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Full Length Research Paper

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

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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 inbreeded (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.

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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

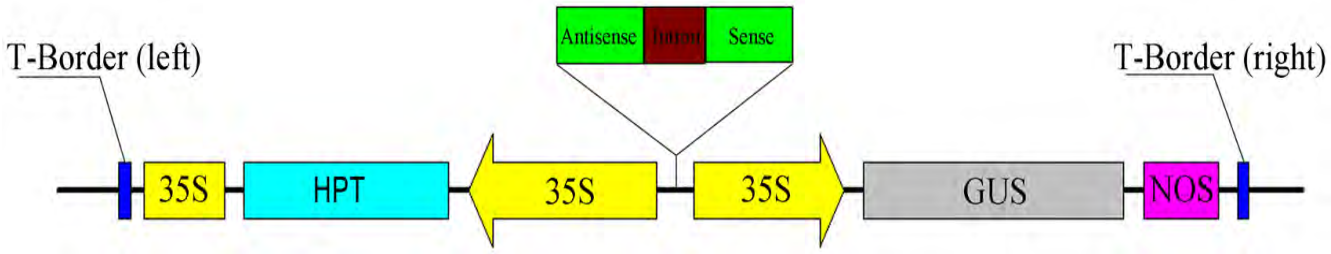

Figure 1. Construction of plant expression vector pCAMBIA1301-RNAi. HPT, hygromycin phosphotransferase; GUS, β-glucuronidase; NOS, nopaline synthase promoter, 35S 35Spromoter, Antisense-Intron-Sense Stem-loop structure.

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 *Xba*I and *Sal*I of multiple cloning sites in pCAMBIA1301 and a terminator was inserted between *Sac*I and *Eco*RI.

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 *CUC3*f (5′-ATTACYTTYTACTTAGCTTCCAAAR-3′) and *CUC3*r (5′-CCTTTGTAGAAMACMARYGTYTTCTTC-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 *Xho*I and *Spe*I in antisense orientation, while the sense *AvCUC3* conservative region was inserted into the same vector using *Bam*HI and *Xba*I. 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^{-1} and 1.0 mg BAP L^{-1} was used as co-culture medium. Co-culture medium with hygromycin B 50 mg hygromycin B L^{-1} and 500 mg carbenicillin L^{-1} was used for screening and differentiation, while MS containing 0.2 mg IBA L^{-1} and 500 mg carbenicillin L^{-1} 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_3PO_4, 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: ACTCACCGCGACGTCTG / HPTr: TTCCTTTGCCCTCGGACG) was designed to detect T1 generation of transgenic plants. The size of PCR product was approximately 1100 bp.

