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Brassinolide Influences the Regeneration of Adventitious Shoots from Cultured Leaf Discs of Tobacco

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When several concentrations of brassinolide (BL) were added to a shoot induction medium (SIM) that contained only BA, re-differentiation of adventitious shoots from tobacco leaf discs was unaffected at low BL levels (10^{-10}~10^{-8} M), but was inhibited at higher concentrations. In comparison, when BL was applied without BA, only cell expansion occurred and no shoots formed. The determination time for shoot formation was shortened at low BL concentrations, but their formation was postponed (i.e., time was lengthened) at higher concentrations. Elongation of shoots incubated for 30 d was unaffected at low BL concentrations, but was inhibited as that amount increased. *NTH1*, a tobacco homeobox gene that is expressed in the central zone of the tobacco shoot apex, showed greater expression levels in the SIM over time, and its expression was stronger in media treated with low concentrations of BL compared with the SIM control at the same time point. Expression of *NTH1* was postponed at higher BL concentrations. In conclusion, at low concentrations, brassinolide has no effect on shoot formation. However, it inhibits their formation at high concentrations when cytokinin is included in the media.

Keywords: Brassinosteroid, cytokinin, *NTH1*, shoot regeneration, tobacco

Organ differentiation is determined by the auxin to cytokinin ratio (Skoog and Miller, 1957). The former is involved in cell expansion and division, vascular differentiation, apical dominance, and root formation (Michalzuk et al., 1992). Through its interaction with auxin, cytokinin is involved in adventitious root and shoot formation (Miller et al., 1955), leaf senescence, vascular development, and lateral bud formation (Mok and Mok, 1994). The determination times associated with the process of shoot or root differentiation and related phenomena have been investigated by pretreating tissues with auxin and cytokinin, then transferring them to hormone-free media (Ramage and Williams, 2004; Christianson and Warnick, 1983). This 'time', defined as when organ formation is observed in plant segments, can differ among individuals within the same species (Ramage and Williams, 2004). In addition, no plant growth-regulating substance is needed after that determination occurs (Komamine et al., 1992).

Brassinosteroids (BRs), which have structures similar to those of animal steroid hormones, are widely distributed in the plant kingdom, where they regulate growth and development (Mandava, 1988). About 40 steroid compounds have been isolated since a brassinosteroid was first obtained from the pollen of *Brassica napus*, and since one of them, brassinolide (BL), was used for BR research (Grove et al., 1979). Dwarf mutants that are defective in BR biosynthesis have been isolated and characterized and their synthetic pathways have been described (reviewed by Choe, 2005). Such mutant plants are short and have reduced fertility; a prolonged life span; and leaves that are dark-green, curled, and round when grown in the light but which, under darkness, have an abnormal phenotype that is similar to photomorphogenetic mutants (Kwon and Choe, 2005). BRs are involved in cell division and expansion, vascular differentiation, root growth, and senescence (Clouse and Sasse, 1998). The effect of endogenous levels has been investigated by exogenously treating BR-deficient or -insensitive mutants with brassinosteroids (Fujioka and Yokota, 2003). BR functions in root growth (Müssig et al., 2003), stimulating lateral root initiation and development (Bao et al., 2004) and enhancing the curvature of corn roots when plants are treated with auxin (Kim et al., 2000). Both BR and gibberellins (GA) promote shoot elongation, but their cellular mechanisms differ (Gregory and Mandava, 1982). Therefore, BRs are considered to be plant hormones along with auxins, GA, and cytokinins (Goda et al., 2002). Although the effect of BR on light or stress responses has been reported, few findings have been published concerning its influence on the differentiation and development of shoots.

Interactions between BR and auxin have been studied intensively (Halliday, 2004; Choe, 2007). The synergistic response in ethylene biosynthesis that is induced by BR first and auxin later has been shown in mung bean seedlings (Arteca et al., 1988). Brassinosteroids induce many auxin-regulated genes (Goda et al., 2002, 2004), and both hormones have interplay in their transcriptome and biosynthesis (Halliday, 2004). However, little research has been conducted on the relationship between BRs and cytokinins.

