Cloning of a *Nicotiana plumbaginifolia* protoplast-specific enhancer-like sequence

Marie Horth, Ioan Negrutiu¹, Arsène Burny, Marc Van Montagu² and Luis Herrera-Estrella^{2,3}

Département de Biologie Moléculaire, Université Libre de Bruxelles, B-1640 Rhode-St-Genèse, ¹Laboratorium voor Plantengenetica, Vrije Universiteit Brussel, B-1640 St.-Genesius-Rode and ²Laboratorium voor Genetica, Rijksuniversiteit Gent, B-9000 Gent, Belgium.

³Present address: Centro de Investigacion y Estudios Avanzados, Irapuato, GTO, Mexico

Communicated by M. van Montagu

We have isolated a 1.5-kb plant DNA fragment (called insert 7) from *Nicotiana plumbaginifolia* DNA that contains a protoplast-specific enhancer-like sequence. The presence of this sequence on a plasmid carrying a chimeric *nos-npt-II* gene conferring kanamycin resistance to plant cells, produces an overexpression of the *npt-II* gene during at least eight days after protoplast transformation. This effect on the expression of the *nos* promoter was independent of the orientation and was observed both on circular and linearized plasmids. On the contrary, insert 7 had no influence when present on another plasmid (*in trans*) in cotransformation experiments. The overexpression of the *nos-npt-II* gene due to the presence of insert 7 on the transforming plasmid is correlated with a higher level of synthesis of the corresponding RNA. Insert 7 did not affect the level of expression of the *nos-npt-II* gene in stably transformed calli, or in regenerated plants. However, the overexpression was again detected in protoplasts prepared from leaves of stably transformed plants. This 1.5-kb plant DNA fragment contains highly repetitive DNA sequences, specific to *N. plumbaginifolia*. However, the enhancer-like activity is localized on a 600-bp unique sequence of insert 7. Insert 7 had no detectable effect on the transient expression of another gene, the nopaline synthase gene present at a longer distance on the same plasmid.

Key words: enhancer-like sequence/transformant/over-expression

Introduction

The most efficient system for introducing foreign genes into plants is the natural gene transfer system of the Ti plasmid: the T-DNA is transferred to plant cells and integrated mostly at a single locus (Budar *et al.*, 1986) in the plant genome. This system has already been used to stably integrate several genes in plant chromosomes and to study their regulation (Herrera-Estrella *et al.*, 1984; Dean *et al.*, 1985; Morelli *et al.*, 1985; Simpson *et al.*, 1985; Spena *et al.*, 1985).

Efficient integration of foreign DNA by transformation of plant protoplasts with purified plasmid DNA is also possible (Pazkowski *et al.*, 1984; Hain *et al.*, 1985). This is the method of choice for plants which poorly interact with *Agrobacterium* such as most monocotyledonous plants (Lörz *et al.*, 1985; Potrykus *et al.*, 1985). Both procedures require the selection of stable transformants and the culture of sufficient quantities of

transformed callus tissue or regenerated plants for analysis of gene expression, and, therefore, take several months.

A faster procedure for studying the role of regulatory sequences consists in transient expression experiments. They have been very useful for the study of expression of transformed foreign genes, both in animal cells (Banerji *et al.*, 1983; Graham *et al.*, 1973) and in plant cells (Fromm *et al.*, 1985; Koncz and Schell, 1986; Werr and Lörz, 1986). In these experiments plasmid DNA was directly introduced into plant protoplasts and the expression of the reporter gene present on the plasmid could be determined one day after transformation.

We have used this method to study the influence of several plant DNA fragments on the transient expression of the chimeric *nos-npt-II* gene. One of these fragments, a 1.5-kb *N. plumbaginifolia* DNA fragment, allowed an overexpression of the *nos-npt-II* gene during at least eight days after transformation. This overexpression can be explained by either one of three hypotheses: (i) the plant DNA fragment could behave as an origin of replication and, therefore, increase the copy number of the plasmid and enhance the NPT-II activity; (ii) the plant DNA fragment has a regulatory effect, as an enhancer sequence, and allows a higher transcription and expression of the target gene and (iii) the plant insertion is able to stabilize the plasmid in the transformed plant cells, and therefore, stabilizes also the NPT-II activity.