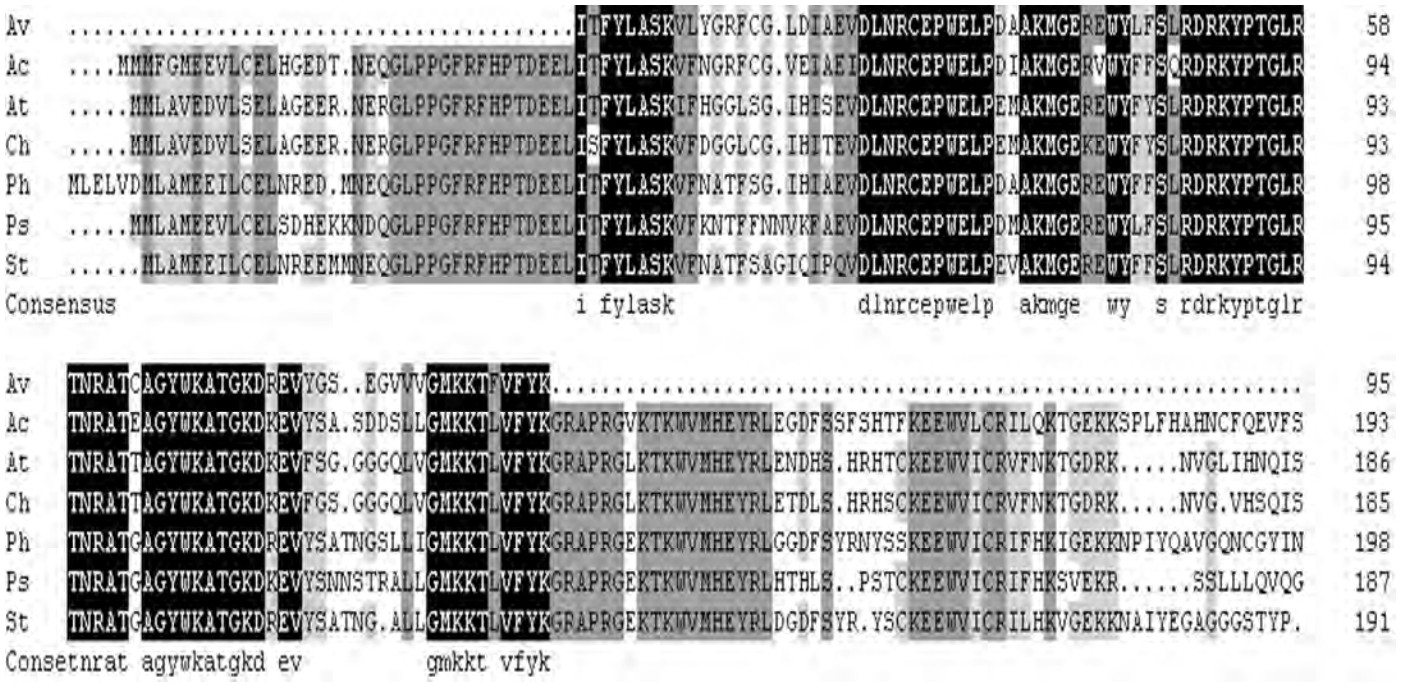

Figure 2. Comparison of the deduced amino acid sequence of *AvCUC3* conservative region with its homologues. Av, *Apocynum venetum*; Ac, *Aquilegia coerulea* (ACL14364); At, *Arabidopsis thaliana* (NP_177768); Ch, *Cardamine hirsuta* (ACL14365.1); Ph, *Petunia x hybrida* (BAF41989); Ps, *Pisum sativum* (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 (*CUC3*f / *CUC3*r and ACTINf: CAACTGGGACGATATGGAG / ACTINr: TACGGCCACTG-GCGTATAGG). 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:

