Cytokinin as a positional cue regulating lateral root spacing in Arabidopsis

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Abstract

The root systems of plants have developed adaptive architectures to exploit soil resources. The formation of lateral roots (LRs) contributes to root system architecture. Roots of plants with a lower cytokinin status form LR primordia (LRP) in unusually close proximity, indicating a role for the hormone in regulating the positioning of LRs along the main root axis. Data obtained from cytokinin-synthesis mutants of Arabidopsis thaliana combined with gene expression analysis indicate that cytokinin synthesis by IPT5 and LOG4 occurring early during LRP initiation generates a local cytokinin signal abbreviating LRP formation in neighbouring pericycle cells. In addition, IPT3, IPT5, and IPT7 contribute to cytokinin synthesis in the vicinity of existing LRP, thus suppressing initiation of new LRs. Interestingly, mutation of CYP735A genes required for trans-zeatin biosynthesis caused strong defects in LR positioning, indicating an important role for this cytokinin metabolite in regulating LR spacing. Further it is shown that cytokinin and a known regulator of LR spacing, the receptor-like kinase ARABIDOPSIS CRINKLY4 (ACR4), operate in a non-hierarchical manner but might exert reciprocal control at the transcript level. Taken together, the results suggest that cytokinin acts as a paracrine hormonal signal in regulating root system architecture.

Key words: Arabidopsis thaliana, cytokinin, lateral root, lateral root spacing, root branching, root system architecture.

Introduction

Lateral roots (LRs) form the main part of plant root systems and are important to optimize the ability of a root system to acquire soil nutrients and water. In Arabidopsis, LRs are formed along the main root axis from xylem pole pericycle cells (PCs) according to certain regularities, such as that lateral root primordia (LRP) usually do not form adjacent or opposite to each other (De Smet et al., 2006; Dubrovsky et al., 2006; reviewed by Dastidar et al., 2012). Xylem pole PCs are developmentally primed to form competent sites for LR formation along the longitudinal axis. This pre-pattern is established by an endogenous clock-like mechanism, termed the LR clock (Moreno-Risueno et al., 2010). However, not all competent cells develop into LRP or LRs and the mechanisms patterning the local spacing of LRP remain largely uncharacterized. It appears that auxin has a role in regulating the distance between two successive LRP through the BDL/IAA12-MP⁄ARF5 signalling module (De Smet et al., 2010) and transcriptional regulators named PLETHORA3 (PLT3),

Abbreviations: LR, lateral root; LRP, lateral root primordium; LRFC, lateral root founder cell; PC, pericycle cell; BA, 6-benzylaminopurine; tZ, trans-zeatin.

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PLT5, and PLT7 (Hofhuis et al., 2013). Furthermore, the receptor-like kinase Arabidopsis CRINKLY4 (ACR4) has been reported to act non-cell autonomously to prevent neighbouring PCs from lateral root initiation (LRI) (De Smet et al., 2008). It has been hypothesized that members of the signalling peptide family GOLVEN (GLY)/ROOT GROWTH FACTORS/CLE-LIKE interact with ACR4 to initiate a signalling cascade (Meng et al., 2012; Fernandez et al., 2013).

Cytokinins are another class of plant hormones playing a crucial role in regulating LR formation and growth, acting as a negative regulator of early processes by preventing the establishment of an auxin gradient required for LR formation (Laplaze et al., 2007; Marhavy et al., 2011). The cytokinin metabolism and signalling pathways of A. thaliana have been elucidated and comprise ~60 genes (reviewed by Sakakibara, 2006; Werner and Schmülling, 2009; Kieber and Schaller, 2014). In brief, the first and rate-limiting step in cytokinin biosynthesis is catalysed by isopentenyl transfersases (IPT), and the subsequent formation of trans-zeatin (tZ) metabolites from isopentenyl adenine (iP) metabolites is catalysed by hydroxylase enzymes (cytochrome P450 CYP735A), encoded by two predominantly root-expressed genes (Takei et al., 2004a; Kiba et al., 2013). The final step in the synthesis of iP- and tZ-type cytokinins is the release of active cytokinins from their nucleotide precursor forms by cytokinin nucleoside 5′-monophosphate phosphoribohydrolases, called LONELY GUY (LOG) (Kurakawa et al., 2007). Cytokinin breakdown is catalysed in a single step by cytokinin oxidase/dehydrogenase (CKX) enzymes (Werner et al., 2006). All of these metabolic enzymes are encoded in Arabidopsis by gene families with several members. The cytokinin signal is perceived through three membrane-located histidine kinase receptors (named AHK2, AHK3, and CRE1/AHK4) (Inoue et al., 2001; Yamada et al., 2001) and transmitted by phosphotransmitter proteins (AHPs) (Hutchison et al., 2006) to the nucleus, where type-B response regulators (type-B ARRs) activate downstream genes specifying cytokinin action (Mason et al., 2005). In Arabidopsis one member of the AHP family, AHP6, lacks the canonical phospho-accepting His residue and acts as a negative regulator of cytokinin signalling (Måhönen et al., 2006).

