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ORIGINAL RESEARCH

Effects of Brassinolide with Naphthalene Acetic Acid on the Formation of Adventitious Roots, Trichome-Like Roots and Calli from Cultured Tobacco Leaf Segments, and the Expression Patterns of *CNT103*

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Abstract We treated cultured tobacco leaf segments with brassinolide (BL) and naphthalene acetic acid (NAA) and determined that optimum concentrations of NAA for adventitious root, trichome-like root, and calli formation were, respectively, 10^{-6}, 10^{-5}, and 10^{-4}M. In the adventitious root formation group, the number and length of adventitious roots were increased at lower concentrations of BL; however, they became trichome-like roots at higher levels of BL. The trichome-like root formation group showed better development when a low concentration of BL was added. However, at higher concentrations of BL, trichome-like root production was reduced, forming calli instead. In the calli formation group, more calli were formed at low BL concentrations and after persistent exposure to BL regardless of BL concentration, and the size of the leaf segments increased. The *CNT103* gene, which is expressed at the root tips showed increased levels of expression at BL concentrations up to 10^{-9}M and decreased levels of expression at BL concentrations over 10^{-9}M in the adventitious roots, trichome-like roots, and calli formation groups.

Keywords Tobacco · Adventitious root · NAA · Brassinolide · *CNT103*

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Since Brassinosteroids (BRs) were found in the organic solvent extract of pollen from *Brassica napus* (Mitchell et al. 1970; Grove et al. 1979), it has been shown that BRs can be isolated from many dicots, monocots, and some green algae (Yopp et al. 1981). BRs stimulate the expression of a diversity of genes (Müssig et al. 2002) and hence have multiple activities such as lamina inclination, leaf epinasty, pollen tube elongation, flowering (Schlagnhaufer & Arteca 1991), root elongation (Sasse 1994), vascular formation, delay of abscission, and stress resistance (Mandava 1988). In addition, BRs interact with auxin in many cases (Mandava 1988).

Exposure of intact plants or segments to BRs with auxin led to increased leaf blade flection (Yopp et al. 1981), geotropism (Meudt 1987; Kim et al. 2000), root elongation (Roddick and Guan 1991; Clouse 1996; Müssig et al. 2003; Mouchel et al. 2006), and emergence of lateral roots (Bao et al. 2004). These results indicate that BRs activate auxin (Mandava 1988; Sasse 1999), and it has been assumed that BRs plus auxins can induce expression of many auxin-regulated genes (Goda et al. 2002, 2004).

Auxins promote root elongation at low concentrations and inhibit at high concentrations (Burström 1969; Evans et al. 1994). However, at the proper concentration, auxins induce lateral root formation by stimulating division of pericycle cells (Charlton et al. 1996); that is, endogenous auxin, which is produced in the shoot apex, translocates to the root tip, which enhances main root elongation. Then, extra auxin moves upward via the PIN protein (Benjamins et al. 2005) to the root cortex (Blakeslee et al. 2005), and accumulated auxin induces dedifferentiation of pericycle cells, which emerge as lateral roots (Casimiro et al. 2001). Thus, extra auxin in apical roots accumulates and causes pericycle cells to develop lateral roots in common root, but