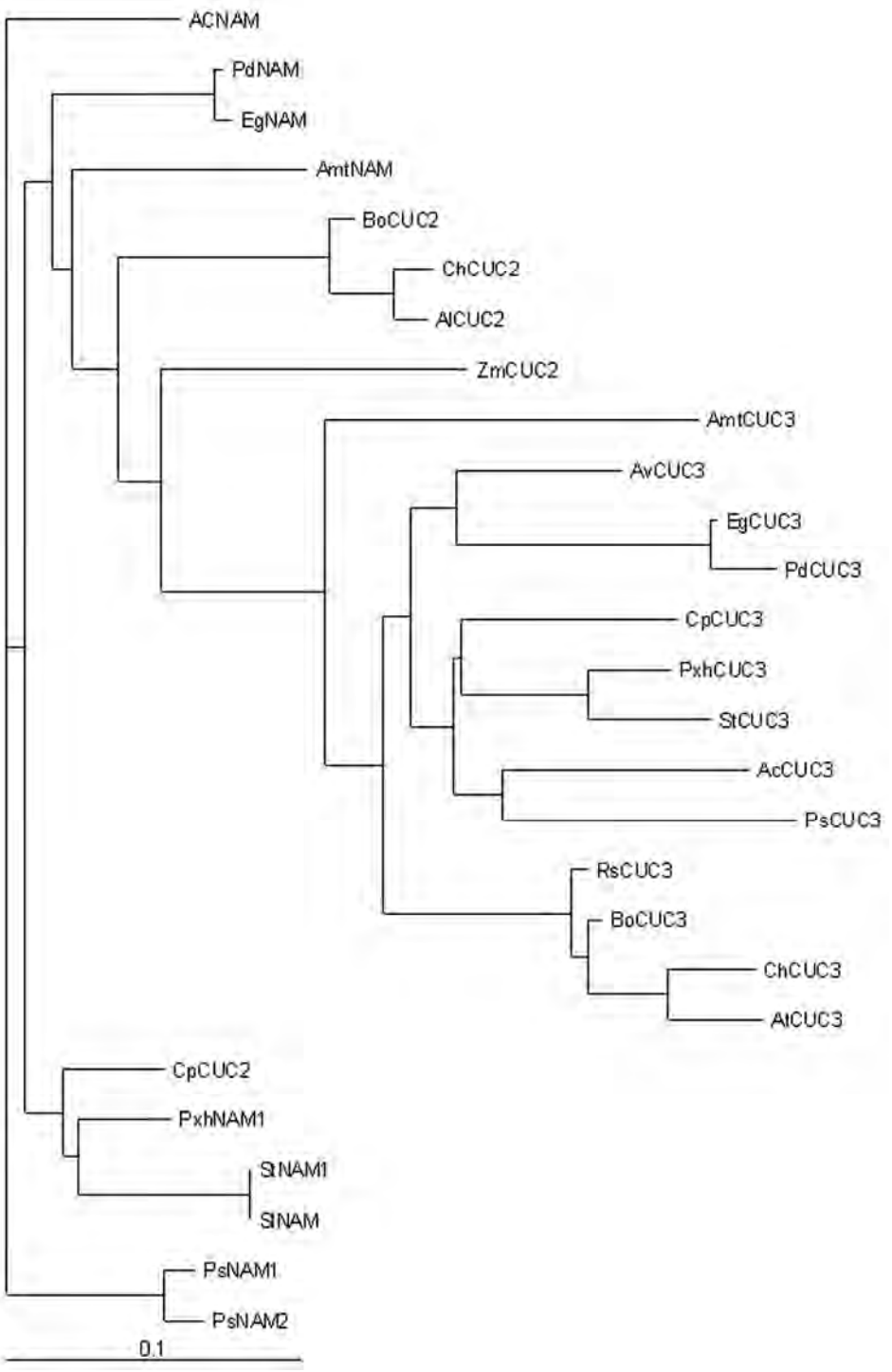

Figure 3. Phylogenetic analysis of the NAM/CUC3 proteins. Species' name abbreviations are as follows: Ac, *Aquilegia coerulea*; Al, *Arabidopsis lyrata*; Amt, *Amborella trichopoda*; At, *Arabidopsis thaliana*; Av, *Apocynum venetum*; Bo, *Brassica oleracea*; Ch, *Cardamine hirsute*; Cp, *Carica papaya*; 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

