

Shoot formation from root tip region: a developmental alteration by *WUS* in transgenic tobacco

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Abstract We examined the effect of ectopic expression of *WUS* on the morphology of tobacco seedlings and the segments in vitro. *WUS* was amplified from *Arabidopsis* cDNA and introduced into the tobacco genome under the transcriptional control of the β -estradiol-inducible expression system. When 1-week-old transgenic seedlings were cultured in the presence of β -estradiol, only the root tip region developed bulbous tissues followed by shoot formation and plant regeneration, suggesting its applicability for improving the strategy of micropropagation in recalcitrant species. Evident abnormality was not observed in the cotyledons, hypocotyl nor root except for the tip. However, ectopic *WUS* seemed to be functional in those parts through the observation of gene expression and the behavior of cultured segments. Small root segments with a root tip treated with β -estradiol also showed bulbing but no shoots unless exogenous cytokinin was supplied. These findings suggest the existence of unknown factors regulating ectopic *WUS* function in the seedling.

Keywords Ectopic expression · *Nicotiana tabacum* · Root tip · Shoot formation · Transdifferentiation · *WUSCHEL*

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Introduction

Since the early observations of in vitro regeneration from somatic cells (Skoog and Miller 1957; Steward et al. 1958; Takebe et al. 1971), cytokinin and auxin have been always critical factors to induce meristematic cells leading to regeneration. However, recently a number of reports demonstrated that regeneration from somatic cells could be induced by ectopic expression of various genes coding for transcription factors (reviewed by Zhang 2003), including *LEAFY COTYLEDON 1 (LEC1)*, Lotan et al. 1998), *LEAFY COTYLEDON 2 (LEC2)*, Stone et al. 2001), *LEC1-LIKE* (Kwong et al. 2003), *WUSCHEL (WUS)*, Laux et al. 1996; Zuo et al. 2002; Gallois et al. 2004) and *BABY BOOM (BBM)*, Boutilier et al. 2002). These suggest a possibility of establishing an alternative method for regeneration of general plant species without using phytohormones.

A reliable expression system of a transgene, using a chimeric transcription factor named XVE, was developed by Zuo et al. (2000). The gene coding for the chimeric transcription factor was constructed by fusing the DNAs coding for the DNA-binding domain of the bacterial repressor LexA, the transactivating domain of VP16 and the regulatory region of the human estrogen receptor. The XVE transcription factor was activated by exogenous estrogen and then it increased the expression level of the transgene under the control of a promoter consisting of eight copies of the LexA operator fused upstream of the (-46)35S minimal promoter. The XVE system was successfully applied to screening for a gene whose ectopic expression causes somatic embryogenesis (Zuo et al. 2002). The gene isolated through the screening was identified as *WUS* (Laux et al. 1996). In *Arabidopsis* zygotic embryogenesis, *WUS* is expressed in the 16-cell-stage

embryo prior to meristem development within the region that originates the apical meristem (Mayer et al. 1998).

In this study, we examined the effect of ectopic expression of *WUS* and *LEC2* on the morphology of tobacco seedlings and the segments using the XVE system.

Materials and methods

Molecular manipulation and plant materials

The DNA clones coding for *WUS* and *LEC2* were obtained by PCR using first stranded cDNAs synthesized from mRNA prepared from inflorescence of *Arabidopsis thaliana* var. Columbia. Specific primers for *WUS* and *LEC2* were designed from the available sequence data supplied by DDBJ (<http://www.ddbj.nig.ac.jp/>) as follows; 5'-ctcgagCTTATTTACCGTTAACTTTGTGAA (small letters indicate *Xho* I restriction site) and 5'-actagtCACATAACGAGAGATAACTAGTTAAC (small letter, *Spe* I site) for *WUS*, and 5'-gtcgacAATGGA-TAACTTCTTACC CTTTCC (small letter, *Sal* I site) and 5'-actagtCTAT TTTTCATAAGGGGGTTATG (small letter, *Spe* I site) for *LEC2*. The PCR fragments of *WUS* (size: 1,208 bp) and *LEC2* (size: 1,332 bp) were directly cloned into the vector, pER8 coding XVE (Zuo et al. 2000). The sequences of the resulting constructs were confirmed by DNA sequencing and then transferred into *Agrobacterium tumefaciens* (strain LBA4404) by electroporation. *Agrobacterium*-mediated transformations of *Nicotiana tabacum* cv. Samsun were performed with leaf discs as previously reported (Kyo et al. 2000). The resulting transgenic tobacco plants were selected in the presence of 50 mg l⁻¹ of hygromycin and grown to mature plants to harvest seeds. They were referred to as *XVE::WUS* and *XVE::LEC2* lines, respectively. We obtained six and five lines transgenic for *WUS* and *LEC2*, respectively. Among them three *XVE::WUS* lines and two *XVE::LEC2* lines showed abnormality in morphology in the presence of β -estradiol (Sigma-Aldrich, MI, USA). One representative of each of these lines was selected and backcrossed with wild tobacco to harvest their seeds for experiments.

