PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2012) **367**, 1469–1478
doi:10.1098/rstb.2011.0233

Research

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

Agnieszka Bielach[1,2], Jérôme Duclercq[1,2], Peter Marhavý[1,2] and Eva Benková[1,2,*]

[1]*Department of Plant Systems Biology, VIB, Technologiepark 927, 9052 Ghent, Belgium*
[2]*Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Ghent, Belgium*

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 aboveground 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].

* Author for correspondence (eva.benkova@psb.ugent.be).

Electronic supplementary material is available at http://dx.doi.org/10.1098/rstb.2011.0233 or via http://rstb.royalsocietypublishing.org.

One contribution of 18 to a Theme Issue 'Root growth and branching'.

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). Whereas priming is under the control of IAA28, ARF5, ARF6, ARF7, ARF8 and ARF19, founder cell specification and progress towards formative division resulting in LRI require the regulatory modules IAA14–ARF7 and IAA14–ARF19, acting in parallel with IAA12–ARF5 [21,22].

As the LR organogenesis programme is activated repetitively over time in simultaneously growing primary roots, the question arises as to the nature of the mechanisms spatio-temporally controlling these recurrent initiations. Oscillations of the auxin activity in the protoxylem cells of the basal root meristem were found to correlate with the subsequent LRI in a more proximal zone of the root [23,24]. Priming has been proposed as one of the earliest events that predetermines the LRI through regular auxin activity peaks [23]. Regular root bending as a consequence of the gravity-sensitive root behaviour generates auxin maxima in the pericycle cells at the convex bent side and activates LRI. Therefore, these auxin oscillations might be triggered by mechanisms that depend on the auxin reflux controlling the root gravity response [23,25] and/or the mechanical deformation of tissues by stretching during the curve formation [6]. Conceptually different is the hypothesis considering existence of endogenous oscillatory mechanisms. Regular oscillations in a set of genes including transcriptional regulators detected in the roots might be the primary mechanism recurrently activating processes that result in LRI [24].

Besides auxin, several other plant hormones have been found to regulate the LR organogenesis [26–29], among which cytokinin exhibits one of the strongest inhibitory effects. Any increase in cytokinin activity, either by exogenous manipulation of cytokinin levels [30,31] or endogenous modulation of the activity of genes involved in cytokinin metabolism, results in changed LRI frequencies and developmental defects [31,32]. Suppression of the cytokinin signalling pathway either by interfering with the receptor *ARABIDOPSIS HISTIDINE KINASE4* (*AHK4*)/*CYTOKININ RESISTANT1* (*CRE1*) and its homologues *AHK2* and *AHK3,* or the positive components, the B-type *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, typically enhances LR organogenesis [33,34].

The opposing contributions of both auxin and cytokinin pathways to regulate the LR organogenesis put special demands on the mechanisms that balance their activities and mediate their interaction. Auxin control over the cytokinin signalling repressors *ARR7* and *ARR15* transcription appear to be critical for proper early embryo root pole establishment [35]. Similarly, auxin and cytokinin activities in the shoot apical meristem are counterbalanced through transcriptional regulation of *ARR7* and *ARR15* by *AUXIN RESPONSE FACTOR5*/*MONOPTEROS* (*MP*) [36]. Besides crosstalk between 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 controlled leaf initiation and positioning and LR organogenesis [41,42]. Although there are several hints at the molecular nature of the auxin–cytokinin crosstalk, our knowledge on the key players is still very limited. Hence, novel approaches are necessary to identify the molecular components of the auxin–cytokinin interaction network.

Here, we describe a forward genetic screen as an approach to characterize intersections 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 *p*rimordia on *a*uxin and *c*ytokinin (*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* [43] and *cre1–12* [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. Four-day-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, non-mutated *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 stereomicroscope 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 ImageJ 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 AOBS (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 S1*a*). 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 S1*b*). 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 S1*b*). Thus, 1 μM IAA and 7 μM BAP applied together were used to screen for mutants initiating LRs under these restrictive conditions (figure 1*a*,*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 *p*rimordia on *a*uxin and *c*ytokinin (*pac*; figure 1*c*).

(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 2*a*).

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 2*b*).

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 2*c*).

