Genetic approach towards the identification of auxin–cytokinin crosstalk components involved in root development

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Phytohormones are important plant growth regulators that control many developmental processes, such as cell division, cell differentiation, organogenesis and morphogenesis. They regulate a multitude of apparently unrelated physiological processes, often with overlapping roles, and they mutually modulate their effects. These features imply important synergistic and antagonistic interactions between the various plant hormones. Auxin and cytokinin are central hormones involved in the regulation of plant growth and development, including processes determining root architecture, such as root pole establishment during early embryogenesis, root meristem maintenance and lateral root organogenesis. Thus, to control root development both pathways put special demands on the mechanisms that balance their activities and mediate their interactions. Here, we summarize recent knowledge on the role of auxin and cytokinin in the regulation of root architecture with special focus on lateral root organogenesis, discuss the latest findings on the molecular mechanisms of their interactions, and present forward genetic screen as a tool to identify novel molecular components of the auxin and cytokinin crosstalk.

Keywords: auxin; cytokinin; forward genetic screen; lateral roots

1. INTRODUCTION

Root is a complex organ necessary to fix the above-ground plant body to the soil and to enable uptake of water and nutrients from the soil. Although the root is established already during embryogenesis at the basal pole of the embryo, the root system starts to develop massively during the post-embryonic plant life. The root system architecture results from two parallelly occurring processes, primary root growth and recurrent branching. In this manner, the plant root occupies the surrounding soil niche and uses the available nutritional resources. Thus, the recurrent initiation of the lateral root (LR) organogenesis by plants is the key process in the dynamic formation of the functional root system.

In Arabidopsis thaliana, LRs originate in the pericycle cell layers adjacent to the xylem pole [1,2]. After certain pericycle cells have acquired founder cell properties, LR primordia are initiated by several anticlinal divisions and they develop as a consequence of coordinated cell division and differentiation. Later on, the LR primordia emerge from the parent root, mainly by cell elongation. The LR meristem, of which structure and function are similar to the primary root meristem, is then activated [3].

The positive role of auxin at all stages of the LR organogenesis, including initiation and development, is well established. Accumulation of auxin and activation of auxin responses in individual pericycle cells stimulate founder cell specification and LR initiation (LRI) [4,5]. This positive impact of auxin on LRI was evidenced by modulation of auxin levels [4,6] as well as of auxin responses [7–9]. In the later developmental phases of LR primordia, auxin distribution gradients with maxima at the primordia tips determine the proper primordia organogenesis [10]. This auxin gradient is generated by the concerted action of AUXIN RESISTANT1 (AUX1)–like AUX1 (LAX) auxin influx carriers [11], PIN-FORMED (PIN) auxin efflux carriers [12–16], and members of the multi-drug-resistant/P-glycoprotein subfamily of ATP-binding cassette proteins [17]. When polar auxin transport is disturbed genetically or by chemical inactivation, the development of the LR primordia is severely affected [10,18,19]. Besides its action as an important patterning factor, auxin also controls the interaction between the LR primordia and the neighbouring tissues and mediates the non-invasive emergence of LR primordia through adjacent tissue layers by stimulation of cell wall-remodelling genes expression [20].

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Although auxin seems to be a general regulator through the different phases of the LR organogenesis, the transduction cascade and downstream response might be very specific for every developmental phase and mediated through specific pairs of auxin signalling components, such as AUX/INDOLE-3-ACETIC ACID (AUX/IAA) repressors and AUXIN RESPONSE FACTORS (ARFs). Thus, auxin likely interacts with cytokinin pathways to regulate the LR organogenesis [41] and cytokinin also interferes with the auxin distribution by modulating the polar auxin transport activity. This mode of interaction is particularly important for root apical meristem maintenance and LR organogenesis [31,37–40]. In the root apical meristem, the auxin–cytokinin crosstalk generates a feedback regulation mechanism that balances the auxin and cytokinin signalling pathways. Thus, when auxin is distributed by modulating the polar auxin transport activity, the feedback regulation mechanism that balances the auxin and cytokinin signalling pathways, cytokinin also interferes with the auxin distribution by modulating the polar auxin transport activity. This mode of interaction is particularly important for root apical meristem maintenance and LR organogenesis [31,37–40]. In the root apical meristem, the auxin–cytokinin crosstalk circuit is mediated through the AHK3 receptor and the downstream transcription factor ARR1 that adjusts the expression of the IAA3/SHORT HYPOCOTYL2 (SHY2) auxin signalling repressor and attenuates the expression of several PIN genes as a consequence [37]. In addition to the transcriptional control, cytokinin also impacts on the PIN1 intracellular trafficking [40–42]. This regulatory mode is important in view of the recent identification of the molecular components of the auxin–cytokinin interaction network.

