

AXR1 is involved in BR-mediated elongation and *SAUR-AC1* gene expression in Arabidopsis

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Abstract Limited information is available concerning the interactions between the brassinosteroid (BR) and auxin signaling pathways. The expression pattern of the *SAUR-AC1* gene, an early auxin-inducible gene in Arabidopsis, was studied in response to brassinolide (BL), in the presence of a BR-biosynthesis inhibitor, in a BR-deficient mutant, and in combination with auxin. The results suggested that the *SAUR-AC1* gene is regulated by BRs independently of auxin levels, and that it is important in BR-mediated elongation. The *axr1* (auxin insensitive 1) mutant was less sensitive to BL-induced elongation and BL-induced *SAUR-AC1* expression, suggesting that a ubiquitin ligase-mediated system is involved in BR-mediated elongation. © 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Brassinosteroid; Auxin; *SAUR-AC1*; AXR1; Ubiquitin system

1. Introduction

Brassinosteroid (BR), a steroidal plant hormone, has a wide range of physiological effects on plant growth and development [1–3]. A dwarf stature is the most remarkable phenotype of BR-deficient mutants. Longitudinal cell size [1] and cell number [4] are greatly reduced in these mutants, indicating the importance of BRs for plant tissue elongation. However, the signal transduction system regulating BR-mediated tissue elongation is poorly understood. Physiological studies have demonstrated synergistic effects of BRs and auxins on elongating tissues and cells in both dicots and monocots [3,5]. Recent DNA microarray studies have revealed that certain auxin-related genes are regulated by BR [6–8], but the molecular mechanism connecting these hormones awaits elucidation.

SAUR (small auxin up RNA) genes were reported originally in soybean. *SAUR* mRNA accumulates within minutes of treatment with auxin, but not in response to treatment with other plant hormones (i.e. cytokinin, abscisic acid, gibberellin,

and ethylene) [9]. The expression of *SAUR* genes occurs in specific tissues and at specific developmental stages, e.g. in elongating hypocotyls or epicotyls [9–11]. In contrast to auxin, BR did not rapidly induce *SAUR* genes, but induction occurred after the beginning of cell elongation in soybean [12,13]. Free indole-3-acetic acid (IAA) levels decreased in BR-treated soybean epicotyls [13]. Based on these observations, it was proposed that auxin biosynthesis and *SAUR* gene expression are not required for initial elongation in BR-treated tissue. *SAUR-AC1* is the best-characterized *SAUR* gene in Arabidopsis [14] and has characteristics similar to the soybean genes. Experiments using transgenic plants harboring a *SAUR-AC1* promoter- β -glucuronidase (*GUS*) fusion gene have suggested that *SAUR-AC1* mRNA is localized in the cotyledons and primary leaves in light-grown seedlings [15] and that the gene is induced by exogenous auxin in the hypocotyls [16]. We recently demonstrated that brassinolide (BL) induces *SAUR-AC1* faster than has been reported previously for *SAUR* genes in soybean [12], making *SAUR-AC1* one of the earliest BR-inducible genes [8]. *SAUR-AC1* showed significant induction within 30 min of BL treatment, and the transcripts gradually increased up to a 12-h treatment. Auxin induction of *SAUR-AC1* is impaired in the auxin insensitive mutant *axr1* [17], which encodes a protein related to the ubiquitin-activating enzyme E1 [18]. The *axr1* mutant shows defects in auxin-mediated growth and development [19] and it has been proposed that AXR1 is a positive regulator of the auxin signaling pathway, modulating the activity of the E3 ubiquitin ligase SCF^{TIR1}.

We used the *SAUR-AC1* gene, which is commonly regulated by BL and IAA, as a molecular marker to study the synergism and interactions of these two hormones. We also used the *axr1* mutant to study the involvement of the ubiquitin system in the two hormonal signaling pathways. The molecular mechanism of BR-mediated cell elongation and the interactions between the BR and auxin signaling pathways are discussed, based on our results.

