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Inhibition of tobacco axillary bud differentiation by silencing CUP-SHAPED COTYLEDON 3

Jian Sun¹,², Haiyan Jia¹,², Xiaodong Wang¹, Xiaofan Yuan¹ and Bing Zhao¹*

¹National Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China.
²Graduate University of Chinese Academy of Sciences, Beijing 100049, China.

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Desuckering is a time-consuming, laborious and expensive process in tobacco production. To resolve this problem, pCAMBIA1301-RNAi (an RNAi vector) was constructed based on the conservative region of CUP-SHAPED COTYLEDON 3 (CUC3) in Apocynum venetum (AvCUC3), a key gene in the differentiation of axillary bud. Nicotiana tabacum has been transformed using vector pCAMBIA1301-RNAi mediated by AGL0 through the leaf disc method. A down-regulation (60 to 95%) of CUC3 mRNA level was observed in positive tobacco transgenic lines. Lines whose CUC3 gene had been suppressed exhibited some important phenotypes, such as leaf fusion, leaf curl and incomplete leaf. CUC3 in N. tabacum was silenced successfully by RNA interference (RNAi), which suggested a high homology between AvCUC3 and N. tabacum CUC3. An EST (FG152687) of N. tabacum that had the highest homology (72%) with AvCUC3 was found by retrieving GenBank database and this EST might be a fragment of CUC3 gene of N. tabacum.

Key words: Apocynum venetum, cup-shaped cotyledon 3 (CUC3), conservative region, RNA interference (RNAi), Nicotiana tabacum.

INTRODUCTION

Topping is an important agronomic practice in tobacco production that diverts the energy and nutrients absorbed by the plants to leaves rather than reproductive organs (Rao et al., 2003). Dormant axillary buds also called suckers, become active after topping (Taylor et al., 2007) and they must be removed periodically to gain full benefit of topping. Desuckering, which can be done by manual and chemicals methods (Taylor et al., 2007; Mahmood et al., 2007), is a time-consuming, laborious and expensive process. With the development of biotechnology, gene engineering has been widely applied in plant breeding and many transgenic tobacco lines were successfully inbred (Eltayeb et al., 2007; Lee et al., 2007). Therefore, desuckering is redundant if the differentiation of axillary bud could be suppressed by molecular genetics methods.

The formation of axillary buds are regulated by the activation of axillary meristems (AM) (Tantikanjana et al., 2001), which is largely determined genetically (Aida et al., 2002). The mutants defective in shoot branching makes it possible to isolate the genes related with AM formation. The well-known regulators for AM formation are Petunia NO APICAL MERISTEM (NAM) (Souer et al., 1996), Arabidopsis LATERAL SUPPRESSOR (LAS) (Greb et al., 2003) and Antirrhinum majus CUPULIFORMIS (CUP) (Weir et al., 2004). Another group of genes that possess the regulatory function of AM formation are CUP-SHAPED COTYLEDON (CUC) 1, 2 and 3 (Takada et al., 2001; Aida et al., 1997; Vroemen et al., 2003). All the three CUC genes are expressed in the boundary of shoot organ primordia and encode transcription factors of the NAC-domain class. Research towards Arabidopsis suggested that they are responsible for meristem formation throughout the life cycle (Hibara et al., 2006).

CUC genes can be subdivided into two clades, the NAM and CUC3 clade (Zimmermann and Werr, 2005; Adam et al., 2011). CUC1 and CUC2, which belong to
the NAM clade, are involved in the formation of AM through regulating the expression of LAS (Raman et al., 2008). CUC3, which was first isolated from Arabidopsis, was classified into the CUC3 clade. Same as eudicots, CUC3-related proteins in monocot species also expressed at the boundaries between organs and are functionally equivalent with eudicots (Zimmermann and Werr, 2005; Oikawa and Kyozuka, 2009; Adam et al., 2011). Its function is of high similarity and partially redundant with that of CUC1 and CUC2 (Vroemen et al., 2003). Unlike CUC1 and CUC2, CUC3 however promotes the formation of AM either through the regulation of LAS or via an LAS-independent pathway. Furthermore, the expression of CUC3 is not suppressed by miR164, a negative regulator of CUC1 and CUC2 (Raman et al., 2008). In view of the potential application in shoot-branching regulation, new tobacco varieties without suckers would be obtained if the endogenous CUC3 gene in tabacum could be suppressed. In the previous study, we cloned the conservative region of CUC3 in Apocynum venetum (AvCUC3) and constructed a RNA interference (RNAi) expression vector based on this conservative sequence (unpublished result). Because of the evolutionary conservation and high homology among CUC3 genes in different species, the RNAi expression vector may play its role in tobacco. In this study, this expressed vector was transformed to Nicotiana tabacum through leaf disc method using Agrobacterium AGL0 and phenotypes of the obtained transgenic tobacco were observed.

