Brassinosteroids Promote Root Growth in Arabidopsis

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Although brassinosteroids (BRs) are known to regulate shoot growth, their role in the regulation of root growth is less clear. We show that low concentrations of BRs such as 24-epicastasterone and 24-epibrassinolide promote root elongation in Arabidopsis wild-type plants up to 50% and in BR-deficient mutants such as *dwf1-6* (*cbb1*) and *cbb3* (which is allelic to *cpd*) up to 150%. The growth-stimulating effect of exogenous BRs is not reduced by the auxin transport inhibitor 2,3,5-triiodobenzoic acid. BR-deficient mutants show normal gravitropism, and 2,3,5-triiodobenzoic acid or higher concentrations of 2,4-dichlorophenoxyacetic acid and naphtaleneacetic acid inhibit root growth in the mutants to the same extent as in wild-type plants. Simultaneous administration of 24-epibrassinolide and 2,4-dichlorophenoxyacetic acid results in largely additive effects. Exogenous gibberellins do not promote root elongation in the BR-deficient mutants, and the sensitivity to the ethylene precursor 1-aminocyclopropane-1-carboxylic acid is not altered. Thus, the root growth-stimulating effect of BRs appears to be largely independent of auxin and gibberellin action. Furthermore, we analyzed BR interactions with other phytohormones on the gene expression level. Only a limited set of auxin- and ethylene-related genes showed altered expression levels. Genes related to other phytohormones barely showed changes, providing further evidence for an autonomous stimulatory effect of BR on root growth.

Recently, the presence of brassinosteroids (BRs) was demonstrated in Arabidopsis, maize (*Zea mays*), pea (*Pisum sativum*), and tomato (*Lycopersicon esculentum*) roots (Kim et al., 2000; Yokota et al., 2001; Bancos et al., 2002; Shimada et al., 2003). In general, the levels of BRs such as castasterone in roots are clearly below the concentrations in shoots, and roots appear to require active BRs to a much lesser extent than shoots. In agreement with the detection of BRs in roots, genes involved in BR biosynthesis (Bancos et al., 2002) and genes involved in BR signaling (Friedrichsen et al., 2000) are expressed in roots. These findings suggest that BRs are important regulatory substances in roots.

Previous studies merely analyzed the effects of exogenously supplied BRs on root growth. To this end, different systems such as shoot cuttings (analysis of adventitious root formation), cultured excised roots, root segments, and seedling roots (Roddick and Guan, 1991) were used. In general, BRs inhibit adventitious root formation even though root induction has also been reported occasionally (e.g. Sathiyamoorthy and Nakamura, 1990). Root cuttings were used widely to analyze BR effects on main root elongation and lateral root formation. This system can provide valuable hints in determining whether roots respond directly to BRs or indirectly via primary effects on the shoot. Both inhibitory effects (e.g. Guan and Roddick, 1988; Roddick et al., 1993) and stimulating effects (e.g. Romani et al., 1983) were observed.

However, potential cross talk with other phytohormones (which may be provided by the shoot and transported into the root) and the nutrient flux are interrupted in this situation. Excised root cultures, which were derived from root cuttings, are afflicted with similar drawbacks. Therefore, the analysis of seedlings and intact plants corresponds to the most natural situation. 24-Epibrassinolide (EBL) showed inhibitory effects on root formation in mung bean (*Vigna radiata*), wheat (*Triticum aestivum*), maize (Roddick and Ikekawa, 1992), tomato (Guan and Roddick, 1988), Arabidopsis (Clouse et al., 1993), and soybean (*Glycine max*) seedlings (Hunter, 2001).

In contrast, a weak increase of root length was observed in dark-grown cress (*Lepidium sativum*) roots (Yopp et al., 1981) and maize primary roots (Kim et al., 2000). The conflicting results may be explained by culture conditions (e.g. light- or dark-grown plants) and in particular by the mode, level, and time of exposure to BRs. The exogenous application of steroids bears limited resemblance to the in vivo situation where roots would respond to endogenous BRs either synthesized within the root or imported from the shoot. Exogenous phytohormones override endogenous signaling networks, and an impaired balance of pathways may be detrimental. Concordantly, auxin (e.g. Leyser et al., 1996), cytokinin (CK; e.g. Bertell and Eliasson, 1992; Cary et al., 1995), ethylene (e.g. Cary et al., 1995), abscisic acid (for review, see Pilet, 1998), and jasmonate (Staswick et al., 1992) all have the potential to inhibit root elongation, although at the same time, other processes such as auxin-induced lateral root formation (Zolman et al., 2000) and auxin- or ethylene-induced root hair elongation (Pitts et al., 1998) can occur.

The BR-deficient *dwf1-6* mutant (Kauschmann et al., 1996), the BR-deficient *cbb3* mutant (which is allelic to the *cpd* mutant; Szekeres et al., 1996), and *CPD*-antisense plants (Schlüter et al., 2002) were used in the present study. In comparison with mutants affected in reactions specific to BR biosynthesis (such as *dwf4* and *cbb3/cpd*), the *dwf1-6* mutant has a rather mild phenotype. The phenotypic changes of *CPD*-antisense plants were intermediate between BR-deficient mutants such as *dwf1-6* and *cbb3/cpd* and the wild type. The combination of different BR-deficient genotypes provides a means to exclude changes, which are restricted to a specific genotype.

In this article, we show: (a) Positive or negative BR effects on root growth occur according to the applied BR concentration. Low concentrations of exogenous BRs stimulate root growth in wild-type plants and normalize the root length deficit of BR-deficient mutants. Higher concentrations are inhibitory. (b) Exogenous auxins stimulate root growth of BR-deficient plants but do not normalize root length. (c) The auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) does not interfere with BR-induced root growth. (d) BRs and auxin stimulate root growth largely addi-tively. (e) A limited number of phytohormone-related genes display altered transcript levels in roots of the BR-deficient *dwf1-6* mutant. In summary, we provide evidence for an autonomous growth-stimulating effect of BRs in roots largely independent from other phytohormones.