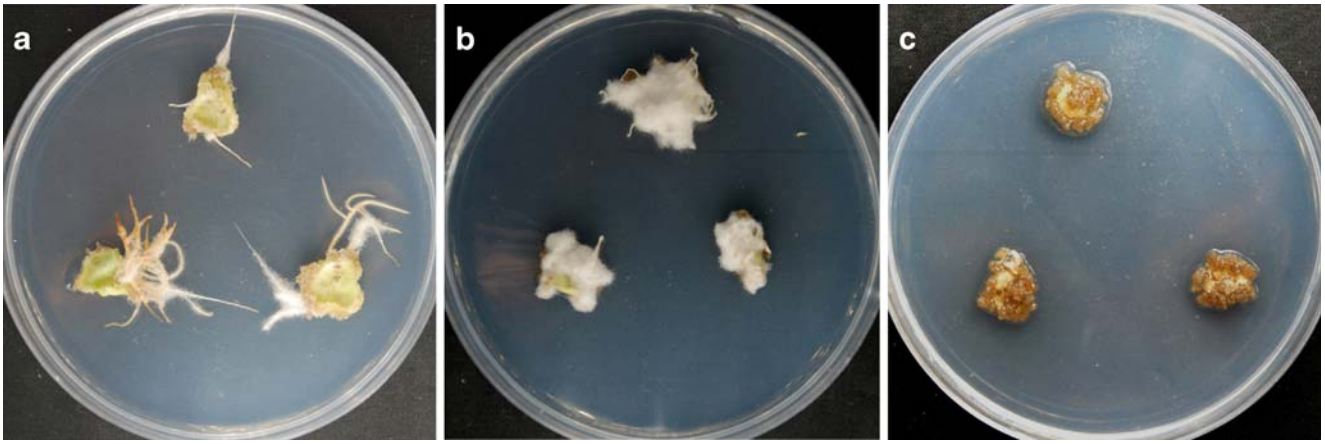

Fig. 1 Effect of NAA on the formation of adventitious roots, trichome-like roots, and calli from leaf segments of tobacco on MS agar media for 4 weeks. **a** Adventitious roots (10^{-6}M NAA); **b** trichome-like roots (10^{-5}M NAA); **c** calli (10^{-4}M NAA)

accumulated auxin in cuttings develops adventitious roots due to callose induction in the sieve plate of wounded sieve tube (Gus'kove et al. 1985; Liu and Reid 1992). Adventitious roots are formed directly from parenchyma cells by dedifferentiation (Blackesley et al. 1991) or via calli (Lovell and White 1986; Hartmann et al. 1990).

In general, the development of adventitious roots from cultured plant segments proceeds by similar mechanisms leading to the emergence of adventitious roots from cuttings; auxin plays a central role in inducing adventitious roots (Han et al. 1999). Even though studies of the effects of BRs on plant organ differentiation have accomplished little, there are