The experiments presented here enabled us to conclude that the 1.5-kb plant DNA fragment produced the overexpression of the *nos-npt-II* gene by a regulatory effect, similar to an enhancer sequence. This effect was observed only in protoplasts and not in stably transformed plants.

Results

Searching for plant origins of replication that would allow autonomous replication of a plasmid in plant cells, we studied the influence of several plant DNA fragments on the transient expression of a chimeric selectable gene, after direct protoplast transformation with plasmid DNA. These plant DNA fragments (8 from *N. plumbaginifolia*, 2 from *T. aestivum*, and 1 from *V. faba*) were considered as good candidates for plant origin of replication sequences as they have been isolated as autonomous replication sequences (ARS) in yeast cells (Horth *et al.*, in preparation).

Influence of a 1.5-kb N. plumbaginifolia DNA fragment on the transient expression of the chimeric nos-npt-II gene

The plant DNA fragments were each cloned in the unique *EcoRI* site of pLGVneo-nos (see Figure 1), at a distance of 0.8 kb upstream of the *nos-npt-II* gene, and their influence on the transient expression of this chimeric gene was studied after transformation of protoplasts isolated from leaves of *N. plumbaginifolia* or *N. tabacum* SR1. Protoplasts were cultured without selection pressure until extracts were assayed for NPT-II activity.

As shown in Figure 2A, one candidate for a plant origin of replication, a 1.5-kb *N. plumbaginifolia* fragment (named insert 7) promoted an overexpression of the *nos-npt-II* gene for at least

Fig. 1. Construction of pLGVneo-nos-ins7. Plasmid pLGVneo1103 has been described by Hain *et al.* (1985). It is a derivative of pBR322 and contains a dominant selective marker gene conferring resistance to kanamycin to plant cells. The selective marker gene is a chimeric gene consisting of the neomycin synthase promoter sequences, followed by the coding sequences of neomycin phosphotransferase of Tn5, and finally the polyadenylation site of the octopine synthase gene (Herrera-Estrella *et al.*, 1983). The HindIII fragment 23 of pTiC58 containing the complete neomycin synthase gene was purified by electroelution and ligated to pLGVneo1103 to form plasmid pLGVneo-nos. EcoRI plant DNA fragments were introduced into the unique EcoRI site of pLGVneo-nos to form plasmid pLGVneo-nos-insX.

eight days after protoplast transformation, when compared with the expression directed by the original plasmid pLGVneo-nos. Figure 2 presents two times courses of the NPT-II activity in protoplasts transformed with plasmid pLGVneo-nos-ins7. When insert 7 was present on the plasmid, NPT-II activity is clearly enhanced. It should be mentioned that none of the other plant DNA fragments had an influence on the transient expression of the *nos-npt-II* gene (data not shown).

Overexpression of the *nos-npt-II* gene due to the presence of insert 7 on the plasmid, was observed after transformation of *N. plumbaginifolia* and *N. tabacum* SR1 protoplasts, and both after DNA uptake mediated either by PEG and CaCl₂ (Pazkowski *et al.*, 1984) or after electroporation (Fromm *et al.*, 1985) (Figure 2B). We also compared the NPT-II activity in protoplasts transformed by pLGVneo-nos, pLGVneo-nos-ins7, or plasmid pLDI in which the *npt-II* gene is under control of a stronger plant promoter, the T_R promoter of the Ti plasmid (Velten *et al.*, 1984), than the *nos* promoter. As shown in Figure 2B, the level of NPT-II activity in protoplasts transformed by

pLGVneo-nos-ins7 is considerably higher than in protoplasts harbouring pLDI or pLGVneo-nos. The relative NPT-II activities were measured by counting the radioactivity of each spot. The results showed that the relative NPT-II activities observed with pLGVneo-nos-ins7, pLGVneo-nos, and pLDI are 20, 1 and 2 respectively.

NPT-II activity obtained after protoplast transformation with circular or linearized pLGVneo-nos or pLGVneo-nos-ins7 were compared: no difference in expression was detected (Figure 2C). Furthermore, it was observed that the effect of insert 7 on the expression of the *npt-II* gene is independent of the orientation (Figure 2C). However, the overexpression effect was not observed when the insert was present on another plasmid (pBR322) co-transformed together with the pLGVneo-nos plasmid. Insert 7 has thus no effect on expression of the *nos-npt-II* gene when present *in trans* (Figure 2C).