RESULTS

Exogenous BRs Stimulate Root Growth

The levels of active BRs such as castasterone in Arabidopsis roots are significantly lower than the levels in shoots (Bancos et al., 2002; Shimada et al., 2003). As a consequence, we applied low concentrations of EBL and 24-epicastasterone (ECS) to consider physiological conditions. Both EBL and ECS stimulate root elongation. The growth-stimulating effect of ECS is most pronounced in the BR-deficient *cbb3* mutant: 10 nM ECS raises root length to the level of mock-treated wild-type plants, and higher ECS concentrations (such as 50 and 500 nM) still stimulate root growth in the *cbb3* mutant (Fig. 1). Ten micro-molar ECS clearly stimulates root growth of wild-

Figure 1. Low levels of exogenous BRs stimulate root growth. Plants were grown on vertically oriented plates in the presence of EBL (top) and ECS (bottom), respectively. Root length was measured 20 ± 1 d after sowing. Relative root lengths are given as the percentage of root length of untreated wild-type plants. Error bars = ±SE.
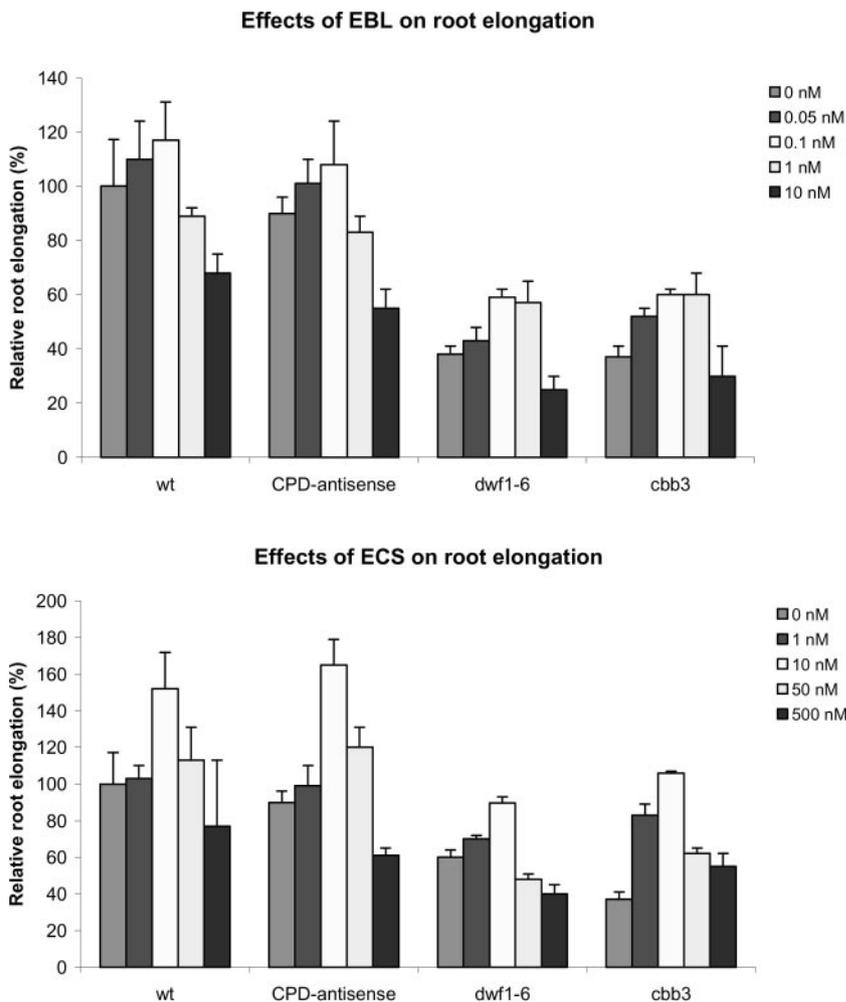
type plants, and 0.05 to 0.1 nM EBL consistently showed a tendency to promote root growth in wild-type plants in a series of three independent experiments. Higher EBL concentrations (≥1 nM) inhibit root growth of wild-type plants (Fig. 1). In contrast, 1 nM EBL clearly stimulates root growth of the *cbb3* mutant, and higher concentrations such as 10 nM EBL are necessary to inhibit root growth.

These findings indicate a positive effect of physiological levels of BRs on root growth. BRs act inhibitory if a threshold level is exceeded. The threshold level depends on the biological activity of the applied BR. For instance, the critical concentration is exceeded earlier with EBL in comparison with ECS. The threshold level also depends on the genotype and is reached later in the *dwf1-6* and *cbb3* mutants, most likely due to low endogenous BR levels.

Auxin Effects on Root Elongation of BR-Deficient Plants

Numerous previous studies pointed to an interaction of BRs and auxins in above ground organs. For example, BR activity was demonstrated in auxin assays (e.g. Grove et al., 1979; Yopp et al., 1981; Takeno and Pharis, 1982; Katsumi, 1985). A positive interaction of BR and auxin in roots could provide the basis for the impaired root growth of BR-mutants. We analyzed the effects of 2,4-dichlorophenoxyacetic acid (2,4-D) and naphtaleneacetic acid (NAA) on root elongation of wild-type plants and BR-deficient mutants. 2,4-D and NAA differ in their transport properties (Delbarre et al., 1996). 2,4-D uptake requires an influx carrier, but 2,4-D is not secreted by an efflux carrier. NAA enters cells by passive diffusion but has its accumulation level controlled by the efflux carrier. Acropetal movement in the root of indole-3-acetic acid (IAA) delivered from the shoot has been implicated in the control of root elongation and lateral root growth, whereas basipetal movement of IAA from the root tip is required for gravity response and has been suggested to affect the initial cell divisions in lateral root initiation.

We found in a series of three independent experiments that low concentrations of both 2,4-D and NAA show a tendency to stimulate root elongation in wild-type plants. The growth-stimulating effects of 2,4-D and NAA are slightly more pronounced in the *dwf1-6* and *cbb3* mutants (Fig. 2). Higher concentrations of 2,4-D and NAA (≥10 and ≥50 nM, respectively) clearly inhibit root growth of both wild-type and BR-deficient plants. Thus, the BR-deficient mutants have no reduced sensitivity to either 2,4-D or NAA, suggesting that auxin influx and efflux are not
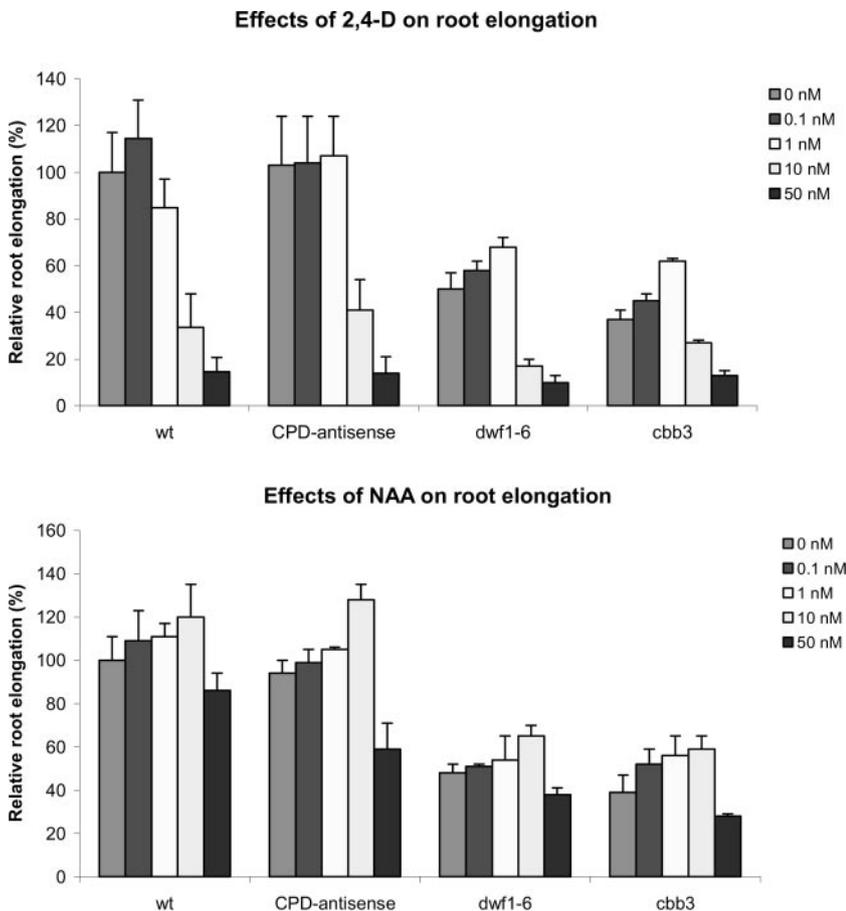

