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Ectopic Expression of *WOX5* Dramatically Alters Root-tip Morphology in Transgenic Tobacco

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ABSTRACT

This paper examines the effects of ectopic expression of *WUSCHEL* (*WUS*)-*RELATED HOMEOBOX5* (*WOX5*) on the morphology of transgenic tobacco plantlets and seedlings under the control of a chemical-inducible expression system. In the presence of the expression inducer, β-estradiol, the root-tip region developed a swelling of callus tissue from which adventitious shoots emerged. Except at the root tip itself, no abnormality was evident in any other parts of treated plantlets such as in the leaf, stem or other parts of the root. Transgenic seedlings also generated ectopic shoots from similar root swellings after treatment with the inducer. Expression analyses reveal that *WOX5* may induce transdifferentiation of root-tip cells that possess competence for the formation of an apical shoot meristem. It is suggested that *WOX5* may execute a common functional regulation in transgenic tobacco as previously reported in the case of *WUS*, during the process of adventitious shoot formation, and from the root-tip region.

Keywords: adventitious shoot formation, C-terminal domains, *Nicotiana tabacum*, quiescent center, transdifferentiation

INTRODUCTION

During plant embryogenesis, the establishment of the apical-basal axis is a critical stage. In the early period of this stage in *Arabidopsis*, a detailed genomic approach has revealed that the *WUSCHEL* (*WUS*)-*RELATED HOMEOBOX* (*WOX*) family members are expressed and that they are potentially involved in the region-specific transcription programs in single precursor cells (Haecker *et al.* 2004). After embryogenesis, plant body development originates from pluripotent stem cells, which exist in the two stem cell niches, of the shoot and root apical meristems. *WUS*, encoding a homeodomain transcription factor, is expressed in the organizing center (OC) of the shoot apical meristem (SAM) and functions to maintain the stem cells undifferentiated and also to induce expression of *CLAVATA3* (*CLV3*), which in turn restricts the size of the *WUS* expression region (Mayer *et al*. 1998; Schoof *et al*. 2000; Brand *et al.* 2000). Likewise, in the root apical meristem (RAM), a signal from the quiescent center (QC) is necessary to maintain the undifferentiated stem-cell pool (van den Berg *et al*. 1997; Spradling *et al*. 2001) and *WOX5* is expressed in QC (Haecker *et al.* 2004). It has been found that the expression pattern of *WOX5* in QC is similar to *WUS* in the SAM, and that they function similarly for stem-cell maintenance in *Arabidopsis* (Mayer *et al.* 1998; Sarkar *et al.* 2007).

XVE is a chimeric transcription factor and was used to develop a reliable expression system of a transgene (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 (X), the transactivating domain of VP16 (V) and the regulatory region of the human estrogen receptor, hER (E). The XVE transcription factor was activated by exogenous estrogen and then it increased the transgenic expression level, under the control of a promoter consisting of eight copies of the LexA operator, which is fused upstream of the (-46)35S minimal promoter (Zuo *et al.* 2000). Zuo *et al.* (2002) successfully applied the XVE system to screening for a gene, whose ectopic expression causes somatic embryogenesis and the isolated gene through the screening was identified as *WUS* (Laux *et al.* 1996). A previous report described the effects of *WUS* expression on the morphology of transgenic tobacco seedlings and their segments *in vitro* under the control of the XVE system (Rashid *et al.* 2007). Notably, a developmental change was observed in the root-tip region of an *XVE::WUS* seedling, the induction of a swelling of callus tissue from which a shoot finally emerged (Rashid *et al.* 2007). Additionally, a similar morphological response was also detected when a *WUS* homolog, *Nicotiana tabacum WUS* (*NtWUS*) was cloned and overexpressed in transgenic tobacco seedlings (Rashid and Kyo 2009). This study investigates the effects of ectopic expression of *WOX5* on the morphology of transgenic tobacco plantlets and seedlings under the control of the XVE system. It provides evidence that *WOX5* is involved in a common functional regulation similarly as *WUS* and induces adventitious shoot formation from the tobacco root-tip region.