Seedling morphology

Seeds of the transgenic lines were sterilized, sown on LS (Linsmaier and Skoog 1965) medium with 0.8% agar and hygromycin (50 mg l⁻¹) and kept in the dark at 25°C for 1 week. Seedlings showing normal root growth on the medium were selected and transferred to LS agar medium with or without β -estradiol to observe their morphological abnormality.

Segment culture

Young seedlings were cut into segments; cotyledon, hypocotyl with or without shoot apical meristem (SAM), root and root tip with root apical meristem (RAM). These segments were cultured on LS agar medium supplemented with β -estradiol and/or 6-benzyladenine (BA) at 25°C in the dark for 1 month.

Expression analysis

Young seedlings were cultured on LS liquid medium with or without β -estradiol. After the treatment with β -estradiol (for 24 h or 5 days) seedlings were harvested and cut into segments; cotyledon with SAM, hypocotyl and root with RAM. These segments were immediately homogenized for RNA extraction followed by RT-PCR according to the method previously reported (Kyo et al. 2000). The sequences of specific primers for RT-PCR were 5'-CTTATTTACCGTTAACTTTGTGAA and 5'-GGYGGCAACTAYACTAGRAGTGG for *WUS*; 5'-GCTCTGGATATGCCGACTTC and 5'-GTAGCCAGCAGCATGTCGAAG for *XVE*; and 5'-TCCTCCTCCTATGATGATGCCT and 5'-TTCACATCAACCTCCTCTTCAGA for *NTH15* (Tamaoki et al. 1997).

Results

Seedling morphology

The 1-week-old seedlings transgenic for *WUS* gene (*XVE::WUS*) were transferred onto LS agar medium with or without β -estradiol. Within 3 days, the seedlings formed bulbous tissues at their root tip regions in the presence of β -estradiol but not in its absence (Fig. 1a). Under the 16 h light/8 h dark condition, the bulbous tissues generated green spots within 2 weeks, and they developed to shoots in the following culture (Fig. 1c–e). In the dark condition also, bulging in root tip regions and regeneration of shoots were observed (Fig. 1f–h), though the frequency of shoot formation was lower than that in a light condition. These results suggest that light is not required for the bulging but promotes the shoot formation. The developed shoots were detached from original tissue and cultured on LS agar medium under the light condition (Fig. 1i, j). They generated roots and developed to adult plants with fertility (Fig. 1j, k). Their offspring contained individuals that show abnormalities in the presence of β -estradiol as their mother plants (data not shown).

When seedlings of *XVE::LEC2* line were cultured on the medium with β -estradiol, primary roots curved and became thicker within 3 days. The roots continued to grow and