Figure 2. BR-deficient plants display normal responses to 2,4-D (top) and NAA (bottom). Plants were grown on vertically oriented plates, and root length was measured 20 ± 1 d after sowing. Relative root lengths are given as the percentage of root length of untreated wild-type plants. Error bars = ±SE.

impaired and that proper responses to auxin occur. Another evidence for a normal auxin transport in the *dwf1-6* and *cbb3* mutants is their proper gravitropic response, which is blocked in mutants such as *aux1* (Marchant et al., 1999) and *pin2* (Müller et al., 1998).

IAA moves out of plant cells through an efflux carrier apparatus that is sensitive to synthetic inhibitors. These inhibitors include *N*-naphtylphthalamic acid and TIBA, which elicit similar effects (Sabatini et al., 1999). The root growth of *CPD*-antisense, *dwf1-6*, and *cbb3* mutants is affected by TIBA. Similar to wild-type plants, the mutants show a 50% to 60% reduction of root length in the presence of 100 μM TIBA (Fig. 3). This result indicates that the auxin promoted growth is not impaired by BR deficiency.

Conversely, TIBA does not interfere with the BR-induced growth. The root length of wild-type plants grown in the presence of 10 nM ECS and 100 μM TIBA is comparable with the root length of mock-treated plants, and 10 nM ECS slightly overcompensates the inhibitory effect of TIBA in the mutants. Thus, BRs clearly stimulate root growth in the presence of TIBA. The degree of root growth stimulation conferred by 10 nM ECS is similar in the presence and the absence of 100 μM TIBA (Figs. 1 and 3). This finding is confirmed by the analysis of ECS-induced root elongation in the presence of different concentrations of TIBA. ECS stimulates root growth in the presence of TIBA in a dose-dependent manner, and the extent of ECS-induced growth is not affected by TIBA (Fig. 4).

Further evidence for an auxin-independent mode of action of BR comes from the simultaneous administration of EBL and 2,4-D. In case BR and auxin act independently, stimulation and inhibition of root growth conferred by both phytohormones should be additive. In fact, the inhibitory effects of 5 nM 2,4-D and 5 nM EBL were largely additive in wild-type plants (Fig. 5). The growth-promoting effects of both phytohormones were tested in BR-deficient backgrounds because stimulatory effects are weak in wild-type plants. In line with the finding that TIBA
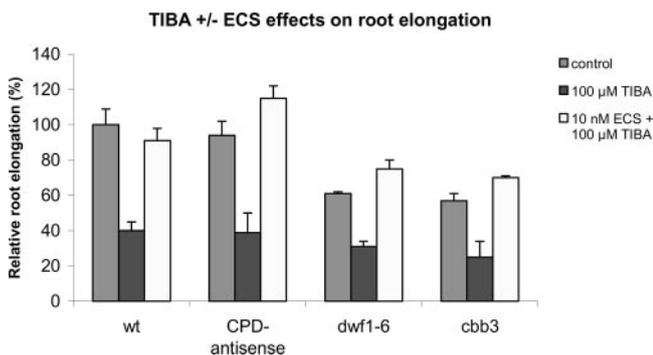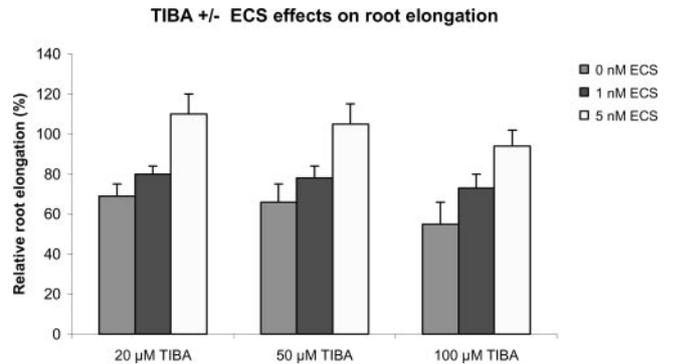

Figure 4. The growth-stimulating effect of ECS is not diminished by 2,3,5-triiodobenzoic acid (TIBA). *CPD*-antisense plants were grown on vertically oriented plates, and root length was measured 20 ± 1 d after sowing. Relative root lengths are given as the percentage of root length of untreated wild-type plants grown in parallel. Error bars = ±SE. Similar results were obtained for wild-type, *dwf1-6*, and *cbb3* plants (data not shown).

does not interfere with BR-induced growth, low concentrations of EBL and 2,4-D additively stimulate root elongation in the *cbb3* (Fig. 5) and *dwf1-6* mutants (data not shown).

Effects of GA and 1-Aminocyclopropane-1-Carboxylic Acid (ACC) on Root Elongation of BR-Deficient Plants

GA-deficient mutants such as *ga1-3* show a drastically reduced root system. GA treatments can normalize the root length in this mutant (Fu and Harberd, 2003). To check GA effects on BR-deficient mutants, *CPD*-antisense, *dwf1-6*, and *cbb3* plants were grown in the presence of different concentrations of GA_4/GA_7. However, GA did not stimulate root elongation in wild type or in the BR-deficient mutants (Fig. 6). Higher GA concentrations caused inhibition of growth, and the dose response curves of BR-deficient mutants were similar to the dose response curve of wild-type plants.

The ethylene sensitivity of BR-deficient plants was checked by means of treatments with the ethylene precursor ACC. The mutants responded in a similar way as the wild-type plants (Fig. 7). This observation indicates that BR deficiency does not alter ethylene responses.

Analysis of Phytohormone Interactions on the Gene Expression Level

The phytohormone and TIBA treatments shown above suggest a growth-stimulating effect of BRs, which does not depend on either auxin or GA. Another approach to reveal potential interactions with other phytohormones is the analysis of gene expression profiles. We searched for phytohormone-related genes with altered transcript levels in roots of the *dwf1-6* mutant. To this end, expression profiles of wild-type and *dwf1-6* root material were established

Figure 3. Effects of 100 μM 2,3,5-triiodobenzoic acid (TIBA) in the presence and absence of 10 nM ECS. Plants were grown on vertically oriented plates, and root length was measured 20 ± 1 d after sowing. Relative root lengths are given as the percentage of root length of untreated wild-type plants. Error bars = ±SE.