MATERIALS AND METHODS

Molecular manipulation and transformation in tobacco

The DNA clones coding for *WOX5* were obtained by PCR using first stranded cDNAs synthesized from total RNA prepared from the root of *Arabidopsis thaliana* (ecotype Columbia). The gene-specific primers were designed from the available sequence data supplied by DDBJ (http://www.ddbj.nig.ac.jp/): *WOX5*-sense, 5′-ctcgagATGTCTTTCTCCGTGAAAGGTCG-3′ (lowercase letters are the *Xho*I restriction site) and *WOX5*-antisense, 5′-actagtTTAA AGAAAGCTTAATCGAAGATCTAAT-3′ (lowercase letters are the *Spe*I restriction site). The RT-PCR fragment of *WOX5* (size: 660 bp) was sequenced and cloned directly into the vector pER8 harboring the XVE system (Zuo *et al.* 2000).

The pER8 vector harboring the *WOX5* cDNA was transferred into *Agrobacterium tumefaciens* (strain LBA4404) via electroporation. The *Agrobacterium*-mediated transformation of *Nicotiana*

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tabacum 'Samsun' was performed using leaf discs of mature plants, as previously reported by (Yamaji and Kyo 2006). Fifteen transgenic plants were selected based on their hygromycin-resistance (Wako, Japan, 50 mg L^{-1}) and initially, all of them were treated with β-estradiol (Sigma-Aldrich, MI, USA) to observe abnormalities in their morphology. Among them, abnormalities were detected in 10 transgenic plantlets, showed an inhibition of normal root elongation and gradually generated pseudo-bulbous-like tissues at their root tip regions. All of those abnormal plantlets were grown to mature plants and self-crossed to harvest their seeds for experiment. In this study, they are referred to as *XVE::WOX5* lines and data has been presented using just one representative line.

Morphological observations

All 15 transformants (hygromycin resistant) were transferred into fresh LS (Linsmaier and Skoog 1965) liquid medium (aseptically into 9 cm Petri dishes) and cultured in the light (16-h photoperiod, 25°C) for the next 2 weeks. When sufficient roots had generated, β-estradiol was aseptically added to some of those Petri dishes (containing an individual plantlet in LS liquid) at a final concentration of 10 μM. The morphological response of the β-estradiol-treated plantlets was compared with the control (no addition of β-estradiol) and one representative result from each treatment condition was photographed.

Seeds of the transgenic line were sterilized, sown on LS solid 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 response.

Expression analyses

Young seedlings of the *XVE::WOX5* line were cultured on LS liquid medium with or without β-estradiol. After 7 days' treatment, seedlings were harvested and cut into segments; cotyledon with SAM, hypocotyl and root with RAM. These tissues were homogenized for total RNA extraction and subsequently RT-PCR was conducted using the protocol previously described by Rashid *et al.* (2007). The sequences of specific primers for RT-PCR were: sense, 5′-CTCGAGATGTCTTTCTCCGTGAAAGGTCG-3′ and antisense, 5′-ACTAGTTTAAAGAAAGCTTAATCGAAGATCTAAT-3′ for *WOX5* (http://www.ddbj.nig.ac.jp/); sense, 5′-GCTCTG GATATGGCCGACTTC-3′ and antisense, 5′-GTAGCCAGCAGC ATGTCGAAG-3′ for *XVE* (Zuo *et al*. 2000); sense, 5′-ATGGAA GCTGCTCAACAACAAAAC-3′ and antisense, 5′-TTAAGGGGA ATTAGGAGATCTGCC-3′ for *NtWUS* (Rashid and Kyo 2009); sense, 5′-TCCTCCTCCTATGATGATGCCT-3′ and antisense, 5′-TTCACATCAACCTCCTCTTCAGA-3′ for *NTH15* (*Nicotiana tabacum homeobox15*; Tamaoki *et al.* 1997). The RT-PCR products were fractionated through agarose (1%) gel electrophoresis.