First hints that cytokinin could provide positional information for LRs came from an analysis of cytokinin-deficient CKX transgenic plants that showed defects in LR spacing (Werner et al., 2003). In addition, a higher LR density has been noted repeatedly on plants with a lower cytokinin content or signalling (Werner et al., 2003, 2010; Mason et al., 2005; Miyawaki et al., 2006; Riefler et al., 2006; Laplaze et al., 2007; Heyl et al., 2008; Bielach et al., 2012; Chang et al., 2013). More specifically, local ectopic expression of a CKX gene in lateral root founder cells (LRFCs) caused a higher density of LRs. This indicated that LRFCs could be the source of a cytokinin signal (Laplaze et al., 2007) that might be perceived in the zones of high cytokinin sensitivity in the vicinity of LRP (Bielach et al., 2012). Here, the role of cytokinin in controlling LR positioning was analysed in more detail, and relevant cytokinin metabolism and signalling genes identified. It is proposed that inhibitory concentrations of cytokinin originate in and around existing LRP, and their establishment requires IPT and LOG4 genes. The relationship between this pathway and the ACR4 pathway in controlling rhizotaxis is also discussed.

### Materials and methods

#### Plant material and growth conditions

All A. thaliana plants used in this study were of the Columbia (Col-0) ecotype. Seeds of acr4 (acr4-2, acr4-2 crr2-2 acr4-2 crr4; De Smet et al., 2008), ipt (ipt3-2, ipt5-2, ipt7-1, ipt3-2 ipt5-3, ipt3-2 ipt7-1, ipt3-2 ipt5-3 ipt7-1; Miyawaki et al., 2006), log4-3 (Kuroha et al., 2009), and cyp735a1-1, cyp735a2-2, cyp735a1-1 cyp735a2-2 (Kiba et al., 2013) mutants were kindly provided by Iye De Smet, Tatsuo Kakimoto, and Hitoshi Sakakibara, respectively. The marker gene lines pCKX:GUS (Werner et al., 2003), pIPT:GFP (Takei et al., 2004b), pLOG:GUS (Kuroha et al., 2009), pCYP735A2:GUS (Kiba et al., 2013), ACR4::H2B:YFP (Gifford et al., 2003), CycB1;1:GUS (Beeckman et al., 2001), TCSn:GFP (Zürcher et al., 2013), and DR5:GUS (Usami et al., 1997) were obtained from Hitoshi Sakakibara, Gwyneth C. Ingram, Laurent Laplaze, Bruno Müller, and the Nottingham Arabidopsis Stock Centre, respectively. 35S:CKX1, the type-B ARR gene mutant lines (arr1-3 arr10-5, arr1-3 arr11-2, arr1-3 arr12-1, arr1-3 arr12-5, arr1-2 arr11-2, and arr1-2 arr12-1), and the cytokinin receptor double mutants akh2-5 akh3-7, akh2-5 crel-2, and akh3-7 crel-2 were described previously (Werner et al., 2003; Mason et al., 2005; Riefler et al., 2006).