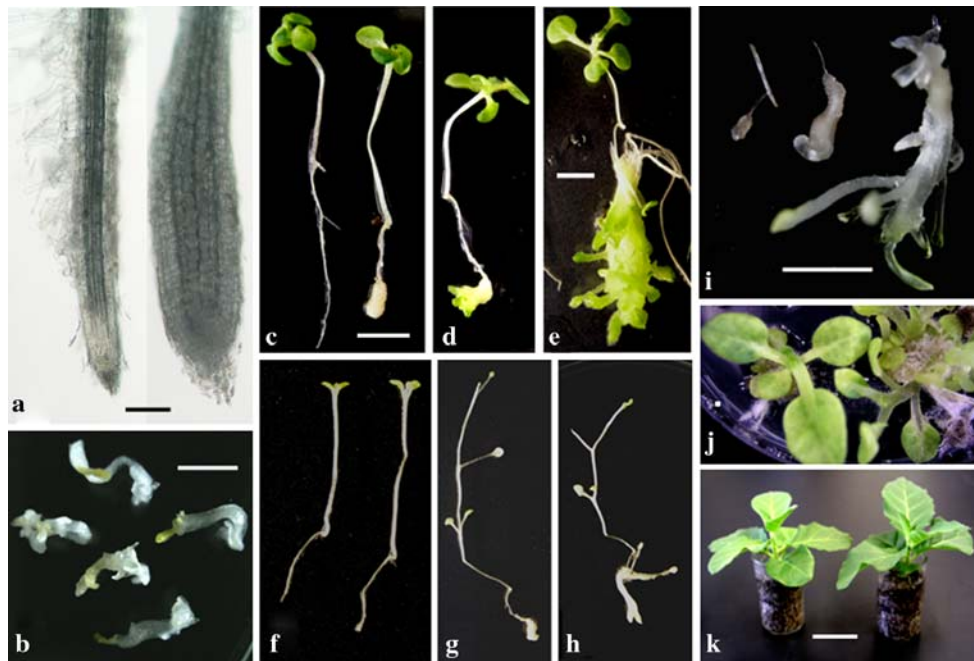


Fig. 1 Aspects of the response of the seedling transgenic for *XVE::WUS* to β -estradiol. **a** The root tip region after a 3-days culture on the medium without (*left*) and with $1 \mu\text{M}$ β -estradiol (*right*). *Bar* $150 \mu\text{m}$. **b** Seedlings with severe abnormality, developed from the seeds sown on the medium with β -estradiol and kept for 18 days in the dark. *Bar* 10mm . **c–h** Seedlings cultured on the medium with β -estradiol for 5 days (**c** and **f**), 10 days (**d** and **g**) and 21 days (**e** and **h**). The seedlings on the left in **c** and **f** were cultured on the medium without β -estradiol as controls. The seedlings were kept in the light/dark cycle (**c–e**) and dark (**f–h**) condition, respectively. **c**, **d** and **f** are

shown at the same magnification and the *bar* in **c** shows 10mm . **e**, **g** and **h** are shown at the same magnification and the *bar* in **e** shows 10mm . **i–k** Shoot formation from bulbous tissue generated on the root tips and their growth to plants. **i** Different stages of bulbous tissue and shoot generated from root tip regions of seedling cultured on the medium with the inducer. *Bar* 5mm . **j** Regenerated shoots were detached from original plate and cultured for one month on LS agar medium without the inducer in a 9-cm dish. **k** Plants grown on the soil in glass pots. *Bar* 50mm

coiled themselves generating long root hairs. Gradually those long root hairs covered the coiled root region and cotton-like structures appeared (Fig. 2). Also in this line, morphological abnormality was limitedly observed only in the root tip region of treated seedlings. Stone et al. (2001) reported that over expression of *LEC2* in transgenic *Arabidopsis* induced somatic embryogenesis around the SAM. However, in our study, no regeneration was observed from those seedlings.

In both transgenic lines, morphological abnormalities were observed in their root tip regions but not in the aerial parts even when treated with β -estradiol during the first period of culture (Figs. 1, 2). When the seedlings were imbibed under liquid medium with various concentrations of β -estradiol (0.1 – $10 \mu\text{M}$), the morphological abnormality was limited to the root tip region (data not shown).