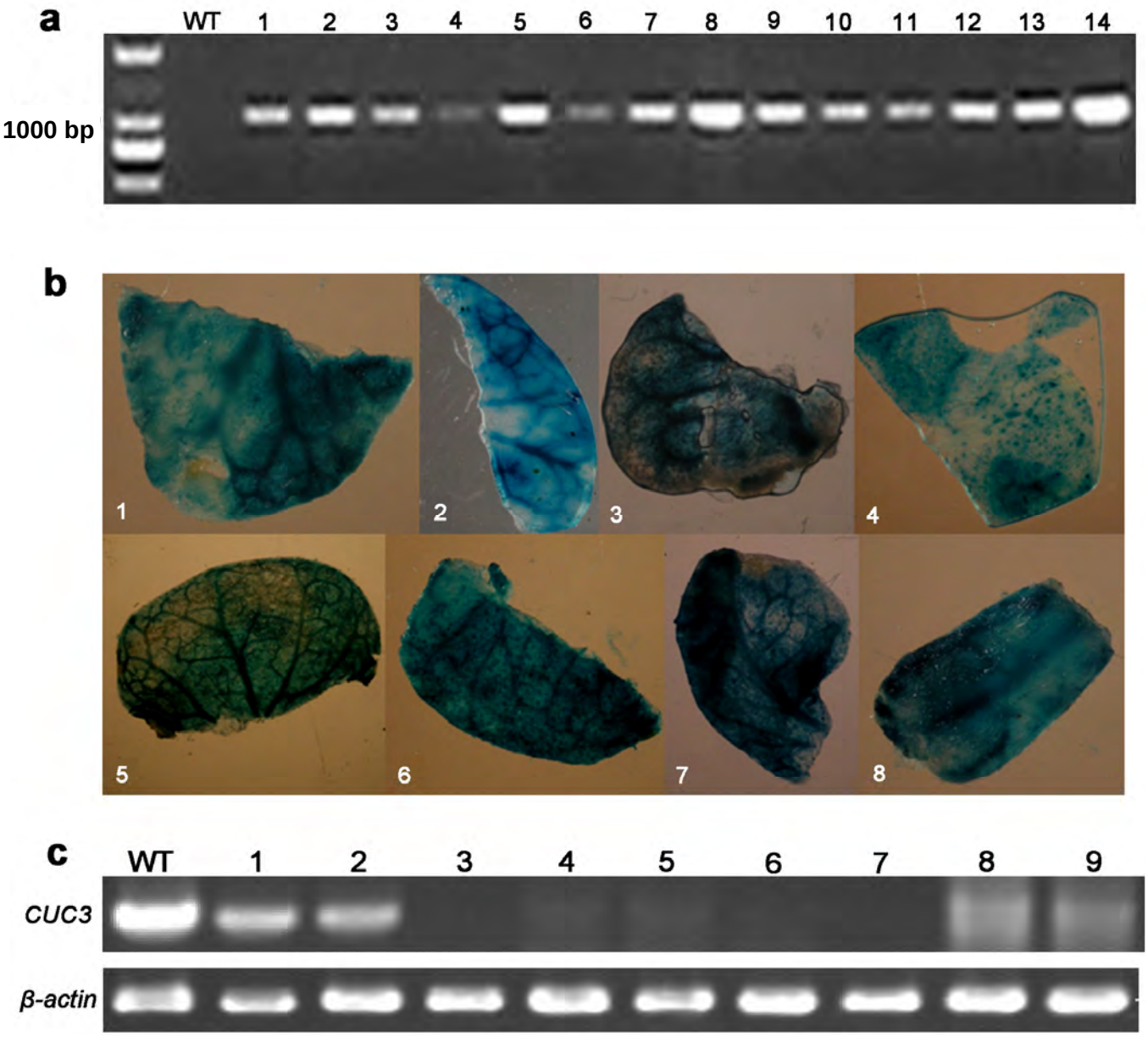

Figure 4. Detection of the integration and expression of exogenous genes in transgenic *N. tabacum* (only partial results were exhibited). (a) PCR identification of transgenic lines. Lanes 1 to 14 shows RNAi transgenic lines. WT Wild type; (b) GUS histochemical assay of transgenic *N. tabacum:* lanes 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).