RESULTS AND DISCUSSION

Morphological response to β-estradiol

Using the transgenic plantlets (T1 generation) of the *XVE::WOX5* line, we observed the morphological response after treatment with or without the inducer, β-estradiol. In the presence of the inducer, an abnormality was observed only in the root-tip region (**Fig. 1**). In the beginning, the treated root-tip region became enlarged and generated a swollen structure within 4 days (data not shown). These abnormal root-tip regions developed swollen tissues in the period following treatment and started greening when replaced under light conditions (**Fig. 1B**). Subsequently, green shoots developed on the swollen root-tip region (**Fig. 1C**). No detectable abnormality was observed in other regions of treated plantlets, such as in the leaves, stem or roots except at the root tip (**Fig. 1B, 1C**) while control plants (without the β-estradiol treatment) developed normally showing no abnormalities, even in the root-tip region (**Fig. 1A**).

Similarly, the 7-day-old seedlings of the *XVE::WOX5* line were transferred onto LS agar medium with or without β-estradiol. Within 3 days, the seedlings generated swollen tissues in their root-tip regions in the presence of β-estradiol but not in its absence (**Fig. 2A**). The swollen tissue gradually enlarged and, after placement in light conditions, developed green shoots (**Fig. 2B**). However, no significant differences were observed between dark and light culture conditions except that ectopic shoot formation from the swollen, root-tip regions was quicker in the presence of light (data not shown).

These results are similar to observations previously reported in transgenic seedlings possessing *XVE::WUS* (Rashid *et al*. 2007) or *XVE::NtWUS* (Rashid and Kyo 2009). In these studies, the β-estradiol-treated root tip also generated swollen tissue and green shoots subsequently developed in the transgenic seedlings possessing *WUS* or *NtWUS*. Moreover, such an abnormality was never observed in the absence of the inducer in transgenic seedlings of

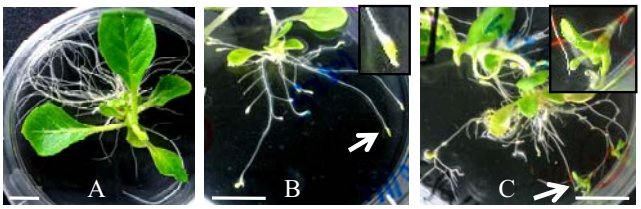

Fig. 1 Morphological response to β-estradiol of tobacco plantlets (T1 generation) transgenic for *XVE::WOX5*. The small, transgenic plantlets (hygromycin resistant) were transferred to LS liquid medium and cultured for two weeks under light conditions to induce sufficient root formation. After this, β-estradiol (10 μM) was added (**A**: day 0 of treatment) and culture continued under light conditions. Within two weeks of treatment by the inducer, the root-tip regions developed swellings which later became green in the presence of light (**B**: day 14 of treatment). In the later culture, ectopic shoots were generated from the swollen root-tip regions (**C**: day 23 of treatment). In both **B** and **C**, the arrows indicate the regions that are enlarged in the insets. Scale bars indicate 1 cm. All cultures were conducted aseptically in 9 cm Petri dishes.

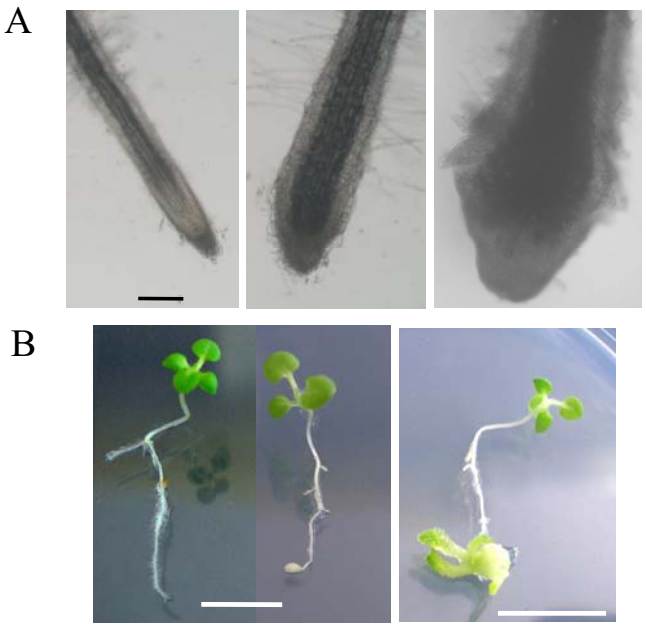

Fig. 2 Morphological features of transgenic seedlings of *XVE::WOX5* line. (**A**) The root-tip region of treated seedling in LS liquid medium without (control, left) or with (middle) β-estradiol (10 μM) for 72 hrs. Within 7 days of treatment with β-estradiol, the root tip developed a swollen structure which gradually grew still larger (right). Scale bar 150 μm. (**B**) Transgenic seedlings showing normal growth (left) or swollen tissue development in the root-tip region (middle) when cultured in the absence or presence of β-estradiol (10 μM) for 10 days. With continued culture, ectopic shoot formation occurred from the swollen root-tip region (day 21, right). Scale bars 1 cm.

