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# Phenotype of Transgenic Tobacco Plants (*Nicotiana tabacum* cv. Petit Havana SR-1) Expressing *1724orf13* Gene of *Agrobacterium rhizogenes* strain MAFF301724

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*Abstract*

*Nicotiana tabacum* cv. Petit Havana SR-1 transgenic plants expressing ORF13 of *Agrobacterium rhizogenes* strain MAFF301724 under different promoters displayed plant morphology abnormalities. They were small, with short and variable internodes lengths; leaves were small, asymmetric, rounded, wrinkled and dark green; flowers were short, and irregularly shaped. This phenotype was also exhibited, similar, but not completely the same, to those of hairy root syndrome, indicating that expression of ORF13 influences plant development.

Keywords: ORF13, *Agrobacterium rhizogenes* strain MAFF301724, transgenic plants, morphology abnormalities

## Introduction

The T-DNA in *Agrobacterium rhizogenes* Ri plasmids is either integral or consists of two fragments, TL- and TR-DNA. The agropine-type *A. rhizogenes* strain A4 has two distinct T-DNA regions, designated TL-DNA and TR-DNA, which are separated in the Ri plasmid by a non-transferred region of about 15 kb (Jouanin, 1984; White *et al*., 1985). Regarding to the oncogenes on the pRiA4 TL-DNA, it was reported that the *A. rhizogenes* Ri plasmid TL-DNA contains four loci, rooting locus A, B, C and D (*rolA, rolB, rolC* and *rolD* genes) (White *et al*., 1985). In addition, ORF13, 13a, and 14 have been reported; however, their products have not been identified completely. Studies on transgenic plants containing various pRi T-DNA gene combinations have shown that three T-DNA genes, *rolA*, *rolB*, and *rolC* play a key role in hairy root induction on tobacco plants (Cardarelli *et al*., 1987; Spena *et al*., 1987). Among these genes, *rolB* plays a central role in inducing roots on most plant hosts. With regard to *orf13* gene, the nucleotide sequence of this gene is highly conserved in Ri plasmids (Hansen *et al*., 1991), and in *Nicotiana tabacum* and in *N. glauca* two plants counterparts were found (*torf13* and *NgORF13*) (Aoki *et al*., 1994; Frundt *et al*., 1998). ORF13 proteins have grouped in the plast (Levesque *et al*., 1988) or rolB family (Otten and Schmidt, 1998), defined on the basis of low and partial protein homologies. This group includes Agrobacterium pTi 6b T-DNA oncogenes, *rolB, rolC, iaaM*, and other less well-known T-DNA genes that remain to be investigated (Helfer *et al*., 2002; Levesque *et al*., 1988; Otten and Schmidt, 1998). Several reports showed that these genes cause the abnormal growth and morphology of roots and shoots (Cardarelli *et al*., 1987; Spena *et al*., 1987; Lemcke and Schmulling, 1998). The

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molecular mechanisms by which these genes cause abnormalities are unknown, except in the case of the *iaaM* gene.

The T-DNA of pRi1724 also contains the plant oncogenes *rolA, B, C, orf13,* and *orf14* (Tanaka et al., 1994), and *mikimopine synthase* (*mis*) genes (Suzuki *et al.*, 2001). However, there is no report about the *1724orf13*, so far. Therefore, a study of the expression of the *1724orf13* has undertaken. In order to investigate the expression of *1724orf13* in tobacco plants, the corresponding open reading frame of *1724orf13* that has been placed under the control of its native promoter, 35S promoter of the cauliflower mosaic virus, and the heat-shock promoter (HSP 18.2) was introduced into tobacco plant by using Agrobacterium-mediated transformation. Some transgenic tobacco plants have been produced, and this report showed the observations on these transgenic plants.

## Materials and Methods

### *Bacterial and tobacco plant strain*

*Agrobacterium rhizogenes* strain MAFF301724 containing mikimopine-type Ri plasmid (pRi1724) (Tanaka et al., 1994) was used as an origin of *1724orf13* gene. *A. rhizogenes* strain DC-AR2, a kanamycin-sensitive mutant derived from strain MAFF301724 (Tanaka et al, 1993a), and *A. tumefaciens* strain LBA4404 were used for binary vectors hosts. *Escherichia coli* strain DH5 was used for cloning. The resulting bacteria were used to transform / infect wild type *Nicotiana tabacum* cv. Petit Havana SR-1.