**MATERIALS AND METHODS**

**Plant materials, bacterial strains and vector**

Aseptic seedlings of A. venetum and N. tabacum were germinated in MS solid medium from the surface sterilization seeds according to the procedure of Caetano-Anollés et al. (1990). Agrobacterium tumefaciens strain AGL0 was inoculated in YEB liquid medium containing 50 mg Rif /L and shaken for 48 h at 28°C. The binary vector used in the experiment was pCAMBIA1301 containing hygromycin phosphotransferase (HPT) and β-glucuronidase (GUS) gene. A 35S CaMV promoter was inserted between XbaI and SacI of multiple cloning sites in pCAMBIA1301 and a terminator was inserted between SacI and EcoRI.

**Gene cloning and sequence analysis**

Total RNA was isolated from A. venetum using TRizol reagent (Invitrogen) according to the manufacturer’s protocol (Simms et al., 1993). Total RNA (1 mg) was reverse-transcribed using cDNA Synthesis Kit. Based on the conservative regions of the known CUC3, a pair of degenerate primers CUC3f (5'- ATTACYYTTTACCTAGCTTCAAAR-3') and CUC3r (5'- CCTTTGTAGAAAMACMARYGTYTTTCTTC-3') were designed. The PCR product was inserted into the T-vector and sequenced. Sequence similarity searches were performed using the BlastP programs with default parameters in protein sequence databases provided by the NCBI server. Multiple sequence alignments of similar sequences were carried out using Clustal X with default parameters (Thompson et al., 1994). The phylogenetic analysis was carried out by the neighbor-joining method and the phylogenetic tree was constructed using PHYLIP ver. 3.69.

**Construction of plant expression vector and plant transformation**

The AvCUC3 conservative region was inserted into pUCC-RNAi vector using XhoI and SpeI in antisense orientation, while the sense AvCUC3 conservative region was inserted into the same vector using BanHI and XbaI. The stem-loop structured fragment was double digested and further cloned into a binary vector pCAMBIA1301 (pCAMBIA1301-RNAi) and used for plant transformation (Zhai et al., 2008). pCAMBIA1301- RNAi (Figure 1) was then introduced into A. tumefaciens strain AGL0 and infected N. tabacum by the leaf disc method (Li et al., 2008). MS containing 0.1 mg NAA L<sup>-1</sup> and 1.0 mg BAP L<sup>-1</sup> was used as co-culture medium. Co-culture medium with hygromycin B 50 mg hygromycin B L<sup>-1</sup> and 500 mg carbenicillin L<sup>-1</sup> was used for screening and differentiation, while MS containing 0.2 mg IBA L<sup>-1</sup> and 500 mg carbenicillin L<sup>-1</sup> was used for rooting.

**Transformation detection by GUS histochemical staining and PCR**

To detect the expression of GUS, small pieces of N. tabacum leaves were immersed in staining solution (50 mM Na<sub>2</sub>PO<sub>4</sub>; pH 7.0; 0.1% Triton X-100; 0.1% X-Gluc) and vacuum-infiltrated before incubated at 37°C overnight. Samples were then decolorized with 95% ethanol and rinsed with water for several times before being observed under microscope. According to the sequence of HPT in pCAMBIA1301 vector, a pair of specific primers (HPTf: ACTCAACCAGGCAGCTCAG / HPTr: TTCTTTGGCCCTGGACG) was designed to detect T1 generation of transgenic plants. The size of PCR product was approximately 1100 bp.
Figure 2. Comparison of the deduced amino acid sequence of AvCUC3 conservative region with its homologues. Av, *Apocynum venetum*; Ac, *Aquilegia coerulea* (ACL14364); At, *Arabidopsis thaliana* (NP_177768); Ch, *Cardamine hirsuta* (ACL14365.1); Ph, *Petunia x hybrida* (BAF41989); Ps, *Pisum sativum* (ACL14366); St, *Solanum tuberosum* (ACL14367).

