Analysis of Transgenic Tobacco with Overexpression of Arabidopsis WUSCHEL Gene

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Abstract: The Arabidopsis WUSCHEL (WUS) gene plays a key role in the specification of the stem cells in the shoot apical meristem (SAM). A cDNA of WUS has been amplified with the RT-PCR approach from Arabidopsis. The plant overexpression vector was constructed. It was driven by a dual enhanced CaMV35S promoter. The construct was transformed into tobacco (Nicotiana tabacum L.) via Agrobacterium mediation. Dramatic phenotypic changes appeared in the WUS overexpression transgenic plants. Aberrant cell divisions and ectopic organogenesis could be found in almost every aerial parts of the transgenic tobacco except the meristems and the inner two floral whors. The data showed a highly conserved function of WUS in tobacco, and suggested that WUS is involved in organogenesis. The leaves were malformed, which strongly matched those only described previously for plants grown in the presence of polar auxin transport inhibitors. It suggested a possible function of WUS in leaf development. These results provide useful information for functional analysis of WUS and important biotechnological implication as well.

Key words: WUSCHEL; Nicotiana tabacum; overexpression; phenotypic analysis

1 Materials and Methods

1.1 Plant and bacteria

Nicotiana tabacum L. cv. W38 and Arabidopsis thaliana L. Wassilewskija-2 ecotype, as well as bacteria of Agrobacterium tumefaciens strain GV3101 (pMP90) (Koncz and Schell, 1986) were used in this study.

1.2 Construction of overexpression vector

Total RNA was extracted using the TRIZOL kit (Gibco BRL, USA) from aerial parts of Arabidopsis plants. First-strand cDNAs were synthesized by reverse transcription kit (TaKaRa, Japan), open reading frame (ORF) of WUS was amplified using primers P1 (5’-TCTTGGTACCATGGACGCCGCCACAGCATCAG-3’) and P2 (5’-TCTTGGAGCTCTAGTTCAGACGTAGCTCAAG-3’), which were designed according to the sequence information of WUS (Mayer et al., 1998). The PCR products were cloned into pGEM-T vector (Promega, USA) and sequenced.

The observation of the constitutively overexpression of WUS in Arabidopsis is difficult, as it would preclude recovery of the seedlings (Schoof et al., 2000), an alteration is the use of the inducible system (Zuo et al., 2002). Here we constitutively overexpressed WUS in tobacco under the drive of the dual enhanced CaMV35S promoter. The transgene caused dramatic phenotypic changes, which provided useful information for functional analysis of WUS.
overexpression vector pBKB. The constructs were examined by PCR and KpnI/SacI double digestion. Plasmid extraction, digestion, electrophoresis, ligation and Escherichia coli transformation were according to Sambrook et al. (1989).

1.3 Plant transformation and identification

pBKB and pKAN-35S were introduced by electroporation into Agrobacterium strain GV3101. Transformation of tobacco leaf discs was performed as described previously (Horsch et al., 1985). The transformed plants were selected by kanamycin. Transgenic plants were transferred into greenhouse at about 25 °C under natural light.

The positive lines were identified with tissue PCR as described by Klimyuk et al. (1993). Level of WUS expression was detected by RT-PCR. Total RNA from leaf, stem and flower was obtained respectively with the same method mentioned above. After quantification one microgramme of RNA was used in every RT-PCR reaction with One Step RNA PCR kit (TaKaRa, Japan) and primers P1 and P2.

1.4 Phenotypic analysis

For conventional scanning electron microscopy (SEM), fresh materials were prepared as described by Chen et al. (2000), and examined with Hitachi S-2460 scanning electron microscope (Japan). The images were photographed on Lucky 120 films.

Other photographs were taken with Sony DSC-F707 digital still camera (Japan).

2 Results

2.1 WUS cloning and transformations

A cDNA of WUS gene was obtained with the RT-PCR method, which was identified as 899 bp in length with a 879 bp ORF. The construct of pBKB was confirmed by PCR and KpnI/SacI double digestion (data not shown). The transgenic plants screened by kanamycin were identified with PCR amplification (Fig.1A), and target fragments were obtained from transformed but not from control plants. It showed that the target gene had been integrated into the genome of transformed tobacco. The transcripts were detected further with RT-PCR (Fig.1B). Again the target fragments were obtained from each parts of transgenic plant detected but not the control plant.

2.2 Phenotypic characterization of transgenic tobacco plants

Eight independent lines of plants with ectopic WUS gene were obtained, they displayed a wide range of altered phenotype as early as at the in vitro regeneration stage. When the seeds were in-seminated directly in soil, almost all of the T1 progeny of transgenic plant showed severe defects and did not develop beyond the seedling stage. Some wild-type seedlings developed normally in T1 plants. This suggested that transgenic plants is lethal at young seedling stage. To overcome this difficulty, the transgenic seeds were germinated and cultured on MS medium till the seedlings had 4 leaves and the roots developed well. A higher viability of T1 plants was obtained by this method and their phenotype was consistent with T0 plants. The higher viability of T0 and T1 plants by in vitro culture could be due to the transgene having more defective effects on the seedlings and the adult shoots having a better tolerance.