Effects of EBL + 2,4-D: Inhibition of root growth (WT)

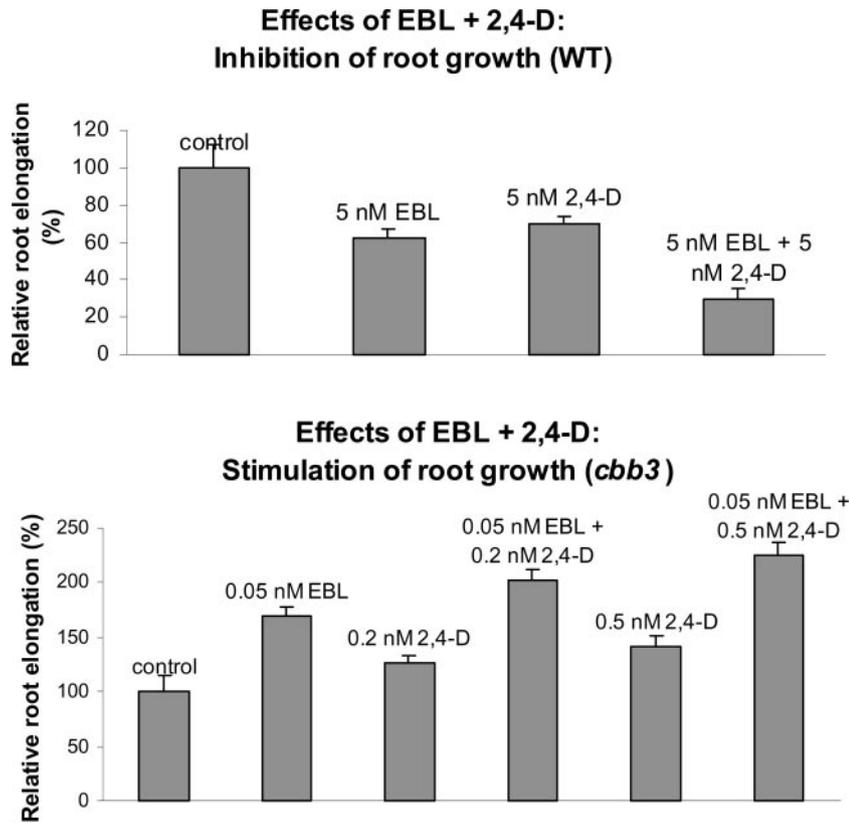## Effects of EBL + 2,4-D: Stimulation of root growth (cbb3)

Figure 5. EBL and 2,4-D effects on root growth are additive. Plants were grown on vertically oriented plates, and root length was measured 20 ± 1 d after sowing. Inhibitory effects (top) and stimulatory effects (bottom) are shown for wild-type and *cbb3* plants, respectively. Relative root lengths are given as the percentage of root length of mock-treated wild-type and *cbb3* plants, respectively. Error bars = $\pm$SE.

by means of Affymetrix ATH1 microarrays. To suppress the effects of biological variability, large pools of plants raised three times independently in a hydroponic system were used for RNA isolation and target synthesis. The targets were checked by means of Test 3 arrays and northern-blot analysis, and only bona fide targets were hybridized to ATH1 arrays. The qualitative and quantitative outcome of the Affymetrix Microarray Suite Version 5.0 software was used to identify genes with altered transcript levels in the *dwf1-6* mutant. The information used included the detection *P* values calculated through single ar-

ray analyses, the change *P* values, and signal log ratios determined through comparison analysis, respectively. The detection *P* value was applied to filter out genes with absent calls (underlying parameters: $\alpha_1 = 0.05$ and $\alpha_2 = 0.065$). Simultaneously, the change *P* value and the signal log ratio algorithms were applied in pair-wise comparisons to identify regulated genes with high reliability. Differences in expression of selected genes were confirmed by means of real-time reverse transcriptase (RT)-PCR experiments. The plant material for real-time RT-PCR was raised two times independently and was also
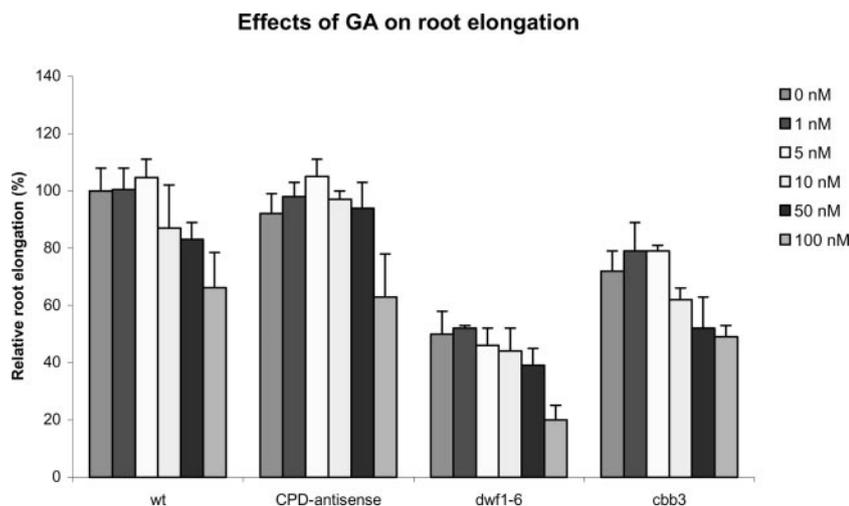
Effects of GA on root elongation

Figure 6. GA does not induce root elongation in BR-deficient plants. Plants were grown on vertically oriented plates, and root length was measured 20 ± 1 d after sowing. Relative root lengths are given as the percentage of root length of untreated wild-type plants. Error bars = $\pm$SE.
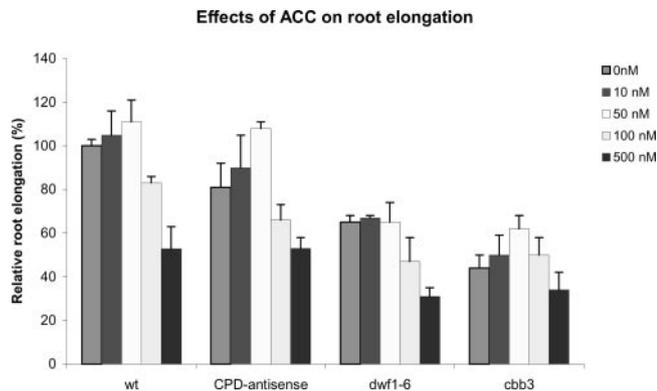
Effects of ACC on root elongation

Figure 7. BR-deficient plants exhibit normal responses to the ethylene precursor ACC. Plants were grown on vertically oriented plates and root length was measured 20 ± 1 d after sowing. Relative root lengths are given as the percentage of root length of untreated wild-type plants. Error bars = ±SE.

independent from the plant material used for ATH1 array hybridizations.