2. Materials and methods

2.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild type (WT) in this study. The BR-deficient mutant *det2-1* [20] and the auxin-insensitive mutant *axr1-3* [19] were also used. Transgenic plants harboring a *SAUR-AC1*-promoter-*GUS* fusion gene were produced by Gil and Green [15]. For histochemical analyses, a homozygous *axr1-3* mutant carrying the *SAUR-AC1*-promoter-*GUS* fusion gene

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Abbreviations: BR, brassinosteroid; BL, brassinolide; IAA, indole-3-acetic acid; Brz, brassinazol; GUS, β -glucuronidase; DMSO, dimethyl sulfoxide; RTQ-RT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; EBR, 24-epibrassinolide

was produced by crossing. Seedlings were grown for 7 days at 22°C under continuous light in half-strength Murashige and Skoog [21] liquid medium (Gibco BRL, Grand Island, NY, USA), supplemented with 1.5% (w/v) sucrose and with shaking at 120 rpm. The seedlings were treated with hormones or mock-treated with 0.1% dimethyl sulfoxide (DMSO) in the medium. The lengths of petioles and hypocotyls were measured using NIH image software (Version 1.61, National Institutes of Health).

2.2. Real-time quantitative (RTQ) RT-PCR

The transcript abundance of *SAUR-AC1* was analyzed using TaqMan RTQ-RT-PCR, which was undertaken as described previously [8,22].

2.3. Histochemical localization of GUS activity and GUS activity assay

Histochemical GUS staining was performed [23] by incubating whole seedlings in GUS staining buffer containing 50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% (v/v) Triton X-100, 2% (v/v) DMSO, and 0.2 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Glc) at 37°C for 5 h.

3. Results

3.1. Regulation of *SAUR-AC1* gene expression by BR and auxin

To study the effects of endogenous IAA or BR on *SAUR-AC1* gene expression, we compared the level of *SAUR-AC1* transcripts in WT seedlings with that in BR-deficient *det2* seedlings. The *det2* mutant seedlings have a lower endogenous BR content [24] and a higher endogenous IAA content [25] per fresh weight than do WT seedlings. The level of *SAUR-AC1* transcripts in *det2* was approximately one-third the level in WT (Fig. 1), suggesting that the lower endogenous BRs down-regulated *SAUR-AC1* gene expression in the *det2* mutant.

In previous reports, *SAUR-AC1* was induced by exogenous auxin within 1 h of treatment [14,17]. To confirm the effects of exogenous auxin on *SAUR-AC1* gene expression, WT seedlings were treated with 10^{-6} – 10^{-8} M IAA. At 10^{-6} – 10^{-7} M IAA, transcript accumulation was apparent within 15 min, and the induction was transient, reaching a maximum between 15 min and 1 h (data not shown). In contrast, 10^{-8} M IAA had little effect on *SAUR-AC1* gene expression (Fig. 2).

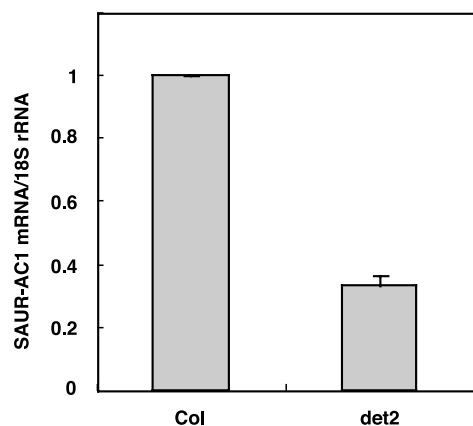


Fig. 1. Abundance of *SAUR-AC1* transcripts in WT and *det2* seedlings. WT (Col-0) and *det2* seedlings were grown for 7 days. Transcript levels are presented as values relative to those at 0 h, defined as 1, after normalization to 18S ribosomal RNA levels. Data are means $\pm$ standard errors from three independent plant samples.