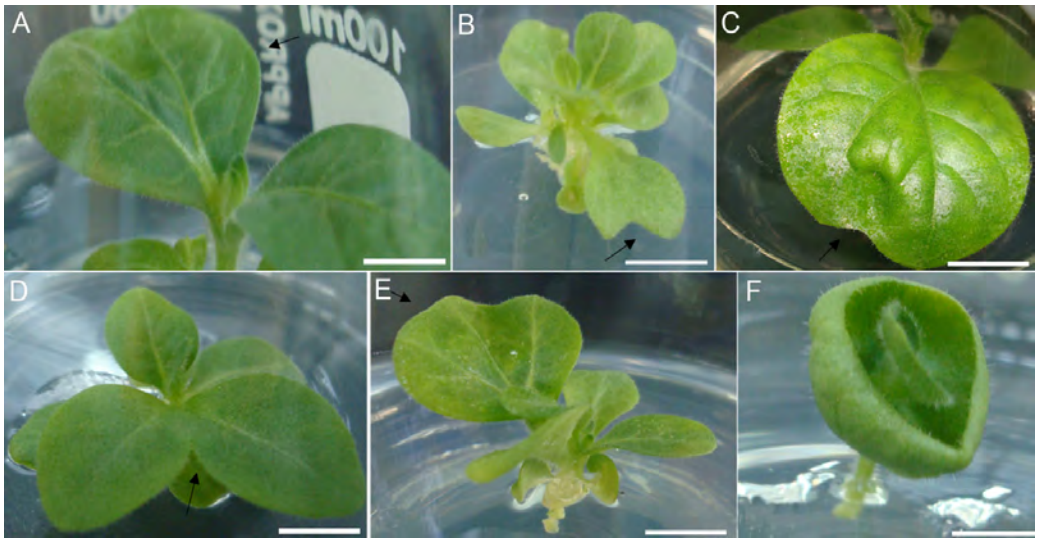

Figure 5. Leaf fusion phenotype of the transgenic plants. **A to D,** Fusion of two leaves; **E** fusion of three leaves; **F** fusion of cup-shaped cotyledon.

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.