### *Plasmid construction*

The *1724orf13* with its own promoter and terminator was isolated from pRTE7.6 (7.6kb EcoRI fragment of pRi1724 containing core-T-DNA) (Tanaka et al., 1994) by digestion with *Sma*I and *Msc*I and was subcloned into pBluescript SK+. The *Sma*I-*Eco*RI fragment from this subclone was then inserted into the corresponding site in the binary vector pBIN19 (Bevan, 1984), resulting in pB13. This binary vector construct was introduced into *A. tumefaciens* strain LBA4404.

To construct orf13 driven by 35S promoter, the full sequence of *1724orf13* (594bp) was amplified from 7.6kb *Eco*RI fragment of pRi1724 (pRTE7.6) by polymerase chain reaction (PCR) using KOD-Plus-DNA polymerase (TOYOBO Co., Osaka, Japan) with a set of primer (5'-ATCCATGGCTCGTTATTTCGGC-3' and 5'-ATGCGGCCGCTA TTCCAACAGG-3' (GENSET KK, Kyoto, Japan; underlining indicate *Nco*I and *Not*I sites). The amplified fragment was double-digested with *Nco*I and *Not*I and then inserted into the corresponding site in pUC18, between the CaMV35S promoter and the nopaline synthase gene (*nos*) terminator. A DNA sequencing was performed, to confirm the full nucleotide sequence of *1724orf13*, using DYEnamicTM ET terminator cycle sequencing premix Kit, and a set of primers (5'-ACTATCCTTCGCAAGACC-3' and 5'-GGCAACAGGATTCAATC-3') (GENSET KK, Kyoto, Japan). A 1.2kb *Hind*III-*Eco*RI fragment containing the CaMV35S promoter, full sequence of *ORF13* and the *nos* terminator was transferred into the binary vector pBIN19. The binary vector construct (pB13.35S) was introduced into *A. tumefaciens* strain LBA4404 by electroporation.

Construction of orf13 driven by heat-shock promoter (HSP 18.2) was performed as follows: a fragment containing HS 18.2 promoter sequence was amplified from pTT101 by polymerase chain reaction (PCR) using KOD-Plus-DNA polymerase (TOYOBO Co., Osaka, Japan) with a pair of primers, M13 reverse (5'-CAGGAAACAGCTATGAC-3') and M13 forward (5'-GTTGTAAAACGACG-

GCCAGT-3') primers (GENSET KK, Kyoto, Japan). The amplified fragment was double-digested with *Hind*III and *Xba*I and then inserted into the corresponding site in pB13.35S, after removing the CaMV35S promoter fragment. The resulting construct was named pB13.HSp and was introduced into *A. tumefaciens* strain LBA4404 by electroporation.

*Plant cells transformation and growth conditions*

The resulting bacteria were used to transform/infect wild type *Nicotiana tabacum* cv. Petit Havana SR-1 by the leaf disk method. Aseptic tobacco plants germinated from the seeds sterilized with 10% sodium hypochlorite were cultured on 1:4 strength of Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) solidified with 1% agar. To obtain and cultivate transgenic tobacco plants, the method described by Suzuki et al. (2001) was employed, as follows: tobacco leaf segments (ca. 8 mm x 8 mm) excised from the aseptic plants were inoculated with an overnight culture of *A. tumefaciens* harboring pB13, pB13.35S and pB13.HSp vectors treated with 10 uM acetosyringone and cultured on MS medium containing 1 mg/L cytokinine, 0.1 mg/L auxin, and 100 mg/L kanamycin sulfate solidified with 0.2% Gelrite (Wako Pure Chemical Industries, Osaka, Japan). To eliminate Agrobacterium, 500 mg/L vancomycin (Sigma Chemical Co., St Louis, MO, USA) and 500 mg/l carbenicillin (Sigma Chemical Co.) were added to the MS medium. Shoots obtained from the above plates were transferred into MS medium supplemented with claforan (500 mg/L), kanamycin (200 mg/L) with no plant growth regulators and cultured in the 16 hours photoperiod at 25°C.