Expression of the transgenes

We examined the expression of transgenes in different parts of *XVE::WUS* seedlings cultured on the medium with or without β -estradiol. As shown in Fig. 3, the constitutive



Fig. 2 The seedling morphology of *XVE::LEC2* line cultured on LS agar medium with or without (control) β -estradiol. *Bar* 10mm

expression of *XVE* gene was detected (amplified fragment size: approx. 600bp) in all parts of treated and untreated seedlings. In contrast, expression of *WUS* gene was de-

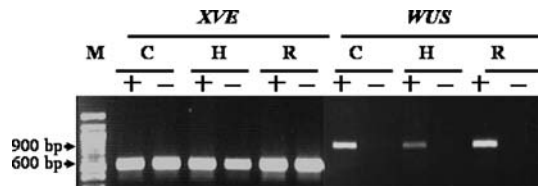


Fig. 3 Expression analysis of transgenes in *XVE::WUS* seedlings. Young seedlings were cultured in the presence (+) or absence (-) of β -estradiol for 24 h. cDNAs were separately prepared from the three types of segments; cotyledon with SAM (C), hypocotyl (H) and root with RAM (R). The approximate length of amplified transcript is indicated at the left. M: 0.1 kbp DNA ladder

tected (amplified fragment size: approx. 900 bp) only in the seedlings treated with β -estradiol. In the presence of β -estradiol, *WUS* was expressed in all parts of the seedling, but as described above, morphological abnormality was observed only in the root tip region. These results suggest that the *WUS* function in the root tip region was not suppressed for an unknown reason, although it was suppressed in other regions.

Segment culture

Segments from *XVE::WUS* seedlings were cultured as described in **Materials and methods**. In the absence of β -estradiol, cotyledons, hypocotyl and root segments generated adventitious or lateral roots, and root segments with RAM continued to grow (Fig. 4a–e). In the presence of β -estradiol, both root generation and the root growth were suppressed (Fig. 4f–j) probably due to the expression of *WUS* though the translation for *WUS* was not examined. Figure 5 shows the frequency of root generation and root growth in each tissue summarizing the results in this experiment (20 segments each). The suppression was observed in each part in the presence of the inducer. Using the segments from non-transgenic seedlings, the suppression was not observed in the presence of the inducer (supplementary Fig. 1).

Bulbing of the root tip region was observed in the presence of β -estradiol (Fig. 4j) as in the seedlings (Fig. 1c, f), but no shoot regeneration was observed even after 2 months of culture. This suggested that shoot for-

Fig. 4 Segment culture of *XVE::WUS* seedlings. The upper (a–e) and lower (f–j) lanes represent segment cultures under the absence or presence of β -estradiol, respectively. Young seedlings were cut into segments; cotyledon (a, f), upper part of hypocotyl with SAM (b, g), lower part of hypocotyl (c, h), upper part of root (d, i) root tip with RAM (e, j) and cultured for 1 month. Bars 5 mm

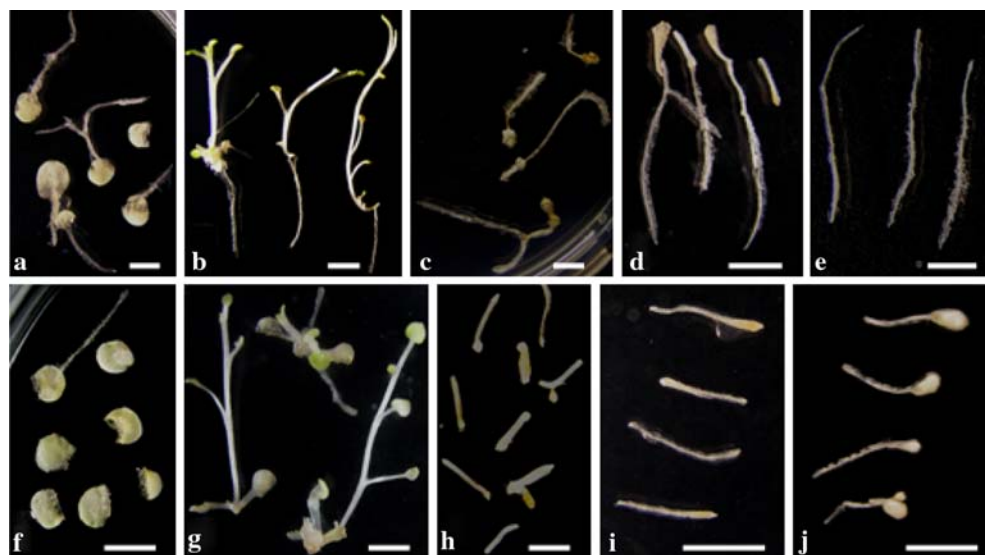


Fig. 5 An inhibitory effect of *WUS* expression on root generation and root growth. In each type of segment, the frequency indicates the percentage of the segments generating roots or growing roots against the total number of segments in the presence and absence (control) of the inducer