WUS (Rashid *et al.* 2007), *NtWUS* (Rashid and Kyo 2009) or *WOX5* (this paper). Taken together, the results in this report suggest that the functional regulation of *WOX5* in transgenic tobacco may be similar to *WUS* or *NtWUS*, involving RAM cells from their identity in the process of swelling (transdifferentiation) and, finally, in the induction of adventitious shoot formation (developmental alteration).

Expression analyses of transcripts

We examined the expression of transgenes in different regions of *XVE::WOX5* seedlings, cultured on the same medium with or without β-estradiol for 7 days. As shown in **Fig. 3**, the inducible expression of *WOX5* was detected (amplified fragment size: approx. 660 bp) in all parts of transgenic seedlings after being treated with β-estradiol. In contrast, *XVE* was constitutively expressed (amplified fragment size: approx. 600 bp) in all parts of treated and untreated seedlings. These results indicate that treatment with β-estradiol induced *WOX5* in all regions of the seedling but, as described above, morphological abnormality was observed only in the root-tip region. We speculate that WOX5 was functional only in the root-tip region although gene expression was detected in all other regions of the treated seedlings (**Fig. 3**).

We also examined the subsequent changes at the molecular level, during the formation of swollen tissue in the root-tip region of *XVE::WOX5* seedlings. Within one week of treatment with β-estradiol, a detectable expression of *NtWUS* (amplified fragment size: approx. 1 kbp) was induced in the swollen tissue of the root but it was never possible to detect this in the root-tip region of control seedlings (**Fig. 3**). However, *NtWUS* was constitutively expressed in the shoot-apex region, containing the SAM, which is consistent with our previous observations (Rashid and Kyo 2009).

In the study carried out by Tamaoki *et al.* (1997), the mRNA localization of *NTH15* was detected at the predicted position of new-leaf formation in wild tobacco and it was, therefore, identified as a marker gene for the SAM region, in which ectopic expression caused abnormality in tobacco leaf morphology accompanied with increases in cytokinin level. In this study, we also investigated *NTH15* expression in different regions of *XVE::WOX5* seedlings by RT-PCR. The constitutive expression of *NTH15* (amplified fragment size: approx. 600 bp) was detected in the aerial regions (**Fig. 3**), similar to the previous report of Tamaoki *et al.* (1997). Interestingly, *NTH15* was inducibly expressed in the swollen root-tip region, after treatment with β-estradiol, indicating that cells specific to SAM were generated in the swollen tissue within 7 days of β-estradiol treatment (**Fig. 3**).

During globular embryo patterning, asymmetric cell division in the hypophysis generates the quiescent center (QC) at the upper side and the lower daughter cells give rise to the columella stem cells (Laux *et al.* 2004). It has been suggested that the early defects in QC development often lead to abnormal differentiation of the neighboring stem-cell precursors (Willemsen *et al.* 1998). After embryogenesis, the QC consists of a group of non-dividing cells and it is essential to maintain the pluripotent, undifferentiated, stem cells by local signalling (van den Berg *et al.* 1997; Sabatini *et al.* 2003). The QC is marked by the expression of *WOX5* (Wysocka-Diller *et al.* 2000; Haecker *et al.* 2004) and detailed investigations of the functional regulation of QC have revealed that it is analogous to that performed by the OC in SAM and both stem cell niches of the root and of the shoot pole share some molecular and developmental similarities (van den Berg *et al.* 1995; Malamy and Benfey 1997; Laux *et al.* 2004). Considering the available information, our results suggest that the ectopic expression of *WUS* (Rashid *et al.* 2007), *NtWUS* (Rashid and Kyo 2009) and *WOX5* (this paper) could induce a specific transition at the molecular level of root-tip cells which might facilitate the attainment of the potential for developing a SAM.