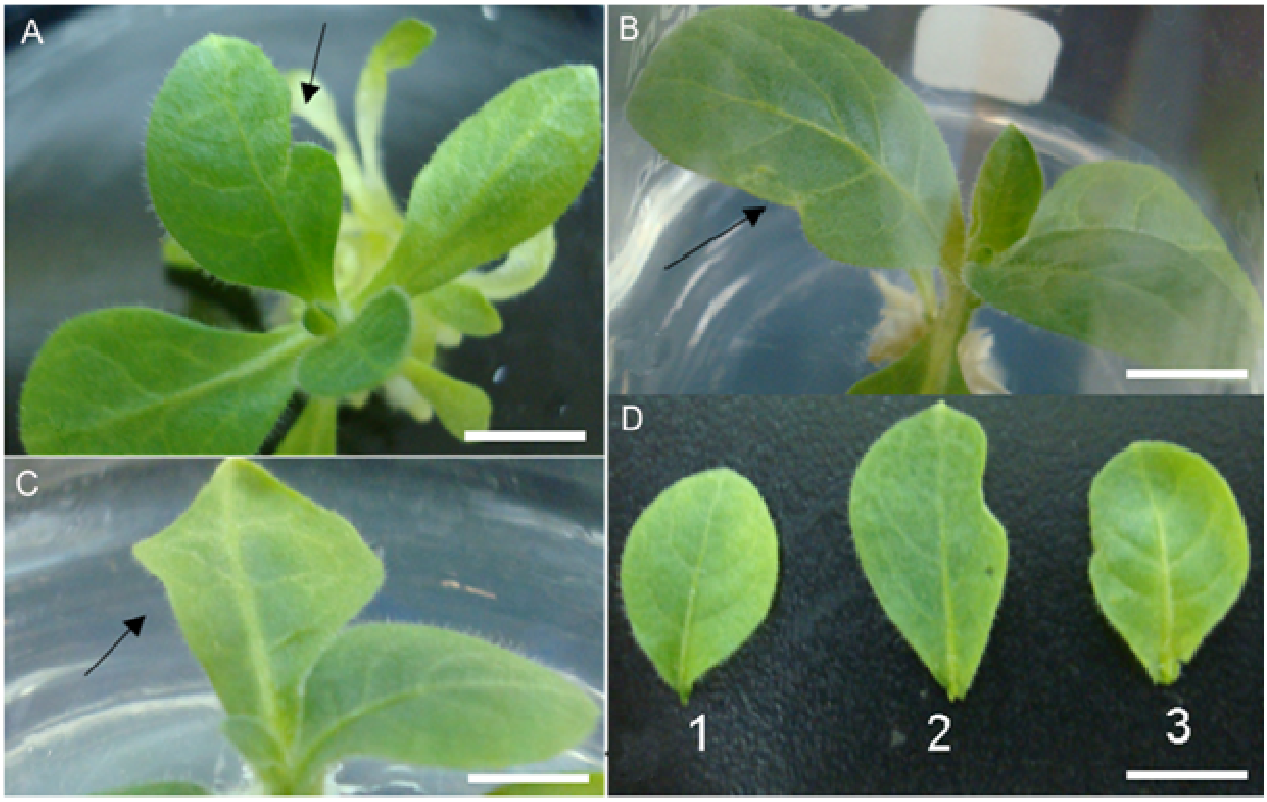

Figure 7. Incomplete leaf of the transgenic plants. **A and B,** Leaf incompleteness on one side; **C,** leaf incompleteness on more than one side; **D,** comparison between the transgenic plants and wild type (1: Wild type; 2 and 3: transgenic plants).

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 TI 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

Figure 8. Two sequences alignment between *AvCUC3* conservative region and an EST of *N. tabacum* (found by Blastn programme with *AvCUC3* conservative sequence in GenBank database).

researches, the differentiation of axillary buds in transgenic tobacco will be suppressed when topping is served as an induction signal.

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REFERENCES

Adam H, Marguerettaz M, Qadri R, Adroher B, Richaud F, Collin M, Thuillet AC, Vigouroux Y, Laufs P, Tregear JW (2011). Divergent expression patterns of *miR164* and *CUP-SHAPED COTYLEDON* genes in palms and other monocots: implication for the evolution of meristem function in angiosperms. Mol. Biol. Evol. 28(4): 1439-1454.

Aida M, Ishida T, Fukaki H, Fujisawa H, Tasaka M (1997). Genes involved in organ separation in *Arabidopsis*: an analysis of the *cup-shaped cotyledon* mutant. Plant Cell. 9(6): 841-857.

Aida M, Vernoux T, Furutani M, Traas J, Tasaka M (2002). Roles of *PIN-FORMED1* and *MONOPTEROS* in pattern formation of the apical region of the *Arabidopsis* embryo. Development, 129(17): 3965-3974.

Blein T, Pulido A, Vialette-Guiraud A, Nikovics K, Morin H, Hay A, Johansen IE, Tsiantis M, Laufs P (2008). A conserved molecular framework for compound leaf development. Science, 322(5909): 1835-1839.

Caetano-Anolles G, Favelukes G, Bauer W (1990). Optimization of surface sterilization for legume seed. Crop Sci. 30(3): 708-712.

Eltayeb AE, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahara T, Inanaga S, Tanaka K (2007). Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. Planta, 225 (5): 1255-1264.

Greb T, Clarenz O, Schäfer E, Müller D, Herrero R, Schmitz G, Theres K (2003). Molecular analysis of the *LATERAL SUPPRESSOR* gene in *Arabidopsis* reveals a conserved control mechanism for axillary meristem formation. Genes Dev. 17(9): 1175-1187.

Hibara K, Karim M, Rezaul T, Shinobu T, Furutani M, Aida M, Tasaka M (2006). Arabidopsis *CUP-SHAPED COTYLEDON3* Regulates Postembryonic Shoot Meristem and Organ Boundary Formation. Plant Cell. 18(11): 2946-2957.

Hobbs SLA, Kpodar P, DeLong CMO (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. 15(6): 851-864.