Seeds were surface sterilized and plated on solid medium (half-strength Murashige and Skoog [MS] medium, 1% sucrose, and 0.9% agar, pH 5.7) and stratified at 4°C for 3 d in the dark before germination. After stratification, all plates were placed vertically strength Murashige and Skoog [MS] medium, 1% sucrose, and 0.9% agar, pH 5.7) and stratified at 4°C for 3 d in the dark before germination. After stratification, all plates were placed vertically under white light (~100 μmol m−2 s−1) in long-day conditions (16 h light/8 h darkness) at 22°C. For cytokinin treatment, seedlings were germinated and grown on half-strength MS medium with or without 6-benzylaminopurine (BA) for 11 d.

#### Microscopic analyses

Histochemical staining for β-glucuronidase (GUS) activity was performed essentially according to Jefferson et al. (1987) as modified by Hemerly et al. (1993). Primary root length was measured on digital images of the plates using ImageJ 1.40 software (http://rsb.info.nih.gov/ij/). For recording the GUS staining pattern and LRP organization, samples were cleared and mounted according to Malamy and Benfey (1997). All samples were analysed by differential interference contrast microscopy (Zeiss Axioskop). The number of PCs between neighbouring LRP was counted in a shootward direction beginning with the LRP closest to the primary root tip.

For fluorescence microscopy, seedlings were mounted in water under glass coverslips for yellow fluorescent protein (YFP) or green fluorescent protein (GFP) signal analysis using a Leica TCS SP5 confocal laser scanning microscope. For YFP fusion protein, a wavelength of 517 nm and a filter of 526–568 nm were used for excitation and signal analysis, respectively. The fluorescence signal of GFP was analysed with a 488 nm argon laser in combination with a 500–530 nm filter set.

#### Quantitative real-time reverse transcription PCR

Total RNA was extracted from roots of 11-d-old Arabidopsis plants with the TRizol method as described by Brenner et al. (2005). cDNA synthesis, quantitative PCR (qPCR), and data analysis were performed according to Werner et al. (2010), UBC10 (At5g53300) and PDF1 (At3g22800) were used as reference genes to normalize expression levels. Primer sequences for all genes analysed are listed in Supplementary Table S1.
Results

Positioning of LRP is altered in cytokinin-deficient plants

First, LR spacing in cytokinin-deficient 35S:CKX1 transgenic Arabidopsis seedlings (Werner et al., 2003) was compared with acr4 mutant seedlings known to have a LR positioning defect (De Smet et al., 2008) and wild-type seedlings under the same growth conditions. In wild-type roots almost no LRP or emerged LRs initiated from immediately adjacent or opposite sites (Fig. 1A, B). Among 373 LRI events in 15 wild-type roots, only five LRP (1.3 ± 0.6%) were located in immediate proximity (Fig. 1J). In contrast, in acr4 mutant plants LRP initiated from adjacent or opposite sites (Fig. 1C, D), which is consistent with previous results (De Smet et al., 2008). In total, 39 LRP out of 348 LRI events (11.0 ± 1.9%) were formed from these aberrant positions (Fig. 1J). Similarly, 76 LRP out of 811 LRI events (9.1 ± 0.9%) were located immediately adjacent or opposite to each other in 35S:CKX1 plants (Fig. 1J). There was phenotypic variability in the defective spacing pattern. In some instances stretches of several PCs showed cell division activity (Fig. 1E) and closely spaced LRP were formed either on the longitudinal axis (Fig. 1F) or on the opposite side of the axis (Fig. 1G). Closely spaced LRP mostly belonged to the same developmental stage or differed by just one stage (according to Malamy and Benfey, 1997), indicating that they were induced at a similar time (Fig. 1F, G). Albeit less frequent, cytokinin-deficient plants also sometimes formed clusters of closely spaced LRP of different growth stages (Fig. 1H). Rarely, several closely spaced LRP emerged and appeared as clustered LRs (Fig. 1I). However, the proportion of emerged neighbouring LRs was low, suggesting that most of the LRP at aberrant positions arrest during development and do not contribute much to the overall root architecture. Notably, the primary roots of acr4 mutants were only about 15% shorter than those of wild type (data not shown) and those of 35S:CKX1 transgenic plants were even longer (Werner et al., 2003), indicating that the shorter distance between LRP is not correlated with the length of the primary root.