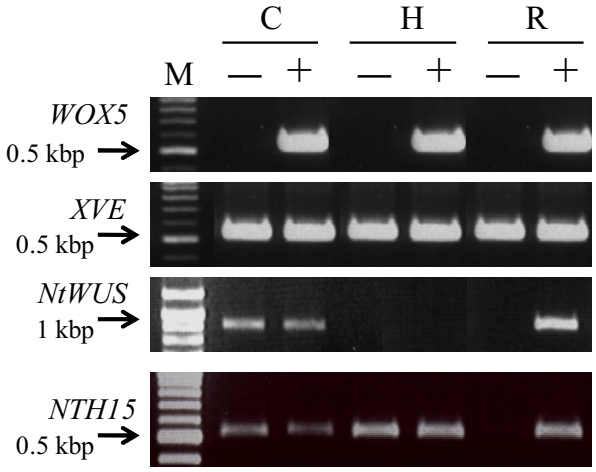

Fig. 3 Expression analyses of transcripts in *XVE::WOX5* seedlings. The one-week-old transgenic seedlings were treated in LS liquid medium in the absence (-) or presence (+) of the inducer, β-estradiol (10 μM) for 7 days. cDNAs were separately prepared from three types of segments: from cotyledons with SAM (C), from hypocotyls (H) and from roots with RAM (R). The corresponding names of the amplified transcripts with their approximate lengths are indicated at left. M: 100 bp DNA ladder.

WOX5 shares common motifs with WUS and NtWUS at their C-termini

Using the available sequence data supplied by DDBJ (http://www.ddbj.nig.ac.jp/), the amino acid sequences of WUS, NtWUS and WOX5 (**Fig. 4**) were analyzed. The open reading frame (ORF) of WOX5 consists of a short peptide sequence of 182 amino acids (Sarkar *et al*. 2007), whereas 292 and 309 amino acids are present in WUS (Kieffer *et al*. 2006) and NtWUS (Rashid and Kyo 2009), respectively. When they are aligned, their homeodomain regions generate 57.9% identities (**Fig. 4**). Kieffer *et al.* (2006) discussed the functional importance of three additional domains (acidic domain, WUS box and EAR-like motif) of WUS protein in *Arabidopsis* and its homolog ROA in snapdragon (*Antirrhinum majus*), located at their respective C-terminal regions. This paper has analyzed these three specific domains at the C-terminal region of the WOX5 protein. It was observed that the short peptide sequence of WOX5 also shared some identical residues to these three specific domains at the C terminal region: 62.5% in both acidic domain and WUS box region and 42.5% in the EAR-like motif region (**Fig. 4**).

A detailed analysis of the *Arabidopsis* database for WUS-related sequences revealed 14 ORFs of *WOX* genes that encode putative proteins with homeodomains sharing 38 to 67% identity and 62 to 87% similarity to the WUS homeodomain (Haecker *et al.* 2004). In that study, apart from the homeodomain region, sequence comparison between WUS and the WOX members in *Arabidopsis* revealed the existence of a common WUS box region, consisting of eight residues downstream of the homeodomain. Additionally, an acidic domain was identified upstream of the WUS box region in WUS and also in three additional WOX members including WOX5 (Haecker *et al.* 2004). Furthermore, the EAR-like motif was identified as one of the necessary functional units for the interaction of WUS with other proteins (Kieffer *et al.* 2006). This report shows that the C-terminal region of WOX5 also possesses a similar motif. It is noteworthy that NtWUS also shows some striking similarities with WUS (Rashid and Kyo 2009) and WOX5 (**Fig. 4**), specifically in these three additional domains in the C-terminal region. Taken together, it is suggested that the functional ability of WOX5 may be commonly associated with WUS due to the high similarities in their conserved short motifs at the C-termini. It is thus reasonable to assume that in tobacco cells, either WUS or WOX5 from *Arabidopsis* origin may contribute towards the