Here, we describe a forward genetic screen as an approach to characterize interactions of the auxin and cytokinin signalling pathways. By using LR organogenesis as a model, we designed a mutant screen that specifically targets the interactions between auxin and cytokinin. Mutants were screened that produce LRs after application of auxin simultaneously with inhibiting concentrations of cytokinin. Twenty-two novel mutant alleles, designated pac, were recovered and classified based on their LRI and response to auxin and cytokinin. Important candidates as crosstalk components are considered primarily mutants in which the basal LRI process was not affected and the cytokinin resistance phenotype occurred only in the presence of auxin. Interestingly, detailed characterization of the pac mutant phenotypes suggested that some mutants might represent molecular components that control the cytokinin-dependent expression of the PIN auxin efflux carriers and photomorphogenesis.

2. MATERIAL AND METHODS

(a) Plant material and growth conditions
Ethyl methanesulphonate (EMS)-mutagenized and non-mutagenized transgenic Arabidopsis thaliana (L.) Heynh. lines harbouring PIN1::PIN1:GFP [10, etr1 [44] and etr1–j2 [44] were used. Seeds were sterilized with chloral gas, sown in Petri dishes on 0.8 per cent agar with 1 per cent sucrose-containing 0.5× Murashige and Skoog (MS) medium, stored for 2 days at 4°C, and grown on vertically oriented plates in growth chambers under a 16 L : 8 D cycle photoperiod at 18°C. Seven days after germination, seedlings were harvested and processed.

(b) Ethyl methanesulphonate mutagenesis and screening of mutants
Seeds of transgenic Arabidopsis plants (ecotype Columbia-0) harbouring PIN1::PIN1::GFP were soaked in 0.2 or 0.3 per cent EMS solution for 8 h. M2 seeds were bulk-harvested from approximately 20 M1 plants and pooled. Approximately 600 M2
seedlings from each pool were used for screening. Fourday-old seedlings germinated on 0.5 × MS media supplemented with 1 per cent sucrose were overlaid with 0.5 × MS liquid medium containing 1 μM IAA and 7 μM 6-benzylaminopurine (BAP) and cultivated for 48 h and 72 h, respectively. To record the efficiency of the hormonal treatments in every experiment, nonmutated PIN1::PIN1:GFP seedlings were analysed treated only with control media, supplemented with 1 μM IAA and 1 μM IAA plus 7 μM BAP. The numbers of LR primordia were scored with a fluorescence microscope MZ16F (Leica Microsystems) and mutants with more LR primordia than the control background were selected.

(c) Analyses of root growth, organogenesis of LR primordia and etiolated seedlings

Mutants and control seedlings were grown on 0.5 × MS medium without or supplemented with hormones: 0.1 μM BAP, 50 nM 1-naphthaleneacetic acid (NAA), 1 μM 1-aminocyclopropane-1-carboxylic acid (ACC). Seven days after germination, the plant material was cleared as described [3]. Root lengths were measured on scanned slides. LR primordia were counted with a differential interference contrast microscope BX51 (Olympus). Hypocotyl lengths in etiolated seedlings were analysed after 6 days of cultivation in the dark. Petri dishes with etiolated seedlings were scanned and hypocotyl lengths were measured with the IMAGIt software (http://rsbweb.nih.gov/ij/). At least 20 seedlings were analysed and the experiments were repeated twice independently. For the statistical evaluation, the t-test was done with the EXCEL statistical package. For calculation of the relative change of LRI after hormone treatment, LRI was expressed as the ratio of treated to untreated plants and the ratio of mutant to control plants was calculated. In each case, the total error was propagated [45]. One way analysis of variance combined with Holm–Sidak method was applied to evaluate a statistical significance using SIGMAPLOT software.

