RESEARCH PAPER

Involvement of hormones and KNOXI genes in early Arabidopsis seedling development

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Abstract

Plant hormones control plant development by modulating the expression of regulatory genes, including homeobox-containing KNOXI genes. However, much remains to be elucidated about the interactions involved. Therefore, hormonal regulation of KNOXI gene expression was investigated using hormone applications and an inducible transgenic ipt expression system to increase endogenous cytokinin (CK) levels. Treatments with auxin, abscisic acid (ABA), cytokinins, ethylene, and gibberellin (GA) did not result in ectopic expression of the BP (BREVIPEDICELLUS) gene. However, BP expression was strongly reduced by ABA, increased by auxin treatment (correlating with the initiation of lateral root meristems, which strongly express BP), and did not significantly respond to short-term treatments with the other hormones in whole seedlings. Following short-term ipt activation, organ-specific differential regulation of KNOXI gene expression was observed. While several KNOXI genes were transiently up-regulated to low levels, STM was selectively repressed (especially at low light) in hypocotyls. In cotyledons, activation of CK-responsive genes preceded ipt induction, suggesting that CKs are transported more rapidly than the inducing agent (dexamethasone). Long-term increases in CK levels induced raised levels of several KNOXI transcripts in hypocotyls, correlating with the radial expansion of vascular tissues, the main domains of KNOXI gene expression, suggesting that CKs had little effect on KNOXI promoter activity. No alterations in hormone sensitivity were observed in a bp null mutant. Constitutive BP overexpression caused reductions in the length and number of lateral roots, while the primary root remained unaffected. The transgenic seedlings displayed weak, but significant, alterations in sensitivity to ABA, CK, and 1-amino-cyclopropane-1-carboxylic acid.

Key words: Arabidopsis thaliana, KNOX genes, plant hormones, pOp/LhGR system, regulation of gene expression.

Introduction

Homeodomain proteins were first discovered in Drosophila (Gehring et al., 1994) during investigations of homeotic transformations of the fly’s body plan. Since then, a number of homeodomain proteins have been found in animals, plants, and yeasts. Plant homeodomain proteins participate as transcription factors in the regulation of a number of developmental processes by activating and/or repressing sets of target genes (Chan et al., 1998; Reiser et al., 2000). The first homeobox gene identified in plants was KNOTTED1 (KN1) from maize (Vollbrecht et al., 1991). In Arabidopsis thaliana, the KNOTTED1-like homeobox (KNOX) gene family consists of eight KN1 homologues, of which STM (SHOOT MERISTEMLESS), BP (BREVIPEDICELLUS), KNAT2, and KNAT6 are KNOXI genes, while KNAT3, KNAT4, KNAT5, and KNAT7 are KNOXII genes. KNOXI genes are expressed mainly in the shoot apical meristem (SAM; Lincoln et al., 1994; Long et al., 1996; Laufs et al., 1998; Nishimura et al., 1999) and are required for SAM maintenance, and thus for the establishment of shoot architecture, as inferred mainly from analyses of loss-of-function mutants. In Arabidopsis, stm mutants have displayed complete or
partial absence of the SAM, and consequent reductions in leaf and flower numbers, together with fused cotyledons (Barton and Poethig, 1993; Clark et al., 1996; Endrizzi et al., 1996). The roles of other KNOXI genes in the SAM have not been fully characterized. However, the roles of BP and STM seem to overlap at least partly, since an active BP allele can restore SAM maintenance in *stm asl* (asymmetric leaves1) plants (Byrne et al., 2002). Failure to down-regulate KNOXI gene expression in leaf primordia and mature leaves can cause abnormal leaf development (Ori et al., 2000; Byrne et al., 2003). In addition to their function(s) in the SAM, there is also growing evidence implicating the involvement of KNOXI genes in defining inflorescence architecture (Lincoln et al., 1994; Douglas et al., 2002; Venglat et al., 2002).

Recently, several indications that KNOXI genes have functions in roots have emerged. Notably, it has been reported that BP (Truemit et al., 2006), maize *Rs1* and KNOX4 (Kerstetter et al., 1994), and *Arabidopsis* KNAT2 (Hamant et al., 2002) and KNAT6 (Dean et al., 2004) are all expressed in roots. KNAT6 expression appears to be localized in the phloem of the primary root and the bases of lateral roots, while BP expression has been detected mainly at the bases of lateral root primordia. Potential functions of KNOXI genes in lateral root initiation have also been inferred from increases in the numbers of lateral root primordia caused by the down-regulation of KNAT6 transcript levels (Dean et al., 2004).

Interest in interactions between KNOXI genes and plant hormones has been strongly stimulated by recent research on their co-ordinated involvement in balancing SAM maintenance and organ production. For instance, KNOXI genes have been found to be down-regulated in a SAM region in which highly localized increases in auxin levels occur that are essential for initiation of leaf primordia. Perturbations of the auxin pattern caused by either auxin transport inhibitors (Scanlon, 2003) or mutations in genes encoding the auxin transport facilitators PIN-FORMED1 and PINOID (Furutani et al., 2004) resulted in defects in lateral organ formation. A possible feedback relationship between KNOX proteins and auxin is indicated by data suggesting that KNOX proteins may also inhibit auxin transport (Tsiantis et al., 1999; Trembl et al., 2005). Kuijt et al. (2004) suggested that auxin may regulate the distribution of KNOXI proteins between the cytoplasm and nucleus.