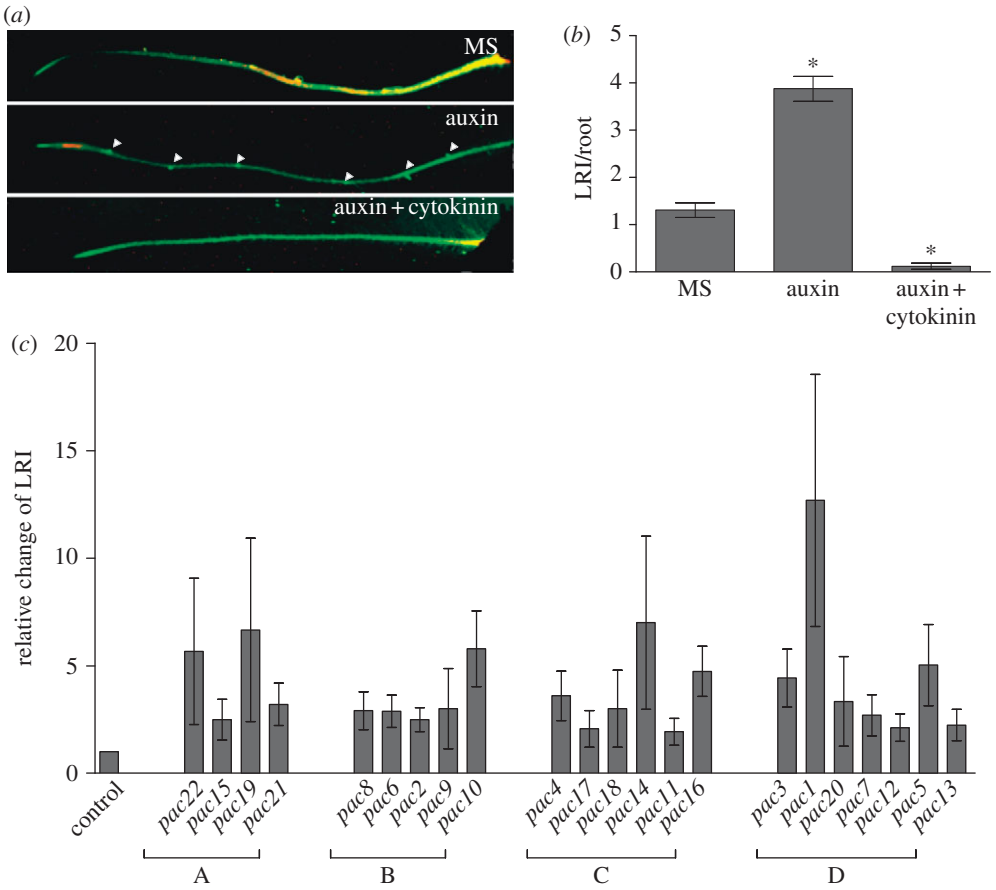

Figure 1. Forward genetic screen for mutants defective in auxin/cytokinin crosstalk. (*a*,*b*) Strong stimulatory effect of auxin (1 μM IAA) application on LRI observed after 48 h on four-day-old *PIN1::PIN1:GFP* seedlings. Simultaneous application of cytokinin (7 μM BAP) counteracted the stimulatory 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.

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 3*a*).

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 3*b*).

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 *cre1* [44] was resistant to cytokinin, but not to ethylene (figure 3*b*,*c*). Interestingly, most *pac* mutants, except *pac8*, were not affected in the response to ethylene or exhibited mild ethylene insensitivity (*pac2*) (figure 3*c*).