The concentration of plant hormones required for organ differentiation differs among species. For example, a high level of cytokinin and low concentration of auxin are needed in the shoot induction medium (SIM) for shoot re-

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[†]Abbreviations: BA, Benzyl adenine; BL, Brassinolide; BRs, Brassinosteroids; *NTH1*, *Nicotiana tabacum* homeobox 1; SAM, Shoot apical meristem; SIM, Shoot induction medium

differentiation (Skoog and Miller, 1957). Because no auxin is necessary for that process in tobacco leaf segments, and because re-differentiation can be induced by BA alone, this system has an advantage in that it can be used to investigate the effects of BRs on shoot re-differentiation.

To examine the role of BR on adventitious shoot formation in tobacco at the molecular level, one must use molecular markers that are involved in shoot bud differentiation. A tobacco homeobox gene, *NTH1* (*Nicotiana tabacum* homeobox 1), that belongs to the Class I knotted 1-type (kn1-type) family is expressed in the shoot apical meristem (SAM). As with animals, such genes in plants may be involved in meristem maintenance and lateral organ formation from the SAM (Jackson et al., 1994; Kerstetter et al., 1994; Nishimura et al., 1999; Sentoku et al., 1999; Morimoto et al., 2005).

NTH1 is up-regulated in the central zone and down-regulated in the peripheral zone of the shoot apex (Nishimura et al., 1999). This expression pattern coincides with that of *NTH15*. NTH9 is expressed in shoots and floral buds while NTH20 is strongly expressed in the peripheral zone (Nishimura et al., 1999). Therefore, *NTH1* is the most appropriate gene with which to analyze the effects of BRs because the rate of shoot bud formation from tobacco leaf segments is proportional to the change in gene expression. Here, we studied how epibrassinolide (epi-BL) affects shoot formation, determination time, and the expression pattern of *NTH1* in tobacco leaf segments.

MATERIALS AND METHODS

Plant Material and Media

Tobacco (*Nicotiana tabacum* L. cv. NT1) seeds were sterilized in a 0.4% (w/v) calcium hyperchloride solution for 15 min, then washed three times (2 min each) with sterile deionized water. Afterward, they were sown in standard Murashige and Skoog (MS) media and incubated for 5 weeks in a growth chamber (16-h photoperiod, 25°C).

Leaf discs (0.8 cm diam.) were used for all experiments. Samples for RNA extraction were harvested in liquid nitrogen and stored at −70°C. The basal medium was MS. Our shoot induction medium (SIM) contained 4.5 μM BA with or without 10^{-10}~10^{-6} M BL. The auxin and cytokinin sources were IAA and BA, respectively. Each experiment was repeated three times.

Analysis of Determination Time for Shoot Regeneration with BA and BL

We investigated the timing of adventitious shoot formation according to the method described by Christianson and Warnick (1983, 1984). To analyze the effect of BL in detail, we observed the determination time and conducted dose-effect experiments using MS media containing 4.5 μM BA, with or without 10^{-10}~10^{-6} M BL. Leaf discs were treated with BA and BR for 5, 6, 7, 8, 9, 10, or 12 d before being transferred to basal MS media. These discs were incubated for 15 d under continuous light at 25°C, and the number of re-differentiated discs or shoot buds was counted to establish the determination time.

Effect of BL on Shoot Elongation from Shoot Buds

To monitor the effect of BR on shoot elongation after the formation of buds, tobacco leaf discs were embedded in SIM and incubated for 12 d under continuous light at 25°C. After the shoots formed, they were transferred to media containing 10^{-10}~10^{-6} M BL and incubated for 18 d. The optimum concentration of BL was determined according to its effect on shoot elongation.