To study the reach of defective LRP positioning, the percentage of LRP separated by zero, one, or up to three PCs was determined in roots of wild-type, acr4, and 35S:CKX1 plants (Fig. 1J). The largest difference between wild type and the mutants was found for a distance of zero PCs, i.e. directly neighbouring cells, and an increased frequency was also observed in the mutants for a LRP distance of up to three PCs (Fig. 1J). In wild type, a total of 21.5% of the LRP had a neighbouring LRP within this distance, while it was 48.0% and 50.0% for acr4 and 35S:CKX1, respectively. All other LRP had a neighbouring LRP at a larger distance. Together, the LR spacing defects in acr4 mutants and 35S:CKX1 plants occurred at a similar frequency and with a similar spatial distribution.

In order to analyse cell division activity during LRP formation, expression of the CycB1:1:GUS reporter marking dividing LRFCs (Casimoro et al., 2001) was compared
between wild type and cytokinin-deficient plants. In wild type, GUS staining was observed only in the LRFCs but not in the neighbouring PCs. In contrast, in cytokinin-deficient roots neighbouring PCs could be activated simultaneously to become LRFCs, which is indicated by the cell division activity following their specification (Fig. 1K). To investigate whether closely spaced LRP establish a normal auxin pattern, the auxin reporter DR5:GUS was analysed in the 35S:CKX1 background. DR5:GUS marked founder cells and subsequent primordium formation in both wild type and neighbouring LRP in 35S:CKX1 plants and displayed similar expression patterns (Fig. 1K). Apparently closely spaced LRP establish a normal cellular division and auxin pattern and lack growth defects seen in other mutants showing spatially altered LRI (Hofhuis et al., 2013).

Cytokinin metabolism and signalling genes are involved in the positioning of LRP

The LR spacing phenotype of cytokinin-deficient plants suggests that the normally occurring inhibition of LRP formation in the vicinity of a LRP that has started to form is abolished. In order to study the spatial organization of the signal generation mediating this inhibition, expression patterns and functions of cytokinin synthesis genes were analysed. The expression of several cytokinin synthesis genes, both IPT and LOG genes, as well as of cytokinin-degrading CKX genes has been reported to occur during LR formation (Werner et al., 2003; Miyawaki et al., 2004; Takei et al., 2004b; Kuroha et al., 2009; Parizot et al., 2010; Kiba et al., 2013). The spatio-temporal expression pattern of these genes during different stages of LR formation was studied in more detail because it has been reported in most cases only for one or two stages. A particularly intriguing expression pattern was shown by the IPT5, LOG4, and CKX6 genes, which were switched on very early during formation of LRP in stage I similar to DR5:GUS (Figs 1K and 2; Supplementary Fig. S1). During further LRP development IPT5 and LOG4 were expressed in most cells until emergence. In the emerged LR the expression of IPT5 became confined to the root tip and LOG4 was strongly expressed in provascular tissue and the LR meristem (Fig. 2). In contrast, CKX6 expression was observed in the vasculature and only in stage I LRP but not in the following stages (Figure S1). The pIPT3:GFP and pIPT7:GFP reporter did not show activity during early LR development. IPT3 was expressed in the PCs and the basal stele of young LRs, IPT7:GFP expression was confined principally to the vascular stele of the primary root (Fig. 2). Expression of reporter genes for LOG3, CKX1 and CKX5 became only visible in the vasculature and at the basis of the emerged LR (Supplementary Fig. S1). Also for pCYP735A2:GUS, no expression was seen in the primordium itself but there was a variable pattern and strength of expression in PCs and vascular tissue on either side of the existing primordia (Fig. 2). The expression pattern

![Fig. 2. Spatio-temporal expression of selected cytokinin synthesis genes during LR development. A developmental sequence of the expression pattern of promoters of cytokinin metabolism genes is shown from left to right starting with stage I LRP to emerged LR. The respective promoter is indicated in the upper left corner of each picture series. Three-day-old seedlings were stained with GUS reaction buffer for 1 h and cleared. GFP expression was analysed in 5-d-old seedlings using a confocal laser scanning microscope. Part of the root staining pattern reporting expression of the cytokinin metabolism genes shown here have been published before (Werner et al., 2003; Takei et al., 2004b; Kuroha et al., 2009; Kiba et al., 2013). Scale bars is 50 μM (this figure is available in colour at JXB online).](https://academic.oup.com/jxb/article-abstract/66/15/4759/484278)
of pCYP735A1:GUS could not be analysed because of its low expression level (Takei et al., 2004a; Kiba et al., 2013). The gene expression analysis showed a specific pattern of cytokinin synthesis gene expression and pointed to the IPT5 and LOG4 genes as being possibly particularly relevant in creating a source of cytokinin in the young primordia. Their expression could establish an inhibitory field preventing LRI in neighbouring cells.