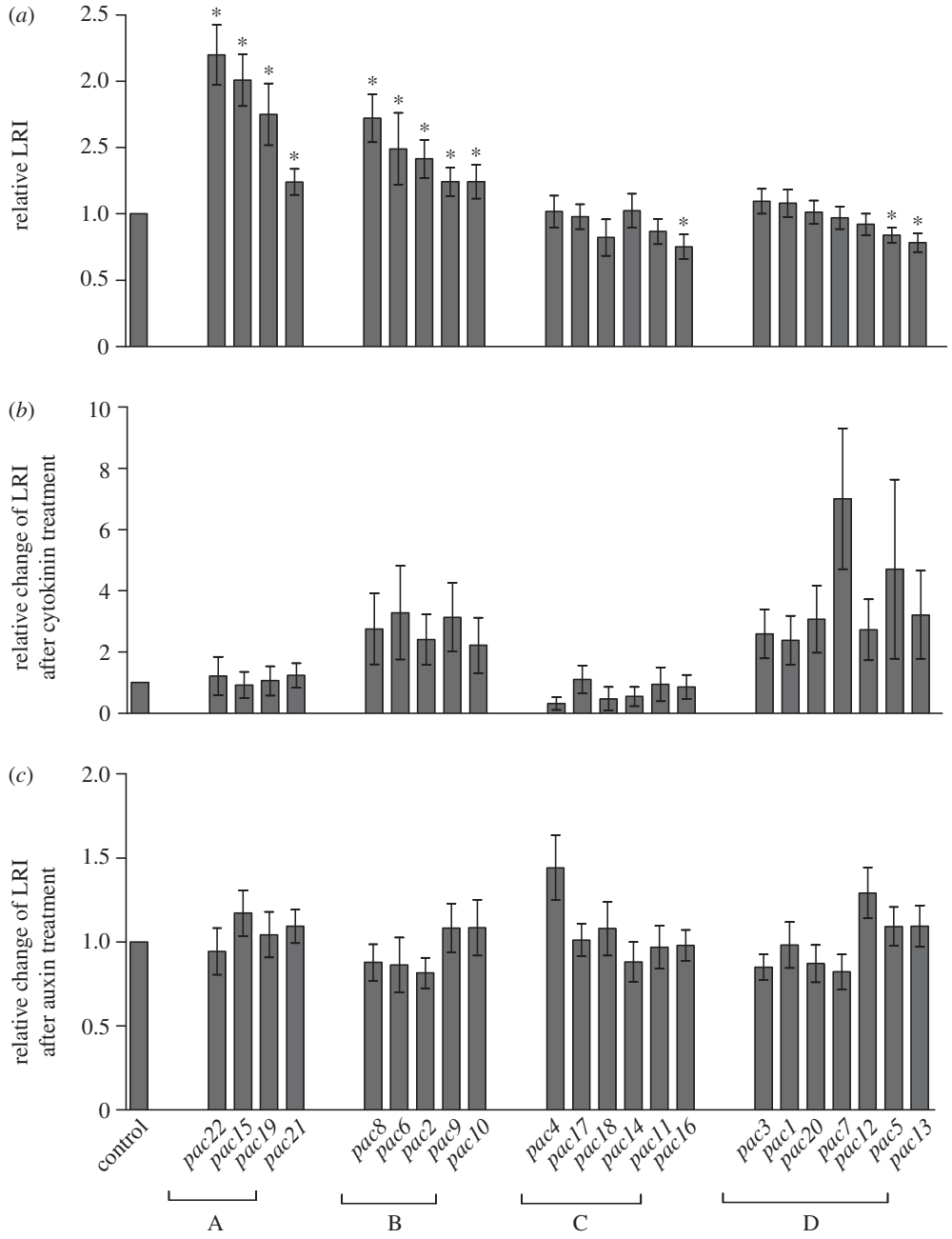

Figure 2. Impact of *pac* mutations on LRI. (*a*) Increased LRI in *pac* mutants of subgroups A and B, but not C and D. (*b*) Reduced LRI cytokinin sensitivity in *pac* mutants of subgroups B and D, but not A and C. (*c*) Increased auxin sensitivity in *pac15* (subgroup A), *pac4* (subgroup C) and *pac12*, (subgroup D) mutants and moderate auxin resistance in *pac2* (subgroup B), and *pac7* (subgroup D). Seven-day-old seedlings were analysed germinated on control media or media supplemented with cytokinin (0.1 μM BAP) or auxin (50 nM NAA; $*p < 0.05$, $n = 20$ seedlings). Error bars mark standard errors. LRI scored as a number of LR primordia per centimetre of root length.