RNA Extraction and Semi-quantitative RT-PCR Analysis

Total RNA was isolated using a BLUE™ total RNA extraction kit (Intron, Republic of Korea). First-strand synthesis was performed for 1 h at 42°C, with 2 μg of total RNA, 1 μL of oligo(dT)$_{15}$ primer (2.5 pmoles μL^{-1}), and 0.5 μL of 10 U μL^{-1} AMV reverse transcriptase (Intron). One microliter of the reverse transcript was used for subsequent amplification. PCR was performed in a reaction mixture containing 1 μL of the reverse transcript reaction, 0.5 μL of Taq polymerase (2.5 U μL^{-1}; Intron), 1 μL of 10 mM dNTP mixture, 3 μL of 25 mM $MgCl_2$, 5 μL of Taq polymerase 10 X buffer (Intron), and 1 μL of 10 pmoles of each primer in a 50 μL reaction. Standard PCR conditions were used (1 cycle of 94°C for 5 min; then 32 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min; with a final extension at 72°C for 1 min). Ten μL was used for agarose gel electrophoresis. Primers for *NTH1* included: forward, 5′-ATGATGGATGAATTGAGCAAA-3′; and reverse, 5′-TTCAAGTGCCAATACTTCCA-3′. The EF-1á gene was used for normalization (forward primer, 5′-TCACATCAACATTGTGGTCATTGGC-3′; reverse primer, 5′-TTGATCTGGTCAAGAGCCTCAAG -3′) as an internal control during the assays.

RESULTS

Effect of BA and BL on Shoot Regeneration

To determine the effective concentrations of BA and BL for shoot regeneration in tobacco, we used discs punched from leaves at 5 weeks after germination. Here, 4.5×10^{-6} M BA in the MS media was the most suitable level, resulting in the regeneration of an average of 20 adventitious shoots from 20 discs (Fig. 1A). No shoots formed upon treatment with 10^{-10}~10^{-6} M BL alone. However, due to cell expansion, the leaf discs became enlarged when the BL concentration was increased (Fig. 2F, H). Based on these results, we used a standard MS supplemented with 4.5×10^{-6} M BA as our SIM in subsequent experiments. When 10^{-10}~10^{-9} M BL was included in the SIM, all 20 leaf discs were re-differentiated (Fig. 1B). However, as that concentration rose, the rate of re-differentiation declined. Treatment with 10^{-8}, 10^{-7}, or 10^{-6} M BL in the SIM produced rates of 90%, 75%, or 50% re-differentiation, respectively, compared with the control. In that control SIM, approximately 3.7 shoot buds were formed from each disc; treatment with 10^{-10} M BL increased that count by about 10%. However, at 10^{-9} M BL, the number of buds formed was the same as that of the control. At 10^{-8}, 10^{-7}, or 10^{-6} M BL, the number of shoot buds was decreased by about 9%, 27%, or 53%, respectively (Fig. 1C). Reductions in re-differentiation rates and the

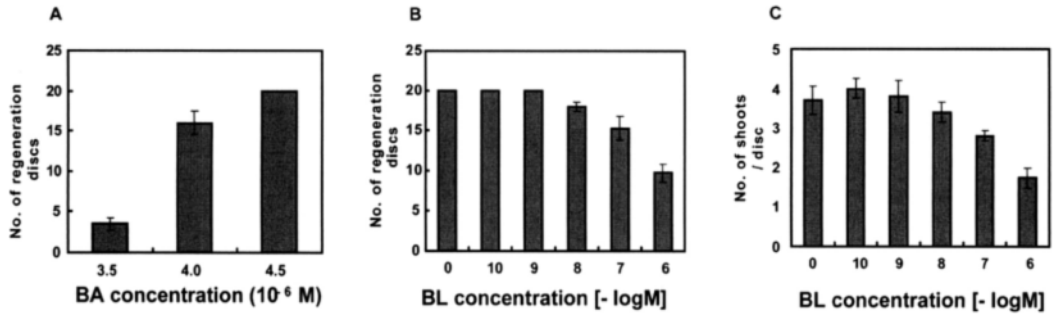

Figure 1. A, Effect of BA on adventitious shoot regeneration from tobacco leaf discs exposed to light for 15 d. **B**, Regenerating effect of BL on discs in SIM. **C**, Effect of BL on adventitious shoot regeneration. Experiments were repeated 3 times, each experiment consisting of 20 replicates. Vertical bars indicate standard errors.