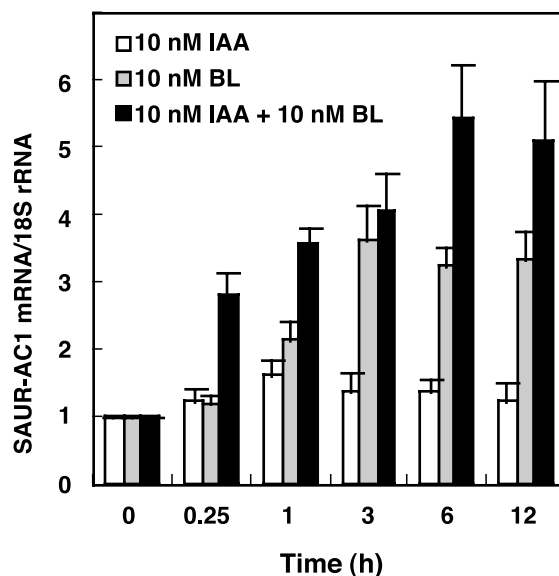


Fig. 2. Synergistic effect of BL with auxin on *SAUR-AC1* gene expression. Seven-day-old light-grown WT seedlings were treated with either 10 nM BL, 10 nM IAA, or 10 nM BL in combination with 10 nM IAA for the indicated time periods. Transcript levels are presented as values relative to those at 0 h, defined as 1, after normalization to the 18S ribosomal RNA levels. Data are means $\pm$ standard errors from three independent hormone treatment experiments.

We previously demonstrated that *SAUR-AC1* was induced by exogenous BL within 30 min in the BR-deficient *det2* mutant [8]. To confirm the effect of exogenous BL on *SAUR-AC1* expression, 7-day-old WT seedlings were treated with 10^{-8} M BL (Fig. 2). The induction kinetics were very similar to those of the *det2* mutant [8], except that the magnitude of the relative induction was less in WT than in the *det2* mutant. Transcript accumulation was apparent within 1 h after BL treatment, the induction was gradual, and the maximum induction level was approximately 3.5-fold the initial level between 6 and 12 h (Fig. 2).

We studied the synergistic effect of BL with IAA on *SAUR-AC1* gene expression. When seedlings were treated with 10^{-8} M BL in combination with 10^{-8} M IAA, *SAUR-AC1* induction was apparent within 15 min, and the maximum induction level was five-fold the initial level at 6 h treatment (Fig. 2). At the same time point, IAA or BL alone did not induce *SAUR-AC1* gene expression. These results indicated that BL and IAA synergistically induced *SAUR-AC1*. At present, we do not find synergism in physiological response, such as increase in fresh weight or hypocotyl elongation in Arabidopsis seedlings.

3.2. Involvement of *AXR1* on BL actions

Clouse et al. reported that, unlike auxin, 24-epibrassinolide (EBR) effectively inhibited root elongation in the *axr1* mutant [26]. On the other hand, Tiriyaki and Staswick recently reported that the EBR inhibition in *axr1* was different from that in WT [27]. Previous studies used continuous EBR treatments to investigate the inhibitory function of BRs on roots; the promoting function of BRs has not been studied in *axr1*. Here we studied the rapid promoting responses to BRs in whole seedlings and in aerial tissues using a transient BL treatment. We used a weak allele of the *axr1* mutant, *axr1-3* [17].