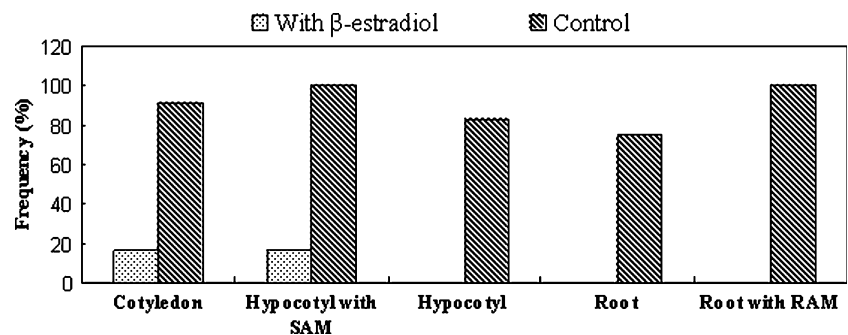
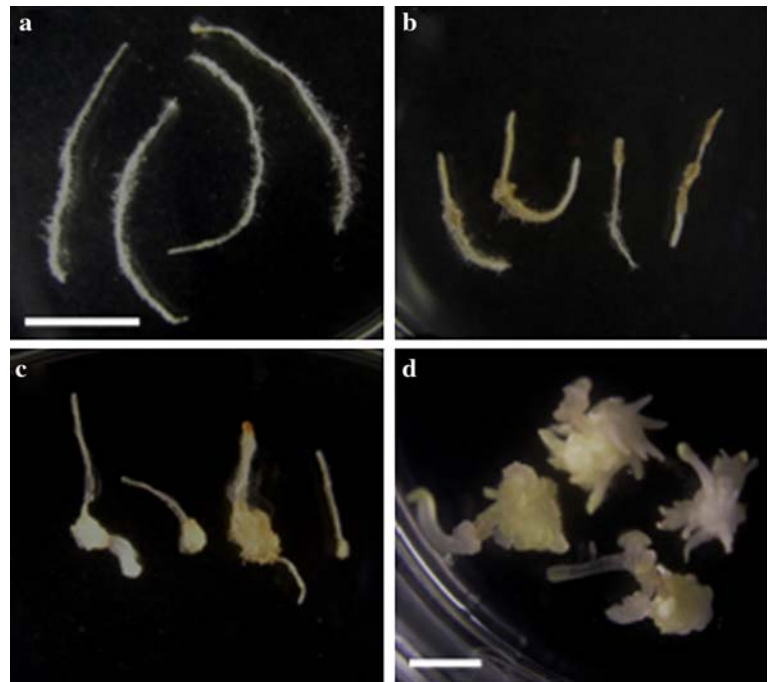


Fig. 6 Effect of β -estradiol and BA on the morphology of the root tip segments with RAM of *XVE::WUS* seedlings. The segments were cultured on the medium with 10 μ M BA (**b**), 1 μ M β -estradiol (**c**), 10 μ M BA and 1 μ M β -estradiol (**d**), and without supplements (**a**). Bars 8 mm



mation requires some endogenous hormones or specific nutrients supplied from the other regions including the aerial parts. We preliminarily examined the effects of auxin, cytokinin, abscisic acid and gibberellin on the growth of the segments from *XVE::WUS* seedlings (data not shown), and found the specific effect of 6-benzyladenine (BA). In the presence of 10 μ M BA and 1 μ M β -estradiol, we observed bulbing of the root tip followed by quick shoot formation (Fig. 6d), but no shoot formation in other conditions (Fig. 6a–c).

After a longer culture period (2 months and more), in the presence of BA (without β -estradiol), we occasionally observed development of calli with leaf primordia. Such calli were generated from the cut-end of root segment but never from the root tip side (data not shown).