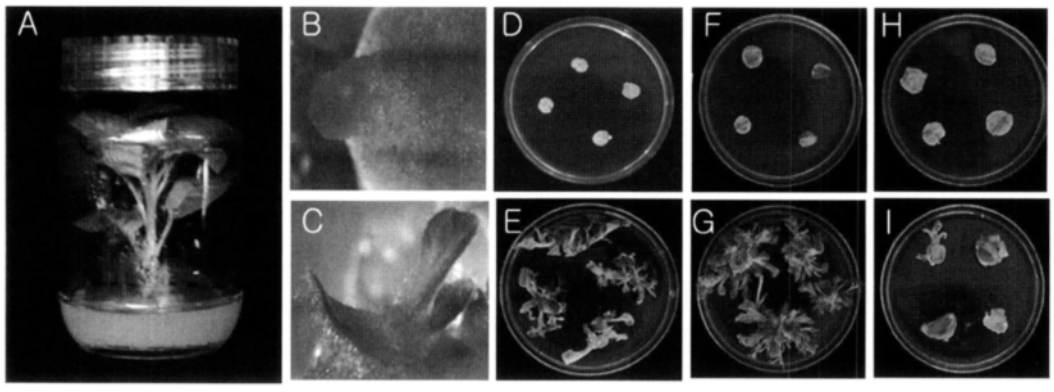

Figure 2. Differentiation patterns of adventitious shoots from tobacco leaf discs treated with 10^{-10}~10^{-6} M BL for Thompson. **A**, Tobacco was incubated for 30 d on SIM. **B**, Shoot buds were formed during incubation for 6 d on SIM. **C**, Adventitious shoot arose after incubation for 12 d. **D-I**, Performance on MS medium alone (**D**), SIM (**E**), MS+10^{-10} M BL (**F**), SIM + 10^{-10} M BL (**G**), MS + 10^{-6} M BL (**H**), or SIM + 10^{-6} M BL (**I**).

number of shoots per disc were significant when treatment was above 10^{-7} BL (Fig. 1B, C).

Analysis of Determination Time for Shoot Regeneration on BA- and BL-containing Media

To examine the effect of BL in detail, we identified the determination time for organ formation, a phenomenon that depended on treatment duration as well as the number of shoots that formed when we used SIM with or without 10^{-10} M to 10^{-6} M BL. Shoot re-differentiation and shoot bud formation started at Day 6 and increased until Day 12. After that point, buds began to develop into shoots (Fig. 3B).