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

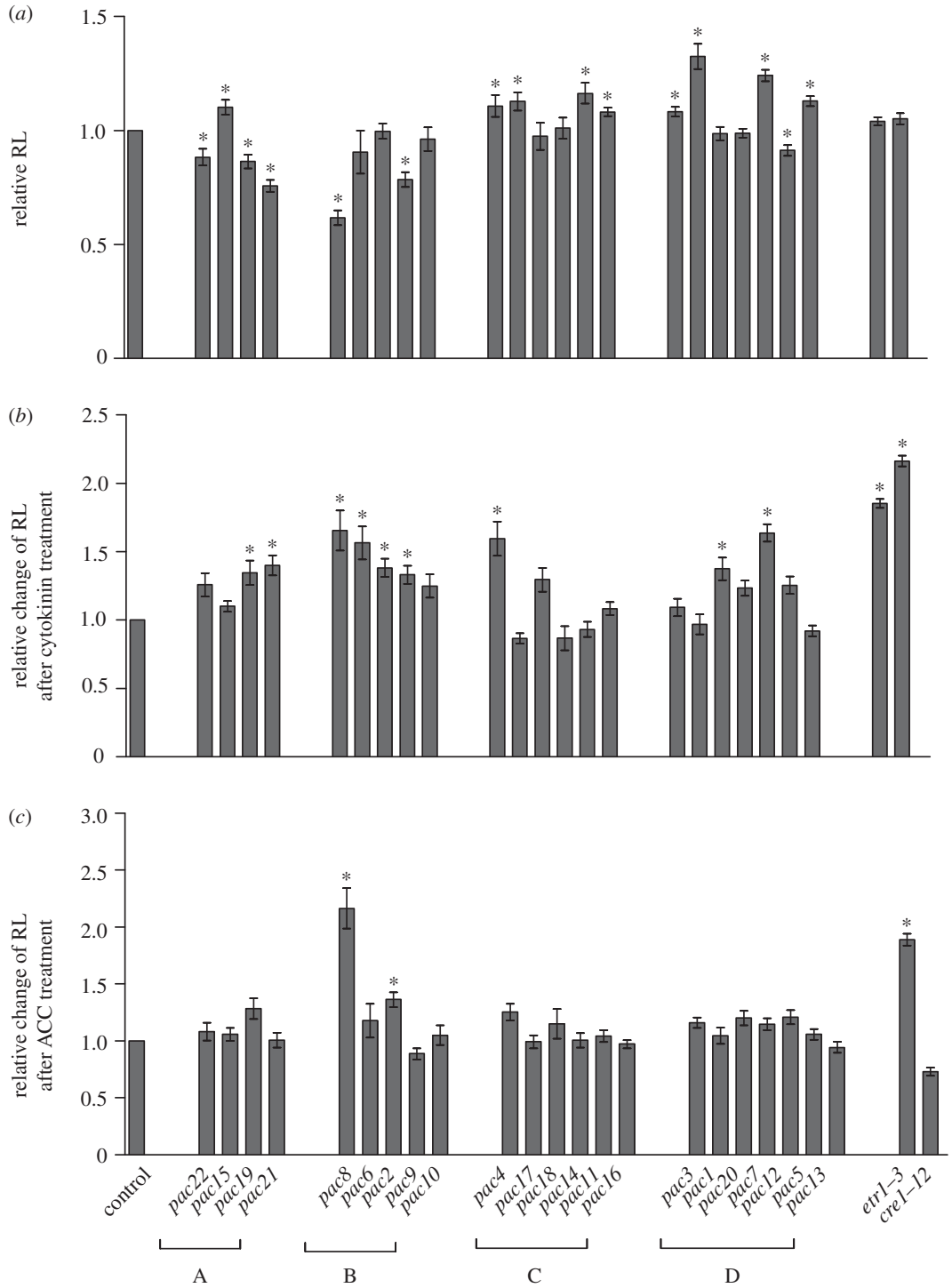

Figure 3. Modulation of root sensitivity to cytokinin but not to ethylene in *pac* mutants. (*a*) Root length analysis of *pac* mutants. Root growth decreased significantly in *pac22*, *pac19* and *pac21* (subgroup A) and *pac8* and *pac9* (subgroup B), but roots elongated in *pac15* (subgroup A), *pac4*, *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.

seedlings (figure 4*a*). 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 4*b*). 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 2*b* and 4*a*). 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.