(d) Analysis of PIN1::PIN1:GFP expression

The cytokinin impact on the PIN1::PIN1:GFP expression was examined in root meristems exposed for 6 h to control medium or liquid 0.5 × MS medium supplemented with 10 μM BAP. At least 10 seedlings were analysed with the confocal laser-scanning microscope TCS SP2 AOPS (Leica Microsystems). The images from the obtained micrographs were processed in Adobe Photoshop.

3. RESULTS

(a) Forward genetic screen for mutants defective in auxin/cytokinin crosstalk

The antagonistic auxin–cytokinin interaction is strongly visible in the regulation of the LR organogenesis. Whereas auxin promotes both LRI and LR development, cytokinin exhibits inhibitory effects [4,30,31,46]. Thus, to identify new molecular components required for balancing the auxin/cytokinin activities, we decided to use the LR organogenesis as a suitable model system. We designed the forward genetic screen to look for mutants that produce LRs when auxin is applied simultaneously with cytokinin at inhibiting concentrations. As best crosstalk candidates, we considered mutants in which the basal LRI process was not affected and the cytokinin resistance phenotype occurred only in the presence of auxin.

To determine the optimal screening conditions, different auxin and cytokinin concentrations were applied, separately or simultaneously, on 4 days old Arabidopsis seedlings (for details, see §2). The LRI was evaluated 48 h and 72 h after treatment (electronic supplementary material, figure S1a). Application of 1 μM IAA enhanced the LRI almost threefold when compared with control seedlings (8 ± 1.4 versus 2.9 ± 0.86 LR primordia per centimetre; electronic supplementary material, figure S1b). When applied simultaneously with cytokinin, 7 μM BAP most efficiently inhibited the auxin-stimulated LRI when compared with 1 or 5 μM BAP (electronic supplementary material, figure S1b). Thus, 1 μM IAA and 7 μM BAP applied together were used to screen for mutants initiating LRs under these restrictive conditions (figure 1a,b). M1 families (1700) of EMS-mutagenized PIN1::PIN1:GFP lines were harvested into 72 pools (approx. 20–25 individuals per pool). Approximately, 600 seedlings from each pool were examined for their sensitivity to auxin/cytokinin and mutants resistant to the hormonal treatment were propagated. From 150 lines selected in the first round of the screen, 22 mutant lines were recovered with obvious resistance to auxin and cytokinin in the next generation and designated primordia on auxin and cytokinin (pac; figure 1c).

(b) Identification of pac mutants defective in auxin–cytokinin crosstalk

To distinguish pac mutants exhibiting an enhanced LRI exclusively under simultaneous auxin/cytokinin treatments from the mutants defective in LRI or altered auxin or cytokinin sensitivity, the LRI phenotypes were analysed thoroughly. Based on the LRI and its cytokinin sensitivity, we grouped the pac mutants into four subgroups: subgroups A (pac22, pac15, pac19 and pac21) and B (pac8, pac6, pac2, pac9 and pac10) exhibited an increased LRI. By contrast, mutants in subgroups C (pac4, pac17, pac18, pac14, pac11 and pac16) and D (pac3, pac1, pac20, pac7, pac12, pac5 and pac13) did not show enhanced LRI (figure 2a).

Based on the cytokinin response, mutants of subgroups B and D were resistant to cytokinin. Whereas in wild-type seedlings germinated on 0.1 μM BAP, the LRI was approximately 80 per cent lower than that of the untreated control, mutants of these two subgroups were able to initiate LRs (figure 2b).

Interestingly, the effect of the pac mutations on the auxin sensitivity was not very pronounced and only a few of the pac mutations modulated the auxin sensitivity. When compared with control seedlings with a 2.5–3-fold increased LRI after auxin (50 nM NAA) treatment, the pac15 (subgroup A), pac4 (subgroup C) and pac12 (subgroup D) mutants showed an enhanced auxin sensitivity, whereas the mutants pac2 (subgroup B) and pac7 (subgroup D) were moderately resistant to auxin (figure 2c).
Visual observation of cleared roots did not reveal any severe defects in LR primordia patterning in pac mutants. However, more detailed analyses using tissue-specific markers are needed for final conclusion on the role of PAC genes in LR primordia patterning.