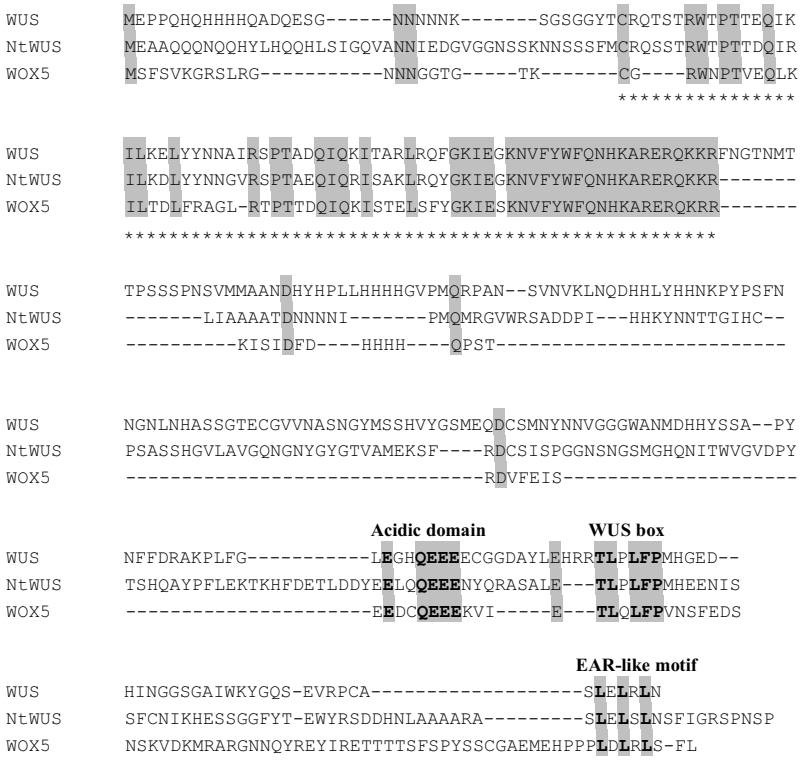

Fig. 4 Multiple alignment of the amino acid sequences of WUS, NtWUS and WOX5. Conserved nucleotides are boxed in black. The homeodomain regions are indicated by asterisks at the bottom panel. Three additional putative domains, the acidic domain, the WUS box and the EAR-like motif, are indicated in their respective tops of C-termini. Dashes correspond to gaps introduced to optimize the alignments.

induction of similar developmental alterations in the root-tip region by regulating a common specificity of interactions. As shown in **Fig. 4**, each of the C-terminal short motifs contains a repeat of one landmark residue: the glutamate (Glu, E) acidic-rich domain (ExxQEEE), leucine (Leu, L) repeat in both WUS box (TLxLFPxx) and EAR-like motif (LxLxL). The existence of such a similar one-repeat architecture with amino acid sequences of WUS (or, NtWUS) and WOX5 at their C-termini may dictate the specificities of their cellular functions.

CONCLUSIONS

In the study carried out by Sarkar *et al*. (2007), it was observed that the over-expression of the WOX5 gene under *WUS* promoter could rescue the premature termination of inflorescence meristems and occasionally floral meristems in the *wus-1* mutant line of *Arabidopsis*. In contrast, *WUS* cDNA expression under the *WOX5* promoter can completely restore QC and stem cells in the *wox5-1* RAM but can never induce adventitious shoots in QC. These results revealed that *WOX5* can substitute for WUS function, though providing a somewhat reduced level of OC signalling (Sarkar *et al*. 2007). Thus, our results also support the idea that ectopic expression of *WOX5* may complement a similar function such as WUS (or NtWUS) for the phenotypic alteration in the tobacco root-tip region under the control of the XVE system. However, the localization of *WOX5* mRNA in both distal and proximal root meristems after β-estradiol treatment and the analysis of the subsequent events, such as starch grain staining in columella cells, remain to be determined. Moreover, evident abnormality was not observed in the aerial regions of *XVE::WOX5* plants even after long-term treatment with β-estradiol (data not shown). This study, therefore, offers insight into the idea of a common regulatory mechanism involving *WOX5* and *WUS* that underlies the formation of tissue swellings in transgenic tobacco root tips. It favors the interpretation that these two regulators are interchangeable at least as far as the process of inducing adventitious shoot formation is concerned.

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