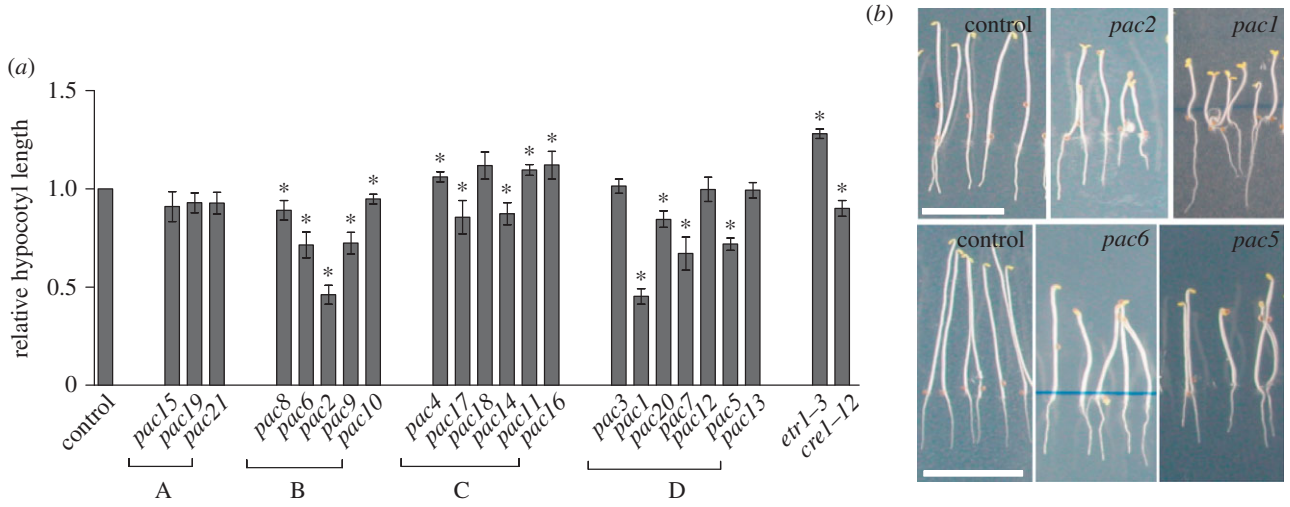

Figure 4. Defects in photomorphogenesis in a subgroup of *pac* mutants. (*a*) Hypocotyl length of etiolated seedlings. *pac* mutants of subgroups B (*pac6*, *pac2* and *pac9*) and D (*pac1*, *pac7* and *pac5*) exhibited a strongly reduced hypocotyl length ($*p < 0.05$, $n = 10$ seedlings). (*b*) Promoted light phenotype, such as short hypocotyl, defective apical hook formation and open cotyledons in dark-grown *pac1*, *pac2*, *pac5* and *pac6* mutants. Error bars mark standard errors. Scale bars = 1 cm.

(e) *A subgroup of* pac *mutations interferes with cytokinin-controlled* PIN1 *expression*

One of the recently revealed important modes of interaction between auxin and cytokinin is the cytokinin-mediated modulation of the polar auxin transport [37–42]. By modifying the expression of *PIN* auxin efflux carriers, cytokinin might influence the cell-to-cell auxin transport and, thus, the auxin distribution required for regulation of different developmental processes, such as LR organogenesis or root meristem activity and size [31,37,38,40]. To examine whether some of the *pac* genes might be involved in these regulatory pathways, the cytokinin-mediated repression of the *PIN1* expression was analysed. The *PIN1-GFP* expression was monitored in control and *pac* mutant roots after cytokinin treatment and compared with untreated roots. As expected, treatment with 10 µM BAP for 6 h dramatically reduced the *PIN1-GFP* signal in roots of control seedlings. Several *pac* mutations (*pac8*, *pac6*, *pac2*, *pac10*, *pac12* and *pac5*) interfered with the cytokinin-mediated repression of the *PIN1* expression (figure 5). Interestingly, these *pac* mutants belonged to subgroups B and D that exhibited a cytokinin-resistant LRI and promoted photomorphogenesis under dark treatment.

We hypothesize that these *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 powerful tools in dissecting the molecular components and mechanisms of the different hormonal signalling pathways, 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 accurately by taking into account the activities of both hormonal pathways in the regulation of common developmental processes.

The forward genetic screen that resulted in the identification of *pac* mutants was aimed at finding the genes that balance the auxin and cytokinin activities 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 organogenesis to proceed and any deficiency in their interaction might be manifested by a defective LR organogenesis. The common feature of all *pac* mutants is the reduced LRI sensitivity to the simultaneous auxin/cytokinin treatment. However, among the *pac* mutations, several subgroups could be recognized according to additional phenotypic characteristics. The *PAC* genes of subgroups A and B are apparently involved in the regulation of LRI because the corresponding mutants exhibit a significantly changed LRI, while the *PAC* genes of the B and D subgroups might contribute to the general cytokinin signal transduction considering their cytokinin-insensitive LRI phenotype. Therefore, the *PAC* genes of these subgroups, 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 *PAC* function is obvious only in the presence of both hormones, hints at the existence of genes that are specifically involved in balancing the auxin–cytokinin activities. Thus, characterization of these *PAC* genes might be an important start point to further investigate the regulatory pathways that mediate the auxin and cytokinin crosstalk.