Based on the detailed LRI phenotype analysis, we identified the subgroup of pac mutants corresponding to our primary requirements. The pac mutants of subgroup C were not affected in LRI and exhibited neither increased cytokinin resistance nor a dramatically changed auxin sensitivity. Thus, they represent the best candidates as crosstalk components that might be involved in the fine-tuning of auxin/cytokinin activities to ensure a relevant developmental output.

(c) Pac mutations modulate root sensitivity to cytokinin

To get insight into the general effect of pac mutations on root growth and cytokinin sensitivity, we analysed root growth on control and cytokinin-supplemented media. Overall, root growth of pac mutants was variably affected. In a few mutants of subgroups A (pac22, pac19 and pac21) and B (pac8 and pac9), root growth was reduced significantly, whereas root length increased moderately in mutants of subgroup C (pac4, pac17, pac11 and pac16) and subgroup D (pac1, pac3, pac12 and pac13) (figure 3a).

The cytokinin sensitivity of pac mutant roots was significantly altered. However, differently from LRI, cytokinin sensitivity was changed not only in mutants of subgroups B and D, but also of subgroup A (pac19 and pac21). Mutants of subgroup C, apart from pac4, did not show any dramatic change in root growth response to cytokinin (figure 3b).

Cytokinin is known to enhance ethylene production. Therefore, part of the cytokinin effects on the root phenotype might be mimicked by ethylene. As a consequence, mutants defective in the ethylene transduction pathway exhibit root growth insensitive not only to ethylene but also to cytokinin [47,48]. To dissect whether some of the pac mutants might be defective in the ethylene-related pathway, we analysed root growth on ACC [49], the precursor of the ethylene biosynthesis. As expected, the ethylene receptor mutant etr1 [43] was resistant to ethylene as well as to cytokinin but the cytokinin receptor mutant crel [44] was resistant to cytokinin, but not to ethylene (figure 3b,c). Interestingly, most pac mutants, except pac8, were not affected in the response to ethylene or exhibited mild ethylene insensitivity (pac2) (figure 3c).

Figure 1. Forward genetic screen for mutants defective in auxin/cytokinin crosstalk. (a,b) Strong stimulatory effect of auxin (1 μM IAA) application on LRI observed after 48 h on four-day-old PIN1::PIN1:GFP seedlings. Simultaneous application of cytokinin (7 μM BAP) counteracted the stimulatory auxin effect. LRI was scored in PIN1::PIN1:GFP seedlings 48 h after treatment with control media (MS) and media supplemented with 1 μM IAA or 1 μM IAA and 7 μM BAP media (*p < 0.05, n = 20 seedlings). (c) pac mutants recovered in the forward genetic screen exhibiting a reduced sensitivity to the simultaneous auxin and cytokinin treatment (p < 0.05, n = 10 seedlings). Error bars mark standard errors. LRI scored as total number of LR primordia per root.
These detailed analyses of root cytokinin and ethylene sensitivity indicate that the mutant screen as designed targeted primarily genes involved in the control of the cytokinin activity and the *pac* mutations do not seem to interfere significantly with the ethylene pathway.

**d. A subgroup of pac mutants exhibits defects in photomorphogenesis**

Next, we examined whether *PAC* genes play a role exclusively in the auxin/cytokinin-controlled LR organogenesis or are involved also in other developmental processes requiring the activity of both hormonal pathways. One such process is seedling development in response to light. In the dark, seedlings undergo skotomorphogenesis and develop long hypocotyls, an apical hook and closed cotyledons. Under light, they adopt photomorphogenesis, resulting in short hypocotyls, open cotyledons and chlorophyll accumulation [50]. Exogenous cytokinin promotes light-grown phenotypes in dark-grown seedlings [51], but auxin enhances hypocotyl elongation and suppression of auxin response seems to be critical in the regulation of photomorphogenic responses [50,52]. When grown in the dark, *pac* mutants from subgroups A and C exhibited no or mild changes in their development, respectively, when compared with control
seedlings (figure 4a). In contrast, pac mutants in subgroups B (pac6, pac2 and pac9) and D (pac1, pac7 and pac5) exhibited strong defects in skotomorphogenesis. These pac mutations promoted the light phenotype, such as shorter hypocotyls, defective apical hook formation and open cotyledons, which might result from the lack of suppression of a photomorphogenic programme (figure 4b). Interestingly, the most affected pac mutants were those belonging to subgroups B and D that were also defective in cytokinin repression of LRI (compare figures 2b and 4a). Dark phenotypes of these pac mutants hint at a link between cytokinin-regulated LRI and photomorphogenesis. An intriguing aspect of this finding is that the lack/malfunction of one molecular factor at the same time decreases the LRI cytokinin sensitivity and stimulates photomorphogenesis in the dark, the phenotype promoted by enhanced cytokinin activity.