No data on interactions between KNOXI genes and abscisic acid (ABA) have been reported. However, KNAT2 and ethylene have been shown to act antagonistically in the regulation of leaf structure and SAM architecture in *Arabidopsis* (Hamant et al., 2002), and numerous, often conflicting, reports on interactions between KNOXI genes and cytokinins (CKs) have accumulated over the last 16 years. The overexpression of *ipt*, a bacterial gene encoding a key enzyme of CK biosynthesis—

isopentenyltransferase—confers a phenotypic syndrome very similar to that of KNOXI overexpression in tobacco, including ectopic shoot meristem formation on leaves (Estruch et al., 1991; Sinha et al., 1993). On the other hand, alterations in levels of several plant hormones, including elevation of CK levels, have been reported in transgenic plants constitutively and ectopically expressing *KN1* homologues from tobacco and rice (Tamaoki et al., 1997; Kusaba et al., 1998). These findings do not provide conclusive proof that CK levels are regulated by KNOX genes, due to the constitutive nature of *KN1* expression. However, two independent groups have recently obtained direct experimental evidence that CKs are involved in mediating control of the SAM by KNOXI genes (Jasinski et al., 2005; Yanai et al., 2005). Activation of several different KNOXI proteins using an inducible system resulted in increases in CKs due to selective up-regulation of AiiPT7, a gene encoding a genuine *Arabidopsis* isopentenyltransferase (Kakimoto, 2001; Takei et al., 2001a). Furthermore, KNOXI proteins have been shown to suppress directly the expression of a key gene of gibberellin (GA) biosynthesis, in the tobacco SAM (Sakamoto et al., 2001). In addition, work by Hay et al. (2002) led to a suggestion that repression of GA activity by KNOXI proteins is a key component of SAM function.

Overall, the published evidence has prompted the hypothesis that KNOXI proteins act as general orchestrators of growth regulator homeostasis at the shoot apex of *Arabidopsis* by simultaneously activating CK and repressing GA biosynthesis, thus promoting meristem activity (Jasinski et al., 2005; Yanai et al., 2005). However, an opposite link between CKs and KNOXI genes has been proposed based on the finding that steady-state mRNA levels of *STM* and *BP* increased in response to activation of *ipt* expression in *Arabidopsis* seedlings (Rupp et al., 1999). The model proposed by the cited authors positioned CKs upstream of *BP* and *STM* in the regulation of SAM establishment and/or maintenance. Taken together, the data obtained from experiments in which KNOXI protein activity and *ipt* expression have been induced indicate that expression of KNOXI gene family members can lead to increases in CK biosynthesis and, in turn, that these genes are up-regulated by CKs, suggesting a positive interdependence between CK and the KNOX family of meristem maintenance genes. However, the proposed feedback loop has been questioned recently based on the finding that *BP* and *STM* were not significantly increased within 48 h of *ipt* induction in 14-d-old *Arabidopsis* seedlings (Craft et al., 2005) and in genome-wide analysis, neither BP nor STM was induced in young seedlings that were either expressing *ipt* (Hoth et al., 2003) or treated with exogenous cytokinin for 24 h (Rashotte et al., 2003). Nevertheless, transient increases were observed in BP and KNAT4 transcripts 6 h after *ipt* induction in young *Arabidopsis* seedlings by Hoth et al.
(2003). This left open the possibility that KNOX gene expression may be highly specifically temporally and spatially regulated by CKs. Further, subtle changes in KNOX gene transcript levels may not have been detected if they did not exceed the thresholds applied in the genome-wide analysis.

In the study presented here, changes in steady-state levels of BP, KNAT2, KNAT6, and STM transcripts in response to both short- and long-term CK treatments were re-examined by quantitative (Q) RT-PCR (reverse transcription-PCR) in individual organs and apical regions of Arabidopsis seedlings cultivated at various light intensities. Combinations of several CK and light treatments were included since some aspects of plant development are influenced by both CKs and light (Su and Howell, 1995; A Reková et al., personal communication), and evidence of a link between light and KNOXII expression has been presented (Serikawa et al., 1997). The spatial regulation of BP expression by CKs was analysed in detail using a BP promoter–uidA fusion construction and in situ mRNA localization, and the effects of CKs were compared with those of other plant hormones. The sensitivity of the Arabidopsis bp mutant and a transgenic line constitutively overexpressing BP to CKs and other plant hormones was also examined. In addition, novel information was obtained on the kinetics of transgene activation in individual Arabidopsis organs using a dexamethasone (DEX)-inducible gene expression system, pOp/LhGR (Craft et al., 2005; Šámalová et al., 2005), together with evidence supporting the view that CK is a long-range rather than a paracrine signal.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 was obtained from the European Arabidopsis Stock centre. pBP-GUS (β-glucoronidase) transgenic plants (lines K3, K4, K9, K14, and K15) and CaMV35S>GR>ipt plants expressing agrobacterial ipt via the glucocorticoid-inducible system (Craft et al., 2005; lines 11 and 13) were kindly provided by Sarah Hake and Ian Moore, respectively. Both of the glucocorticoid-inducible lines (11 and 13) were used to analyse phenotypic changes following DEX applications, and the stronger line (11) was used for determination of transcript levels. 35S-BP (ecotype Nossen), breVIPedicellus (bp) mutant (ecotype Ler), and control lines were obtained from the European Arabidopsis Stock centre. 35S-BP (ecotype Nossen), pBP-GUS, named K3, K4, K9, K14, and K15, were used. Intact plants were vacuum infiltrated with a substrate solution containing 0.1 M phosphate buffer (pH 7.0), 1% Triton X-100, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (X-gluc), then incubated at 37 °C for 2, 5, or 20 h. Chlorophyll was removed from the plants’ tissues using 75% ethanol and they were cleared by immersion in 0.25 M HCl and 20% methanol at 57 °C. The plants were then brought to 50% glycerol in a graded series, and their tissues were photographed using an Olympus BX61 microscope fitted with an Olympus DP50 digital camera. Consistent results were obtained for the five lines, and images obtained from plants of line K14 are presented.