RT-PCR analysis of gene expression

To detect the silencing effect of RNAi on *N. tabacum* CUC3, semi-quantitative polymerase chain reaction (PCR) was performed. Total RNA was extracted from GUS positive plants and first strand of cDNA was generated by reverse transcription. Using reverse transcription products as a template, PCR was conducted with two pairs of primers (CUC3f / CUC3r and ACTINf: CAACTGGGACGATATGGAG / ACTINr: TACGGCCACTGCGTATAGG). The wild type *N. tabacum* was used as a control.

RESULTS

Sequence analysis

Sequence analysis showed that AvCUC3 conservative region was 287 bp, encoding 95 amino acids (Figure 2). The amino acid sequence alignment revealed that the AvCUC3 conservative region had a high sequence identity with known CUC3 proteins. AvCUC3 conservative sequence has 81, 78, 79, 81, 78 and 78% homology with CUC3 protein sequence in *Aquilegia coerulea*, *Arabidopsis thaliana*, *Cardamine hirsute*, *Petunia x hybrida*, *Pisum sativum* and *Solanum tuberosum*, respectively. Phylogenetic analysis was performed to examine the relationship between AvCUC3 and those of related proteins (Figure 3). As expected, AvCUC3 belongs to the CUC3 clade. Within the CUC3 clade, each species possesses only a single gene of this type.

Detection of transgenic *N. tabacum*

In order to verify the integration and expression of exogenous genes in T1 generations, PCR, GUS histochemical assay and RT-PCR were carried out. A pair of primers was designed according to the DNA sequence of HPT to detect the T1 transgenic plants. 30 positive transgenic lines were obtained in 40 independent lines using wild type as control (Figure 4a). GUS staining results (Figure 4b) showed that GUS gene was expressed in leaves of all positive transgenic lines. The blue dye was well distributed in wide regions over the whole leaf surface. However, control samples did not show any blue dye when they were tested with GUS assay (data not shown). Also, there were no differences in aseptic seedlings of T1 generation grown from day 25 to 40 (Figure 4b1 to 8). To test the inhibition effect of RNAi, We detected the tobacco CUC3 in transcriptional level by RT-PCR in all 30 positive transgenic lines. A down-regulation (60 to 95%) of CUC3 mRNA levels compared with wild type plant was observed in all lines (Figure 4c).

Analysis of transgenic tobacco phenotypes

Special phenotypes were observed in T1 generation transgenic plants. They were categorized into three classes according to their phenotypes as follows:
Figure 3. Phylogenetic analysis of the NAM/CUC3 proteins. Species' name abbreviations are as follows: Ac, Aquilegia coerulea; Al, Arabidopsis lyrata; Amt, Amborella trichopoda; At, Arabidopsis thaliana; Av, Apocynum venetum; Bo, Brassica oleracea; Ch, Cardamine hirsute; Cp, Carica papaya; Eg, Elaeis guineensis; Pd, Phoenix dactylifera; Ps, Pisum sativum; Pxh, Petunia x hybrida; Rs, Raphanus sativus; Sl, Solanum lycopersicum; St, Solanum tuberosum; Zm, Zea mays.

1) All seedlings in the first class (8 lines) showed a fusion of two or three leaves and we called this phenotype the leaf fusion phenotype. Leaves of this type shared one petiole with two or three distinct main veins (Figure 5A to
Figure 4. Detection of the integration and expression of exogenous genes in transgenic *N. tabacum* (only partial results were exhibited). (a) PCR identification of transgenic lines. Lanes 1 to 14 shows RNAi transgenic lines. WT Wild type; (b) GUS histochemical assay of transgenic *N. tabacum*: lanes 1 and 2, aseptic seedlings grown for 25 days; lanes 3 and 4, aseptic seedlings grown for 30 days; lanes 5 and 6, aseptic seedlings grown for 35 days; lanes 7 and 8 aseptic seedlings grown for 40 days; (c) RT-PCR analysis of the expression of *CUC3*: WT; Wild type; lanes 1 to 9 represent gene silencing by RNAi.