Insert 7 behaves as an enhancer-like sequence

The overexpression due to the presence of insert 7 on the plasmid pLGVneo-nos could be explained by either one of the following hypotheses: (i) insert 7 could act as an origin of replication, thereby increasing the copy number of the plasmid (gene dosage effect); (ii) insert 7 could stabilize the plasmid in protoplasts and (iii) insert 7 could exert a regulatory effect acting as an enhancer sequence and following a higher rate of transcription and expression of the marker gene.

In order to distinguish between these possibilities we performed the following experiments. An approach to eventually detect replication of the plasmid in transformed protoplasts consisted of cultivating the protoplasts, after transformation, in the presence of ³²P-phosphate during three and six days. After harvesting the protoplasts, total DNA was extracted, electrophoresed on a 0.6% agarose gel, and autoradiographed. The results are shown in Figure 3 (lanes 1–5). The high mol. wt chromosomal DNA had incorporated radioactive phosphate, while no radioactive plasmid could be detected. In a parallel experiment, protoplasts were transformed and cultured for four days. After hydrolysis of residual extracellular DNA by DNase I treatment (Werr and Lörz, 1986) total DNA was extracted as previously, electrophoresed on a 0.8% agarose gel, transferred to nitrocellulose and hybridized to a radioactive probe corresponding to plasmid pLGVneo-nos. The results showed that extrachromosomal plasmid was detectable (Figure 3, lanes 7 and 8). We could thus conclude that, soon after transformation, plasmids pLGVneo-nos and pLGVneo-nos-ins7 were present in transformed protoplasts as non-replicating extrachromosomal elements.

The results presented in Figure 3 (lanes 7 and 8) indicated that plasmid pLGVneo-nos and pLGVneo-nos-ins7 were present in equal amounts in plant cells, four days after protoplast transformation. These data excluded a stabilizing effect of insert 7 on the plasmid pLGVneo-nos. In order to test whether insert 7 exerts a regulatory effect on the expression of the *nos-npt-II* gene, *N. plumbaginifolia* protoplasts were transformed by either pLGVneo-nos or pLGVneo-nos-ins7. After a three day culture, total RNA was extracted (Logemann *et al.*, 1987), denatured in the presence of glyoxal and DMSO (Maniatis *et al.*, 1982), divided into two samples, electrophoresed in a 1% agarose gel and hybridized to a radioactive probe corresponding either to the chimeric *nos-npt-II* gene or to the complete *nos* gene. The results presented in Figure 4 clearly showed that the higher level of NPT-II activity observed in protoplasts transformed by the plasmid containing insert 7, correlated with a higher level of synthesis of the corresponding RNA. This is consistent with an effect of insert 7 on the expression of the *nos-npt-II* gene in transformed

Fig. 2. (A) Two-time courses of NPT-II activity found in protein extracts isolated from *N. plumbaginifolia* cells after transformation by direct gene transfer (Pazkowski *et al.*, 1982) with plasmid pLGVneo-nos or pLGVneo-nos-ins7. The time period (hours or days) between transformation and cell harvest is indicated above the lanes. A positive control consists in a *npt-II*-containing *E. coli* protein extract. The same amounts of protein were loaded on each slot. (B) NPT-II activity found in protein extracts isolated from *N. plumbaginifolia* or *N. tabacum* cells after transformation by electroporation. The transforming plasmid is indicated above the lanes. PC is a positive control consisting of protein extract of a stably transformed *npt-II*-positive plant. The same amount of protein was loaded on each slot. (C) NPT-II activity found in protein extracts isolated from *N. plumbaginifolia* cells after transformation by direct gene transfer. The transforming plasmid is indicated above the lanes. PC is a positive control consisting of protein extract of a stably transformed *npt-II*-positive plant. In the two next lanes, protoplasts were co-transformed with pLGVneo-nos together with pBR322, or with pLGVneo-nos together with pBR322 in which insert 7 has been cloned in the *EcoRI* site. In the last two lanes, protoplasts were transformed with plasmid pLGVneo-nos or pLGVneo-nos-ins7, both linearized by *SmaI*.