To further investigate the role of cytokinin synthesis genes in LR positioning, a detailed analysis was performed using single and higher order mutants. Single ipt mutants showed only weak (ipt3, ipt5) or no (ipt7) LRP positioning defects (Fig. 3A). The double mutants ipt3 5 and ipt3 7 showed an increased proportion of abnormally positioned LRP compared to wild type but only the difference seen for ipt3 5 was statistically significant. The proportion of misplaced LRP increased further to 13.6 ± 2.0% (57 out of 416 LRP in 12 roots) in ipt3 5 7 roots (Fig. 3A). The misplaced LR in ipt mutants displayed a similar pattern to that shown in 35S:CKX1 plants and included different developmental stages ranging from LRP to emerging LR (Supplementary Fig. S2). The log4 mutant also exhibited LR spacing defects (7.0 ± 1.4%) (Fig. 3A).

Single and double mutants of CYP735A genes also showed an abnormal LRP positioning phenotype. The cyp735a1 and cyp735a2 single mutant phenotypes were enhanced in the cyp735a1 a2 double mutant, with 13.9 ± 3.0% of the LRI events located in the immediate vicinity (Fig. 3A). This double mutant contained less than 5% of wild-type levels of tZ-type cytokinins but a 2-fold higher level of iP-type cytokinins (Kiba et al., 2013). Thus the mutant phenotype indicates a particular functional relevance of tZ in regulating LRP spacing.

The LRP spacing defect phenotype was further analysed by counting the PC number between two closely positioned LRP in ipt3 5 7 and log4 roots compared to wild type. Fig. 3B shows that both cytokinin-synthesis mutants had a higher proportion of LRP separated by zero to three PCs (44.5% for ipt3 5 7 and 47.9% for log4) as compared to wild type (22.8%). The largest difference in LRP specification between the cytokinin-synthesis mutants and the wild type was immediately adjacent to an already initiated LR and then declined gradually, showing an almost similar frequency at a distance of three PCs (Fig. 3B). This gradient of activity could possibly be due to a cytokinin flow originating in young LRP and caused by the combined activities of IPT5 and LOG4 that primarily affect the immediately adjacent cells (Fig. 2A). Fig. 4 shows that reduced cytokinin synthesis in and around LRP affects the cytokinin output signal in the zone of high cytokinin sensitivity surrounding LRP (Bielach et al., 2012). The signal of the cytokinin reporter TCSn:GFP (Zürcher et al., 2013) in wild type was high in cells neighbouring LRP but lower in these cells in roots of the cytokinin-synthesis mutant ipt3 5 7. This is consistent with a lower inhibitory activity of cytokinin on LR initiation in these cells.

To investigate whether the cytokinin receptor genes and response regulator genes are involved in the regulation of LR spacing, the double receptor mutants and type-B ARR mutants were analysed. A higher percentage of misplaced LRP as compared to wild type was formed in all three double receptor mutants, but the difference was not statistically
significant (Fig. 3C). Among the type-B response regulator genes, the arr1 12 (6.1 ± 1.6%) and arr10 12 (6.8 ± 2.2%) double mutants showed significantly increased spacing defects compared to wild type (Fig. 3C). These data are consistent with the high degree of functional redundancy of cytokinin receptors and type-B ARR genes in regulating LR development (Chang et al., 2013) and demonstrate that perception and transmission of the cytokinin signal is important to regulate LR spacing.

