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Genetic characterization of a double-flowered tobacco plant obtained in a transformation experiment

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Summary. A leaf-disk transformation experiment was performed with tobacco (*Nicotiana tabacum* L.) using a binary vector and a strain of *Agrobacterium tumefaciens* that carried a wild-type Ti-plasmid, pTiBo542. Although the majority of kanamycin-resistant, transgenic plants was morphologically normal, one of the plants was double-flowered and had a slightly wavy stem and leaves whose edges were bent slightly upwards. The abnormal morphology was controlled by a single, dominant Mendelian gene. Young plants that carried this gene were distinguishable from normal plants at the stage of cotyledons. The homozygotes, with respect to this gene, were more seriously deformed than the heterozygotes. DNA segments derived from the binary vector and from the T_L- and T_R-DNA of pTiBo542 were detected in the double-flowered plant, but the T-DNA genes involved in biosynthesis of phytohormones were absent from the plant. The abnormal morphology, the resistance to kanamycin, and the segments of foreign DNA were genetically linked, and the linkage was very tight, at least between the abnormal morphology and the resistance to kanamycin; the meiotic recombination frequency was less than 0.02%, if recombination occurred at all.

Key words: *Agrobacterium tumefaciens* – Double flower – Morphological mutant – *Nicotiana tabacum* – Transformation

Introduction

Agrobacterium tumefaciens and *A. rhizogenes* are soil bacteria that can genetically transform plant cells with segments of DNA (transfer DNA, abbreviated as T-DNA) from tumor-inducing and root-inducing plasmids (Ti- and Ri-plasmids) to produce a crown gall, which is a plant tumor, and hairy-root syndrome, respectively (Bevan and Chilton 1982; Depicker et al. 1983; Nester et al. 1984). A number of sophisticated plant transformation vectors, based on this naturally occurring gene-transfer mechanism, have been developed, and such vectors are widely employed in plant molecular biology and genetic engineering (Fraley et al. 1986).

A binary vector (An et al. 1988) is one of the most widely used vectors. In a binary system, one Ti-plasmid serves as a helper, and an artificial "T-DNA," containing a selectable marker and genes of interest, is placed on a second, small plasmid, a binary vector. When a wild-type Ti-plasmid is used as a helper plasmid, complicated transformational events may result from the multiple "T-DNAs" in an *Agrobacterium* strain. It is necessary to demonstrate that the "T-DNA" of interest is transferred efficiently and that undesired events, such as inhibition of plant regeneration by the wild-type T-DNA, do not occur at any significant frequency.

For the above-mentioned purpose, a wild-type strain called A281, which carries pTiBo542 (Hood et al. 1984; Komari et al. 1986), has been tested in combination with a common binary vector. During these experiments, a tobacco plant with an unusual morphology, namely, double flowers, was unexpectedly obtained. If such a morphological alteration is associated with the strain-vector combination, the application of the combination will be rather limited. Nonetheless, this transformant was of particular interest, because investigation of this phenomenon may identify additional gene(s) for the morphological control of plants. This report describes a genetic study of this double-flower morphology.

Materials and methods

Plant materials

Nicotiana tabacum L. cv Xanthi NC was used as the starting material for transformation experiments.

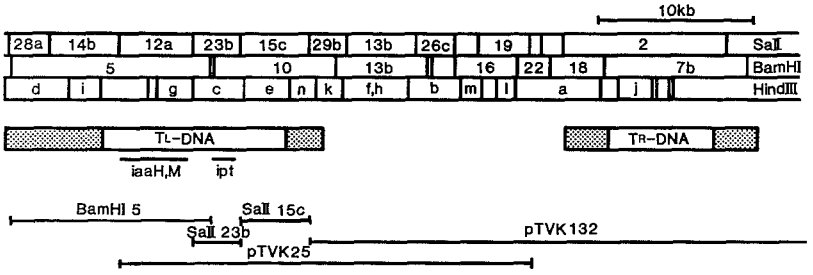

Fig. 1. The T-region of pTiBo542 and the hybridization probes. The restriction map is shown with the *boxes* that represent the extent of the T-DNA and the *bars* that indicate the location of some T-DNA genes. The stippled boxes represent the border regions. The lines below the map show the DNA segments used as hybridization probes

Bacterial strains and plasmids

Agrobacterium tumefaciens A281 (Watson et al. 1975) and the binary vector, pGA472 (An et al. 1985), have been described previously. pGA472 was introduced into A281 by the freeze-thaw method (An et al. 1988). *A. tumefaciens* was grown on AB medium (Chilton et al. 1974) at 28 °C. Cosmid clones, pTVK25 and pTVK132, from pTiBo542 were described previously (Komari et al. 1986).