After planted in soil for three months, ectopic lateral outgrowths appeared on the laminas, the stems, and in the leaf axils of WUS overexpressing plants (Fig.2D, F, H). During flowering phase, ectopic outgrowths also appeared on the receptacles and even the corollas (Fig.2J, L). It should be noted that some of the outgrowths could develop into shoot meristems or flower buds (Fig.2G, H, J). The transgenic plants had flowers with shorter filaments and styli, and the stigmas are smaller than that of wild-type, but none ectopic outgrowth could be found (Fig.2K, L).

The alterrance of leaves was also obvious. From the third or fourth leaf, the young leaves showed reduced expansion and upright position, subsequently, the laminas showed curled phenotypes and rolled up at their fringes, and the leaf vein pattern was also altered (Fig.2C). Sometimes conjoint leaves were formed, and trumpet-shaped
leaves were seen at in vitro stage (data not shown).

To character the ectopic cells described above, the leaf outgrowths were observed by SEM. Results showed that the outgrowths were leaf primordia-like, with small and dense cells resembled the meristematic cells (Fig. 3). The meristems of the plants were examined by histological sections and no evident histological differences were observed between the wild-type and transgenic plants.

3 Discussion

3.1 Overexpression of WUS in tobacco leads to ectopic organogenesis

Owing to the results above, WUS overexpression is sufficient to promote aberrant cell divisions and ectopic organogenesis de novo in differentiated tissue in tobacco (Fig. 2D−L). However, it is reported that WUS overexpression only induces aberrant cell divisions and embryonic cell clusters but not organogenesis in Arabidopsis (Gallois et al., 2002; Zuo et al., 2002). We consider the difference in the phenotypic effect of WUS in tobacco and Arabidopsis reported is due to an enough period of overproduction of WUS protein in tobacco, but not a difference in the molecular function of the WUS gene that maintains SAM activity as reported (Noriko et al., 2003), because the similar multiple shoots phenotype have been observed in Arabidopsis (unpublished data). This result suggested a new definition of the function of WUS in organ formation, that is, WUS is involved in organogenesis. This effect of WUS could also have important biotechnological implications for vegetative propagation from differentiated cells.

3.2 WUS and meristem cells

Several observations suggested that the size of the stem cell population in the SAM and the floral meristem of Arabidopsis are regulated by a negative feedback loop between WUS and CLAVATA3 (CLV3), the stem cell marker gene. In this loop, WUS activates the expression of CLV3, and CLV3 repress WUS expression (Brand et al., 2000;
Schoof et al., 2000). A initially similar self regulating circuitry is established between WUS and AGAMOUS (AG), the floral stem homeotic gene which plays a key role in floral meristem termination and specifies organ identity in whorls 3 (stamens) and 4 (carpels) (Bowman et al., 1989).

The transgenic plants have abnormal flowers with shorter filaments, stylis and smaller stigmas, which indicated an unclear effect of transgene on these two organs, but no ectopic outgrowth was seen (Fig.2L). Histological section analysis of transgenic plant showed that the cells of the meristem still positioned properly (data not shown). Therefore, in spite of the widespread expression directed by the 35S promoter, it seems that WUS can not promote excess cell division or organogenesis in the shoot apical and floral meristems. Similar meristematic phenotype was reported when WUS was overexpressed ectopically in Arabidopsis under the drive of inducible or meristem-specific promoters (Schoof et al., 2000; Lenhard et al., 2001; Lohmann et al., 2001; Zuo et al., 2002). It was known that the level of WUS expression was increased in mutants clv3 and ag because of loss of its suppressor. In these mutants, the enlarged shoot apical and floral meristems in clv3 and indeterminate flowers with papels in ag mutant formed (Bowman et al., 1989; Yanosky et al., 1990; Clark et al., 1995). One conceivable interpretation for this difference is that the similar repressors of WUS exist and functioning in tobacco, as CLV3 and AG in Arabidopsis, and this kind of suppression is strong. This is also proof of the high conservation of the function of WUS in tobacco.

3.3 WUS and auxin

The malformed leaves showed by the WUS-overexpressing plants matched those only described previously for plants grown in the presence of polar auxin transport inhibitors (Liu et al., 1993; Sieburth, 1999). Therefore, these changes probably result specifically from the loss of auxin polar transport or the decrease of auxin synthesis level due to widespread expression of WUS. This observation suggested that WUS may function non-cell-autonomously in leaf development, and auxin is mediated here. Previous study suggested that WUS regulates stem cells and integument initiation in the chalaza by a non-cell-autonomous way, but the mechanism is unknown (Mayer et al., 1998; Gross-Hardt et al., 2002), our results provide a possible hint for this research.

In summary, overexpression of the Arabidopsis WUS gene leads to aberrant cell divisions and ectopic organogenesis in tobacco, and these changes can not be found in the meristems and the inner two floral whorls, suggesting a new definition of the function of WUS in organ formation and a high conservation of its function in tobacco. The malformation of leaves indicated that WUS might function in leaf development and auxin polar transport may contribute to it.

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