ACR4 expression is reduced in roots of cytokinin-deficient plants and the cytokinin status is lowered in acr4 mutants

Because both the cytokinin and ACR4 pathways prevent neighbouring PCs from LRI, the relationship between these pathways was investigated. The expression of ACR4 in roots of 11-d-old seedlings of cytokinin-synthesis and -signalling mutants was analysed. Fig. 5A shows that the abundance of the ACR4 transcripts was lower in the roots of most plants with a reduced cytokinin status. The strongest reduction of ACR4 transcript abundance (~60% reduction compared to wild type) was noted in ipt3 5 7 triple mutants. Further, in wild type, pACR4-driven H2B:YFP expression was observed in the LRP while fluorescence of YFP was hardly detectable in the cytokinin-deficient 35S:CKX1 and ipt3 5 7 background (Fig. 5B). However, ACR4 transcript analysis at different time points following exogenous addition of cytokinin did not reveal a strong influence of cytokinin on the steady-state level of ACR4 mRNA (Brenner et al., 2012 and data not shown). Taken together, this shows that ACR4 expression is reduced in cytokinin-deficient plants but that ACR4 is not a primary cytokinin response gene. The reduced LR spacing in cytokinin-deficient plants might be partly accomplished through lowered expression of ACR4.

The transcript levels of the GLV genes, which encode small signalling peptides that are the putative ligands of ACR4 (Meng et al., 2012; Fernandez et al., 2013), were then investigated. The expression of all five GLV genes that are specifically expressed during early stages of LR development (Fernandez et al., 2013) showed a lower expression in the roots of plants with a globally reduced cytokinin status (Fig. 5C), even though these form more LRP. The strongest reduction of GLV gene transcript levels (~70–80% as compared to wild type) was again observed in ipt3 5 7 triple mutant plants, similar to the strong reduction of ACR4 (Fig. 5A,C). However, in contrast, the cyp735a double mutant and the log4 mutant did not display strong changes of GLV gene expression compared to wild type. It could be that changes in iZ do not affect GLV gene expression and that the local changes of the cytokinin content expected in log4 mutants are diluted in whole root extracts. In any case, cytokinin appears to positively regulate GLV gene expression in roots. However, whether this results in altered ACR4 activity that affects LRI remains to be shown.

The transcript profiles of cytokinin genes were analysed by qPCR in the roots of acr4 mutants, and in double mutants combining acr4 with mutant alleles of two other family members, CRR2 and CRR4 (De Smet et al., 2008). The analysis showed significantly changed transcript levels for several of the cytokinin genes. IPT3 and IPT5 were both downregulated in the acr4 mutants while CKX1 and CKX6 were upregulated (Fig. 5D). Similarly, several genes encoding cytokinin signalling factors were downregulated, including the receptor gene AHC3 and the type-B ARR genes ARR1 and ARR12. Together, the altered expression of cytokinin genes suggests a lower cytokinin status in acr4 mutant roots. Interestingly, CYP735A2 was strongly upregulated in the acr4 ccr2 and acr4 crr4 double mutants, indicating a negative control of the ACR4 system on the synthesis of iZ-type cytokinins (Fig. 5D).

Cytokinin and ACR4 inhibit the initiation of neighbouring PCs through independent non-hierarchical pathways

To further investigate whether cytokinin and ACR4 control the positioning of LR through the same or separate pathways, the LR spacing phenotype of the acr4 35S:CKX1 hybrid seedlings was compared to the parental phenotypes. The hybrid
acr4 35S:CKX1 plants displayed a much higher percentage of aberrantly positioned LRP (20.0 ± 2.9%) than both parents (Fig. 6A). This largely additive effect suggests that cytokinin and ACR4 function at least partly in separate pathways in preventing neighbouring PCs from LRI. Consistently, addition of cytokinin to the growth medium had only a weak effect on the LR spacing defect in acr4 mutants, while it did lower the LR spacing defect by 67% in 35S:CKX1 seedlings (Fig. 6B).