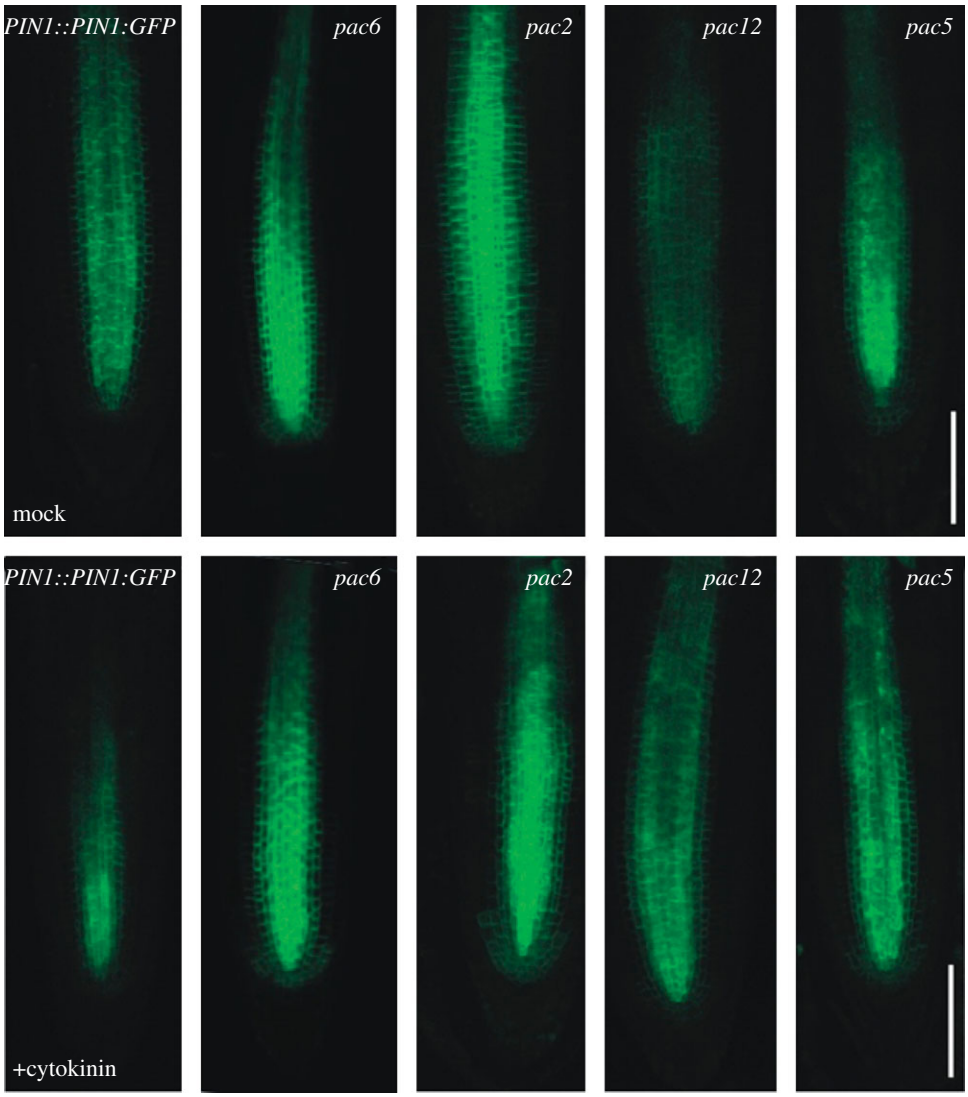

Figure 5. Interference with cytokinin-controlled *PIN1* expression in *pac* mutants. *PIN1::PIN1:GFP* expression in root meristem of *pac* mutants exposed for 6 h to control medium or liquid 0.5× MS medium supplemented with cytokinin (10 μM BAP). The *PIN1* expressions are representative of at least 10 analysed root tips of particular *pac* mutants. *PIN1-GFP* down-regulation on the control media was consistently observed in *pac12*. Scale bars = 150 μm.

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.

We thank Marleen Vanstraelen and Candela Cuesta Moliner for helpful discussions, Jiří Friml for providing material and Martine De Cock and Annick Bleys for help in preparing the manuscript. This work was supported by the European Research Council Starting Independent Research grant (ERC-2007-Stg-207362-HCPO to E.B.).

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