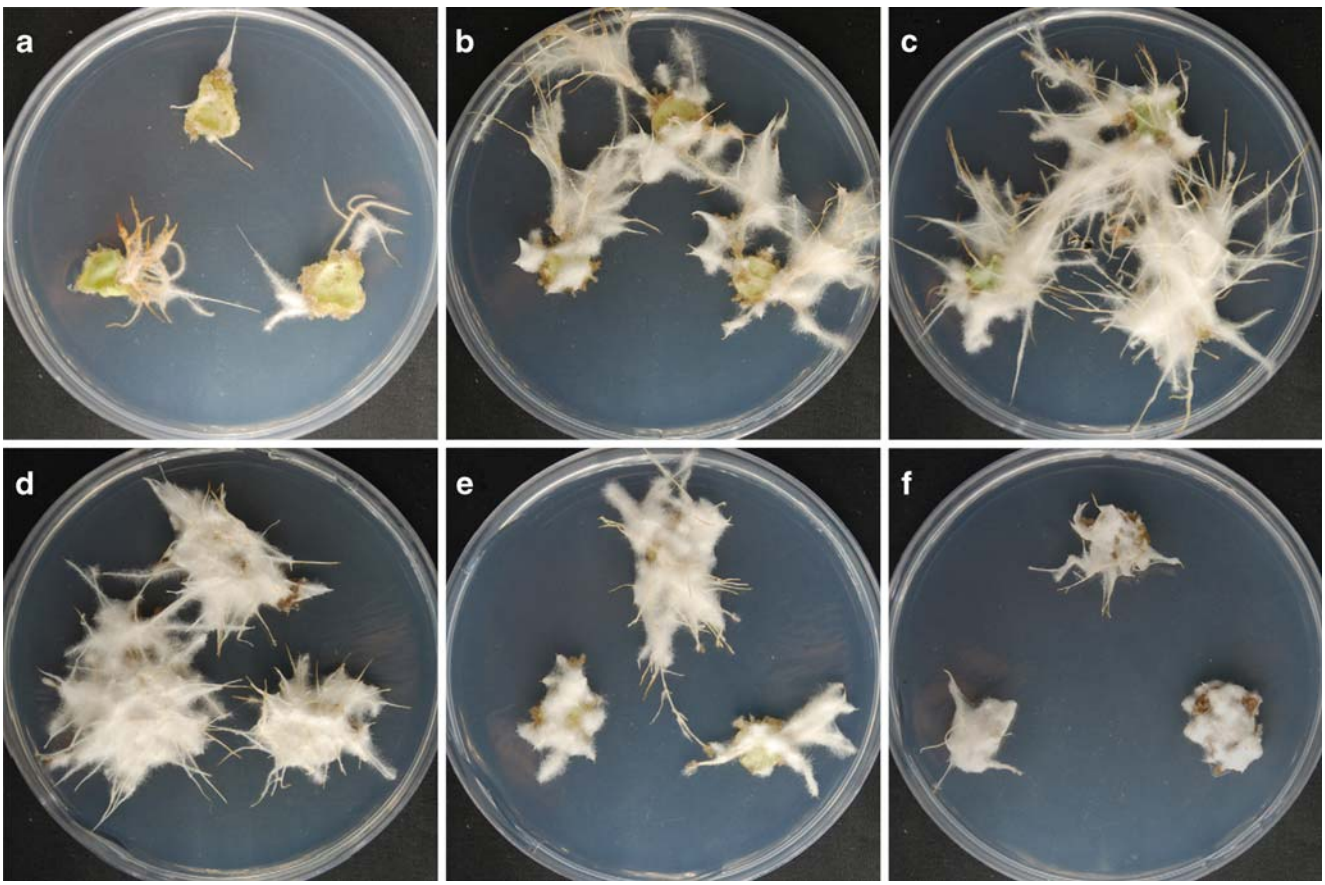

Fig. 2 Effect of BL on tobacco leaf segments cultured in adventitious root formation medium (10^{-6}M NAA) for 4 weeks. **a** control; **b** 10^{-10}M BL; **c** 10^{-9}M BL; **d** 10^{-8}M BL; **e** 10^{-7}M BL; **f** 10^{-6}M BL

