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Novel Marker Gene for Assessment of Behavior of Transgenic Plants in the Field

Masanori TAMAOKI[1]*, Yumio TODA[2], Nobuyoshi NAKAJIMA[1], Akihiro KUBO[2], Mitsuko AONO[2] and Hikaru SAJI[2]

[1] *Biodiversity Conservation Research Project and* [2] *Environmental Biology Division, National Institute for Environmental Studies, 16–2 Onogawa, Tsukuba, Ibaraki 305–8506, Japan*
**Corresponding author* E-mail address: mtamaoki@nies.go.jp

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Abstract

The wide use of transgenic crops has raised some concern about their escape into the field and movement of transgene to unintended hosts. Thus, it is essential to keep track of the transgenic plants in the field. Here, we show that alteration of leaf morphology, which is caused by *knotted1*-type homeobox genes can be a useful marker. We examined transgenic tobacco expressing the homeobox gene that exhibited 'curved', 'wrinkled', or 'dwarf' phenotype whose foliar malformations were easily distinguished from wild-type plants with the naked eye. Expression of the homeobox genes could have various effects on some growth parameters of the host plants. However, the growth of 'curved'-type transgenic plants was not much different from that of wild-type plants, suggesting that the 'curved' phenotype is a useful marker for assessing the whereabouts of transgenes/transformants in the field.

Key words: homeobox gene, leaf malformation, plant growth, risk assessment, transgene movement.

The use of transgenic plants in agriculture and phytoremediation has raised concern about their possible escape from the initial site into the natural field and/or introgression of transgenes into wild relatives through hybridization (Raybould and Gray, 1993). The latter may promote the adaptation of wild-type plants to the natural environment, allowing them to propagate in that environment (Raybould and Gray, 1993; Stewart, 1996). Therefore, it is important to develop a system for monitoring transgenic plants, that is widely applicable and suitable for use in the field. Use of antibiotic- or herbicide-resistance genes as transgenic markers is not suitable for commercial-scale agriculture or phytoremediation because it requires destructive plant tissue sampling and/or time-consuming assays. A system that allows non-destructive detection of the transgene in live plants would be more desirable. Brian *et al.* (1999) proposed the green fluorescent protein (GFP) as a marker for monitoring the transgene in live plants. Although GFP has many advantages over other available markers, it has some faults as an *in vivo* marker for monitoring transgenic plants: One is that the detection of GFP requires some equipment such as UV light illuminator and cut-off filter, and the other is that the fluorescence of GFP can only be observed in the dark.

To overcome these problems, we developed a novel marker for assessment of diffusion of transgenic plants in the field. Here, we propose the use of the *knotted1*-type homeobox gene as a marker for monitoring transgenic plants. This type of homeobox gene was first identified in maize (Vollbrecht *et al.*, 1991), and over-expression of the gene altered leaf morphology in many plants (Lincoln *et al.*, 1994; Tamaoki *et al.*, 1997, 1999; Sentoku *et al.*, 1998; Nishimura *et al.*, 2000). The alteration in leaf morphology is visually distinguishable, making it potentially useful for monitoring transgenic plants in the field. However, for this purpose, introduction of the homeobox gene should have little effect on plant growth. In this study, therefore, we have investigated various growth parameters of transgenic tobacco plants. The advantages of homeobox genes as a marker for monitoring transgenic plants are discussed.

The transgenic tobacco plants used here were generated through the introduction of *CaMV35S::NTH* chimeric constructs, using six kinds of tobacco homeobox genes, *NTH1, NTH9, NTH15, NTH20, NTH22* and *NTH23* (Tamaoki *et al.*, 1997, 1999; Sentoku *et al.*, 1998; Nishimura *et al.*, 2000). Tobacco seeds were sterilized in 5%