Fig. 3. Lanes 1–5, autoradiography of total cellular DNA of *N. plumbaginifolia* cells after transformation with plasmid pLGVneo-nos, pLGVneo-nos-ins7, and of untransformed cells as negative control (NC). After transformation, protoplasts were cultured during three or six days in 10 ml K3 culture medium containing no cold phosphate salts, but supplemented with 35 μ Ci ^{32} P-orthophosphate. Lanes 6–8: Southern hybridization of total cellular DNA of *N. plumbaginifolia* cells three days after transformation with plasmid pLGVneo-nos or pLGVneo-nos-ins7 to a radioactive probe corresponding to the plasmid pLGVneo-nos. Lane 6 is a negative control consisting of protoplasts incubated with the plasmid pLGVneo-nos (but not transformed), treated with DNase; the DNA was isolated as in lanes 7 and 8, and probed with the plasmid pLGVneo-nos. Lanes 9–17: Southern hybridizations of total cellular DNA of *N. plumbaginifolia* calli stably transformed by pLGVneo-nos or pLGVneo-nos-ins7 to a radioactive probe to the plasmid pLGVneo-nos. NC is a negative control corresponding to DNA from untransformed callus.

Fig. 4. Northern hybridization of total RNA extracted from *N. plumbaginifolia* cells transformed by plasmid pLGVneo-nos or pLGVneo-nos-ins7. Nop⁺ is a positive control which consists of total RNA extracted from protoplasts made from leaves of a nopaline-positive plant, submitted to the complete transformation procedure but without plasmid DNA, and cultured exactly as transformed protoplasts. After extraction, RNA samples were divided in two, after electrophoresis in a 1% agarose gel in presence of DMSO and glyoxal, transferred to nitrocellulose, and hybridized either to a radioactive probe corresponding to the chimeric *nos-npt-II* gene or to the nopaline synthase gene. The two DNA probes were similarly labelled to 1×10^8 d.p.m./kg by nick translation with [³²P]dCTP (Maniatis *et al.*, 1982).

Fig. 5. (A) NPT-II activity found in protein extracts in independent calli stably transformed by either pLGVneo-nos or pLGVneo-nos-ins7. The same amount of protein has been loaded in each slot. (B) NPT-II activity found in protein extracts of independently regenerated plants stably transformed by either pLGVneo-nos (plants 5, 6, 7, and 8) or pLGVneo-nos-ins7 (plants 1, 2, 3, and 4). NPT-II activity was tested in protein extracts of leaves, or of 2-day-old protoplasts made from those leaves. The same amount of protein has been loaded in each slot. This figure presents the NPT-II activity in leaves of plants 1, 2, 5, and 6, and not of plants 3, 4, 7, and 8 because of the limiting number of channels available on the gel. However, the NPT-II activity in leaves of all eight plants (plant 1–8) was approximately the same (data not shown).

Fig. 6. Southern hybridization of radioactively purified insert 7 DNA fragment to plant or animal DNAs restricted by *EcoRI*, electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose paper (Maniatis *et al.*, 1982). (A) Autoradiogram obtained after a 24-h exposure. (B) Autoradiogram obtained after 5 days of exposure. (C) Southern hybridization of *N. plumbaginifolia* DNA (channel a) digested by *HindIII* to radioactive probes corresponding to pLGVneo-nos-ins7 or to pLGVneo-nos-ins7*. The two probes were similarly labelled to 1.5×10^8 d.m.p./μg by nick translation. The radioactive probe pLGVneo-nos-ins7* was also hybridized to *N. tabacum* DNA digested by *HindIII* (channel b).

protoplasts. On the contrary, insert 7 had no detectable influence on the transcription of the complete *nos* gene (Figure 4). From this experiment, where the same technique was applied to identify the transcripts of two different genes, it could be concluded that the transcription of the *nos-npt-II* gene was enhanced while the transcription of the *nos* gene was not.

We also tested the influence of insert 7 on nopaline synthase activity in transformed protoplasts. As insert 7 had no detectable effect on the nopaline synthase activity (data not shown), it followed that the influence of insert 7 is limited on a distance inferior to 4 kb, and that pLGVneo-nos-ins7 did not replicate in transformed protoplasts.