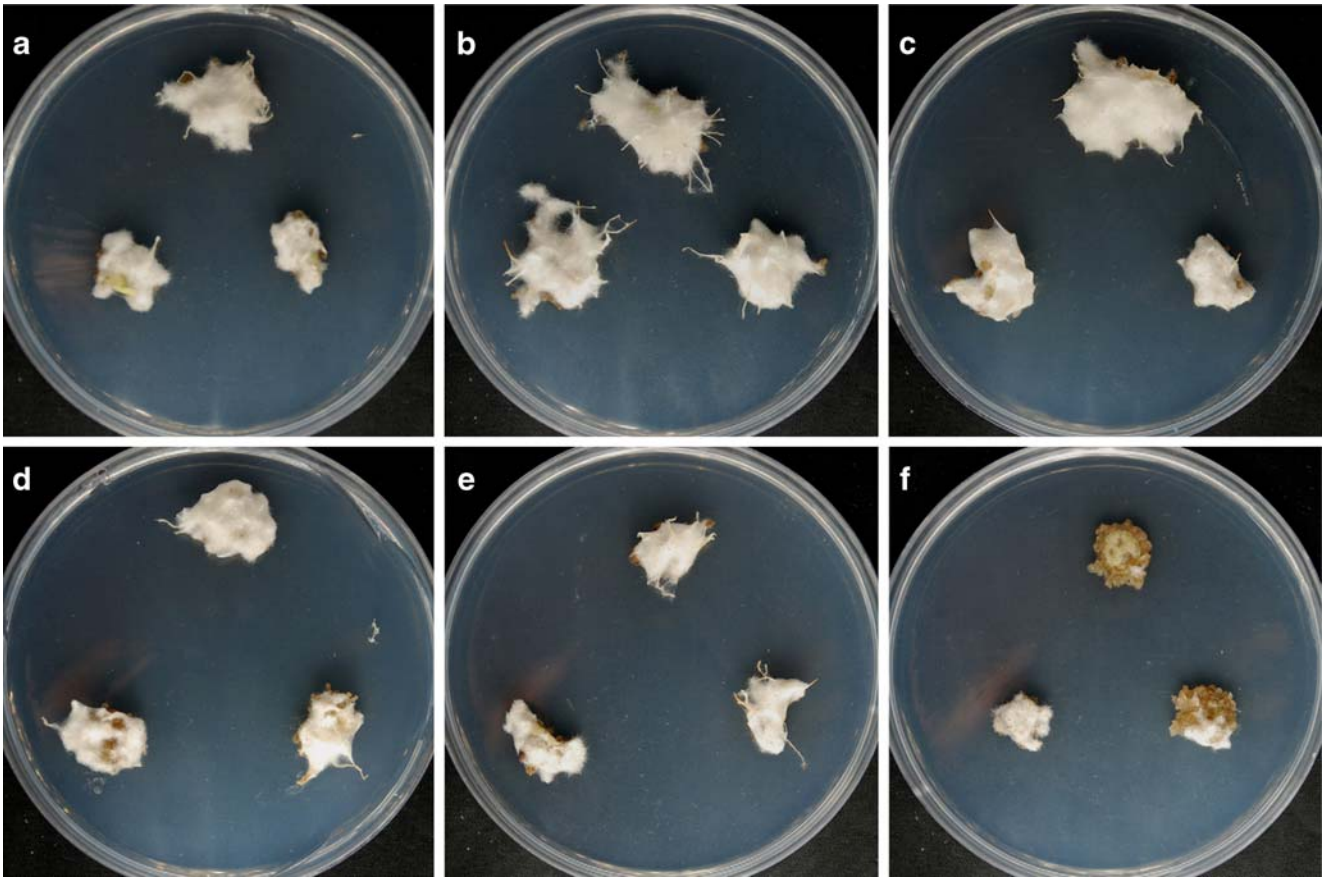

Fig. 3 Effect of BL on tobacco leaf segments cultured in trichome-like root medium (10^{-5}M NAA) for 4 weeks. **a** Control; **b** 10^{-10}M BL; **c** 10^{-9}M BL; **d** 10^{-8}M BL; **e** 10^{-7}M BL; **f** 10^{-6}M BL

a few reports that brassinolides (BL) have influences on shoot formation with cytokinin in tobacco leaf segments (Kim et al. 2008; Song et al. 2009), and auxins affect the emergence of lateral roots (Charlton et al. 1996) and adventitious roots (Jeon et al. 1998). Thus, BRs have effects on adventitious roots, trichome-like roots, and calli formation as well as increasing auxin levels (Kim and Han 2003).

Even though BRs stimulate expression of genes that are involved in plant cell elongation and cell division (Goda et al. 2002; 2004; Mouchel et al. 2006; Song et al. 2009), molecular biological studies of plant organ differentiation at the molecular level have not been fully understood. The *CNT103* gene, which is a tobacco root-tip-specific gene, showed temporary expression in G1 phase during cell division in root apical meristem (van der Zaal et al. 1991). Therefore, we surmise that expression of the *CNT103* gene is needed for the interaction between auxins and BRs in adventitious roots, trichome-like roots, and calli formations because this gene is induced by auxins and is related to cell division in the root tip.

In this study, tobacco leaf segments that can form adventitious roots, trichome-like roots, and calli were used to investigate, through morphological evidence, the effects of BL plus NAA at each phase of differentiation. We also sought to determine *CNT103* gene expression patterns in order to determine BR's effects when given with increasing auxin concentrations.

Material and Methods

Plant Materials and Media

Tobacco (*Nicotiana tabaccum* L. cv. NT1) seeds were sterilized in 0.4% (*w*/*v*) calcium hypochloride solution for 15 min and washed three times with distilled water. Afterward, seeds were sown on standard Murashige and Skoog (MS) medium and incubated for 5 weeks in a growth chamber under long day conditions (16 h light/8 h dark, and 25°C).

All experiments were done using tobacco leaf discs (0.7 cm diameter) for the 5 weeks growing period. Samples for RNA extraction were harvested in liquid nitrogen and stored at −70°C.