sodium hypochloride for 5 min and germinated on a medium [MS salts (Sigma-Aldrich, MO, USA) with 1% sucrose and 0.5% gelangum] under continuous light at 25 ℃. The seedlings were then transplanted to soil and grown at 25 ℃ in a 16-h light/8-h dark cycle. Thirty-two transformants formed abnormal leaves that were classified into three groups (**Fig. 1A**): 'curved (C; 19 plants)', 'wrinkled (W; 9 plants)', and 'dwarf (D; 4 plants)'. Distribution of these phenotypes in transgenic plants carrying *35S::NTH* genes was shown by Nishimura *et al.* (2000). Among these plants, plants transformed with *35S::NTH1*, *35S::NTH20* and *35S::NTH15* were used to investigate 'curved', 'wrinkled' and 'dwarf' phenotype, respectively. Although the degree of leaf malformation differed with the transformant, the transgenic plants could easily be distinguished from the wild-type plants by the leaf morphology (**Fig. 1B**). To assess the inheritance of abnormal leaf morphology with the transgene, we generated F1 hybrid plants between wild-type and transgenic tobacco plants with curved leaves. The F1 hybrid plants had leaf malformations similar to those of the parental transgenic plant (**Fig. 1C**). Plants were analyzed for the presence or absence of the transgenes by PCR. Genomic DNA was isolated from leaves by using Nucleon PhytoPure systems (Amersham Biosciences, Piscataway, NJ, USA), and PCR was performed in a 20 μl reaction mixture containing 10 ng of genomic DNA in a DNA Thermal Cycler (Applied Biosystems, Foster City, CA, USA) programmed for 25 cycles at 94 ℃ for 30 s, 55 ℃ for 30 s, and 72 ℃ for 1 min. Primers for detecting the presence of *35S::NTH1* transgenes were 5'-GATCTAGTAACATAGATGAC-3' and 5'-AACCTTCAGAGGATATGCAG-3'. As a result of PCR analysis, F1 hybrid plants also had transgene, suggesting that the abnormal leaf phenotype in transgenic plants was dominantly inherited with the transgene to their progenies. This indicates that the leaf alteration caused by introducing *35S::NTH* constructs is a useful marker for detecting the propagation of the transgene as well as transgenic plants in the field. The GFP has also been demonstrated to be suitable as an *in vivo* marker for monitoring transgene movement (Brian *et al.*, 1999). However, the fluorescence of GFP is hardly detectable even under UV light. By contrast, no equipment is needed to detect the alteration of leaf morphology and such a phenotype can be investigated under white light, suggesting that the homeobox gene is a more useful marker than GFP for assessment of transgenic plants in the field.

The fresh weight, height, flowering time, flower number and total seed weight of the host plants were measured and compared between wild-type and transgenic plants to determine whether the transgene had any side effects. The average plant fresh weight at 86 days after sowing in wild-type, 'curved', 'wrinkled' and 'dwarf' plants was 116.7 ± 7.0, 92.0 ± 5.9, 45.9 ± 8.8 and 5.5 ± 0.3 g, respectively (**Fig. 2A**). In addition, the average plant height at the end of flowering in wild-type, 'curved', 'wrinkled' and 'dwarf' plants was 101.1 ± 3.6, 126.4 ± 3.3, 114.4 ± 17.8 and 110 ± 1.0 cm, respectively (**Fig. 2B**). These results indicate that the vegetative growth in 'curved' tobacco was not significantly different from that in wild-type tobacco. No significant differences were observed in the day of first flower blooming or in total flower number between wild-type and 'curved' tobacco plants (**Fig. 2C, D**), suggesting that flowering ability is also similar in these plants. These results suggest that the ability of survival of 'curved' tobacco in the field is not much different from that of wild-type tobacco.

By contrast, the total seed weight per plant in 'curved' tobacco (5.3 g) was significantly lower than that in the wild-type tobacco (11.5 g) (**Fig. 2E**). Such difference did not result from the growth of seeds because the seed weight of 'curved' tobacco was higher than that of the wild-type tobacco (**Fig. 2F**). This indicates that the difference in total seed weight between wild-type and 'curved' plants is based on the difference in seed production number per plant. Wild-type tobacco plants continuously formed flowers for two months and almost all flowers produced many seeds. The flower number and period of flowering in 'curved' tobacco were similar to those in the wild-type tobacco, but the flowers of the former plants showed abnormal morphology at the late flowering stage. At this stage, 'curved' tobacco plants formed flowers in which stamens were much shorter than the pistil (**Fig. 2G**). Such abnormal flowers may produce fewer seeds in 'curved' tobacco because self-pollination would be hindered in these flowers. In fact, pods formed in the late flowering stage of 'curved' tobacco contained only a few seeds (data not shown). Is this a fatal point in the use of 'curved' phenotype as a marker for transgene/transgenic plants? Introgression of transgene into wild relatives in the field occurs through hybridization, which may be mediated by pollinators such as insects and/or wind. The seed production in flowers in the primary stage of 'curved' tobacco was not significantly different from that of the wild-type plant (data not shown), suggesting that 'curved' plants at the primary flowering stage was useful as a pollen supplier. In addition, although stamens in flowers of