Figure 3. Modulation of root sensitivity to cytokinin but not to ethylene in pac mutants. (a) Root length analysis of pac mutants. Root growth decreased significantly in pac22, pac19 and pac21 (subgroup A) and pac8 and pac9 (subgroup B), but roots elongated in pac15 (subgroup A), pac4, pac17, pac11, pac16 (subgroup C) and pac1, pac3, pac12, pac13 (subgroup D) mutants. (b) Reduced root cytokinin sensitivity in pac mutants. (c) Root sensitivity to ethylene moderately affected in pac mutants. Seven-day-old seedlings were analysed germinated on control media or media supplemented with cytokinin (0.1 μM BAP) or 1 μM ACC (*p < 0.05, n = 20 seedlings). Error bars mark standard errors.
activity and size. To examine whether processes, such as LR organogenesis or root meristem required for regulation of different developmental pro-
cell auxin transport and, thus, the auxin distribution
efflux carriers, cytokinin might influence the cell-to-
motors of subgroups B (\(\text{pac}6, \text{pac}2\) and \(\text{pac}9\)) and D (\(\text{pac}1, \text{pac}7\) and \(\text{pac}5\)) exhibited a strongly reduced hypocotyl length (\(p < 0.05, n = 10\) seedlings). (b) Promoted light phenotype, such as short hypocotyl, defective apical hook formation and open cotyledons in dark-grown \(\text{pac}1, \text{pac}2, \text{pac}5\) and \(\text{pac}6\) mutants. Error bars mark standard errors. Scale bars = 1 cm.

(e) A subgroup of \(\text{pac}\) mutations interferes with cytokinin-controlled PIN1 expression

One of the recently revealed important modes of inter-
action between auxin and cytokinin is the cytokinin-
mediated modulation of the polar auxin transport [37–42]. By modifying the expression of \(\text{PIN}\) auxin efflux carriers, cytokinin might influence the cell-to-cell auxin transport and, thus, the auxin distribution required for regulation of different developmental pro-
cesses, such as LR organogenesis or root meristem activity and size [31,37,38,40]. To examine whether some of the \(\text{pac}\) genes might be involved in these regulatory pathways, the cytokinin-mediated repression of the \(\text{PIN}1\) expression was analysed. The \(\text{PIN}1\)-GFP expression was monitored in control and \(\text{pac}\) mutant roots after cytokinin treatment and compared with untreated roots. As expected, treatment with 10 \(\mu\)M BAP for 6 h dramatically reduced the \(\text{PIN}1\)-GFP signal in roots of control seedlings. Several \(\text{pac}\) mutations (\(\text{pac}8, \text{pac}6, \text{pac}2, \text{pac}10, \text{pac}12\) and \(\text{pac}5\)) interfered with the cytokinin-mediated repression of the \(\text{PIN}1\) expression (figure 5). Interestingly, these \(\text{pac}\) mutants belonged to subgroups B and D that exhibited a cytokinin-resistant LRI and promoted photomorphogenesis under dark treatment.

We hypothesize that these \(\text{PAC}\) genes might be the components of the pathway that regulates the polar auxin transport and, thus, underlie the control of two distant developmental processes, such as LRI and photomorphogenesis.