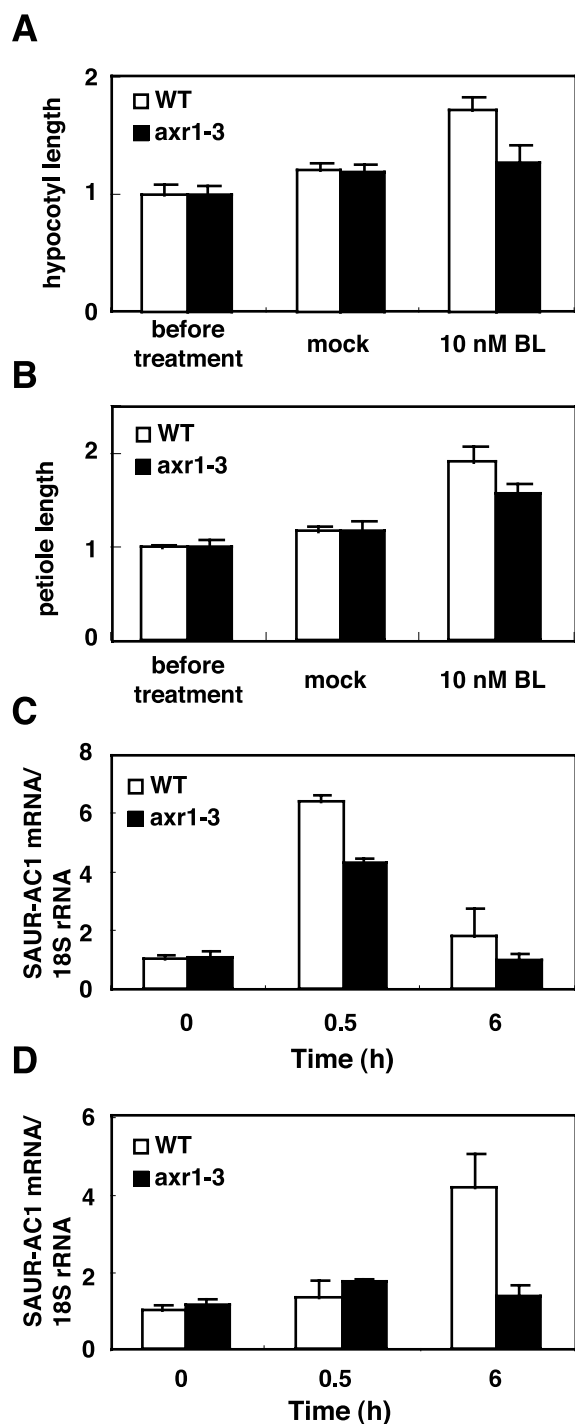


Fig. 3. The induction of the *SAUR-AC1* gene and the elongation of hypocotyls in the *axr1* mutant in response to BL. Seven-day-old WT and *axr1* seedlings were mock-treated or treated with 10 nM BL for 24 h, and the lengths of the hypocotyls (A) and petioles (B) were measured in 30 seedlings before or after treatments. Lengths are expressed relative to those before treatment. The initial lengths of petioles were 1.63 mm for WT and 1.11 mm for the *axr1*. The initial lengths of hypocotyls were 1.56 mm for WT and 1.05 mm for the *axr1*. Seven-day-old WT and *axr1* seedlings were treated with 1 μ M IAA (C) or 10 nM BL (D) for the indicated time periods. Transcript levels are presented as values relative to WT values at 0 h, defined as 1, after normalization to the 18S ribosomal RNA levels. Data are means $\pm$ standard errors from three independent hormone treatment experiments.

Seven-day-old WT or *axr1-3* seedlings were treated with 10 nM BL for 24 h, and the lengths of the hypocotyls and petioles were measured. BL promoted hypocotyl (Fig. 3A) and petiole (Fig. 3B) elongation in WT seedlings. Petiole and hypocotyl elongations in BL-treated seedlings were about 6- and 3.5-fold the elongations in mock-treated seedlings, respectively. Petiole and hypocotyl elongations in response to BL were inhibited in *axr1-3* seedlings.

SAUR-AC1 gene expression in the *axr1* mutant was analyzed in response to BL and IAA. Seven-day-old WT or *axr1-3* seedlings were treated with either 1 μ M IAA or 10 nM BL for specific time periods of the maximum induction (30 min for IAA induction and 6 h for BL induction), which had been determined based on precise dose- and time-dependent induction studies in WT and *det2* seedlings (as described above). Before hormone treatment, the level of *SAUR-AC1* in *axr1-3* seedlings was about the same as that in WT plants (Fig. 3C,D). With a 30-min IAA treatment, the *SAUR-AC1* transcript level in the *axr1* plants was about two-thirds that of the WT plants (Fig. 3C). The auxin-insensitivity of the *axr1* mutant was consistent with previous results [17]. On the other hand, with a 6-h BL treatment, the induction of *SAUR-AC1* gene expression was strongly inhibited in the *axr1* mutant (Fig. 3D). Therefore, the *axr1* mutation reduces induction of *SAUR-AC1* in response to both IAA and BL. The defect that inhibits BL-induced gene expression in *axr1* may be more severe than the defect that inhibits auxin-induced expression (Fig. 3C,D).