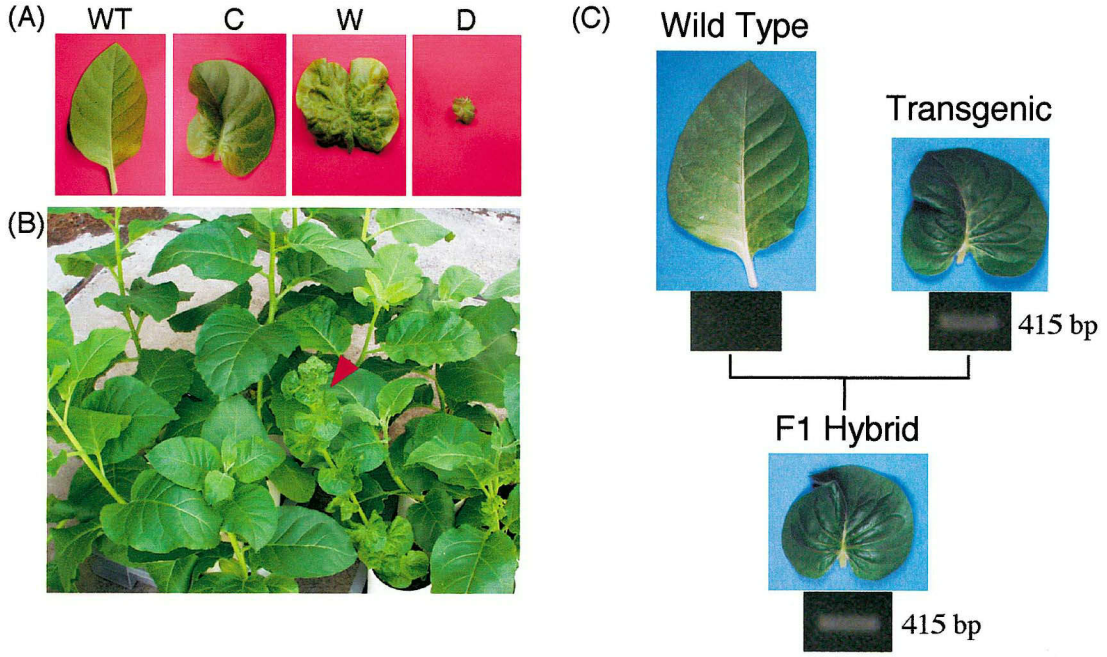

Fig. 1 Phenotypes and heritability of transgenic plants carrying *35S :: NTH* chimeric genes. (A) Leaves from wild-type (WT), 'curved' (C), 'wrinkled' (W) and 'dwarf' (D) tobacco plants. (B) A transgenic plant with the chimeric gene is visually identified among many wild-type plants. Red arrow indicates a transgenic plant with wrinkled leaves. (C) Gene flow from a transgenic plant to a wild-type plant can be visualized with the chimeric gene. F1 hybrid plant was made by artificially crossing between wild-type and transgenic 'curved' tobacco plants. Results of PCR amplification of the transgene are shown under each photograph.