Transformation

Tobacco leaves were surface-sterilized, and 6-mm disks were prepared with a paper punch. The disks were incubated with approximately 10^8 cells of *A. tumefaciens* in 2 ml of liquid medium that consisted of Linsmaier and Skoog (1965) salts and 30 g/l of sucrose (LS-R medium). After 48 h of co-cultivation, the bacteria were washed off and the disks were transferred to LS-R medium supplemented with 0.3 mg/l indole-3-acetic acid, 10 mg/l isopentenyl adenine, 0.9% agar, 250 mg/l cefotaxim, and 100 mg/l kanamycin. Root induction of drug-resistant regenerates was achieved on LS-R agar (0.9%) medium. The plants were then transferred to soil and grown in a greenhouse.

Analysis of transformants

The medium used for germination of the seeds obtained from the transformants was the same as the medium used for induction of roots. When appropriate, 100 mg/l kanamycin was added to the medium for germination of seeds. The procedures for isolation of DNA from plants and for Southern hybridization were those previously described (Komari et al. 1989). The probes used were the 2.1-kb BamHI-HindIII fragment from pGA472, entire pTiBo542, and the cloned DNA fragments from the T-DNA of pTiBo542. A map of the T-DNA that is based on published results (Hood et al. 1984, 1986a, b; Komari et al. 1986; Strabala et al. 1989) and the probe fragments are shown in Fig. 1.

Results

A transformation experiment with leaf disks of tobacco was carried out with *A. tumefaciens* A281 (pGA472). The leaf disks were treated with the bacteria and selected for resistance to kanamycin on shoot-inducing media. The cells transformed with A281 (pGA472) were presumably a mixture of cells that contained the "T-DNA" from pGA472, the wild-type, oncogenic T-DNA, or both types of DNA. However, more than 20 regenerated plants transformed with A281 (pGA472) were easily obtained in a single set of experiments. Therefore, A281 (pGA472) was acceptable as a strain for subsequent experiments.

The majority of plants transformed with A281 (pGA472) were morphologically normal. Among them, one transformant had flowers with a very distinctive morphology, namely, double flowers (Fig. 2A). This plant was designated as TGK120. Two layers of petals were clearly observed in the flowers (Fig. 2B). The outer petals of most of the flowers were folded outwards in the middle, and their ends were hidden behind the sepals. Occasionally, the outer petals were unfolded, and fully open double flowers were observed (Fig. 2A). Other parts of the flowers looked normal. Thus, the additional petals were not the products of petalodization of other floral organs. These flowers produced normal amounts of seeds and outcrossing with other tobacco plants was also possible. Other parts of the plant appeared almost normal, but close examination revealed that the stem was slightly wavy (Fig. 2C) and the edges of the leaves were bent slightly upwards.

When the self-pollinated progeny were examined, two types of plant were segregated. One-fourth (23 out of 90) of the plants were indistinguishable from nontransgenic plants and had normal flowers. The growth of the other 67 plants was severely delayed at the stage of cotyledons (Fig. 2D); the activity of the apical tissues seemed to be retarded initially. The elongation of roots was also not very rapid. Abnormally shaped leaves eventually appeared in the apical regions, but the plants became gradually less and less abnormal as the growth proceeded. Two-thirds (45/67) of these plants became relatively normal and eventually developed double flowers. The morphology of their stems, leaves, and flowers was identical to that described for the initial transformant TGK120.

The remaining one-third of the abnormal offspring (22/67) remained severely deformed (Fig. 2E). Their flowers were also severely deformed and had multiple layers of petals (Fig. 2A). Therefore, the abnormal S_1 progeny fell into two classes, those that were relatively normal and those that were severely deformed. The two classes were indistinguishable at the early stage of growth. The overall segregation ratio for normal and abnormal plants was 1:3, and the ratio for normal to relatively normal to severely deformed plants was 1:2:1 in the S_1 generation. There was no difficulty in obtaining the S_2 progeny from the normal and relatively normal plants. The flowers of the severely deformed plants were

