endocytosis [42]. PIP2 has been shown to regulate ion channel activity. In some cases, PIP2 negatively regulates ion channel activity [43]. Therefore, PLC mediated depletion of PIP2 results in derepression of ion channel activity. PIP2 could have similar functions in Arabidopsis. Possibly, PLC3-regulated PIP2 levels are important for the cycling of AUX1/LAX3. Alternatively, PIP2 could influence auxin influx carrier activity directly.

PLC3 could also regulate appropriate levels of PA in cooperation with DGK. In mammals, PA is involved in membrane trafficking by binding to Arf [15] and the ARF GAP protein ASAP1 [44]. GTPase activity of Arf was stimulated by ASAP1 in a PA-dependent manner. A similar mechanism has been described for Arabidopsis AGD7 and Arf1, suggesting that this mechanism is conserved across kingdoms and hence could play a role in the subcellular trafficking of AUX1/LAX3.

Both PIP2 and PA are potentially involved in regulating PIN1-mediated auxin efflux by binding PDK1/PID [24, 25]. Interestingly, PDK1 phosphorylates many protein kinases \textit{in vitro} [24]. It could be that one ore more PDK1 targets regulate AUX1/LAX3 localization and/or activity. We could not detect a difference in the

\textit{PLC3 is important for lateral root formation • 141}
levels of PA, PIP$_2$ or PIP between $plc3$ and wild-type after $^{32}$P-labeling of seedlings (data not shown). Since $PLC3$ is expressed in the phloem only, any local differences could have been below detection limits.

Subcellular fractionation followed by enzyme assays has demonstrated that PLC activity is predominantly localized in the plasmamembrane [39], in agreement with our hypothesis. To study the subcellular localization of PLC3, translational fusions with RFP were expressed under the control of its native promoter in the $plc3$-2 background or overexpressed behind the 35S promoter in wild-type plants. $pPLC3::RFP$:PLC3 complemented the $plc3$-2 lateral-root phenotype (Supplemental figure 1), indicating that RFP:PLC3 is functional. Preliminary CLSM experiments with $T_2$ plants failed due to low fluorescence. This is in contrast with $pPLC3::GUS$:YFP, which could be visualized by CLSM. Possibly, RFP:PLC3 is more rapidly turned over than GUS:YFP. Similar differences in fluorescence between transcriptional and translational fusions have been reported with several transcription factors [45]. Nonetheless, overexpression of RFP:PLC3 (Fig. 6a) and PLC3:RFP (data not shown) resulted in plasma membrane-localized fluorescence. Fluorescence was apparent in the cytosol as well, probably as a result of the high expression level. RFP:PLC3 did not appear to cycle between the plasma membrane and endosomes as BFA treatment did not induce its accumulation in multivesicular bodies as observed with AUX1:YFP [40].

In order to test the dependency of AUX1 and LAX3 localization on PLC3, experiments were designed to pharmacologically and genetically interfere with PLC3 function. $AUX1$:YFP and $LAX3$:YFP reported lines were crossed with $plc3$-2 or treated with PLC inhibitors. However, the phloem in which PLC3 is exclusively expressed, could not be imaged with high enough resolution to yield conclusive data. To obtain a better resolution, we are currently implementing a two-photon imaging technique.
If PLC3 regulates the function of AUX1 and/or LAX3, then plc3 should be epistatic to aux1 and/or lax3. The phenotype of plc3 is comparable to aux1 and lax3, and intermediate compared to the aux1 lax3 double mutant which hardly develops any lateral roots [46]. These phenotypes allow us to test the genetic interactions between plc3, aux1 and lax3. If PLC3 regulates AUX1 or LAX3, then at least one of the plc3 aux1 or plc3 lax3 double mutants should have a more severe phenotype than the single mutants containing a PLC3 wild-type allele. We are currently in the process of constructing these double mutants.

Materials and methods

Plant material used in this study

PLC3 T-DNA insertion mutants were obtained from the SALK collection (table 1) [47] and homozygous plants were selected by PCR using gene specific primers in combination with the left border primer LBa (table 1). aux1-7 [48] pAUX1::AUX1:YFP [49] and DR5rev::GFP [33] have been described previously.

Table 1. T-DNA insertion lines used in this study

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* LBa gives a product in both directions

Table 2. Primers used in this study

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RT-PCR
Total RNA was extracted as described previously [50]. 5 μg of RNA was converted to cDNA using oligo-dT18 primers, dNTPs, and SuperScript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer’s instructions. PLC3 and TUBULIN α4 were PCR amplified for 40 and 30 cycles respectively with gene specific primers (table 2).