4. DISCUSSION

Forward genetic screens have proved to be very power-
ful tools in dissecting the molecular components and mechanisms of the different hormonal signalling path-
ways, including those of auxin and cytokinin [53–56].
To assess the hormonal crosstalk and to identify the molecular components that mediate the pathway interactions, genetic screens have to be designed accu-
rate by taking into account the activities of both hormonal pathways in the regulation of common development processes.

The forward genetic screen that resulted in the identification of \(\text{pac}\) mutants was aimed at finding the genes that balance the auxin and cytokinin activi-
during LR organogenesis. LR organogenesis is a very suitable model for such screens because both auxin and cytokinin contribute to its regulation from the earliest stage on (for review, see [57]). As both auxin and cytokinin interact antagonistically, proper crosstalk is particularly important for the LR organo-
genesis to proceed and any deficiency in their interaction might be manifested by a defective LR organogenesis. The common feature of all \(\text{pac}\) mutants is the reduced LRI sensitivity to the simultaneous auxin/cytokinin treatment. However, among the \(\text{pac}\) mutations, several subgroups could be recognized according to additional phenotypic characteristics. The \(\text{PAC}\) genes of subgroups A and B are apparently involved in the regulation of LRI because the corre-
sponding mutants exhibit a significantly changed LRI, while the \(\text{PAC}\) genes of the B and D subgroups might contribute to the general cytokinin signal trans-
duction considering their cytokinin-insensitive LRI phenotype. Therefore, the \(\text{PAC}\) genes of these sub-
groups, although undoubtedly important factors in the regulation of LR organogenesis, might not be necessarily the components that control directly the auxin–cytokinin interaction. Importantly, the identification of the subgroup C, in which the lack of the \(\text{PAC}\) function is obvious only in the presence of both hor-
mones, hints at the existence of genes that are specifically involved in balancing the auxin–cytokinin activities. Thus, characterization of these \(\text{PAC}\) genes might be an important start point to further investigate the regulatory pathways that mediate the auxin and cytokinin crosstalk.

Figure 4. Defects in photomorphogenesis in a subgroup of \(\text{pac}\) mutants. (a) Hypocotyl length of etiolated seedlings. \(\text{pac}\) mutants of subgroups B (\(\text{pac}6, \text{pac}2\) and \(\text{pac}9\)) and D (\(\text{pac}1, \text{pac}7\) and \(\text{pac}5\)) exhibited a strongly reduced hypocotyl length (\(p < 0.05, n = 10\) seedlings). (b) Promoted light phenotype, such as short hypocotyl, defective apical hook formation and open cotyledons in dark-grown \(\text{pac}1, \text{pac}2, \text{pac}5\) and \(\text{pac}6\) mutants. Error bars mark standard errors. Scale bars = 1 cm.

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In addition to the LR organogenesis, both auxin and cytokinin are involved also in the regulation of other developmental processes (for review, see [58–61]), including the developmental switch between photomorphogenesis and skotomorphogenesis (for review, see [62]), but, as for the LR organogenesis, the mechanisms underlying their communication is unknown. Recent results [41] have revealed that cytokinin might be an important integrator of the light and auxin pathways. Lack of leaf initiation in dark-grown tomato meristems can be rescued by application of cytokinin. In the dark, PIN1, the key auxin transporter that ensures the proper hormone distribution underlying phyllotaxis [63], is internalized. Cytokinin might compensate for light treatments and stabilize PIN1 on the membranes. These results imply a scenario in which light activates the cytokinin signalling that, in turn, alters the auxin distribution important for the proper phyllotaxis through the modulation of the polar auxin transport activity. Interestingly, mutants of subgroups B and D, besides the reduced sensitivity of LRI to auxin/cytokinin and cytokinin treatments, exhibit additional defects in skotomorphogenesis manifested by dark-insensitive seedling development and cytokinin-insensitive PIN1 expression. These PAC genes hint at common regulatory mechanisms that might underlie the auxin and cytokinin interactions important not only for LR organogenesis, but also, simultaneously, for other auxin/cytokinin-regulated processes, such as seedling development controlled by darkness and light. Mutant phenotypes imply that part of such a regulatory mechanism might be executed through modulation of the auxin transport. Thus, the PAC genes of subgroups B and D represent promising candidates for additional factors that integrate cytokinin, auxin and light pathways in the regulation of plant development.

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