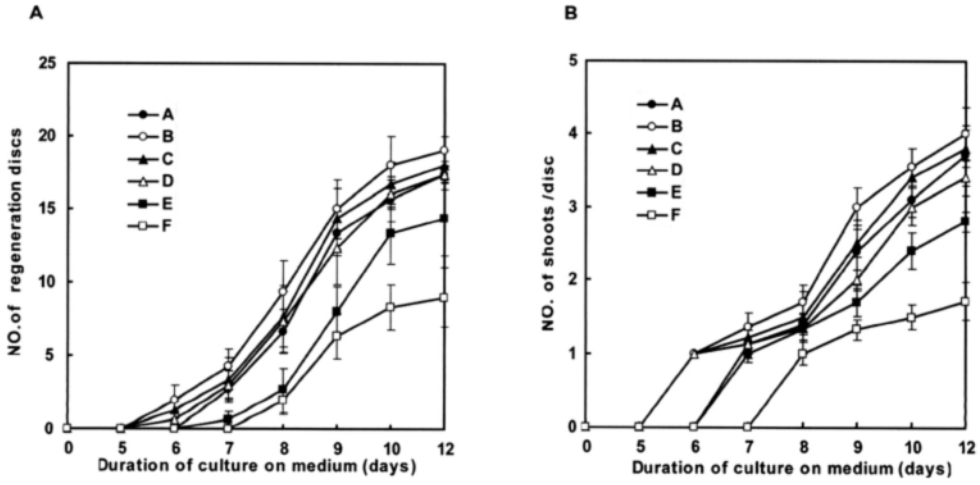

Figure 3. Analysis of determination time for adventitious shoot regeneration. Tobacco leaf discs were cultivated in SIM with 10^{-10}~10^{-6} M BL for 5, 6, 7, 8, 9, 10, or 12 d before being transferred to basal media (BM). Afterward, culturing periods totaled 15 d. **A**, Regenerating effect of BL. **B**, Effect of BL on adventitious shoot regeneration. Experiments were repeated three times, each experiment consisting of 20 replicates. Vertical bars indicate standard errors. [A, SIM; B, SIM+10^{-10} M BL; C, SIM+10^{-9} M BL; D, SIM+10^{-8} M BL; E, SIM+10^{-7} M BL; and F, SIM+10^{-6} M BL.]

The time at which 16 shoots had re-differentiated was also determined, being about 10 d in the control SIM and about 9.4 d in the SIM with 10^{-10} M BL. However, the time for shoot formation decreased as the BL concentration increased; with 10^{-7} M BL, that time lengthened to about 10.5 d (Fig. 3A). The process of shoot-bud formation also was postponed. In a separate examination, we recorded the time at which three shoot buds had formed: 9.6 d in the control vs. 9.0 d in media containing 10^{-10} M BL. This time lengthened as the BL concentration increased (Fig. 3B). At low concentrations, shoot re-differentiation and shoot-bud formation were not much affected, and the time required for those processes was not shortened very much (Fig. 3). However, at higher concentrations, re-differentiation and bud formation were decreased and the time for both was lengthened (Fig. 3).

Effect of BL on Shoot Elongation after Shoot Bud Formation

To monitor the effect of BL on growth after shoot buds had formed, we included BL (10^{-10}~10^{-6} M) in the SIM in which adventitious shoots developed. Leaf discs that were loaded on the SIM for 12 d produced adventitious shoots after they were transferred to the MS media (Fig. 3A). Buds appeared to be most active when discs were incubated on SIM for 12 d (Fig. 3B). We also studied how BL affected the elongation of shoot buds formed on leaf discs that had been incubated on SIM for 12 d, and subsequently incubated those buds on BL-containing media for 18 d (Fig. 4). Compared with their performance on the control medium, shoots did not elongate much more on media supplied with 10^{-10} M BL. As that concentration increased, the tendency to elongate was diminished, and an approximately 50% decline in elongation was observed at 10^{-6} M BL.

Expression Level of the *NTH1* Gene after BL Treatment

We evaluated the expression of *NTH1* (*Nicotiana tabacum* homeobox 1) at the time point when adventitious shoots were formed. This gene was expressed in the shoot apical meristem when adventitious shoots re-differentiated. When 10^{-10} to 10^{-6} M BL alone was used for treatment, no expression occurred (data not shown). This result was comparable to our finding that no organs formed in media containing just BL (Fig. 2). Expression increased after 8 d in SIM without any BL (Fig. 5), which implied that *NTH1* expression is induced by the cytokinin included in the SIM. When 10^{-10} M BL was added, expression also was detected at 8 d, and transcript levels were higher than those measured in SIM that lacked BL (cf., Fig. 5A and Fig. 5B). In SIM with 10^{-6} M BL, expression was noted after 10 d, which was 2 d later compared with its expression in SIM with or without 10^{-10} M BL (Fig. 5C). This expression pattern was comparable to our results shown in Figures 1 and 3.