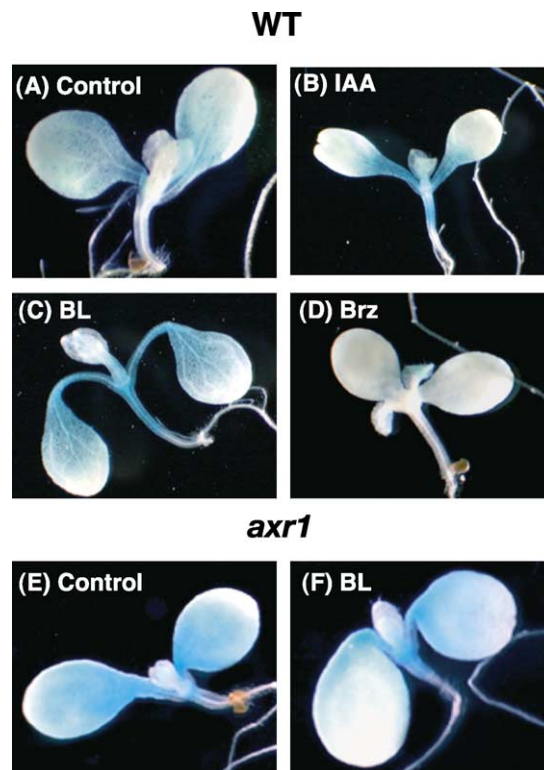


Fig. 4. Histochemical GUS activity in *SAUR-AC1* promoter-*GUS* transgenic Arabidopsis seedlings. Seven-day-old WT seedlings transgenic for a *SAUR-AC1* promoter-*GUS* gene were mock-treated (A), treated with 1 μ M IAA (B) or 10 nM BL (C) for 12 h, or treated with 3 μ M Brz (D) for 36 h. Seven-day-old *axr1-3* seedlings transgenic for a *SAUR-AC1* promoter-*GUS* gene were mock-treated (E) or treated with 10 nM BL (F) for 12 h.

3.3. Histochemical analysis of *SAUR-AC1* gene induction

To investigate the organ specificity of *SAUR-AC1* gene expression, especially in response to BRs, WT seedlings that were transgenic for a *SAUR-AC1* promoter-*GUS* fusion gene were treated with hormones for 12 h and then stained for GUS activity. Histochemical staining was performed for only 5 h to allow observation of early-staining regions. In control plants, GUS activity was observed in cotyledons and in petioles (Fig. 4A), but not in roots (data not shown). These results were consistent with previous reports [15,16]. When seedlings were treated with IAA, GUS activity was induced in the hypocotyl (Fig. 4B). When seedlings were treated with BL, petiole and hypocotyl elongations were promoted with concomitant GUS staining in these organs (Fig. 4C). This result suggested that BL regulates *SAUR-AC1* gene expression at the transcriptional level. To study the effect of endogenous BRs on *SAUR-AC1* gene expression, transgenic seedlings were treated with the specific BR biosynthesis inhibitor, brassinazole (Brz) 220 [28] for 36 h and were then stained for GUS activity. GUS staining was markedly reduced by the Brz treatment (Fig. 4D). The staining was recovered following BL treatment (data not shown).

Seven-day-old *axr1* seedlings transgenic for the *SAUR-AC1* promoter-*GUS* gene were mock-treated or treated with BL for 12 h and observed for GUS staining. The organ specificity of *SAUR-AC1* gene expression in mock-treated *axr1* seedlings was similar to that in WT (Fig. 4E). On the other hand, the BL-treated *axr1-3* seedlings showed insensitivity in induction of GUS staining (Fig. 4F, compare to Fig. 4C).