Insert 7 is a protoplast-specific enhancer-like element

After transformation with plasmid pLGVneo-nos of pLGVneo-nos-ins7 by direct gene transfer, protoplasts were cultured on kanamycin-containing medium and stably transformed clones

were isolated. Southern hybridization on total DNA extracted from those stably transformed clones, revealed that both plasmids were integrated in chromosomal DNA (Figure 3, lanes 9–17).

As shown in Figure 5A, expression level of the *npt-II* gene in calli transformed by pLGVneo-nos was roughly the same as in calli transformed by pLGVneo-nos-ins7. The difference in NPT-II activity between independent clones might reflect differences in the genomic environment of the integrated plasmid. The same results were obtained in both types of regenerated plants: the NPT-II activity tested in leaves of two plants of each type is presented in Figure 5B. In total, four plants of each type (plants 1, 2, 3, and 4, transformed by pLGVneo-nos-ins7, and plants 5, 6, 7, and 8, transformed by pLGVneo-nos) were tested for NPT-II activity in leaves, stems, and root tips (data not shown), and the level of NPT-II activity was approximately the same in each type of plant. These data were at variance with those obtained in transient expression experiments where overexpression of *npt-II* gene was observed in the presence of insert 7. An hypothesis that would conciliate these apparently inconsistent results would consist of the fact that insert 7 could only promoter higher expression of *npt-II* gene in protoplasts. In order to test this hypothesis, protoplasts were isolated from leaves of plants transformed by pLGVneo-nos (plants 5–8) or pLGVneo-nos-ins7 (plants 1–4), and the level of NPT-II activity in protoplasts and in original plants was compared. As shown in Figure 3B, NPT-II activity detected in plants transformed by pLGVneo-nos (plants 5 and 6) was roughly the same as in plants transformed by pLGVneo-nos-ins7 (plants 1 and 2). On the contrary, in protoplasts made from those plants, the enhancement effect of insert 7 on the NPT-II activity is again clearly detected. These results confirmed the enhancer-like function of insert 7 and precisied the protoplast-specific influence of insert 7 on *npt-II* gene expression.

Characterization of the 1.5-kb *N. plumbaginifolia* DNA fragment

In an attempt to characterize the 1.5-kb plant insert, several plant and animal total DNAs were extracted, restricted with *EcoRI*, and after electrophoresis in an agarose gel, transferred to nitrocellulose and hybridized to a radioactive probe corresponding to the purified 1.5-kb *N. plumbaginifolia* fragment. As shown in Figure 6, insert 7 hybridized strongly to *N. plumbaginifolia* DNA, weakly to *N. tabacum* DNA, and not at all to other plant or animal DNAs tested. Moreover, insert 7 appeared to contain highly repetitive DNA sequences. Results from *in situ* hybridizations of insert 7 to *N. plumbaginifolia* chromosomes revealed that these repetitive sequences were present on every chromosome (data not shown).

In order to more precisely localize within the 1.5-kb insert 7, the region responsible for the enhancer-like behavior, purified insert 7 was digested with exonuclease III and nuclease S1, and blunt-end cloned in *EcoRI*-digested pLGVneo-nos. One of the generated fragments, a 600-bp sequence called insert 7*, was shown to exhibit the enhancer-like activity (Figure 2B) and to lack repetitive elements (Figure 6C). Insert 7* did not hybridize to *N. tabacum* DNA (Figure 6C).

Discussion

Our results show that a 1.5-kb *N. plumbaginifolia* DNA fragment cloned in the plasmid pLGVneo-nos induced overexpression of the reporter *nos-npt-II* gene in transient expression assays. This increased expression was observed until at least eight days after transformation but could not be detected in stably trans-

formed calli or in transgenic plants. However, the overexpression effect due to the presence of insert 7 appeared again in protoplast made from leaves of stably transformed plants. The influence of insert 7 on the expression of the *nos-npt-II* gene was thus protoplast-specific.

Overexpression of the chimeric *npt-II* gene was not due to an increased stability or to the autonomous replication of the plasmid. It should be mentioned that we cannot exclude that a small proportion of the plasmid population present in protoplasts did replicate; however, if a low level of replication occurred, it was not detectable by either one of the experimental tests presented here.