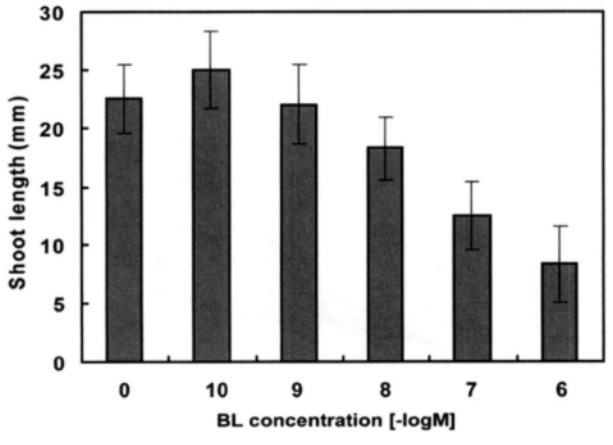

Figure 4. Effect of BL during adventitious shoot elongation. Tobacco leaf discs were cultured for 12 d in SIM with 10^{-10}~10^{-6} M BL, then transferred to media supplemented with various hormones and inhibitors for 18 d of culturing. Experiments were repeated three times, each experiment consisting of 20 replicates. Vertical bars indicate standard errors.

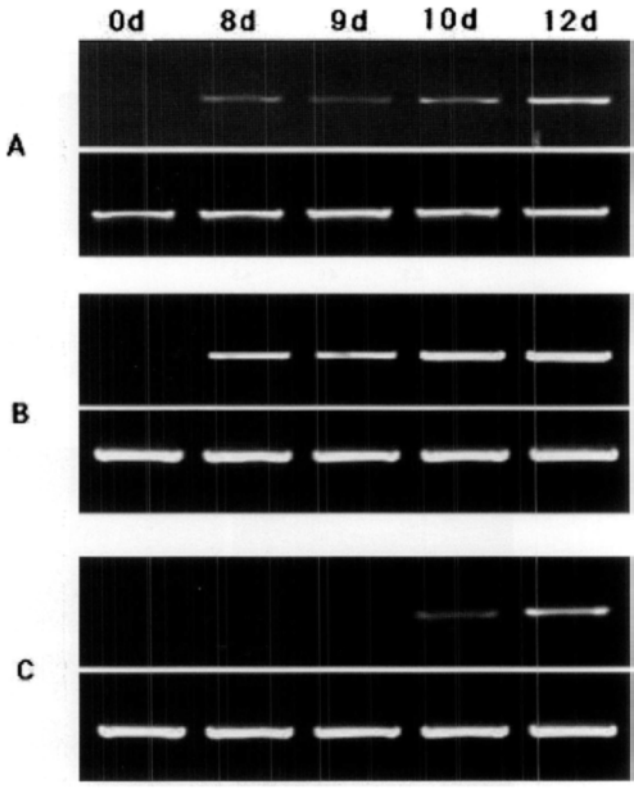

Figure 5. Role of *NTH1* expression in formation of adventitious shoots from tobacco leaf explants. Total RNA was prepared with combination of MS + 4.5×10^{-6} M BA and 10^{-10}~10^{-6} M BL. Explants were cultured on media for 8, 9, 10, or 12 days (indicated by 'd'). EF1-α was used to show relative quantity of cDNA in each lane (lower part). **A**, MS+BA alone; **B**, MS+BA+10^{-10} M BL; **C**, MS+ BA +10^{-6} M BL.

DISCUSSION

Different plant hormones influence growth and development via cross-talk. In spite of their various known effects on a wide range of species, few reports have been published on the role of brassinosteroids (BRs) in shoot formation. Therefore, we aimed to characterize those effects at the tissue-culture and molecular levels.