In situ hybridization

The 644 bp long fragment from the 5’ end of BP cDNA was used to synthesize sense and antisense probes. The cDNA clone was synthesized using the specific primers: IBPsh (5’-CTC TAG TGC CTT TTT GAT TTC ATA GAT-3’) and IBPsh (5’-GCA TAG TTC ATG GAT TTT AGC CCT C-3’), and incorporated into the SpeI site of pBluescript SK–. The specificity and orientation of the inserts were verified by sequencing. The sense and antisense RNA probes were labelled with digoxigenin-11-UTP using T3 and T7 polymerase (Roche) and the hybridized probes were detected using anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche). Sections 5 μm thick were cut after tissues had been fixed in 4% formaldehyde and embedded in ParaplastPlus (Fisher Scientific). Pre-treatment of the sections and in situ hybridization were performed according to Lincoln et al. (1994). Very slight non-specific staining was detected in leaves, with both sense and antisense probes.

Quantitative RT-PCR and data analysis

To perform RT-PCR, total RNA was isolated from plant tissues frozen in liquid nitrogen using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Duplicates of RNA samples were isolated and analysed in all but a few cases, including the following types of samples from seedlings in which ipt was activated throughout the whole cultivation period: hypocotyls (5 μmol m−2 s−1/200 nM DEX; 56 μmol m−2 s−1/200 nM DEX) and apices (5 μmol m−2 s−1/200 nM DEX and 5 μmol m−2 s−1/500 nM DEX). RNA samples were treated with DNase I (Top-Bio, Czech Republic) to digest contaminating DNA, and the efficiency of the DNase treatment was checked by PCR amplification of actin, using non-transcribed RNA as a template. First-strand cDNA was prepared using SuperScript II reverse transcriptase (Invitrogen) and the oligo(dT) (Hradilová et al., 2005), together with evidence supporting the view that CK is a long-range rather than a paracrine signal.

To 60 seedlings were used for measurements of morphological characteristics and statistical analysis. The significance of morphological differences between different plants was evaluated by t-tests (deeming differences to be significant if P < 0.05).

Histochemical analysis of GUS activity

To minimize the possibility of positional effects of transgenic insertions influencing the obtained results, five independent lines of pBP-GUS, named K3, K4, K9, K14, and K15, were used. Intact plants were vacuum infiltrated with a substrate solution containing 0.1 M phosphate buffer (pH 7.0), 1% Triton X-100, 5 mM potassium ferrocyanide, and 0.1% 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), then incubated at 37 °C for 2, 5, or 20 h. Chlorophyll was removed from the plants’ tissues using 75% ethanol and they were cleared by immersion in 0.25 M HCl and 20% methanol at 57 °C. The plants were then brought to 50% glycerol in a graded series, and their tissues were photographed using an Olympus BX61 microscope fitted with an Olympus DP50 digital camera. Consistent results were obtained for the five lines, and images obtained from plants of line K14 are presented.
hybridization (Fig. 1O, P, and Supplementary Fig. S1E, F), chemical analysis was confirmed by in situ JXB was not detected at all (Supplementary Fig. S1 available at BP developed lateral roots, staining. In two- and three-cell layer primordia, the expression was detected in all cells of developing primordia and roots in whole-mount preparations and hypocotyl GUS staining was found in vascular tissues of hypocotyls bases of mature leaves as well as leaf primordia (Fig. 1). Expression in the subapical region of the shoot and the longitudinal sections of the shoot apex visualized BP expression in Arabidopsis seedlings. Whole-mount histochemical analysis of young seedlings grown on MS indicated that BP was strongly expressed in the shoot apex. GUS staining and in situ mRNA hybridization of longitudinal sections of the shoot apex visualized BP expression in the subapical region of the shoot and the bases of mature leaves as well as leaf primordia (Fig. 1). GUS staining was found in vascular tissues of hypocotyls and roots in whole-mount preparations and hypocotyl cross-sections (Fig. 1D, M, Q). Interestingly, strong BP expression was also observed throughout the course of lateral root development. At the one-cell layer stage, GUS staining was detected in all cells of developing primordia and the nearest cells of the pericycle within 2 h of staining. In two- and three-cell layer primordia, the staining was distributed in all cells, while at later stages it became restricted to the lateral root base. In fully developed lateral roots, BP expression was restricted to a peripheral ring of cells at their bases or, in some cases, was not detected at all (Supplementary Fig. S1 available at JXB online). The expression pattern revealed by histochemical analysis was confirmed by in situ mRNA hybridization (Fig. 1O, P, and Supplementary Fig. S1E, F at JXB online).

Hormonal regulation of BP expression patterns

To assess the effects of key plant hormones on BP expression, 6-d-old pBP-GUS seedlings were transferred to liquid medium supplemented with: the gibberellin GA$_3$ (10 µM and 100 µM); the auxins 2,4-diphenoxycetic acid (2,4-D; 1 µM and 10 µM) and naphthalene acetic acid (NAA; 1 µM and 10 µM); the ethylene precursor 1-amino-cyclopropane-1-carboxylic acid (ACC; 1 µM); the cytokinins benzyladenine (BA; 10 µM and 100 µM), trans-zeatin (t-Z; 10 µM and 100 µM), and abscisic acid (ABA; 10 µM and 100 µM); incubated for 24 h and subsequently analysed for GUS activity (Fig. 2). Auxin treatment induced no major changes in BP expression in the aerial parts of the seedlings, but a dramatic increase in BP expression was observed in their roots (Fig. 2C, D). This increase was due to BP expression in the developing lateral root primordia and became apparent concomitantly with their initiation. Treatment with ABA at both tested concentrations resulted in dramatic reductions in GUS staining in the shoots and roots (Fig. 2E, F), GA, CKs, and ethylene had no apparent effects on GUS staining at the end of the 24 h treatments, and thus presumably no effects on BP expression patterns (not shown). These hormonal effects on BP expression were confirmed by Q RT-PCR; no statistically significant changes in steady-state levels of BP transcripts compared with untreated seedlings were found following CK and ethylene treatments (not shown), but their levels increased by 35% and decreased by 70%, respectively, following treatments with 1 µM 2,4-D and 100 µM ABA (Fig. 2G).