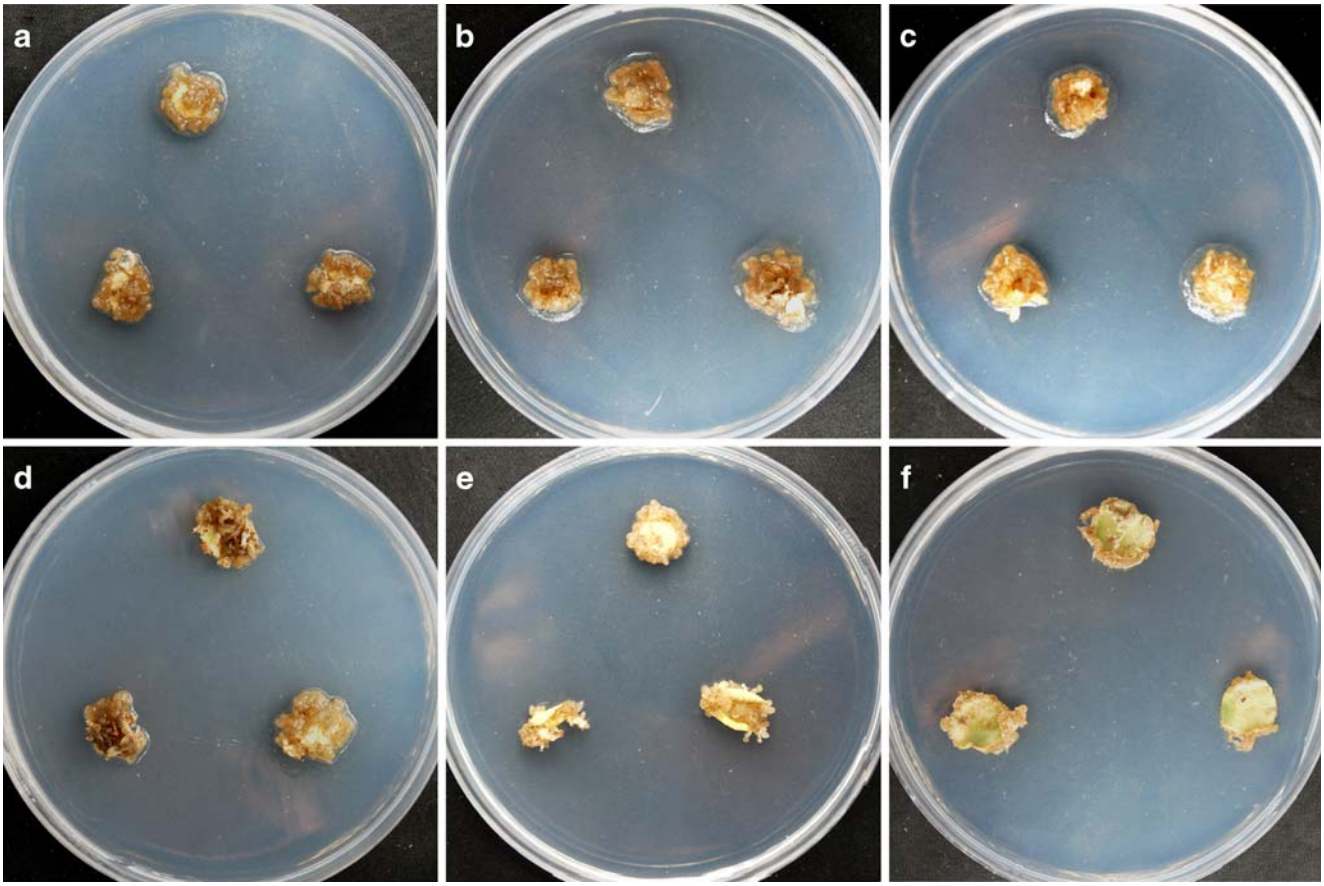

Fig. 4 Effect of BL on tobacco leaf segments cultured in calli medium (10^{-4}M NAA) for 4 weeks. **a** Control; **b** 10^{-10}M BL; **c** 10^{-9}M BL; **d** 10^{-8}M BL; **e** 10^{-7}M BL; **f** 10^{-6}M BL

Effects of NAA plus BL on Adventitious Roots, Trichome-Like Roots and Calli Formation

We investigated the effects of different NAA concentrations on adventitious roots, trichome-like roots, and calli formation from tobacco leaf discs. To analyze the effect of BL in detail, we used MS media containing 10^{-7} to 10^{-3}M NAA, with or without 10^{-10} to 10^{-6}M brassinolide (BL). We also observed leaf discs incubated for 4 weeks under continuous darkness and at 25°C. Each experiment was repeated three times.

RNA Extraction and PCR

Total RNA was isolated essentially as described by (Jurgen et al. 1987). cDNA synthesis was done for 1 h at 42°C in a water bath, with 10 μg of total RNA, 1 μL of oligo$(dT)_{15}$ primer (2.5 pmol μL^{-1}), and 0.5 μL of 10 U L^{-1} AMV reverse transcriptase (Intron).

PCR was done in a reaction mixture containing 1 μL of the reverse transcript reaction, 0.2 μL of Taq polymerase (1 U μL^{-1}; Intron), 2 μL of 10 mM dNTP mixtures, 2 μL of Taq polymerase 10× buffer (Intron), and 1 μL of each primer in a 20-μL reaction. The following PCR conditions were used: one 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 5 min. Primers for *CNT103* included a forward primer, 5′-ATGGCAGAAGTGAAGTTGCT-3′, and a reverse primer, 5′-TTTGGGAGCGGAAAT-AGAAG-3′. PCR products were extracted using a gel extraction kit (NucleoGen, Korea) and agarose gel electrophoresis and then transformed into the *Escherichia coli* DH5αstrain by the pGEM®-T easy vector (Promega, USA), which inserted the cDNA.