The ATH1 chip represents 24,000 genes. Two hundred fifty-seven genes displayed stronger expression in the wild type (present in the wild type, change P value < 0.01, signal log ratio ≥ 0.9), and 66 genes displayed stronger expression in the *dwf1-6* mutant (present in the mutant, change P value < 0.01, signal log ratio ≥ 0.9). Most genes with altered transcript levels were not characterized hitherto. A complete set of expression data are downloadable at our Web page (http://www.mpimp-golm.mpg.de/BR_reg_gene_expression/).

Similar to the situation in above ground organs (Bancos et al., 2002; Müssig et al., 2002), the steroid 22-hydroxylase *DWF4* is clearly BR down-regulated, and the *CPD* gene shows a tendency to increased transcript levels in the mutant also. This finding is in agreement with the proposed negative feedback inhibition of BR biosynthesis.

In roots of the *dwf1-6* mutant, only few auxin-related genes display significantly altered transcript levels (Tables I and II). The *NIT3* gene (encoding an enzyme involved in IAA biosynthesis; Kutz et al., 2002) shows higher transcript levels in the mutant. *IAA14* (Fukaki et al., 2002), *IAA2*, and a putative auxin-responsive GH3-like gene show weaker expression in the mutant. Thus, the expression data indicate a potential weak link between BR and auxin action in the root. A positive interaction with ethylene may be more obvious (Table I). Several genes of the ethylene response (such as *ERF2* and *ERF5*, encoding activators of GCC box-mediated gene expression [Fujimoto et al., 2000]) and ethylene biosynthesis (e.g. ACC oxidases) show weaker expression in the *dwf1-6* mutant (Table I; Web page). Genes related to GA, CK, ABA, jasmonate biosynthesis, signaling, and response did not show significantly altered transcript levels (see web page).

DISCUSSION

BRs Promote Root Growth

BR-deficient mutants such as *dwf1-6* and *cbb3* show 40% to 60% shorter roots than wild-type plants. Previous studies predominantly reported inhibitory effects of BRs on root growth (e.g. Guan and Roddick, 1988; Roddick and Ikekawa, 1992), and this inhibitory effect was used to identify or characterize BR-insensitive mutants (Clouse et al., 1996; Koka et al., 2000; Li et al., 2001; Montoya et al., 2002). In this article, we show that the BR effects on root growth are strongly dependent on the BR concentration used. Exogenous BRs stimulate root growth at low concentrations. Inhibition of root growth occurs at higher BR levels, which exceed a certain level. The EBL dose response curve (Fig. 1) corroborates previous results that showed stimulation of root growth of 7-d-old seedlings by 0.1 and 0.5 nM EBL (Clouse et al., 1996). It takes higher concentrations of biologically less active BRs (such as ECS) to exceed this level, and the *dwf1-6* and *cbb3* mutants tolerate higher concentrations, most likely due to reduced endogenous BR levels.

Interactions with Other Phytohormones

Expression analysis of aerial part of plants revealed a positive BR effect on the expression of a large number of auxin-related genes. Genes such as *TCH4* (Xu et al., 1995); *SAUR6B* (Zurek et al., 1994); and *IAA2, IAA3, IAA13, IAA19, IAA22,* and *SAUR-AC1* (Goda et al., 2002; Müssig et al., 2002) are regulated by BRs and auxin. The expression data indicate a potential weak BR-auxin cross talk in roots also. The *IAA2* gene, the *IAA14* gene, and a GH3-like gene show weaker expression in the *dwf1-6* mutant than in the wild type. This finding could indicate reduced auxin levels, reduced auxin sensitivity, or altered auxin distribution in roots of BR-deficient plants. However, the *NIT3* gene, which is involved in auxin biosynthesis, shows stronger expression in the mutant, and root growth of BR-deficient mutants is strongly inhibited by TIBA or higher concentrations of 2,4-D and NAA. These observations argue against impaired auxin responses in the mutants, and BRs may exert a direct regulatory effect on auxin-regulated genes.

The BR-induced root elongation is not diminished by TIBA (Figs. 2–4), and 100 nM EBL drastically inhibits root growth of the auxin-insensitive *axr1* mutant (Clouse et al., 1993). Furthermore, EBL and 2,4-D effects on root growth are largely additive (Fig. 5). These findings provide further evidence for an auxin-independent effect of BRs on root elongation.

Low levels of 2,4-D and NAA slightly promote root growth in wild-type and BR-deficient plants (Fig. 2). Conceivably, the growth-stimulating effect of auxin could be due to a stimulation of BR biosynthesis. To

Table I. *Phytohormone-related genes: stronger expression in wild-type plants*

All transcripts meet the presence criterion in the wild-type expression profile after hybridization of an Affymetrix ATH1 array. The change *P* values are <0.01 (indicating an increase according to the change algorithm of the MAS 5.0 software). Real-time RT-PCR fold change values were calculated from average threshold cycle (C_T) values (see "Materials and Methods"). The table shows average C_T values ($\pm$SE) from three replicates at each experiment for both the gene of interest and *eEF1α*.