Results

BP expression patterns in Arabidopsis seedlings

Transgenic lines harbouring the uidA gene driven by the BP promoter (pBP-GUS; Ori et al., 2000) were used to obtain the first insights into the hormonal regulation of BP expression in Arabidopsis seedlings. Whole-mount histochemical analysis of young seedlings grown on MS indicated that BP was strongly expressed in the shoot apex. GUS staining and in situ mRNA hybridization of longitudinal sections of the shoot apex visualized BP expression in the subapical region of the shoot and the bases of mature leaves as well as leaf primordia (Fig. 1). GUS staining was found in vascular tissues of hypocotyls and roots in whole-mount preparations and hypocotyl cross-sections (Fig. 1D, M, Q). Interestingly, strong BP expression was also observed throughout the course of lateral root development. At the one-cell layer stage, GUS staining was detected in all cells of developing primordia and the nearest cells of the pericycle within 2 h of staining. In two- and three-cell layer primordia, the staining was distributed in all cells, while at later stages it became restricted to the lateral root base. In fully developed lateral roots, BP expression was restricted to a peripheral ring of cells at their bases or, in some cases, was not detected at all (Supplementary Fig. S1 available at JXB online). The expression pattern revealed by histochemical analysis was confirmed by in situ mRNA hybridization (Fig. 1O, P, and Supplementary Fig. S1E, F at JXB online).

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Short-term effects of CKs and light on KNOXI transcript levels

The glucocorticoid-inducible transgene expression system pOp/LhGR (Craft et al., 2005; Sámalová et al., 2005) was employed to study changes in expression of KNOXI genes in response to increased levels of endogenous CKs in Arabidopsis seedlings. To investigate short-term effects of increased CK levels, seedlings of CaMV35S>Gr ipt line 11 (Craft et al., 2005) were grown on MS plates under the 82 µmol m$^{-2}$ s$^{-1}$ light intensity regime. At appropriate time points, seedlings were transferred to plates containing MS supplemented with 500 nM DEX to induce ipt expression for 3, 6, 10, 15, and 24 h by the end of a 10 d growth period. The apical part (hereafter referred to as the apex), cotyledons, hypocotyls, and leaves were dissected out from the harvested seedlings, and the abundance of the ipt, ARR5, BP, KNAT2, KNAT6, and STM transcripts in them was analysed by Q RT-PCR. Pronounced increases in ipt transcript levels were observed, initially in roots, in which ~30% of saturation response levels were reached after 3 h, and saturation levels after 6 h of induction. Ipt levels increased in the other tissues after further lag periods, reaching levels close to saturation in hypocotyls after 10 h, and in the apex and cotyledons after 15 h (Fig. 3). Levels of ARR5 transcripts, which are rapidly induced by CKs (D’Agostino et al., 2000), and have been shown to be proportional to CK levels in many cases, were used to monitor increases in CK levels. DEX
treatment increased the abundance of *ARR5* transcripts in all organs investigated, regardless of site-specific *ipt* activation, within 6 h of DEX application, although the level of induction was relatively low in roots. In cotyledons, *ARR5* transcript levels rose prior to those of *ipt* transcripts, suggesting that CKs are transported or translocated much more rapidly than DEX into cotyledons. Hoth *et al.* (2003) reported *KNAT4* to be transiently up-regulated after 6 h of *ipt* activation. Therefore, *KNAT4* and *KNAT3*, another member of the *KNOXII* gene family, were included as additional reporters of increased CK levels in cotyledons. *KNAT3* and *KNAT4* transcripts peaked in cotyledons after 6 h and 10 h of DEX treatment, respectively (Fig. 3D). Thus, their increases clearly preceded increases in *ipt* transcript levels, in a similar manner to those of the *ARR5* transcript.
In apices, \(BP\) transcript levels increased \(\sim2.0\)-fold from 6 h after induction. Slightly weaker increases \(\sim1.7\)-fold; Supplementary Table S1 at \(JXB\) online), with comparable kinetics, were observed for \(KNAT2\) and \(KNAT6\) transcripts. Slight increases in \(STM\) transcript levels became apparent within 10 h of DEX treatment (Fig. 3).

No consistent, significant effects of DEX treatment on the expression of \(KNOXI\) genes were observed in either cotyledons or roots (Supplementary Table S1 at \(JXB\) online).

In hypocotyls, \(BP\), \(KNAT2\), and \(KNAT6\) transcript levels fluctuated within relatively narrow ranges without any clear decreasing or increasing trends. In contrast, \(STM\) transcript levels declined after 10 h of DEX treatment, to only \(\sim50\)% of those found in non-treated seedlings after 24 h treatment (Fig. 3B; Supplementary Table S1 at \(JXB\) online). The repression was dramatically enhanced \((STM\) transcript levels falling to only \(8\)% of untreated control levels) when the treatment was carried out under a low light intensity regime \((5\ \mu\text{mol}\ \text{m}^{-2}\ \text{s}^{-1})\). Interestingly, no similar effect of decreased light intensity on transcript levels falling to only \(8\)% of untreated control levels) when the treatment was carried out under a low light intensity regime \((5\ \mu\text{mol}\ \text{m}^{-2}\ \text{s}^{-1})\). Interestingly, no similar effect of decreased light intensity on transcript levels falling to only \(8\)% of untreated control levels) when the treatment was carried out under a low light intensity regime \((5\ \mu\text{mol}\ \text{m}^{-2}\ \text{s}^{-1})\). Interestingly, no similar effect of decreased light intensity on transcript
levels was found for the other members of the KNOXI gene family (Fig. 3B; Supplementary Table S1 at JXB online). According to A Rekova et al. (personal communication), ipt induction in seedlings grown under low light intensity conditions does not result in greater increases in CK levels compared with induction under standard light intensities. Thus, this effect cannot be explained by CK levels being higher in seedlings grown under low light intensity conditions.