4. Discussion

In our previous report, the BL-induction kinetics of the *SAUR-AC1* gene were similar to those of genes involved in cell elongation and cell wall organization [8]. Here, we confirmed the induction kinetics in WT seedlings and found that the *SAUR* induction kinetics in WT are similar to those in *det2*. It is interesting to note that these induction kinetics are also similar to the BL-induction kinetics of *IAA3* [8], *IAA5* and *IAA19* genes [25]. The expression of the *SAUR-AC1* gene in the *det2* mutant (Fig. 1), as well as in response to Brz treatment (Fig. 4), strongly suggested that the *SAUR-AC1* gene is regulated not only by exogenous BL, but also by endogenous BRs. We have observed that endogenous IAA levels per gram fresh weight do not increase when Arabidopsis WT seedlings or BR-deficient *det2* seedlings were treated with BL [25]. Furthermore, there were fewer *SAUR-AC1* transcripts in the *det2* mutant seedlings than in the WT (Fig. 1), even though endogenous IAA levels per gram fresh weight were higher in the *det2* seedlings than in the WT [25]. These observations indicate that BRs regulate the expression of *SAUR-AC1* independently of auxin levels.

In the presence of a low concentration of IAA (10^{-8} M), which has little effect on *SAUR-AC1* gene expression, BL induced a quicker and greater *SAUR-AC1* expression than did BL alone (Fig. 2). The synergistic induction of the *SAUR* gene by BR with auxin was reported to occur with a longer treatment (2 h) in soybean [12]. The synergistic effects of BL with IAA on the *SAUR* expression suggest that BRs may regulate *SAUR* gene expression through independent pathways and not through auxin. The independent auxin and BR signaling pathways may cross-talk upstream of the

transcription of the *SAUR* gene, and this may account for the synergism of the two hormones. On the other hand, the organ specificity of BL-induced *SAUR-AC1* expression correlated well with the BL-induced elongating region (Fig. 4). BL inducibility was lost in the *axr1* mutant, which is less sensitive to BL-induced elongation. Based on a series of *SAUR-AC1* expression analyses, we propose that *SAUR-AC1* is important for BR-mediated elongation and can be used as a marker gene to study the interaction of BR and auxin signaling pathways.

The auxin insensitive mutant *axr1* has been used to study the auxin signaling system. Recent studies, however, reported that AXR1 is involved in multiple ubiquitin ligase-mediated processes in the photomorphogenic response [29] as well as in the jasmonate response [27]. Here we demonstrated that the *AXR1* gene is required for both BL-mediated elongation and BL-mediated *SAUR-AC1* induction (Figs. 3 and 4), suggesting that a ubiquitin ligase-mediated process is required for BR-mediated growth promotion. In the auxin signaling pathway, the AXR1-ECR1 heterodimer (E1) activates and conjugates the ubiquitin-related protein RUB1 to cullin subunit (At-CUL1) of SCF^{TIR1} (E3) ligase via the RUB1-conjugating enzyme (RCE1) (E2) [30,31]. Arabidopsis has another RCE1-like protein (RCE2) and four additional cullins (AtCul2-5) [31]. There are therefore open questions as to whether BR signaling involves SCF^{TIR1} or another E3 complex, AtCUL1 or another cullin subunit, or RCE1 or another E2. Recently, He et al. reported that BL treatment stabilizes a positive regulator of BR signaling, Bzr1, which is localized in nuclei and degraded by the proteasome [32]. The expression of the *SAUR-AC1* gene may be regulated positively by such a regulator that is stabilized in a BL-dependent manner. Recent studies revealed that gibberellin [33,34] and jasmonate [35] signaling pathways are also regulated by the E3 ubiquitin ligase.

In conclusion, BR-induced expression of *SAUR-AC1* gene correlated well with BR-induced elongation. The reduced BL sensitivity of *axr1* might be due to the deficiency in BR-mediated *SAUR-AC1* induction. We propose that the *AXR1* gene functions as a shared component of the BR and auxin signaling pathways and that a ubiquitin ligase-mediated system is involved in BR-mediated tissue elongation and *SAUR-AC1* gene expression.

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