**Discussion**

The positioning of LRP along the *Arabidopsis* main root defines a usually regular rhizotactic pattern and LRP do not form in close proximity to each other. The hormone cytokinin functions in suppressing the formation of LRP close to existing ones and thus acts as a positional cue regulating the distance between LRP. The positional signal is generated by locally and developmentally controlled precise tuning of cytokinin synthesis gene expression. The lower distance of LRP in cytokinin-synthesis mutants is hypothesized to be due to a local inhibitory cytokinin gradient originating from the LRFCs, and to cytokinin produced in PCs neighbouring LRFCs. This paracrine action of cytokinin, a combination of non-cell autonomous and cell autonomous activity, inhibits the formation of LRP in PCs neighbouring existing LRP. This hypothesis is supported by (i) the activation of cytokinin synthesis genes early during LRP development and their expression in neighbouring PCs; (ii) the fact that disruption of these genes causes aberrant positioning of LRP, with the strongest effect in the immediate vicinity of existing LRP; and (iii) a gradual decrease of the inhibitory cytokinin action in wild type with increasing distance from LRFCs. The proposed activity of cytokinin is summarized in a model (Fig. 7).

The expression pattern of cytokinin synthesis genes showed that *IPT5* and *LOG4* were expressed very early during LR initiation (Fig. 2) (Miyawaki et al., 2004; Kuroha et al., 2009). However, *TCS:GFP* reporter expression revealed that cytokinin signalling is strongly repressed during early phases of LR development (Bielach et al., 2012), indicating that the
signal generated by IPT5 and LOG4 in the initiated LR was suppressed or diffused laterally. Considering the strong enhancement of abnormal LR positioning when IPT5 is mutated in addition to IPT3 and IPT7 and, in particular, the LR positioning defect caused by LOG4 mutation alone (Fig. 3A,B), it is possible that cytokinin synthesized by IPT5 and LOG4 in the LRFCs is transmitted to neighbouring PCs to prevent LRI. This is in agreement with the lowest frequency of new LR immediately adjacent to existing ones in wild type and their gradually increasing frequency with increasing distance from existing LRP (Fig. 3B). Consistently, the strongest increase of aberrantly positioned LRP in ipt3 5 7 and log4 mutants was found immediately adjacent to existing ones (Fig. 3B). Further, expression of a cytokinin-degrading CKX gene in the LRFCs reduces the distance from new neighbouring LRP, which is in line with a suppressive function of LRFC-derived cytokinin in neighbouring cells (Laplaze et al., 2007). In accordance with the idea of a cytokinin signal coming from LRFCs was the finding that a cytokinin output signal is detected few hours after LRI in adjacent cells (Bielach et al., 2012). However, the authors did not report any cell-to-cell movement of a cytokinin signal. An alternative possibility to explain the gradual difference in response of PCs neighbouring LRFCs to a suppressive cytokinin signal would be differences in their cytokinin sensitivity. However, a strongly enhanced percentage of LRP immediately adjacent to existing LRP was not found in mutants retaining single cytokinin receptors (Fig. 3C), although they generally show a higher LRP and LR density (Chang et al., 2013). In any case, there is an additional relevant role of cytokinin synthesis genes (IPT3, IPT7) expressed in pericycle tissue adjacent to existing LRP. This argues for a combined action of LRP-derived cytokinin and cytokinin synthesized in neighbouring cells (Fig. 7).

Recently, other cases of cytokinin action over a short distance involving LOG4 have been reported. Cytokinin production by LOG4 in embryonic xylem precursor cells has been shown to induce procambial cell proliferation in neighbouring cells, thus regulating growth and patterning during vascular development in a non-cell-autonomous manner (De Rybel et al., 2014; Ohashi-Ito et al., 2014). In addition, a computational model has demonstrated that cytokinin generated by LOG4 may signal over several cell layers in the shoot apical meristem and act together with CLV signalling as a positional cue within the stem cell niche (Chikaramane et al., 2012). This indicates that the final activating step during cytokinin biosynthesis has a pivotal role in establishing local cytokinin gradients and, more specifically, suggests that LOG4 has an important role in cellular patterning.

The analysis of cyp735a mutants indicated that iZ has strong activity in suppressing the formation of LRP. This reveals a novel local function of the CYP735A genes known for their importance in synthesizing iZ-type cytokinin for root-to-shoot communication (Kiba et al., 2013). CYP735A2 promoter-driven reporter gene activity was present in the cells surrounding the LRP but not in the LRP itself. CYP735A2 is one of the genes most strongly downregulated by auxin in an ARF7/ARF19-dependent manner in the root (Parizot et al., 2010). Unfortunately, due to the low expression of CYP735A1, there was no similar information to evaluate its
behaviour in this context. However, it could be that iP synthesized by IPT5 and LOG4 in LRFC reaches neighbouring cells by diffusion and/or transport and is then converted to IZ.