Because shoot re-differentiation can be induced without auxin, we investigated how BL influences shoot formation

by adding BL to shoot induction media containing just BA (as a hormonal source). At a certain range of BL concentrations (10^{-10}~10^{-9} M), the re-differentiation rate for shoot buds was either unchanged or only slightly increased. However, that rate decreased at concentrations higher than 10^{-8} M (Fig. 1B, C). When this result was considered alone, brassinolide seemed to influence shoot re-differentiation. However, when BL was applied without BA, re-differentiation did not occur, and only cell expansion was observed (Fig. 2F, H). Similar expansion has been reported with auxin treatment (Jones et al., 1988). Adventitious roots can be formed in response to certain concentrations of auxin, although here we observed no organ differentiation, except cell expansion, when leaf discs were treated with BL alone. Even though the process of organ differentiation is too complicated simply to reveal the characteristic functioning of BR, we conclude that treatment with BL alone is related to cell expansion of the leaf disc, and that BL and BA together are involved in organ differentiation.

Interestingly, low concentrations of BL shortened the time for shoot re-differentiation and formation, while the time required was lengthened at high concentrations (Fig. 3). Shoot buds were formed after 6 d in any SIM with BL, except when BL was applied alone. However, these treatments differed in their times of shoot re-differentiation. For example, shoots formed 1 d earlier (i.e., on Day 5) in SIM containing 10^{-10} M BL compared with SIM that was supplemented with BA but not BL. In SIM enhanced with 10^{-10} M BL, a 1-day delay was observed (i.e., shoots formed on Day 7) compared with the SIM control.

The determination time was 12 d for the elongation of shoots incubated on SIM after they were treated for 18 d with different BL concentrations. We monitored shoot elongation for a total of 30 d. Little growth was stimulated at low BL concentrations, and was inhibited at higher concentrations (Fig. 4). This result was comparable to that for shoot formation. Sasaki (2002) has shown that brassinosteroids make more cells responsive, i.e., shifting their sensitivity to cytokinin for shoot regeneration. We demonstrated here that cytokinin also increased their sensitivity to BR.

We also investigated the effect of BL at the molecular level. Because *NTH1* is expressed within the central zone of the shoot bud (Nishimura et al., 1999), we performed RT-PCR to check its transcript levels each day after the shoot was formed. When BA was applied alone, *NTH1* expression was elevated (Fig. 5A); at 10^{-10} M BL, expression was increased after 8 d of treatment, to higher levels compared with the SIM control (Fig. 5B). At 10^{-6} M BL, *NTH1* expression appeared after 10 d, which was later than that from either BA treatment (SIM) or 10^{-10} M BL (Fig. 5C). This expression pattern was comparable to the results obtained from our examination of shoot re-differentiation, i.e., both determination time and the induction time for *NTH1* expression were delayed as the BL concentration increased.

Brassinosteroids enhance the expression of endogenous cytokinins (Gaudinova et al., 1995). In addition, cytokinin increases the transcription level of a *cpd* gene during trachea development in *Zinnia* (Yammamoto et al., 2007). Therefore, a mutual interaction may exist through the regulation of these hormonal levels. However, cytokinin itself is indispensable in terms of the shoot re-differentiation seen here. This implies that sensitivity to BR is dependent on the level of cytokinin in the tissue.

In conclusion, the time required for shoot-budding is shortened and shoot growth is improved by low BL concentrations, whereas such growth is inhibited at higher concentrations. Treatment with BL alone stimulates the expansion of leaf discs but has no effect on any organ formation or *NTH1* expression. However, transcript levels increase when both hormones are utilized, with expression being dependent upon BR concentration. This phenomenon is correlated with the shoot regeneration process. To further understand the mechanism for this stimulatory role of BR on shoot formation, it would be necessary to apply other plant hormones or light simultaneously with BR, then investigate their effects.

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