**Effects of long-term CK treatments on BP expression patterns**

To investigate long-term changes in BP expression patterns in response to treatment with exogenous CKs and constitutive increases in endogenous CK levels via induction of ipt expression, the resulting patterns were visualized histochemically and by in situ RNA hybridization of BP transcripts, respectively. The histochemical analysis was performed with pBP-GUS seedlings grown on MS medium supplemented with BA (0.1–10 μM) for 14 d (Fig. 1), and for the in situ RNA analysis CaMV35S>GR>ipt (line 11) seedlings grown on MS medium supplemented with 500 nM DEX for up to 12 d were used. Both approaches gave essentially identical results. In apices, no differences in the BP expression patterns between treated and non-treated seedlings were observed. Thus, BP expression remained restricted to the subapical region of the shoot apex, and the bases of leaf primordia and mature leaves with no expansion or contraction of BP expression in the SAM (Fig. 1K, L, O, P). At the hypocotyl–root junction, no staining associated with developing lateral root primordia was detected in the CK-treated seedlings, in accordance with the suppression of lateral root primordium formation in the presence of CKs. In contrast, staining associated with vascular tissues expanded in BA-treated seedlings in a concentration-dependent manner, without any noticeable change in staining intensity. Thus, increases in the expansion of GUS staining appeared to be correlated with the periclinal expansion of vascular tissues observed in CK-treated seedlings (Fig. 1D–F). In roots, GUS staining decreased in CK-treated seedlings co-ordinately with the suppression of lateral root formation. No ectopic BP expression was observed as a consequence of CK treatment in leaves (Fig. 1J).

**Morphological alterations of CaMV35S>GR>ipt seedlings in response to increased CK levels at various light intensities**

To modulate endogenous CKs to various levels, as judged by the severity of morphological alterations, CaMV35S>GR>ipt (line 11) seedlings were grown on media supplemented with DEX at concentrations of 70, 200, and 500 nM for 14 d under the 82 μmol m⁻² s⁻¹ light intensity regime. Weak, but consistent, morphological alterations typical of increased CK levels were observed in seedlings grown on media supplemented with 70 nM DEX, including slight reductions of their root system and biomass of their aerial parts, and slight widening of their hypocotyls. However, neither deposition of anthocyanins nor chlorosis was observed. In contrast, strong reductions in the root system, cotyledons, and leaf rosette, accompanied by pronounced widening of the apex base and hypocotyls, and anthocyanin accumulation and chlorosis, were found in seedlings grown on media supplemented with 500 nM DEX. Seedlings grown on media supplemented with 200 nM DEX displayed an intermediate phenotype.

The hypocotyl widening was reminiscent of the effects of KNAT2 overexpression in Arabidopsis (Hamant et al., 2002). To investigate possible interactions between CKs, KNOXI genes, and light in hypocotyls, the CK-over-producing seedlings were cultivated at four different light intensities, which resulted in distinct morphological responses in the hypocotyls and apices. In general, the extent of the morphological alterations found at a particular DEX concentration decreased with decreasing light intensity, as did its dose dependence (Supplementary Fig. S2 at JXB online).

**Modulation of KNOXI transcript levels in response to long-term CK action at various light intensities**

Steady-state transcript levels of the individual members of the KNOXI gene family determined in seedlings grown under the conditions outlined above are summarized in Supplementary Table S2 at JXB online, and exemplified (using BP expression data) in Fig. 4. The roots were excluded from this analysis because it was not feasible to collect sufficient root tissues for standard Q RT-PCR. BP transcripts were not detectable in cotyledons and leaves. In apices and hypocotyls, the strength of the CK effect on BP expression depended on the light intensity provided during their cultivation. In apices of seedlings grown under the 82 μmol m⁻² s⁻¹ and 56 μmol m⁻² s⁻¹ light intensity regimes, BP transcript levels increased with increases in the DEX concentration, but no significant increases in BP transcript levels, compared with uninduced controls, were observed in seedlings grown under the 16 μmol m⁻² s⁻¹ and 5 μmol m⁻² s⁻¹ regimes. Similarly, BP transcript levels increased significantly with increases in DEX concentration in hypocotyls of seedlings grown under the 16, 56, and 82 μmol m⁻² s⁻¹ regimes, but no significant differences in BP transcripts from control levels were observed in seedlings grown under the 5 μmol m⁻² s⁻¹ regime.

The histochemical analysis of BP expression patterns in pBP-GUS seedlings grown for 14 d on medium supplemented with BA (Fig. 1) indicated that increases in BP
expression may reflect increases in the size of the apex and hypertrophy of vascular tissues caused by CKs rather than increases in BP promoter activity. Therefore, the correlations between BP transcript levels and apex and hypocotyl width were investigated. As hypothesized, a strong correlation between the apex size (represented by the width of the apex base) and BP transcript levels was found in seedlings grown under the 82 l mol m$^{-2}$ s$^{-1}$ and 56 l mol m$^{-2}$ s$^{-1}$ regimes (correlation coefficients $r = 0.89$ and $r = 0.82$, respectively), and between hypocotyl width and BP transcript levels in seedlings grown under the 82, 56, and 16 l mol m$^{-2}$ s$^{-1}$ regimes (correlation coefficients $r = 0.94$, $r = 0.98$, and $r = 0.81$, respectively).

Similarly, increases in the steady-state levels of KNAT2 and KNAT6 transcripts appeared to correlate with increases in the proportions of vasculature in the cotyledons and leaves of seedlings cultivated in the presence of DEX. For instance, the proportions of vasculature to total cotyledon area were 2.7±0.14% in untreated seedlings of line 11, and 5.2±0.57% in seedlings of this line cultivated in the presence of 500 nM DEX (Supplementary Fig. S3 at JXB online). Similar results were observed in leaves (not shown).