Affymetrix Identification	ATH1 Array: Wild Type vs. *dwf1-6*	Annotation, Description	Real-Time RT-PCR: Wild Type vs. *dwf1-6*	
			Experiment no. 1	Experiment no. 2
	Fold change		*Fold change, C_T values*	
		Ethylene-related genes		
253259_at	5.3	At4g34410, putative ethylene-responsive element binding factor	nd[a]	nd
258727_at	3.5	At3g11930, putative ethylene-responsive protein	nd	nd
258133_at	3.0	At3g24500, putative ethylene-responsive transcriptional coactivator	nd	nd
248799_at	2.0	At5g47230, ethylene-responsive element-binding factor 5 (*ERF5*)	1.3	3.1
			WT: $C_{T\ ERF5}$ 23.19 $\pm$ 0.19; $C_{T\ eIF}$ 17.2 $\pm$ 0.05	WT: $C_{T\ ERF5}$ 21.15 $\pm$ 0.36; $C_{T\ eIF}$ 17.33 $\pm$ 0.13
			dwf1-6: $C_{T\ ERF5}$ 23.38 $\pm$ 0.12; $C_{T\ eIF}$ 16.99 $\pm$ 0.07	*dwf1-6*: $C_{T\ ERF5}$ 22.68 $\pm$ 0.32; $C_{T\ eIF}$ 17.21 $\pm$ 0.16
265948_at	2.0	At2g19590, putative ACC oxidase	2.2	3.7
			WT: $C_{T\ ACCox}$ 25.19 $\pm$ 0.2; $C_{T\ eIF}$ 17.2 $\pm$ 0.05	WT: $C_{T\ ACCox}$ 25.62 $\pm$ 0.12; $C_{T\ eIF}$ 17.33 $\pm$ 0.13
			dwf1-6: $C_{T\ ACCox}$ 26.1 $\pm$ 0.15; $C_{T\ eIF}$ 16.99 $\pm$ 0.07	*dwf1-6*: $C_{T\ ACCox}$ 27.4 $\pm$ 0.15; $C_{T\ eIF}$ 17.21 $\pm$ 0.16
248794_at	1.7	At5g47220, ethylene responsive element-binding factor 2 (*ERF2*)	2.8	7.9
			WT: $C_{T\ ERF2}$ 22.44 $\pm$ 0.17; $C_{T\ eIF}$ 17.2 $\pm$ 0.05	WT: $C_{T\ ERF2}$ 21.49 $\pm$ 0.14; $C_{T\ eIF}$ 17.33 $\pm$ 0.13
			dwf1-6: $C_{T\ ERF2}$ 23.69 $\pm$ 0.11; $C_{T\ eIF}$ 16.99 $\pm$ 0.07	*dwf1-6*: $C_{T\ ERF2}$ 24.36 $\pm$ 0.1; $C_{T\ eIF}$ 17.21 $\pm$ 0.16
257922_at	1.7	At3g23150, ethylene receptor (*ETR2*)	nd	nd
		Auxin-related genes		
264929_at	4.9	At1g60730, putative auxin-induced protein	nd	nd
262099_s_at	2.1	At1g59500, putative auxin-regulated protein GH3	6.0	2.2
			WT: $C_{T\ GH3}$ 19.96 $\pm$ 0.3; $C_{T\ eIF}$ 15.28 $\pm$ 0.34	WT: $C_{T\ GH3}$ 23.05 $\pm$ 0.04; $C_{T\ eIF}$ 17.87 $\pm$ 0.05
			dwf1-6: $C_{T\ GH3}$ 21.97 $\pm$ 0.35; $C_{T\ eIF}$ 14.7 $\pm$ 0.07	*dwf1-6*: $C_{T\ GH3}$ 23.3 $\pm$ 0.1; $C_{T\ eIF}$ 17.01 $\pm$ 0.13
245593_at	1.9	At4g14550, Aux/IAA protein family (*IAA14*)	2.3	1.8
			WT: $C_{T\ IAA14}$ 19.52 $\pm$ 0.08; $C_{T\ eIF}$ 15.28 $\pm$ 0.34	WT: $C_{T\ IAA14}$ 22.8 $\pm$ 0.1; $C_{T\ eIF}$ 17.87 $\pm$ 0.03
			dwf1-6: $C_{T\ IAA14}$ 20.14 $\pm$ 0.48; $C_{T\ eIF}$ 14.7 $\pm$ 0.07	*dwf1-6*: $C_{T\ IAA14}$ 22.79 $\pm$ 0.12; $C_{T\ eIF}$ 17.01 $\pm$ 0.13
257766_at	1.7	At3g23030, Aux/IAA protein family (*IAA2*)	1.7	2.6
			WT: $C_{T\ IAA2}$ 17.7 $\pm$ 0.03; $C_{T\ eIF}$ 15.28 $\pm$ 0.34	WT: $C_{T\ IAA2}$ 20.8 $\pm$ 0.2; $C_{T\ eIF}$ 17.33 $\pm$ 0.37
			dwf1-6: $C_{T\ IAA2}$ 17.9 $\pm$ 0.1; $C_{T\ eIF}$ 14.7 $\pm$ 0.07	*dwf1-6*: $C_{T\ IAA2}$ 22.07 $\pm$ 0.07; $C_{T\ eIF}$ 17.21 $\pm$ 0. 6

[a] nd, Not determined.

check this, wild-type plants were established in a hydroponic culture system, and 1 nM 2,4-D was added to the liquid medium. *CPD* and *DWF4* mRNA levels were not altered 3 and 24 h after treatments (data not shown), suggesting that auxin does not influence BR biosynthesis.

In contrast to BR-induced root growth, BR-induced gravitropic curvature in maize primary roots is auxin dependent. Kim et al. (2000) have shown that the activation of the gravitropic response by BRs is nullified by application of TIBA. However, the *CPD*-antisense, *dwf1-6*, and *cbb3* plants show normal gravitropic responses on vertical plates, calling the necessity of BRs for the gravitropic response into question and suggesting functional auxin influx and efflux carriers to be present despite the BR deficiency in these mutants (Müller et al., 1998; Marchant et al., 1999).

An impaired auxin transport is also indicated by the administration of different auxins, which possess different transport properties. For instance, root growth of the *aux1* mutant is less sensitive to auxins requiring carrier-mediated uptake (2,4-D and IAA), but the dose response curves for wild type and *aux1* root elongation in the presence of varying concentrations of 1-NAA are identical (Marchant et al., 1999). However, roots of BR-deficient plants do not show reduced sensitivity to either 2,4-D or 1-NAA in comparison with wild-type plants. For instance, 10 nM

2,4-D and 50 nM NAA clearly inhibit root elongation in all genotypes (Fig. 2). We also did not find significantly altered transcript levels of genes involved in the auxin transport (see Web page).

Another root growth-promoting phytohormone is GA. Roots of GA-deficient mutants (e.g. *ga1-3* from Arabidopsis [Fu and Harberd, 2003] and *na* from pea [Yaxley et al., 2001]) are shorter than those of the wild type. GA treatment normalizes the root length of *ga1-3* mutant plants to wild-type levels, showing the role of GA in the regulation of root growth (Fu and Harberd, 2003). Auxin is necessary for GA-mediated control of root growth because attenuation of polar auxin transport by means of *N*-naphtylphthalamic acid or decapitation of plants restrains GA-induced root growth (Fu and Harberd, 2003). GA treatments do not normalize root length of the *dwf1-6* and *cbb3* mutants. The dose response curves of BR-deficient mutants are identical to the dose response curve of wild-type plants (Fig. 6).

First hints to GA and BR signaling cross talk were derived from the finding that *bri1-201* seedlings have drastically reduced transcript levels of the GA-repressed *GA5* gene and elevated transcript levels of the GA-inducible *GASA1* gene. BR treatment of *cpd* seedlings resulted in decreased *GASA1* and increased *GA5* expression, showing that BRs affect the expression of the *GA5* and *GASA1* genes antagonistically to GA (Bouquin et al., 2001). However, no GA-related

Table II. *Phytohormone-related genes: stronger expression in the dwf1-6 mutant*

All transcripts meet the presence criterion in the *dwf1-6* expression profile after hybridization of an Affymetrix ATH1 array. The change *P* values are <0.01 (indicating an increase according to the change algorithm of the MAS 5.0 software). Real-time RT-PCR fold change values were calculated from average C_T values (see "Materials and Methods"). The table shows average C_T values (±SE) from three replicates at each experiment for both the gene of interest and *eEF1α*.