*RT-PCR analysis*

Total RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) analysis were performed as described previously (Tanaka et al., 1998). One m of total RNA treated with RQ1 RNase-free Dnase (Promega, Madison, WI, USA) and a 17-mer oligo-dT primer were mixed and added with ReverTraAceR reverse transcriptase (TOYOBO Co.). The reaction mixture was then incubated at 42°C for 1 hr to synthesize complementary DNA (cDNA) pool. The cDNA pool was then used as template to amplify a part of 1724orf13 cDNA (205 bp) using a set of primers (5'-CTTGCCAATTGCCAGTATGG-3' and 5'-GCTCGTTATTTCGGCAGTAG-3') with ExTaq polymerase (Takara Bio Inc.). As a control, a part of b-tubulin 1 cDNA (187 bp) was amplified from the same cDNA pool as a template using a set of primers (5'-GATGGAGTTCA CCGAGGCAGAA-3' and 5'-TGTCAATGCCAAAGGACCA TCA-3'). Thirty PCR cycles at 98°C for 10 sec, 56°C (for *1724orf13*) or 64°C (for b-tubulin 1) for 30 sec, and 72°C for 30 sec were performed. The amplified DNA fragments were electrophoresed in the 2 % agarose gel. The electrophoresis images were imported to personal computer by CCD video camera system (ATTO, Tokyo, Japan) using Densitograph software (ATTO) and brightness of amplified bands were analyzed by NIH image software (National Institute of Health, USA).

Results

The 1724orf13 transgenic plants showed a variety of characteristic alterations. The over expression of *ORF13* gene under the control of CaMV35S promoter resulted in slowly grow shoots with necrotic, and the shoots died after several days. It seems that the over expression inhibited the development of the shoot. The abundant products of *1724orf13* gene probably caused toxicity to the cells.

The expression of *1724orf13* on tobacco plant under the control of its native promoter shows alterations in morphogenesis and development. One of the independently transformed lines for *1724orf13* under the control of its native promoter (L1np) exhibited developmental abnormalities. The plant was dwarf, probably due to a reduced internodes length (Figure 1A and B). The leaves were wrinkling and round with alteration on leaf apex (Figure 1C, D and E) and leaf margin (Figure 1D). They were smaller than those of untransformed tobacco plants (Figure 1C, right). The leaf apexes of the transgenic plants were folded, which seems caused by inhibited development of veins or midrib, while the leaf blade continually grew (Figure 1C, D and E). Besides, formation of spikes (protrusions between minor veins) on both adaxial (Figure 1D) and abaxial (Figure 1E) of leaves were also found.

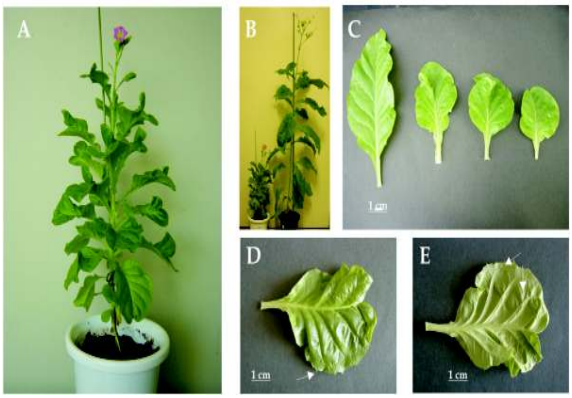


Figure 1. 1724orf13 transgenic tobacco plants under the control its native promoter. A and B right: transgenic plants; B left: untransformed tobacco plant; C, D, and F: leaves of transgenic plant and those of untransformed one (right); D: Spikes formation on the adaxial and abaxial (E) of leaves.

Flowers of the transgenic had shorter flower tube (Figure 2E) compared to the flower of untransformed plants, with short stamens (Figure 2A, B, D and F lower). Moreover, petals that grew outside from the sepals were also observed (Figure 2E). Concerning to the spikes, Stieger *et al.* (2004) shows transverse sections of tomato leaves expressing *orf13* T-DNA *A. rhizogenes* that were made at the site of spike formation showed the palisade parenchyma consisted of multiple cell layers with isodiametric cells, whereas in the control leaves the palisade parenchyma consisted of one cell layer with elongated cells.