Growth of seedlings
Seeds from comparable seed batches were used. A maximum of 100 μl seeds was put in an eppendorf tube which was placed in a dessicator together with a beaker containing 100 ml household bleach to which 3 ml concentrated HCl was added. After 3 hours, the eppendorf tubes were placed in a flow cabinet to allow the chloride gas to evaporate. Seeds were placed on plates containing 2.2 g/l Murashige and Skoog medium, pH 5.7 (KOH) and 1% sucrose (w/v), supplemented with auxins where indicated. Plates were kept in the coldroom (4°C) for 3 days to promote uniform germination. Next, the seeds were allowed to germinate by placing the plates in a climate room kept at 22 °C and 70% humidity with a 16-hour photoperiod. After 8-9 days, lateral roots were counted under a dissecting microscope and the length of the root was indicated with a marker. Plates were scanned and the length of the primary root was quantified with Object Image software. The lateral root density was obtained by dividing the number of lateral roots by the length of the primary root.

DNA manipulation
To generate the pPLC3::GUS:YFP fusion, a 2437 bp upstream region of AtPLC3 (At4g38530) was amplified from genomic DNA using PLC3promHindIIIfw 5’-CCC AAG CTT CAA GTC GCC GAA CGA GAC ATC -3’ and PLC3promNheIrev 5’- CTA GCT AGC TCT TCT TCT TCT TAC TTG TTA G -3’ and cloned in a HindIII/XbaI digested pJV-GUSYFP. The PLC3::GUS:YFP
cassette was transferred to pGreen0179 using NotI resulting in pGreen179-PLC3::GUSYFP. To generate pGreen179-PLC3::PLC3, the AtPLC3 orf was amplified from the full-length cDNA clone pda16929 obtained from the RIKEN bioresource center [51, 52] using the primers PLC3NheIfw 5’- CTA GCT AGC ATG TCG GAG AGT TTC AAA GTG TG -3’ and PLC3BglIIrev 5’- GAA GAT CTT CAA CGA AAC GTA TAA GGA G -3’ and transferred to a NheI/BamHI digested pGreen179-PLC3::GUSYFP resulting in pGreen179-PLC3::PLC3. To generate pGreen17935S-RFP-PLC3, mCherry (kindly provided by Roger Tsien, San Diego, USA) was amplified using FPfwXbal 5’- GCT CTA GA A TGG TGA GCA AGG GCG AG -3’ and FP_2xGAEcoRIrev 5’- GGA ATT CGG AGC TGG TGC TGT GAG CAA GGG CGA GGA G -3’ and PLC3 was amplified using PLC3_2xGAEcoRIfw 5’- GGA ATT CGG TGC TGG AGC TAT GTC GGA GAG TTT CAA AGT GTG -3” and PLC3BglIIrev to introduce a 4xGA linker between mCherry and AtPLC3. Both PCR fragments were ligated into pJV35S digested with XbaI/BamHI. The 35S-mCherry-AtPLC3 cassette was transferred to pGreen179 using NotI. All PCR products were verified by DNA sequencing. For transformation into A. thaliana, constructs were transferred to the Agrobacterium tumefaciens strain GV3103.

GUS staining

Transgenic seedlings containing pPLC3::GUS:YFP were grown for indicated times and transferred to a solution containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-glucuronic acid (X-gluc), 50 mM phosphate buffer pH 7.0 and 0.1% TX-100. Vacuum was applied for two min and the seedlings were incubated overnight at 37°C. The next day, the solution was replaced by 100% ethanol to destain the tissue.
Confocal laser scanning microscopy

For confocal microscopy, Arabidopsis seedlings were grown for 5 days at 21 °C and transferred to object slides containing a fixed coverslide and ½ MS supplemented with 1% (w/v) sucrose. Microscopy was performed on a Zeiss LSM 510 CLSM (Confocal laser scanning microscope) (Carl Zeiss GMBH, Jena, Germany). For imaging YFP or YFP and RFP we used confocal configurations as described before [53].

Acknowledgments

We would like to thank Dorus Gadella and Erik Manders of the Centre for Advanced Microscopy for using their microscopes, Remko Offringa for advice and sending us DR5::GFP seeds, Malcolm Bennett for AUX1:YFP seeds, Norbert Vischer for advice on quantifying root elongation, Robert Schuurink for helpful discussions and Zillah Kaptein for technical assistance.

References


PLC3 is important for lateral root formation
Supplemental table 1. Relative expression of *LAX* genes and *PLC3* in Arabidopsis root tissues as published by Brady *et al.* [36].

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