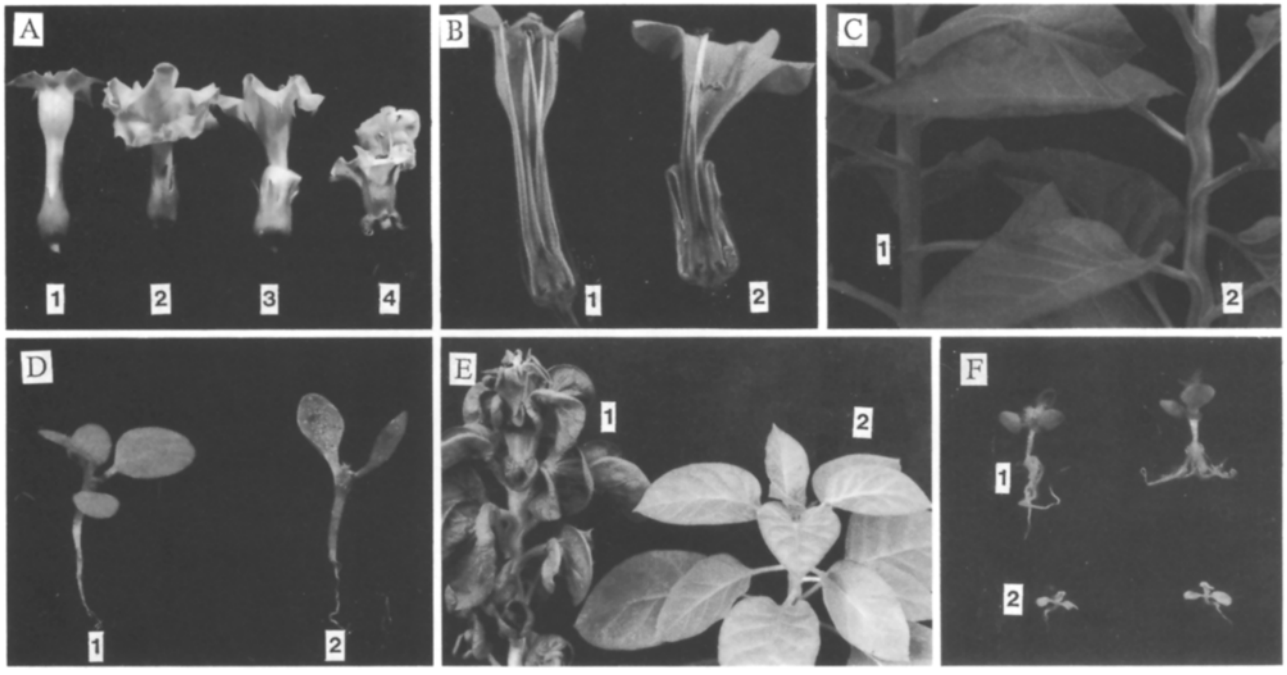

Fig. 2A–F. Morphology of normal and double-flowered plants. **A** Flowers of a normal plant (*1*), a heterozygote (*2* and *3*), and a homozygote (*4*), with respect to the gene that controls the double flower. **B** Longitudial sections of a normal flower (*1*) and a double flower (*2*). **C** Stems of a normal plant (*1*) and a double-flowered plant (*2*). **D** A normal plant (*1*) and a double-flowered plant (*2*) at an early seedling stage. **E** A homozygote (*1*) and a heterozygote (*2*) before flowering. **F** Double-flowered (*1*) and normal (*2*) seedlings grown on kanamycin-containing medium

Table 1. Segregation of the resistance to kanamycin (Km) and the abnormal morphology

Generation	No. of plants					χ^2	P
	Km^r abnormal	Km^s abnormal	Km^r normal	Km^s normal	Total		
S_1 of TGK120	308	0	0	106	414	0.081[a]	0.75–0.90
F_1 of wild-type × TGK120	2,761	0	0	2,718	5,479	0.337[b]	0.50–0.90

[a] and [b] tested for 3:1 and 1:1 ratios, respectively

so abnormal that S_2 seeds were obtained from only 5 of 22 plants.

When the self-pollinated progeny were grown on kanamycin-containing media, the normal seedlings were kanamycin-sensitive, the abnormal ones were kanamycin-resistant (Fig. 2E), and the ratio of sensitive to resistant seedlings was 1:3 (Table 1). The S_2 progeny were also analyzed. It turned out that the relatively normal class of double-flowered plants was all heterozygous with respect to resistance to kanamycin and the abnormal morphology; normal, kanamycin-sensitive offspring and abnormal, kanamycin-resistant offspring were segregated in the ratio of 1:3. All of the members of the S_2 generation from the five severely deformed S_1 plants were exclusively abnormal, kanamycin-resistant plants, indicating that the severely deformed plants were homozygous. This assignment of genotype was consistent with the segregation ratio.