Affymetrix Identification	ATH1 Array: *dwf1-6* vs. Wild Type	Annotation, Description	Real-Time RT-PCR: *dwf1-6* vs. Wild Type	
			Experiment no. 1	Experiment no. 2
	Fold change		*Fold change, C_T values*	
		Auxin-related genes		
252677_at	1.9	At3g44320, hydrolysis of indole-3-acetonitrile to indole-3-acetic acid (*NIT3*)	2.9	3.0
			WT: $C_{T\ NIT3}$ 26.16 ± 0.09; $C_{T\ eIF}$ 17.2 ± 0.05	WT: $C_{T\ NIT3}$ 23.93 ± 0.19; $C_{T\ eIF}$ 17.33 ± 0.13
			dwf1-6: $C_{T\ NIT3}$ 24.39 ± 0.26; $C_{T\ eIF}$ 16.99 ± 0.07	*dwf1-6*: $C_{T\ NIT3}$ 22.21 ± 0.13; $C_{T\ eIF}$ 17.21 ± 0.16
		BR-related genes		
252184_at	2.5	At3g50660, steroid 22-hydroxylase (*DWF4*)	2.2	1.9
			WT: $C_{T\ DWF4}$ 23.87 ± 0.19; $C_{T\ eIF}$ 17.2 ± 0.05	WT: $C_{T\ DWF4}$ 23.8 ± 0.04; $C_{T\ eIF}$ 17.33 ± 0.13
			dwf1-6: $C_{T\ DWF4}$ 22.54 ± 0.06; $C_{T\ eIF}$ 16.99 ± 0.07	*dwf1-6*: $C_{T\ DWF4}$ 22.77 ± 0.08; $C_{T\ eIF}$ 17.21 ± 0.16
250752_at	1.3	At5g05690, steroid 23-hydroxylase (*CPD*)	nd[a]	1.9
				WT: $C_{T\ CPD}$ 24.38 ± 0.1; $C_{T\ eIF}$ 17.8 ± 0.07
				dwf1-6: $C_{T\ CDP}$ 23.76 ± 1.0; $C_{T\ eIF}$ 18.1 ± 0.32

[a]nd, Not determined.

genes (including the *GA5* and *GASA1* genes) show significantly altered transcript levels in the *dwf1-6* mutant. Thus, the BR effect on root elongation most likely is independent from GA.

Putative BR Effects on Ethylene Production and Responses

The elongation of roots is inhibited by ACC and ethylene (Cary et al., 1995). This also holds true for BR-deficient plants (Fig. 7). Higher concentrations of auxin inhibit root growth as well, suggesting a common mechanism (for review, see Casson and Lindsey, 2003). In fact, auxin stimulates ethylene biosynthesis, and elevated ethylene levels inhibit root elongation potentially via the inhibition of polar auxin transport. This mechanism constitutes an auxin-ethylene feedback loop.

Likewise, BRs may stimulate ethylene biosynthesis and trigger ethylene responses in roots. BRs are known to stimulate the production of ethylene in shoots and roots (e.g. Schlagnhaufer et al., 1984; Arteca et al., 1985; Woeste et al., 1999; Yi et al., 1999; Arteca and Arteca, 2001). In line with these observations, our expression data point to a positive BR effect on genes involved in ethylene biosynthesis and ethylene response (Table I; Web page). The positive BR effect on ethylene production could have an impact on the auxin-ethylene feedback loop. However, this cannot account completely for the BR-induced growth inhibition because EBL clearly inhibits root growth in the *axr1* mutant (Clouse et al., 1993). A direct BR-ethylene feedback loop may exist, which specifically interferes with BR transport, BR biosynthesis, or BR responses.

A close ethylene interaction in roots was shown for CKs also. Synthetic CKs inhibit root growth, and this effect is coupled to ethylene (e.g. Bertell and Eliasson, 1992; Cary et al., 1995; for review, see Casson and Lindsey, 2003). Transgenic plants with reduced CK content display enhanced root growth (Werner et al., 2001). However, there is little evidence for a BR-CK interaction, and *dwf1-6* roots did not show significantly altered transcript levels of CK-related genes. Furthermore, the *dwf1-6* mutant shows decreased rather than increased transcript levels of genes involved in the ethylene response or ethylene biosynthesis. Therefore, the reduced root growth of BR-deficient plants most likely is not related to CKs.

Conclusion and Outlook

Our experiments have shown that BRs promote root elongation. This effect appears to be largely independent from auxin and GA. In future experiments, we intend to analyze the inhibitory effect of higher concentrations of BRs, which may depend on ethylene. The simultaneous application of high concentrations of BRs and ethylene biosynthesis inhibi-

tors (such as aminoethoxyvinyl-Gly) or ethylene signaling inhibitors (such as silver thiosulfate) and the analysis of BR effects on ethylene mutants allows verification of the proposed BR-ethylene interaction.

The Affymetrix expression profiles and real-time RT-PCR experiments (Table I; Web page; data not shown) indicate altered expression levels of the *SCARECROW* transcription factor (Di Laurenzio et al., 1996; stronger expression in *dwf1-6*), the *ROOT HAIRLESS 1* gene (Schneider et al., 1998; stronger expression in *dwf1-6*), and *SOLITARY-ROOT/IAA14* gene (weaker expression in *dwf1-6*; Fukaki et al., 2002). These genes are required for the radial organization of the Arabidopsis root, formation of root hairs, and involved in lateral root formation, respectively. Thus, BRs may play a role in root differentiation. Although BR-deficient plants produce lateral roots and root hairs, it may be worthwhile to carry out a detailed morphological analysis of the root system (in particular, of the root meristem).

MATERIALS AND METHODS

Hydroponic Growth Conditions

Arabidopsis cv 24 (wild type) and the BR-deficient mutant *dwf1-6* (*cbb1*; Kauschmann et al., 1996) were grown hydroponically in modified 0.25× Hoagland medium. Two different conditions were applied to establish plants. Black 0.5-mL reaction tubes (Treff AG, Degersheim Switzerland) were filled with agar-solidified medium (0.8% [w/v] agar in 0.25× modified Hoagland medium), and the bottom of the tube was cut after the agar became solid. The medium composition was 1.5 mM $Ca(NO_3)_2$, 0.28 mM KH_2PO_4, 0.75 mM $MgSO_4$, 1.25 mM KNO_3, 0.5 µM $CuSO_4$, 1.0 µM $ZnSO_4$, 5 µM $MnSO_4$, 25 µM H_3BO_3, 0.1 µM Na_2MoO_4, 50 µM KCl, 3 mM MES-KOH (pH 5.7), and 20 µM FeEDTA. Single seeds were placed on top of the agar-filled tubes, and the tubes were placed into holes in the cover of boxes filled with medium. Thus, light entry into the medium was largely prohibited. Boxes were transferred into a greenhouse, and the medium was changed weekly. Plants were harvested 24 d after sowing. This method was used for raising the material for microarray hybridizations.

Alternatively, plants were raised in one-half-concentrated Murashige and Skoog medium supplemented with 1% (w/v) Suc and solidified with 0.7% (w/v) agar under a 16-h day (140 µmol m^{-2} s^{-1}, 22°C)/8-h night (22°C) night regime. Plants were transferred to boxes filled with 0.25× modified Hoagland medium after 10 d of growth and then treated as described above. This method produces larger plants and was applied for auxin treatments of wild-type plants and for production of wild-type and *dwf1-6* plant material for real-time RT-PCR.