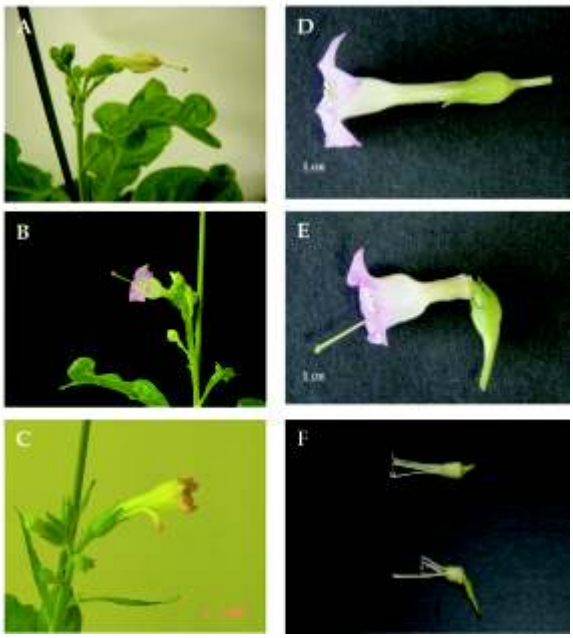


Figure 2. Flower of 1724orf13 transgenic tobacco plants under the control its native promoter. A, B, E, and F lower, are flowers of transgenic plants, which show shorter staments. C, is asymmetrical flower of transgenic plant. F, is flower of untransformed (upper) and transgenic (lower) plants after removing the petals and sepals.

The other independent line for *1724orf13* with its native promoter (L2np) showed wrinkled leaves with similar spikes formation on adaxial of leaves. This line grew normally as the untransformed plants, and did not show dwarf phenotype. Height and internodes length were much the same as those of the control (untransformed) plants. From these transgenic plants, it was

found that at the flowering time showed the asymmetric flowers (Figure 2C).

Transformed lines for *1724orf13* under the control of heat-shock promoter (HSP) also showed normal growth as the untransformed plants. These transgenic plants had leaves which were increased the length-width ratio (Figure 3B to D) with petioles that fused to the main stem. It was also clearly observed formation of spikes on the adaxial of leaves (Figure 4A), and it frequently was also formed on petals and sepals (Figure 4B). The transgenic plants also showed alteration morphology of the flower (Figure 3F) compared to the control (Figure 3E).

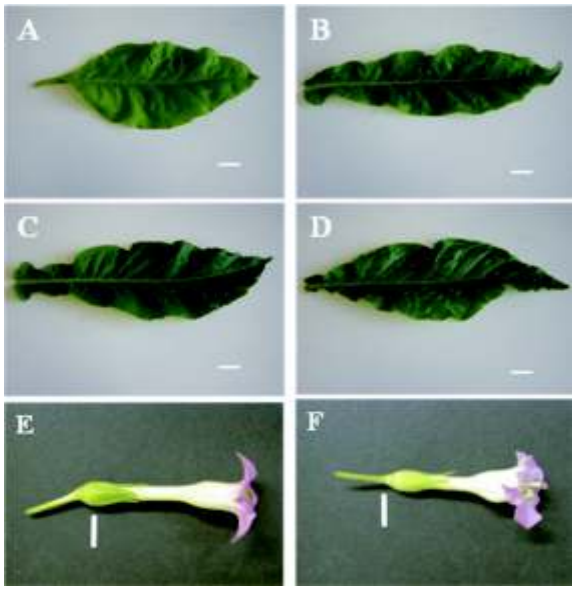


Figure 3. The Transgenic leaves show increasing the length-width ratio (B), (C) and (D). (A) and (E) are leaf and flower of untransformed plant. (F) is flower of transgenic plant. *Scale bars 1 cm.*


Figure 4. Spikes formation. The spikes were found on leaves (A), and also on flowers (B). Scale bars 1 cm