In apices of the seedlings grown in the presence of DEX, there was no clear correlation between levels of transcripts of any of the other KNOXI genes and DEX concentrations. However, in hypocotyls, KNAT2 and KNAT6 transcript levels followed trends similar to those found for BP, except that they showed clear increases in DEX-induced seedlings relative to control levels, even in seedlings grown under the 5 μmol m$^{-2}$ s$^{-1}$ light intensity regime. Interestingly, a distinct pattern of gene regulation was observed for STM transcript levels, which were higher than control levels in DEX-induced seedlings under the 82 μmol m$^{-2}$ s$^{-1}$ and 56 μmol m$^{-2}$ s$^{-1}$ regimes, unaffected by DEX in seedlings under the 16 μmol m$^{-2}$ s$^{-1}$ regime, and progressively declined with increasing DEX concentrations in seedlings grown under the 5 μmol m$^{-2}$ s$^{-1}$ regime (Supplementary Table S2 at JXB online).

Effects of knock-out mutation and constitutive overexpression of BP on sensitivity to hormone treatments

To investigate further the possible role(s) of BP in hormone signalling, BP knock-out mutant (bp; Douglas et al., 2002; Venglat et al. 2002) and CaMV35S-BP transgenic seedlings (Lincoln et al., 1994) were grown on MS media supplemented with BA (5 nM to 10 μM), 2,4-D (1 nM to 10 μM), NAA (1 nM to 10 μM), ACC (5 nM to 20 μM), GA$_3$ (50 nM to 50 μM), and ABA (50 nM to 1 μM) for 12 d. No significant developmental differences between bp and control seedlings grown on MS and hormone-supplemented media were observed (not shown). However, significant differences were found in development and responses to exogenous hormones between CaMV35S-BP and wild-type seedlings (Fig. 5). In 12-d-old CaMV35S-BP seedlings grown on MS medium, the hypocotyl length was reduced (2.3±0.3 cm compared
with 3.8±0.7 cm in the wild type) while the primary root was slightly elongated (7.34±0.88 cm compared with 6.71±0.91 cm in the wild type). However, the numbers and lengths of lateral roots were reduced in CaMV35S-BP seedlings, which released 13.4±3.3 lateral roots compared with 17.4±2.6 in the wild type, and the longest lateral roots were 0.79±0.27 cm and 1.32±0.41 cm in CaMV35S-BP and the wild type, respectively. The numbers and lengths of the other lateral roots were also reduced in CaMV35S-BP seedlings (Fig. 5C).

The roots of CaMV35S-BP and wild-type seedlings displayed distinct differences in sensitivity to exogenous CK treatment. The inhibition of primary root growth was more severe, and the severity of the inhibition increased more rapidly with increasing CK levels, in CaMV35S-BP seedlings. The lengths of the main root of CaMV35S-BP seedlings reached 70–80% of wild-type lengths in the presence of 25–100 nM BA, but only 35% in the presence of 10 μM BA (t-test, P<10⁻¹²; Fig. 5E). The main root of CaMV35S-BP seedlings was also found to be more sensitive to ACC, although the differences between the transgenic and wild-type seedlings were less pronounced, albeit still highly significant (t-test, P<10⁻²⁵), at high ACC concentrations. The lengths of the main transgenic roots were 71% of wild-type lengths when the seedlings were grown in the presence of 10 μM ACC (Fig. 5F). Inhibition of lateral root development by BA and ACC was comparable in the transgenic and wild-type seedlings (not shown). ABA also had similar effects on transgenic and wild-type seedlings, except for their lateral root development. In transgenic seedlings, lateral roots were shorter and essentially insensitive to ABA while the growth of lateral roots was inhibited by ABA in wild-type plants in a dose-dependent manner, dropping to the length of the transgenic lateral roots at 1 μM ABA (Fig. 5D). No significant, consistent differences in transgenic and wild-type seedling development were observed on media supplemented with 2,4-D, NAA, or GA₃ (not shown).

Discussion

Plant hormones regulate plant development, in conjunction with environmental signals, by modulating the expression of specific regulatory genes, including KNOXI genes, a family of homeobox-containing genes that have been found in all plant species examined to date. However, much remains to be elucidated about the interactions involved. Therefore, interactions between hormones (auxin, ABA, CK, ethylene, and GA) and KNOXI genes in early Arabidopsis seedling development...
were analysed both by applying exogenous hormone treatments and by using inducible expression of *ipt*, a CK biosynthesis gene. None of the short-term hormone treatments resulted in ectopic expression of the *BP* gene. However, *BP* expression was strongly down-regulated by ABA. Increases in *BP* transcript levels were observed following auxin treatment, correlating with the initiation of lateral root meristems (in which *BP* is strongly expressed). *BP* expression did not significantly respond to short-term treatments with the other hormones when assayed in whole seedlings. Following short-term *ipt* activation, organ-specific differential regulation of *KNOXI* gene expression was observed; several *KNOXI* genes were generally activated to low or moderate levels, but *STM* was specifically repressed in hypocotyls, and reducing the light intensity dramatically strengthened the repression. Long-term increases in CK levels induced increases in *BP*, *KNAT2*, and *KNAT6* transcript levels, mainly in hypocotyls, that apparently correlated with the radial expansion of vascular tissues (the main domains of *KNOXI* gene expression). No changes in hormone sensitivity were observed in *bp*, a loss-of-*BP*-function mutant. Constitutive *BP* expression driven by the CaMV35S promoter resulted in reductions in the length and number of lateral roots, while the primary root remained unaffected. The transgenic seedlings displayed weak, but significant alterations in sensitivity to ABA, CK, and ACC treatments. Interestingly, in cotyledons, activation of CK-responsive genes preceded induction of *ipt*, suggesting that CKs were more rapidly transported than the inducing agent, DEX.