E). Individual lines even exhibited cup-shaped fusion, a complete fusion of the cotyledons at the base (Figure 5F). Except for the fusion leaves, the other leaves showed no significant differences compared with the wild type.

2) Seedlings of the second class (7 lines) exhibited leaf curling phenotype on the first and second pairs of leaves (Figure 4) and this phenotype was subdivided into three categories according to the curling directions and strength: a) Leaf with slightly inward curling (Figure 6A and B); b) Leaf with severe inward curling (Figure 6C and D) and c) Leaf with outward curling (Figure 6E).

3) Seedling of the third class (5 lines) displayed incomplete leaf phenotype on one (Figure 7A and B) or both sides (Figure 7C) of the leaf margin. Most lines had only one incomplete leaf and 2 to 3 incomplete leaves in one plant rarely occurred. The incompleteness was significant in comparison with wild type leaves (Figure 7D).
Figure 5. Leaf fusion phenotype of the transgenic plants. **A to D,** Fusion of two leaves; **E** fusion of three leaves; **F** fusion of cup-shaped cotyledon.

Figure 6. Leaf curling phenotype of the transgenic plants. **A, B** Leaf with slightly inward curling. **C, D** Leaf with severe inward curling. **E** Leaf with outward curling.
DISCUSSION

Degenerate PCR primers designed on the basis of conserved sequence of *CUC3* allowed the isolation of a gene fragment from *A. venetum*. Multiple sequences alignment and phylogenetic analysis result showed that the gene fragment shared the high similarity in amino acid sequence with known *CUC3* genes. The high identity proved that this gene fragment was derived from CUC3. Phylogenetic analysis based on the conserved NAC domain has shown that *CUC3*-related sequences belonged to an independent clade. In addition, *CUC3* was a single gene in all species that were examined so far (Vroemen et al., 2003; Zimmermann and Werr, 2005; Blein et al., 2008) and our results also confirmed this conclusion. This characteristic avoided the suppression of other genes in NAC domain class, when silencing the *CUC3* gene based on the complementary sequence.

Moreover, to inhibit the differentiation of axillary buds, we constructed an RNAi vector (pCAMBIA1301-RNAi) based on AvCUC3 conservative region and this vector was successfully transformed to tobacco cell mediated by AGL0. GUS staining was observed at different growth periods, which confirmed the expression of foreign gene in transgenic plants. However, the strength of the silencing effects varied from 60 to 95%, indicating that the copy number of exogenous gene may differ among tobacco transformants (Hobbs et al., 1990, 1993).

Transformed by pCAMBIA1301-RNAi vector, different phenotypes were observed in T1 generation of *N. tabacum*, such as leaf fusion, leaf curl and incomplete leaf. Since similar results of other plants were reported in *CUC3* mutation researches (Hibara and Karim, 2006; Weir et al., 2004) combined with the result of RT-PCR, we have good reason to believe that the *CUC3* gene was suppressed in the transgenic tobacco. The mechanism of the suppression was based upon the complementary sequence, which suggested the high homology between the *CUC3* gene of *N. tabacum* and *A. venetum*. To test this hypothesis, GenBank database was retrieved by Blastn programme using AvCUC3 conservative sequence as query sequence. An EST (FG152687) of *N. tabacum* that had the highest homology (72%) with AvCUC3 was found (Figure 8) and it may be a fragment of *CUC3* gene of *N. tabacum*.

In this study, the RNAi fragment expression is driven by a constitutive promoter (CaM35S), which would suppress the expression of *CUC3* gene at all developmental stages. An inducible promoter would be a better replacement, because the target gene will be expressed only when receiving the induction signal. Our study indicated that RNAi technology can suppressed the tobacco endogenous *CUC3* expression. Therefore, if combined with a wound inducible promoter in future...
researches, the differentiation of axillary buds in transgenic tobacco will be suppressed when topping is served as an induction signal.

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