Root Growth Assays

For monitoring effects of different phytohormones on root growth of wild type, *CPD*-antisense (Schlüter et al., 2002), *dwf1-6*, and *cbb3* plants (Kauschmann et al., 1996), plants were grown in one-half-concentrated Murashige and Skoog medium supplemented with 0.8% (w/v) agarose under a 16-h day (140 µmol m^{-2} s^{-1}, 22°C)/8-h night (22°C) regime. Phytohormones and inhibitors were added as described in the text. BRs (CIDtech Research Inc., Cambridge, ON), GA (61.2% GA_4 and 30.1% GA_7, Duchefa, Haarlem, The Netherlands), and TIBA (Duchefa) were solved in MeOH, ACC (Sigma, Taufkirchen, Germany) was solved in water, 2,4-D (Duchefa) in ethanol, and NAA (Duchefa) in dimethyl sulfoxide. All plates of a given experiment (e.g. four different EBL concentrations and the appropriate control) contained the same amount of solvent (e.g. in case of EBL treatment, all plates contained 5 × 10^{-4}% [v/v] MeOH) to rule out solvent effects. Approximately 3 cm of medium was removed after the medium became solid, and seeds were placed on top of the cutting area. Only for the experiments shown in Figure 5, no medium was removed, and seeds were

placed directly on the agar surface. Plates were placed upright, and root length was measured 20 ± 1 d after sowing. All experiments were performed three times (BR treatments [Fig. 1], auxin treatments [Fig. 2], and TIBA treatments [Figs. 3 and 4]) or two times (auxin and BR treatment [Fig. 5], GA treatment [Fig. 6], and ACC treatments [Fig. 7]). Twenty-five plants per experiment, treatment, and genotype were analyzed.

Hybridization of Affymetrix Genome Arrays

Total RNA was isolated by means of the TRIzol Reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The protocol was modified with respect to the RNA precipitation step (1/20 volume 3 M NaOAc was added to increase yield). The quality and quantity was checked using the Bioanalyzer 2100 (Agilent Technologies, Böblingen, Germany) and MOPS-formaldehyde agarose gels. Twenty micrograms of total RNA was used for double-stranded cDNA synthesis (SuperScript Choice System, Invitrogen). Biotin-labeled cRNAs were synthesized using the BioAray High Yield RNA Transcript Labeling Kit (Enzo, New York). All cRNA samples were checked for degradation by gel analysis according to the Affymetrix technical manual. In addition, all targets were checked by hybridizations of Test 3 arrays. Only bona fide probes were used for ATH1 array hybridizations (Affymetrix, Santa Clara, CA). Hybridization, washing, staining, and scanning procedures were performed as described in the Affymetrix technical manual. Expression analysis via the Affymetrix Microarray Suite Version 5.0 software was performed with standard parameters. The output of every experiment was multiplied by a scaling factor to adjust its average intensity to a target intensity of 100. Thus, scaling allows comparisons between any two experiments. To consider biological variability, RNA from three independent experiments was pooled for the synthesis of the Affymetrix target. Selected expression data were verified two times by means of real-time RT-PCR with independent plant material.

Real-time RT-PCR

Total RNA was isolated using the Invisorb Spin Plant RNA kit (Invitek, Berlin). One microgram of total RNA was then reverse transcribed with the Superscript II RT (Invitrogen) in a reaction volume of 28.5 μL to generate first strand cDNA. Every cDNA was checked for contamination with genomic DNA by means of PCR using the intron-spanning primers SPY_fw 5′ gga atc tag ctt tcg att gtt ttt ctg a 3′, SPY_rev 5′ tca aag ttt gga gac aca gct aga cat c 3′, and CCA1_fw 5′ aac agc aac aac aat gca act act gat t 3′, and CCA1_rev 5′ aca aac aga gac aag aga caa gac atg g3′. Occasionally, genomic PCR products appeared after 35 cycles, and these cDNAs were discarded. Real-time RT-PCR was performed with 1 μL of a 1:3.5 dilution of the first strand cDNA reaction and the SYBR Green reagent (Applied Biosystems, Foster City, CA) in a 25-μL volume on a Perkin Elmer Geneamp 5700 machine (PerkinElmer, Boston) using the primers ERF2_fw 5′acggactcct-caaagatgccttc 3′, ERF2_rev 5′ctcctccatcgcccgtaaagttct 3′ (At5g47220), ERF5_fw 5′tgacgttaacggtggagacg 3′, ERF5_rev 5′tgaggagataacggcgaca-gaag 3′ (At5g47230), ACC_ox_fw 5′gtcagccattaccctccttgtcc 3′, ACC_ox_rev 5′ctgaaggccatcatattcatcg 3′ (At2g19590), NIT3_fw 5′ atgatcctactgtctccggag-gtg 3′, NIT3_rev 5′ ccaagatcaagatcagctgtgacg 3′ (At3g44320), GH3_fw 5′ tgtcaagcttggtcaggaatacg 3′, GH3_rev 5′ cgctttgttcttgaaaccagtca 3′ (At1g59500), IAA2_fw 5′ cccgtaagaacaacaacagtgtga 3′, IAA2_rev 5′ ctcta-acgctttgagaagctcgg 3′ (At3g23030), IAA14_fw 5′ tatgtgccaagctacgaggacaa 3′, IAA14_rev 5′ ccaactgcttcagatcccttcat 3′ (At4g14550), DWF4_fw 5′ cagacgat-gatcttttgggatgg 3′, DWF4_rev 5′ agaagaagtctcatgtccggcaa 3′ (At3g50660), CPD_fw 5′ cagagcaactcggtaacgacagg 3′, CPD_rev 5′ gcggtgaaggaaaaca-gagagtg 3′ (At5g05690), eIF1α _fw 5′ ttgacaggcgttctggtaagg 3′, eIF1α _rev 5′ cagcgtcaccattcttcaaaaa 3′ (At5g60390), Ubq10_fw 5′ cacactccacttggtctttgcgt 3′, and Ubq10_rev 5′ tggtctttccggtgagagtcttca 3′ (At4g05320). All primer pairs amplified single products, as shown by the melting temperatures of the amplicons and gel electrophoresis. The use of different control genes (either *eEF1α* or *Ubq10*) did not bias the results with respect to the fold change values. Therefore, only the *eIF1α*-based data are shown in Tables I and II.

Data were normalized to *eIF1α* and then compared according to the formula (considering as example *DWF4*):

$$nC_T = \mathbf{C}_{T\ DWF4} - \mathbf{C}_{T\ eIF1\alpha}$$

$$\Delta C_T = nC_{T\ WT} - nC_{T\ dwf1\text{-}6}$$

$$\text{Fold change } (dwf1\text{-}6 \text{ vs. WT}) = \mathbf{2}^{\Delta CT}$$

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