Expression of *NTH15*

Nicotiana tabacum homeobox 15 (*NTH15*) gene was identified as a marker gene for the SAM region and its ectopic expression caused abnormality in tobacco leaf morphology accompanied with decrease of gibberellin level and increase of cytokinin level (Tamaoki et al. 1997). We examined the expression of *NTH15* in different parts of transgenic seedlings by RT-PCR. In the absence of β -estradiol, the expression was detected in cotyledons with SAM and hypocotyl but not in root (Fig. 7). These expression patterns were similar to the previous result (Tamaoki et al. 1997). However, in the presence of β -estradiol, the expression was detected also in the root generating bulbous tissues, indicating that cells specific to

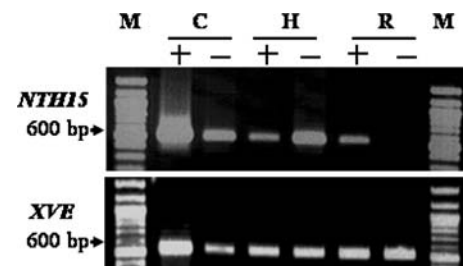


Fig. 7 The expression analysis of *NTH15* and *XVE* in *XVE::WUS* seedlings. Young seedlings were cultured in the presence (+) or absence (–) of β -estradiol for 5 days. cDNAs were separately prepared from the three types of segments; cotyledon with SAM (C), hypocotyl (H) and root with RAM (R). The approximate lengths of amplified sequences are indicated on the left side. M: 0.1 kbp DNA ladder

SAM were generated and the marker gene was expressed in the bulbous tissue within 5 days after the β -estradiol treatment (Fig. 1c, f).

Discussion

Appearance of shoots from root, so called “radical bud”, is observed in a number of genera: for example, *Ipomoea batatas*, *Armoracia rusticana*, *Cirsium arvense*, *Convolvulus arvensis* and *Coronilla varia*, though the radical buds originated from the pericycle and not from the root tip (Rauh 1950). This strategy is very efficient for vegetative reproduction in natural conditions. If such a specific capacity can be given to recalcitrant plant species by

transferring the single gene, it is advantageous for efficient micropropagation. By inducing ectopic expression of *WUS* originated from *Arabidopsis*, we could regenerate fertile plants from the root tip region in tobacco (Fig. 1). Our observation suggests a potential utilization of the gene in the strategy for producing clonal plants from roots in various crops.

Shoot formation from the root tip (not from pericycle) seems to be a rare phenomenon in natural conditions (Wareing 1978) and also in vitro. We observed adventitious shoot formation from the transgenic tobacco root tips though we did not confirm whether the root meristem directly changed to the adventitious shoot meristem. From the viewpoint of plant developmental biology, we are interested in this phenomenon, especially in terms of commitment and transdifferentiation. As shown in Fig. 1a, in the early period of the response to β -estradiol, the root tip first enlarged in size and developed to a clump prior to form one to several shoot buds. When small root segments with RAM were treated with β -estradiol they also developed a clump but no shoot buds unless exogenous cytokinin was supplied (Fig. 6). We speculate that the *WUS* function deprived the RAM cells of their identity in the process of bulbing. This process is distinguishable from the following process of generating SAM, which requires endogenous (in case of whole seedlings) or exogenous (in case of small root tip segments) cytokinins. The canceling of developmental commitment seems to be essential for the transdifferentiation from RAM to SAM.

In *A. thaliana*, ectopic expression of *WUS* successfully induced adventitious organs by the XVE system (Zuo et al. 2002) and a Cre-*loxP*-recombination system, which confers random and mosaic expression pattern in tissues (Gallois et al. 2004). In the former system, adventitious embryos were generated mainly in the root region and the embryos developed to plants. In the latter system, *WUS*-expressing sectors were induced mainly near the root tips and the sectors generated adventitious leaves, embryos (in the presence of NAA) or inflorescences (with cooperative expression of *LEAFY*). However, the development of those adventitious organs did not continue probably because of constitutive expression of *WUS* driven by 35S promoter after Cre-*loxP* recombination. A similar developmental arrest was also reported in the 35S::*WUS* transgenic plants by Zuo et al. (2002).