How could the activity of cytokinin and the regulation of cytokinin synthesis gene be linked to auxin, which is the master regulator of LR development? Some indications come from gene expression studies. The expression of IPT5 is activated during LR formation in an ARF7/ARF19-dependent manner (Parizot et al., 2010) and the LOG4 promoter has been shown to be a direct target of the TMO5-LHW transcription factors acting downstream of the BDL/IAA12-MP/ARF5 auxin response module (De Rybel et al., 2014), acting a bit later than ARF7-ARF19 in LRI (De Smet et al., 2010). Interestingly, the mp mutant shows a LR spacing defect (De Smet et al., 2010) that might be partially due to reduced LOG4 expression. Additional cytokinin genes that are also switched on through the ARF7-ARF19 and TMO5-LHW pathways are two auxin-responsive CKX genes (CKX1, CKX6) (Parizot et al., 2010) and the auxin-responsive negative regulator AHP6 (Ohashi-Ito et al., 2014), which is expressed in LRs from the early stages onwards (Moreira et al., 2013). Activation of these genes in the LRFCs could maintain a low cytokinin status in these cells to avoid a negative impact of cytokinin on their further development. Indeed, occasional periclinal cell division in the LRFC of ahp6 mutants suggests a depressive action of AHP6 on cytokinin signalling in these cells (Moreira et al., 2013). Taken together, the auxin-induced lowering of the cytokinin status in the LRFCs and cytokinin synthesis in neighbouring cells would contribute to a high auxin/low cytokinin ratio in LRFCs, and a low auxin/high cytokinin ratio in the neighbouring cells. Comparisons of DR5 and TCS reporter signals have consistently indicated that auxin and cytokinin response maxima are complementary in the pericycle and during LR development (Benková et al., 2003; Bielach et al., 2012).

A strongly lowered cytokinin status does not release the inhibition of all xylem pole PCs that are developmentally primed to eventually become LRFCs, suggesting that one or several other systems operate in parallel to control LR spacing. One such system is based on ACR4. Expression of ACR4 was lower in the roots of cytokinin-deficient plants, and acr4 mutants had a lower cytokinin status (Fig. 5). A genetic analysis did not reveal a hierarchical order between the cytokinin and the ACR4 pathway (Fig. 6). However, the reciprocal influence on transcript levels indicates that crosstalk exists and suggests that ACR4 has a positive impact on the cytokinin pathway and vice versa. This reciprocal influence might be gradual and not necessarily strongly influence the outcome of the genetic analysis, although the percentage of misplaced LRP was higher than it would be from an additive effect in the acr4 35S:CKX1 hybrids (Fig. 6A). Moreover, the extent of the local reciprocal influence on the transcript levels could not be evaluated as these were measured in whole root extracts. Regardless, one pathway could act through transcriptional regulation in a feed-forward-like manner to enhance the other pathway’s suppression of LRI in neighbouring cells.

Together, the results show the involvement of cytokinin in regulating root system architecture soon after founder cell selection, the first key step in LR formation from a primed cell. Cytokinin is proposed to act locally as a paracrine signal that originates in LRFCs and its neighbouring cells, acting both as a suppressor of LRI and to determine which of the competent sites develop into LR. An instructive signal from LRFCs to neighbouring PCs for LRI would spare resources and help develop an optimal root system. Furthermore, cytokinin may act as an endogenous signal responding to environmental cues such as nutrients and osmotic conditions (Malamy et al., 2005; Ramireddy et al., 2014). In this way it contributes to the developmental plasticity of root architecture, which has to adapt constantly to a highly complex subterranean environment.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Spatio-temporal expression of selected cytokinin metabolism genes during LR development.

Fig. S2. LR spacing is altered in mutants with a lower cytokinin status.

Table S1. Primers used for quantitative real-time reverse transcription PCR.

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