**Effects of short-term hormone treatments on BP expression patterns**

*BP* is expressed in various tissues during both vegetative and generative development (Lincoln et al., 1994). According to these authors, in the vegetative shoot apex *BP* expression is preferentially localized in the peripheral and rib meristem zones, and does not occur at detectable levels in the central zone. During the course of seedling development, *BP* expression extends to the subapical region of the shoot apex and the bases of leaf primordia and mature leaves, where it accumulates in cells adjacent to vascular tissues, and the bases of stipules. *BP* expression is associated with vascular tissues in hypocotyls and roots. The presented results confirm the *BP* expression pattern found in aerial parts of seedlings by Lincoln et al. (1994). In lateral root primordia, *BP* was expressed in all of the cells at the one-cell layer stage, up to the time of lateral root emergence, after which its expression was restricted to the bases of the roots (Supplementary Fig. S1 at *JXB* online). This expression pattern in roots is similar to that of *KNAT6* (in roots, *KNAT2* and *KNAT6* expression has been detected in phloem and at the base of the primary root; Dean et al., 2004), indicating that these *KNOXI* genes may have overlapping functions in the regulation of lateral root development. The *BP* expression pattern was not affected by short-term treatments with CK, ethylene, or GA (Fig. 2). Increased expression levels following auxin treatment were detected in roots, localized in newly initiated lateral root primordia, but this was apparent only after their initiation. Interestingly, ABA treatment resulted in strong reductions in *BP* expression in apices and roots, even though lateral root primordia were formed in ABA-treated seedlings, indicating that *BP* expression is not a key prerequisite for lateral root development. Thus, none of the short-term hormone treatments resulted in ectopic expression of the *BP* gene that would have led to the formation of new structures.

**The pOp/LhGR system as a tool to regulate ipt expression in Arabidopsis seedlings**

The ability of the pOp/LhGR system to control expression of the *ipt* gene has been demonstrated in *Arabidopsis* and tobacco recently (Craft et al., 2005; Šámalová et al., 2005). In the two cited studies, the absence of root growth inhibition indicated that *ipt* expression in CaMV35S>GR>*ipt* lines was stringently repressed in the absence of exogenous inducer, while strong phenotypic changes typical of CK overexpression indicated that *ipt* was efficiently activated in the presence of DEX. Q RT-PCR enabled levels of *ipt* transcripts in individual *Arabidopsis* organs to be quantified before and after DEX treatment. In untreated CaMV35S>GR>*ipt* seedlings, clearly detectable degrees of ‘leakiness’ of the pOp/LhGR system (Supplementary Table S1 at *JXB* online) were found, but the leakiness is insufficient to cause consistent increases in the pools of endogenous CK in uninduced seedlings, according to Hradilová et al. (2007). DEX treatment resulted in the rapid induction of *ipt* expression in the roots of seedlings growing on solid medium (Fig. 3). Similar kinetics have been reported for CaMV35S>GR>uidA seedlings treated in liquid medium (Craft et al., 2005). *Ipt* induction was delayed in the aerial part of the seedlings (Fig. 3). Elevated levels of transcripts of the CK-responsive gene *ARR5* were observed, indicating that the levels of *ipt* induction achieved effectively elevated CK activity. Interestingly, in cotyledons and apices, increases in *ARR5* transcript levels substantially preceded increases in *ipt* transcript levels (Fig. 3). Furthermore, increases in *KNAT3* and *KNAT4* transcripts followed shortly after increases in *ARR5* transcript levels, but their levels had already declined to basal levels again when *ipt* transcript levels peaked. Previously, the *KNAT4* gene has been reported to be transiently up-regulated after 6 h, and to return to basal levels after 24 h of *ipt* induction in *Arabidopsis* seedlings (Hoth et al., 2003).
The striking correlation between the induction kinetics of *ipt* in roots and *ARR5* in cotyledons suggests that *ARR5*, *KNAT3*, and *KNAT4* were activated in cotyledons by CKs originating in roots, which are known to be transported to shoots via xylem vessels at regulated rates (Takei et al., 2001b, 2002). The translocation of CK bases and nucleosides can be mediated by certain members of the purine permease and equilibrative nucleoside transporter families, respectively (Bürkle et al., 2003; Hirose et al., 2005), but seedlings do not appear to possess a transport system that can be recruited to transport DEX at rates comparable with those observed for CKs. This view is consistent with the classical concept of CKs as carriers of information from roots to shoots rather than the restriction of CK functions to paracrine signalling as proposed by Faisst et al. (1997). Low but significant increases in *ARR5* transcript levels in response to *ipt* activation in roots (Fig. 3) are consistent with the finding reported by Werner et al. (2003) that the responsiveness of *ARR5* to CKs is lower in the root than in the shoot.

**Modulation of KNOXI transcript levels by short- and long-term elevation of endogenous CK levels**

No dramatic increases in the expression of *KNOXI* genes have been detected in genome-wide screenings for CK-responsive genes, either in young *Arabidopsis* seedlings expressing *ipt* (Hoth et al., 2003) or in those treated with exogenous CKs for periods ranging from 15 min to 24 h (Rashotte et al., 2003; Brenner et al., 2005), although slight transient increases in *BP* and *KNAT4* have been observed after 6 h of *ipt* induction (Hoth et al., 2003). In the present study, slight, but clearly detectable, organ-specific increases were found in several *KNOXI* gene transcripts in response to short-term elevations of endogenous CKs (Fig. 3; Supplementary Table S1 at JXB online). Interestingly, the responses of *STM* expression to increases in CK levels appear to differ sharply in apices (where they increased) and hypocotyls (where they fell). The reduction in expression in the hypocotyls was dramatically strengthened in seedlings cultivated under low light, but changes in light intensity do not strongly affect changes in CK levels following *ipt* activation, according to A Reková et al. (unpublished results), suggesting that CKs and light may have synergistic effects on *STM* expression.

It has been hypothesized that CKs act upstream of *BP* and *STM*, and the effects of CKs on homeobox genes provide links between the hormones and developmental genes, implying that CKs play roles in the development of the SAM. Evidence for this hypothesis comes from the observation that *Arabidopsis* plants subjected to daily heat shock-induced *ipt* expression for 14 d exhibited substantial increases in the steady-state abundance of transcripts of the *KNOXI* genes *STM* and *BP* (Rupp et al., 1999).