For comparison with the gene expression pattern conferred by Cre-*loxP*-recombination system (Gallois et al. 2004), we preliminarily examined the gene expression pattern by the XVE system using a fusion reporter gene (*H3sGFP*) consisting of synthetic green fluorescent protein gene and tobacco H3 histone gene (Yamaji and Kyo 2006). In the root tip region of tobacco seedlings treated with the inducer, green fluorescence was observed in the nuclei of

the whole organ (supplementary Fig. 2), being very different from the mosaic expression pattern as shown previously (Gallois et al. 2004). The expression pattern of *WUS* in the tobacco root tip seems to be identical to that of *H3sGFP* driven by the XVE system. The XVE system was probably suitable for obtaining regenerants from the adventitious organs. In tobacco *XVE>::WUS* line we observed shoot formation but no somatic embryogenesis as in *A. thaliana*. Whether somatic embryogenesis can be induced by using tobacco orthologs for *WUS* remains to be examined.

In *A. thaliana* (Zuo et al. 2002; Gallois et al. 2004) and tobacco (this paper), the ectopic *WUS* expression induces specific cells possessing the identity of embryonic and/or shoot stem cells. However, depending on the expression manner (level and duration), the expressing area (site and size) and additional factors (phytohormones and other gene expression), the specific cells did not show the same behavior. The factors effective for the behavior of the adventitious stem cells remain to be identified in the future.

As is frequently observed in *A. thaliana* (Zuo et al. 2002; Gallois et al. 2004), somatic embryogenesis or adventitious organogenesis was observed most frequently on root explants or root tip region. In tobacco, as described above, the expression of *WUS* was detected in all parts of tobacco seedlings treated with the inducer (Fig. 3) and, in the segment culture experiment, root generation and root growth were inhibited depending on the possession of the transgene (supplementary Fig. 1) and the presence of the inducer (Figs. 4, 5). Therefore, *WUS* were expressed and the translational products seemed to be functional not only in the root tip but in all parts of the tobacco seedling. Additionally, when seeds of tobacco *XVE>::WUS* lines were sown on the medium with the inducer, germination was delayed and severe abnormality was observed in the whole seedling both in the dark (Fig. 1b) and in the light condition (supplementary Fig. 3). Also in the *XVE>::LEC2* line severe abnormality was observed in the whole seedling when the seeds were sown in the presence of the inducer (data not shown) but when 7-day-old seedlings were treated the abnormality was limited to the root tip region (Fig. 2).

As for the organ-specific and developmental stage-specific responses to the ectopic *WUS* expression, existence of putative co-factors and antagonistic factors were speculated (Zuo et al. 2002). We suggest two possibilities, one is that putative co-factors for *WUS* decreased gradually after the seed germination and finally remained only in the root tip region. As another possibility, a putative antagonistic factor was accumulated in whole seedlings except for root tips. In previous study in *A. thaliana* (Schoof et al. 2000; Lenhard et al. 2002; Lohmann et al. 2001), even if *WUS* was overexpressed around the shoot apical meristem by using meristem-specific promoters somatic embryogenesis

was not induced probably because antagonistic factors regulated the activity of *WUS*, like a negative feedback loop including *CLAVATA1*.

In the *A. thaliana* shoot region, microarray analysis revealed that 104 genes were up-regulated and 44 genes were down-regulated in response to the overexpression of *WUS* (Leibfried et al. 2005). The latter down-regulated genes include four type-A ARR (*Arabidopsis* response regulator) genes, which are known to show a quick response to exogenous cytokinin (D'Agostino et al. 2000), suggesting that the *WUS* function and cytokinin effect are antagonistic to each other in the shoot region. However, as described in the segment culture using tobacco *XVE::WUS* line, the small root segments with *RAM* required both the *WUS* and exogenous cytokinin for generating shoot buds (Fig. 6). At present, it is difficult to trace the context from the primary events to the appearance of markers for the shoot meristem in the root tip region. Our future target is to find some substantial changes on chromatin that are associated with the transdifferentiation of root tip cells induced by ectopic *WUS* function.

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