In order to estimate the linkage between the resistance to kanamycin and the abnormal morphology, a large number of plants from a cross between a wild-type tobacco and the initial transformant TGK120 was analyzed. No plants showing the recombinant phenotype were found among more than 5,000 plants examined (Table 1). This result suggests that the two loci, if not identical, were very close together on the genetic map.

A few morphologically normal transformants were selected and analyzed by Southern hybridization (examples are shown in Fig. 3, lanes b and f). The gene for neomycin phosphotransferase (NPT) was detected in these plants, and the DNA sequence from the Ti-plasmid was absent. This result indicates that the cells that re-

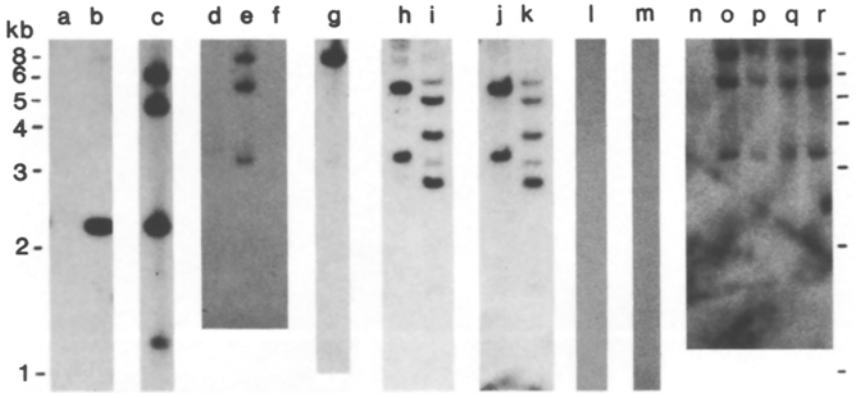

Fig. 3. Southern hybridization. DNA was digested with BamHI (except in the case of lanes i and k) or HindIII (lanes i and k), subjected to agarose gel electrophoresis, transferred to nylon membranes, and hybridized to ^{32}P-labeled fragments of DNA. *Lanes a–c*: DNA from a nontransgenic plant (*a*), from a morphologically normal, transgenic plant (*b*) and from the double-flowered plant (*c*) was hybridized to the NPT probe. *Lanes d–f*: the same DNA as in lanes a, c, and b, respectively, was hybridized to pTiBo542. *Lanes g–m*: DNA from the double-flowered plant was hybridized to pTVK132 (*g*), to pTVK25 (*h* and *i*), to SalI 15c (*j* and *k*), to SalI 23b (*l*), and to BamHI 5 (m). *Lanes n–r*: DNA from the plants in the normal class (*n*), in the severely deformed class (*o* and *r*), and in the relatively normal (*p* and *q*) class of the S_1 progeny of the double-flowered plant was hybridized to pTiBo542. Fragments are identified in Fig. 1

ceived only the "T-DNA" from pGA472 were efficiently selected during the regeneration process.

In the DNA isolated from TGK120, the NPT probe detected four BamHI fragments (6.2, 5.0, 2.3, and 1.2 kb, lane c of Fig. 3). The 2.3-kb fragment is the one expected from the structure of pGA472. The other bands probably resulted from some rearrangements upon transformation. pTiBo542 hybridized to three BamHI fragments (8.0, 5.5, and 3.3 kb, lane e). When cosmid clones from pTiBo542 were used as probes, pTVK132, which contained the T_R-DNA, hybridized to the 8.0-kb BamHI band (lane g), and pTVK25, which contained the T_L-DNA, hybridized to the 5.5- and 3.3-kb BamHI bands (lane h). A map of the T-region of pTiBo542 and the DNA fragments used as probes are shown in Fig. 1. These results indicate that the 8.0-kb BamHI band was from the T_R-DNA and the 5.5- and 3.3-kb BamHI bands were from the T_L-DNA of pTiBo542.