However, more recently, Craft et al. (2005) observed no significant increases in either *STM* or *BP* transcript levels in CaMV35S*>GR>*ipt seedlings or leaf tissues treated with DEX. To explain these differences, Craft et al. (2005) suggested that the heat-treated 4-week-old seedlings studied by Rupp et al. (1999) may have possessed substantially more active axillary meristems (released by increased CKs at this developmental stage) than the controls, thus increasing the amount of meristem tissue in the samples and, consequently, *STM* and *BP* transcript levels. The presented results support and extend those reported by Craft et al. (2005) and Hoth et al. (2003). In addition to effects caused by short-term increases in CKs (see above), changes as a result of long-term CK action in *KNOXI* transcript abundance were observed. However, these changes correlate with morphological alterations, largely increases in the proportions of vascular tissues, caused by CKs and light (Supplementary Fig. S3 at JXB online). Expression of the *KNOXI* genes has been found to be associated with vascular tissues in cotyledons and leaves (Dean et al., 2004). Therefore, the observed increases in the transcript levels may merely reflect increases in the proportions of cells expressing *KNOXI* in seedlings with elevated levels of endogenous CKs rather than increases in the copy numbers of the transcripts per cell.

*BP* has been shown to induce the production of lobed leaves with ectopic meristems when overexpressed in *Arabidopsis* (Chuck et al., 1996), which were presumed to be extensions of serrations. Since CKs can cause leaf serration in *Arabidopsis* (Rupp et al., 1999), a possible explanation of this phenomenon was that it could have been mediated by *BP* in response to CKs, if CKs promote the expansion of *BP* expression from the peripheral zone of the SAM to adjacent regions, especially to leaf primordia and leaves. However, it is demonstrated here that ectopic expression of *BP* in leaf primordia and leaves is not induced either in CK-treated *Arabidopsis* seedlings or in *Arabidopsis* seedlings overexpressing *ipt* (Fig. 1), indicating that *BP* is not a downstream agent in establishment of the serrated leaf phenotype in response to CKs in *Arabidopsis*.

**Responses to hormonal treatments in BP knock-out and overexpressing Arabidopsis seedlings**

Changes in the sensitivity to CKs of seedlings or tissues over- or underexpressing a gene or (more rigorously) its product are commonly used as indicators that the gene is involved in a CK signalling chain, as illustrated by the following examples. The CK receptor CRE1 was identified in a screening experiment for mutants with impaired CK responses, including rapid cell proliferation and shoot formation in tissue culture (Inoue et al., 2001). Plants with a mutation affecting the CK signalling inhibitor AHP6
displayed increased sensitivity to exogenous CKs in an adventitious root formation assay (Mihonen et al., 2006). Silencing of a CrHP1 gene encoding a histidine-containing phosphotransmitter in *Catharanthus roseus* abolished the inductive effect of CK on transcription of *CrRR1*, a gene encoding a type-A response regulator in *C. roseus* (Papon et al., 2004). *ARR1*-overexpressing plants display hypersensitive responses to exogenous CKs, while a loss-of-function mutation in *ARR1* results in CK hyposensitivity (Sakai et al., 2001). In addition, double and higher order mutants in type-A ARRs show progressively increasing sensitivity to CK in various CK assays, indicating that these genes act as negative regulators of CK responses (To et al., 2004).

A link between KNOXI genes and ethylene has been demonstrated by Hamant et al. (2002), who found that ACC treatment repressed KNAT2 expression in wild-type *Arabidopsis* seedlings and suppressed some phenotypic alterations (including hypocotyl elongation, lobed leaves, and epinastic cotyledons) in *KNAT2*-overexpressing transgenic seedlings. No significant differences in sensitivity to exogenous hormone treatments between *bp* and wild-type seedlings were detected. This might reflect partial functional redundancy with other KNOXI genes, as indicated by the partial overlap observed in their expression patterns. Increases in main root sensitivity to CK and ACC in seedlings overexpressing *BP* (Fig. 5) are consistent with the hypotheses that *BP* is involved in the mediation of CK and ethylene responses. However, the differences in the increases indicate that CK and ethylene responses may be mediated by at least two partly independent pathways. Interestingly, the increases were not paralleled in lateral roots. On the contrary, in seedlings overexpressing *BP*, the lateral roots lost sensitivity to ABA while the sensitivity of the main root remained unaltered. Thus, *BP* might represent an integrator of plant hormone stimuli, and may play distinct roles in primary and lateral root development. Analysis of multiple knock-out mutants and conditional overexpressors of KNOXI genes is likely to yield more detailed insights into interactions between KNOXI genes and plant hormones involved in the regulation of plant development.

**Supplementary material**

The following supplementary data are available at *JXB* online.

(i) Figure S1 showing *BP* expression in roots;
(ii) Figure S2 showing effects of endogenously overproduced CKs on seedling morphology;
(iii) Figure S3 showing the morphology of cotyledons in seedlings overproducing CKs; (iv) Table S1 summarizing levels of *ipt*, *ARR5*, *KNAT3*, *KNAT4* and KNOXI transcripts following short-term DEX treatment; (v) Table S2 listing levels of KNOXI and *ARR5* transcripts following long-term DEX treatment.

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**References**


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Nishimura A, Tamaoki M, Sato Y, Matsuoka M. 1999. The expression of tobacco knotted1-type class 1 homeobox genes correspond to regions predicted by the cytostrophological zonation model. The Plant Journal 18, 337–347.


Rupp HM, Frank M, Werner T, Strnad M, Schmulling T. 1999. Increased steady state mRNA levels of the STM and KNAT1 homeobox genes in cytokinin overproducing Arabidopsis thaliana indicate a role for cytokinins in the shoot apical meristem. The Plant Journal 18, 557–563.