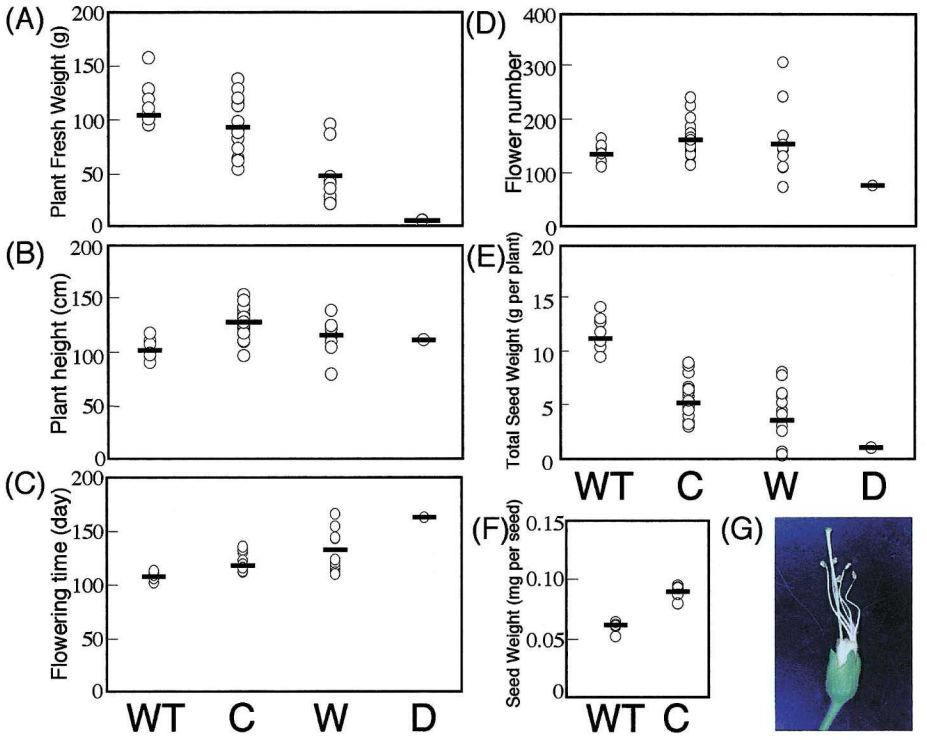

Fig. 2 Various developmental parameters of wild type and transgenic plants. (A) The fresh weight per pant of 3-month-old wild-type and transgenic plants. (B) Plant heights of wild-type and transgenic plants. The plant height was measured after the flowering. (C) The period (day) after sowing to the appearance of the first flower. (D) Flower number per plant. Total flower number was counted after the plant was blighted. (E) Weight of total seeds harvested per plant. (F) Weight of each seed produced by wild-type and 'curved' transgenic plants. The value was calculated from the number of seeds contained in a batch of 50 mg seeds. (G) Inside view of a flower collected from a 'curved' transgenic plant. Each plot in the figures (A-F) represents the value of each plant. The calculated means are indicated as horizontal lines. WT, wild-type ; C, curved ; W, wrinkled ; D ; dwarf.

'curved' tobacco were stunted in the late stage of flowers, the pollen activity in these flowers was well maintained. Indeed, we confirmed the pollen activity of abnormal flowers by out-crossing between wild-type and 'curved' tobacco flowers (**Fig. 1C**). Moreover, the number of pollen grains was not much different between wild-type (259648 ± 3245 per flower) and 'curved' tobacco flowers (247477 ± 2258 per flower). Taken together, 'curved' tobacco may be useful for monitoring transgene movement as a pollen supplier. However, the detailed mechanism of tobacco pollination by pollinators has not been clarified yet, and further studies are necessary to evaluate the availability of our transgenic plants for monitoring transgenes/transformants in the field.

As mentioned above, we showed that 'curved' phenotype is useful for monitoring transgene with little effect on the growth of host plants. The 'curved' phenotype appeared with introducing particular homeobox genes. Indeed, all transgenic plants introduced with *35S::NTH1* showed 'curved' phenotype. Other phenotypes, such as 'wrinkled' and 'dwarf', appeared uncertainly (Nishimura *et al.*, 2000). Moreover, the appearance of 'curved' phenotype was not influenced with the expression level of transgene. In other words, the appearance of 'curved' phenotype was not dependent on insertion position of the transgene. These also suggest that 'curved' phenotype is useful for monitoring transgenes.

In this study, we showed that alteration of leaf morphology with introducing homeobox genes was useful for monitoring transgene movement in tobacco. Such abnormal leaves were also observed in some other plants (such as maize, tomato, *Arabidopsis*, lettuce) with overexpressing homeobox genes (Vollbrecht *et al.*, 1991; Lincoln *et al.*, 1994; Hareven *et al.*, 1996; Frugis *et al.*, 2001). In addition, it has been demonstrated that abnormal leaf morphology in transgenic plants results from altered phytohormone levels (Tamaoki *et al.*, 1997; Frugis *et al.*, 2001). Therefore, alteration of leaf morphology with the overexpression of a homeobox gene would be applicable as a marker for transgene movement for many other plant species.

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