Further hybridization experiments were performed using small, gel-purified fragments of the T_L-DNA. The SalI fragment designated as 15c from pTiBo542, which contained the right-hand end of the T_L-DNA, hybridized to both the 5.5- and 3.3-kb BamHI bands (Fig. 3, lane j). The BamHI fragment 5, which contained the left-hand half of the T_L-DNA including the *iaaH* and *iaaM* genes, and the SalI fragment 23b, which contained the *ipt* gene, did not hybridize to any of the fragments (lanes l and m). When the HindIII digest of the DNA from TGK120 was analyzed, pTVK25 and the SalI 15c showed similar hybridization patterns; they hybridized strongly to bands of 4.9, 3.5, and 2.6 kb, and weakly to bands of 5.9 and 3.2 kb (lanes i and k). These data suggest that only the right-hand end of the T_L-DNA was introduced into TGK120. It is possible that the 2.6-kb HindIII fragment corresponded to the HindIII fragment e of pTiBo542. The other bands appeared to be generated by combinations of the DNA derived from the T-DNA and the plant genome.

The DNA isolated from some of the S_1 progeny was also analyzed by Southern hybridization (Fig. 3); 2, 4, and 15 plants from the normal, relatively normal, and severely deformed classes, respectively, were analyzed. pTiBo542 hybridized to the 8.0-, 5.5-, and 3.3-kb BamHI fragments in the DNA from the two double-flowered classes (lanes o–r), and to no fragments of DNA from the normal class (lane n). The intensity of the hybridization signals in the case of the severely deformed class (lanes o and r) was stronger than that in the case of the relatively normal class (lanes p and q). Therefore, the three BamHI fragments were genetically linked to each other and to the abnormal morphology.

Discussion

A. tumefaciens strain A281 is a so-called "super-virulent" strain, and its host range is wider and its transformation efficiency is higher than those of other strains (Jin et al. 1987). One exceptional product of the system of A281 (pGA472), a double-flowered tobacco plant, was characterized in detail in the present study. Genetic analysis revealed that the unusual morphology was controlled by a single, dominant Mendelian gene. This gene is temporarily designated as *Df* in this discussion. *Df* exhibited a pleiotropic nature; this gene affected the morphology of various parts of the plant, including the flowers, cotyle-

dons, leaves, and stems. To be quite precise, the effect of *Df* was incompletely dominant, because the heterozygotes, *Df*/+, and the homozygotes, *Df*/*Df*, expressed the same characteristics in different magnitudes.

The results of Southern hybridization showed that at least three fragments of foreign DNA, which were derived from pGA472 and from the T_R- and T_L-DNA of pTiBo542, were integrated in the double-flowered plant. The T_L-DNA contained *iaaH* and *iaaM*, which are involved in biosynthesis of auxin, and *ipt*, which is involved in biosynthesis of cytokinin, and it is reportedly tumorigenic (Nester et al. 1984; Komari et al. 1986; Strabala et al. 1989). Therefore, the occurrence of the T_L-DNA in the regenerated plant was not expected. However, further hybridization revealed that only a part of the T_L-DNA, from which these phytohormone genes were absent, was incorporated in the plant.

Df, the kanamycin-resistance gene, and the segments of foreign DNA were genetically linked, and the linkage, at the very least, between *Df* and the kanamycin-resistance gene was very tight; the recombination frequency was less than 0.02%, if any recombination did in fact occur.

Various possible explanations for the nature of *Df* can be considered. The main question is whether this gene was of foreign or cellular origin. Although both the *iaa* genes and the *ipt* gene, which have been reported to affect the morphology of transformed cells (Nester et al. 1984), were absent from the double-flowered plant, it has been reported that some *Agrobacterium*-derived genes, which are not directly involved in synthesis of phytohormones, have the ability to alter plant morphology (Schmulling et al. 1988). Such genes may exist in the T_L- and/or T_R-segments in the double-flowered plant, and they might have played significant roles. Transformation experiments, in which several cloned fragments of DNA from the T_L- and T_R-DNA are introduced into tobacco plants, are currently in progress in our laboratory. The reproduction of the double-flower phenotype has not been accomplished so far, but this approach may lead us to the causative gene(s).

If *Df* is of cellular origin, it might have been generated by "insertional mutagenesis." The generation of *Df* by this mechanism can be considered as a very rare event, and is not necessarily associated with the system of A281 (pGA472). A tobacco mutant, "corolle double X", which had flowers similar to those reported here and was controlled by a recessive gene, was described some 40 years ago (Hitier 1950). The occurrence of such a mutant suggests that possible candidates for *Df* may exist in the tobacco genome. Since the genetic linkage between the kanamycin resistance gene and *Df* was very tight, it may be possible to clone the causative cellular gene(s) by "walking" along the region around the kanamycin resistance gene.

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