Northern Blot Analysis

Northern blot hybridization was performed according to the method of Sambrook et al. (1989). RNA (15μg) was separated on a 1% formaldehyde agarose gel [0.4 g agarose, 34.8 mL diethyl pyrocarbonate (DEPC)-treated water, 1.2 mL 37% formaldehyde, 4 mL 10× MOPS buffer (pH 7, 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA)] in 1× MOPS buffer and transferred to a positively charged nylon transfer membrane, Hybond-N^+ (Amersham Pharmacia Biotech). Hybridization was performed using 32P-labeled cDNA as a probe.

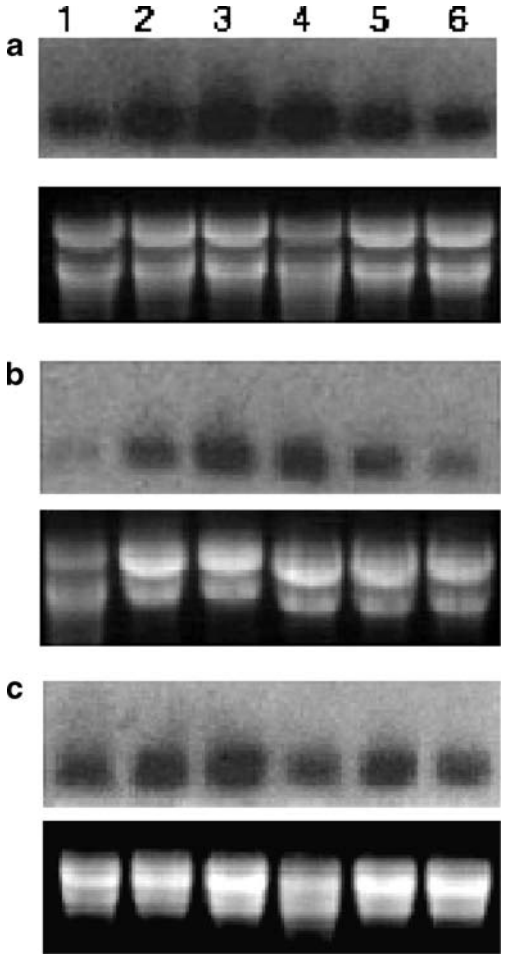

Fig. 5 Northern blot analysis using of tobacco leaf segments treated with NAA and BL for 4 weeks. The blot was hybridized to the P^{32}-labeled cDNA probes prepared by random priming of tobacco leaf clone *CNT103* insert DNA. **a** Adventitious roots; **b** trichome-like roots; **c** calli. *Line 1* Control, *line 2* BL 10^{-10}, *line 3* BL 10^{-9}; *line 4* BL 10^{-8}; *line 5* BL 10^{-7}; *line 6* BL 10^{-6}

Results

Effects of NAA on Adventitious Roots, Trichome-Like Roots and Calli

To induce the growth of adventitious roots, trichome-like roots, and calli formation, tobacco leaf segments were cultured in MS medium with 10^{-7} to 10^{-3}M NAA in the dark at 25°C for 4 weeks. The formation of adventitious roots, trichome-like roots, and calli was the greatest at, respectively, 10^{-6}, 10^{-5}, and 10^{-4}M (Fig. 1).

Effects of BL plus NAA on the Formation of Adventitious Roots, Trichome-Like Roots and Calli

To determine whether BL has effects on the formation of adventitious roots, trichome-like roots, and calli, we cultured the adventitious root formation group with 10^{-6}M NAA, the trichome-like root formation group with 10^{-5}M NAA, and the calli formation group with 10^{-4}M NAA and with 10^{-10} to 10^{-6}M BL at 25°C in the dark. In the adventitious root formation group, after 4 weeks, adventitious root formation was greatest at 10^{-9}M BL; when it is treated with higher concentration than 10^{-9}M BL, numbers of shortened trichome-like roots were produced (Fig. 2).

In the trichome-like root formation group, the rate of production of trichome-like roots was highest at 10^{-9}M BL and was reduced with increasing concentrations of BL. Furthermore, at 10^{-6}M, they became calli (Fig. 3). In the calli formation group, 10^{-9}M BL yielded maximum calli development, and there was decreased production with increasing BL concentrations; at 10^{-6}M, leaf segments become light green and expanded, with a little calli relatively (Fig. 4).