## Discussion

Transgenic plants expressing the *1724orf13* in this study showed some morphology changes similar, but not completely the same, to those of hairy root syndrome, which are exhibiting dwarfism, reduced internodes length, wrinkled leaves, and altered flower morphology (David *et al.*, 1984; Tepfer, 1984). Previous studies suggested that transformation of plants with the *rolA*, *rolB*, *rolC*, or *ORF13* genes individually causes distinct developmental abnormalities and do not show all phenotypic abnormalities of hairy root syndrome. Therefore the synergistic actions of these genes might result in the full phenotype of hairy root syndrome (Schmulling *et al.*, 1988; Lemcke and Schmulling, 1998). Moreover, a study for the ORF13 promoter of pRi8196 showed that the ORF13 promoter is wound inducible (Hansen *et al.*, 1997). Interestingly, without over expression of the gene it could be observed those alterations in the low level expression of the gene. The morphology alterations of an independent line transgenic plant expressing *1724orf13* under control of native promoter showed severe phenotype, although the expression level detected by RT-PCR analysis showed comparable level to other lines (Figure 5 Lane 1).

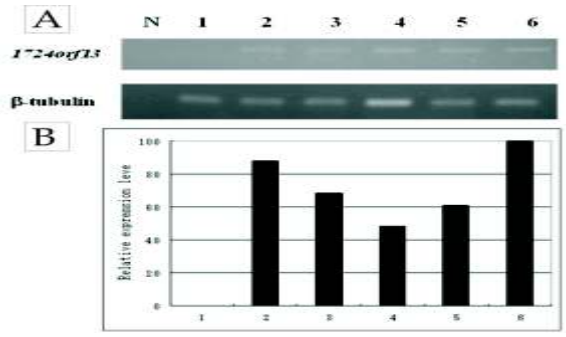


Figure 5. Expression of *1724orf13* in *1724orf13*-transgenic tobacco plants.
A. Accumulation of *1724orf13* mRNAin the leaves of *1724orf13*-transgenic tobacco plants by RT-PCR analysis. A 589 bp of *1724orf13* and ?-tubulin 1 cDNA(187 bp) as an internal control.

B. The graph which indicates the relative expression level (%) of *1724orf13* gene in the leaves of transgenic plant, normalized by the expression level of b-tubulin 1 gene used as a standard. Control is an untransformed plant. Transgenic is *1724orf13*-transformed plant. Lane 1, control; Lane 2, transgenic line np-1; Lane 3, transgenic line np-2; Lane 4, transgenic line hsp-1; Lane 5, transgenic line hsp-2; Lane 6, transgenic line hsp-3.

It had been reported that transformation with the ORFs 13 and 14, in addition to the rolA, B, and C, is necessary for root induction on carrot root disks but not on tobacco plants (Capone, *et al.*, 1989). Therefore, an auxin-like effect has been suggested for ORF13 and ORF14. However, both ORF13 and ORF14 show no homology with auxin biosynthesis genes. Over expression of orf13 homologue of mannopine strain *A. rhizogenes* 8196 in tobacco plants induced altered phenotypes such as round and wrinkled leaves, irregularly shaped flowers, and reduced root system (Hansen *et al.*, 1993). Orf13 of *A. rhizogenes* strain HR1 transgenic plants showed a variety of characteristic alterations include the irregular formation of leaves, irregular leaf venation and a reduced cell number and cell size in the root (Lemcke and Schmulling, 1998). The results indicate that the biological activities of the different ORF13 proteins have been largely conserved, suggesting a relevant role of these gene products for *A. rhizogenes.*

As the conclusion of this research, each transgenic plant expressing *1724orf13* under control of different promoters gave morphology alterations, but the alterations of each line were not completely the same. There were also variations in the morphology of the 1724orf13 transgenic lines obtained from transformation of Agrobacterium containing same plasmid construct. This phenomenon seems to be due to variations in site of insertion of the T-DNA in the genome of transformed cell and variations in the copy number of the T-DNA. Further investigation for gene expression using more lines of 1724orf13 transgenic plants is necessary to get clear *1724orf13* characteristics.

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