Hobbs SLA, Warkentin TD, DeLong CMO (1993). Transgene copy number can be positively or negatively associated with transgene expression. Plant Mol. Biol. 21(1): 17-26.

Lee YP, Kim SH, Bang JW, Lee HS, Kwak SS, Kwon SY (2007). Enhanced tolerance to oxidative stress in transgenic tobacco plants expressing three antioxidant enzymes in chloroplasts. Plant Cell Rep. 26(5): 591-598.

Li M, Li H, Jiang H, Wu G (2008). Establishment of a highly efficient *Agrobacterium tumefaciens*-mediated leaf disc transformation method for *Broussonetia papyrifera*. Plant Cell Tissue Org. Cult. 93 (3): 249-255.

Mahmood M, Shah S, Tariq M, Wadood A (2007). Efficacy of various suckericides for sucker control in flue-cured virginia tobacco. Pak. Tob. 25: 20-24.

Oikawa T, Kyozuka J (2009). Two-step regulation of *LAX PANICLE1* protein accumulation in axillary meristem formation in rice. Plant Cell. 21(4): 1095-1108.

Raman S, Greb T, Peaucelle A, Blein T, Laufs P, Theres K (2008). Interplay of *miR164*, *CUP-SHAPED COTYLEDON* genes and *LATERAL SUPPRESSOR* controls axillary meristem formation in *Arabidopsis thaliana*. Plant J. 55(1): 65-76.

Rao KN, Bangarayya M, Murthy Y, Babu DP (2003). Effect of topping at different growth stages on the yield and quality of fcv tobacco grown in northern light soils of Andhra Pradesh. Indian J. Plant Physiol. 8(1): 48-52.

Simms D, Cizdziel P, Chomczynski P (1993). TRIzol: A new reagent for optimal single-step isolation of RNA. Focus, 15(4): 99-102.

Souer E, van Houwelingen A, Kloos D, Mol J, Koes R (1996). The *no apical meristem* gene of Petunia is required for pattern formation in embryos and flowers and is expressed at meristem and primordia boundaries. Cell, 85 (2): 159-170.

Takada S, Hibara K, Ishida T, Tasaka M (2001). The *CUP-SHAPED COTYLEDON1* gene of *Arabidopsis* regulates shoot apical meristem formation. Development, 128 (7): 1127-1135.

Tantikanjana T, Yong JWH, Letham DS, Griffith M, Hussain M, Ljung K, Sandberg G, Sundaresan V (2001). Control of axillary bud initiation and shoot architecture in Arabidopsis

through the SUPERSHOOT gene. Genes Dev. 15(12): 1577-1588.
Taylor ZG, Fisher L, Jordan D, Smith W, Wilcut J (2007). Management of axillary shoot growth and maleic hydrazide residues with diflufenzopyr in flue-cured tobacco (Nicotiana tabacum). Tob. Sci. 47: 13-21.
Thompson J, Higgins D, Gibson T (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 (22): 4673-4680.
Vroemen CW, Mordhorst AP, Albrecht C, Kwaaitaal MACJ, de Vries SC (2003). The *CUP-SHAPED COTYLEDON3* gene is required for boundary and shoot meristem formation in *Arabidopsis*. Plant Cell. 15(7): 1563-1577.
Weir I, Lu J, Cook H, Causier B, Schwarz-Sommer Z, Davies B (2004). *CUPULIFORMIS* establishes lateral organ boundaries in *Antirrhinum*. Development, 131(4): 915-922.
Zhai J, Liu J, Liu B, Li P, Meyers B, Chen X, Cao X (2008). Small RNA-directed epigenetic natural variation in *Arabidopsis thaliana*. PLoS Genet. 4(4):e1000056.
Zimmermann R, Werr W (2005). Pattern Formation in the Monocot Embryo as Revealed by *NAM* and *CUC3* Orthologues from *Zea mays* L. Plant Mol. Biol. 58(5): 669-685.