Northern Blot Analysis

When we studied the effect of BL plus auxin on *CNT103* gene expression (Fig. 5) in the adventitious root formation group (A), expression was highest at 10^{-9}M BL (lane 3) and gradually decreased at higher concentrations (lanes 4–6). The trichome-like root formation group (B) and the calli formation group (C) showed the highest level of gene expression at 10^{-9}M (lane 3) and also decreased at higher BL concentrations (lanes 4–6).

Discussion

Adventitious roots are formed directly from parenchyma cells by dedifferentiation from normal lateral roots or via calli (Lovell and White 1986). In this study, adventitious roots, trichome-like roots, and calli developed from leaf segments as the concentration of NAA increased. Han et al. (1999) reported that the production of trichome-like roots and calli increased with NAA concentration in *Arabidopsis* leaf segment cultures, and this agreed with our results (Fig. 1). In addition, we assumed that a high concentration of NAA promotes cell division and inhibits differentiation of adventitious roots or trichome-like roots (Hertmann et al. 1990).

Several reports have uncovered that BRs in low concentrations activate auxins to stimulate root elongation (Mouchel et al. 2006) and lateral root formation (Bao et al. 2004; Lee SH 2006), and we obtained similar results—the NAA effect is enhanced by adding 10^{-10} to 10^{-6}M BL. In the adventitious root formation group, when we added BL, more adventitious roots were formed at BL concentrations up to 10^{-9}M, but at or above 10^{-8}M BL, adventitious root formation was reduced, while trichome-like root production

increased (Fig. 2); that is, as BL concentrations rose, adventitious root numbers and elongation increased, but at higher BL concentrations, the number of adventitious roots continued to increase while elongation was reduced. The trichome-like root formation group also showed a greater generation of trichome-like roots as a result of adding 10^{-10} to 10^{-9}M BL, but forming calli and trichome-like root induction was low above 10^{-8}M BL (Fig. 3). When we added BL to the calli formation group, the development of calli was increased until 10^{-9}M BL; however, it decreased at and above 10^{-8}M BL. Significantly, at 10^{-6}M BL, calli production was sharply reduced, and we observed enlargement of leaf segments (Fig. 4). According to previous reports, low concentrations of BRs (below 1 pM) induce root and adventitious root formation; otherwise, high concentrations of BRs inhibit primary root extension and lateral root formation (Roddick and Guan 1991; Sasse 1994; Kim et al. 2005). Also treatment using high levels of BL (10^{-6}M BL) plus 2,4-D produced larger numbers of lateral roots and shortened the main root in transformed intact rice seedling experiments (Song et al. 2009). Our in vitro experiment showed enhancement of the NAA effect when NAA plus 10^{-10} to 10^{-9}M BL were added but showed inhibition of NAA induced trichome-like root or calli formation at 10^{-8}–10^{-6}M BL. In particular, 10^{-6}M BL-induced calli development was almost repressed, but the size of leaf segments expanded, maintaining a light green color. Therefore, we should be more careful in drawing conclusions about the effects of high concentrations of BL because BL interacts with NAA to increase NAA action despite high concentration of BL.

To investigate the effect of BL with NAA at the transcription level, northern blot analysis was done for the *CNT103* gene (Fig. 5), which is involved in cell division at the root tip (van der Zaal et al. 1991). In all control groups, which include the adventitious root formation group (A), the trichome-like root formation group (B), and the calli formation group (C) and a group receiving only NAA treatment, we observed similar *CNT103* gene expression (lane 1). These results indicate that *CNT103* gene expression is not proportional to NAA concentration but is auxin inducible. Furthermore, when BL is added to the NAA treatment groups at 10^{-9}M BL, *CNT103* gene expression is highest (lane 3) and becomes reduced above 10^{-8}M BL (lanes 4–6). This suggests that low concentrations of BL promote *CNT103* gene expression, but BL does not have any significant effects above the optimal concentration. In other words, BL improves NAA activation by enhancing *CNT103* gene expression in tobacco leaf segment culture.

In summary, BL consistently increased NAA functions at each phase and transferred to the next phase. That we found greater expression of *CNT103* gene at lower concentrations of BL and some expression at high BL concentration indicates that BL interacts with a steady level of NAA, promoting NAA action regardless of its concentration.

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