We have shown that insert 7-promoted overexpression of the *nos-npt-II* gene is correlated with a higher level of transcription of the chimeric gene. The influence of insert 7 on the target *nos-npt-II* was independent of the orientation and was abolished when insert 7 was present *in trans*. Insert 7 had no detectable influence on the expression of the nopaline synthase gene, also present on the plasmid, but at a greater distance (4.2 kb) than the *nos-npt-II* gene (0.8 kb). From these results we can conclude that insert 7 has properties comparable to enhancer elements found in animal (Banerji *et al.*, 1983; Weiher *et al.*, 1983) and plant genomes (Timko *et al.*, 1985; Simpson *et al.*, 1986).

The first plant enhancer-like element was isolated by Timko *et al.* (1985) in the 5'-upstream region of a *rbcS* gene (ribulose-1,5-bisphosphate carboxylase small subunit) which could, in both orientations, confer light-inducible expression when fused to homologous or heterologous promoters. Simpson *et al.* (1986) have described a 5' upstream element of a LHCP (light-harvesting chlorophyll *a/b*-binding protein) gene, which presents both enhancer and silencer activities conferring light-inducible and tissue-specific expression, in both orientations, on a heterologous promoter gene system. Kaulen *et al.* (1986) have located a 100-bp enhancer-like element in the 5'-upstream region of a *chs* (chalcone synthase) gene.

It would be interesting in our work to construct new plasmids in which the *nos* gene would be closer from insert 7 than the *nos-npt-II* gene (the inverse situation than in pLGVneo-nos-ins7) and study the influence of insert 7 on both genes after protoplast transformation. Since we did not detect any extrachromosomal plasmid in stably transformed clones, this suggests that there is no replication of a plasmid before integration into chromosomal DNA, using naked DNA protoplast transformation.

We are presently trying to localize more precisely the regulatory sequence present within insert 7 and to establish its nucleotide sequence for comparison with other regulatory sequences. Preliminary results showed that a 600-bp portion of insert 7 exhibits enhancer activity and is a unique sequence. We have shown that insert 7 has the same activity on the expression of the *nos-npt-II* gene in *N. plumbaginifolia* protoplasts as in *N. tabacum* protoplasts. It will be interesting to study its activity in other species, and on other promoters than the nopaline synthase promoter. More precisely, insert 7 could turn out to be useful in the study of relatively weak plant promoters, and in enhancing transient expression of foreign genes in species presenting a low rate of transformation.

To a certain extent, the enzymatic conversion of mesophyll cells into protoplasts could be considered as a stress situation: cells were indeed submitted to an osmotic stress, to particular hormone levels and to partial anaerobiosis by culture in liquid medium. Moreover, the removal of the cell wall could also be a stressful treatment as it has been shown that protoplasts pre-

sent dramatic changes in protein synthesis as soon as they are isolated and incubated in their culture medium (Fleck *et al.*, 1980). In particular, the influence of a high osmotic strength has been documented (Fleck *et al.*, 1983).

In order to verify whether the activity of insert 7 is induced by stress conditions, we plan to study *npt-II* expression in stably transformed plants submitted to stress treatments.

Materials and methods

Protoplast isolation

Protoplasts from leaves of 6-week-old plants from *N. plumbaginifolia* line P1 or *N. tabacum* cv. Petit Havana Line SR1 were isolated as described by Negrutiu (1981) and Nagy and Maliga (1976), respectively, modified by addition of 0.4 M sucrose in the leaf digest enzymatic solution.

N. plumbaginifolia protoplast transformation by direct gene transfer

The transformation method was essentially that of Krens *et al.* (1982): 1-ml aliquots of 2×10^6 protoplasts each in K3 medium supplemented with hormones, were incubated, after a thermic shock (5 min at 45°C) with 13% w/v PEG 6000 (pH 9.0), 15 µg of supercoiled plasmid DNA (from a 1 mg/ml stock), and 50 µg calf thymus DNA for 30 min at 26°C. After stepwise dilution with F medium, protoplasts were pelleted (5 min at 100 g), resuspended in 20 ml K3 medium and cultured in this medium until harvested, washed twice in K3 medium, pelleted, and finally frozen in liquid nitrogen before protein, DNA, or RNA extraction. In co-transformation experiments, protoplasts were incubated as before with 15 µg of each plasmid.

For stable transformation experiments, after 10–12 days culture in K3 medium, cells were diluted to 3000 colonies per dish of 10 ml MAPOA medium (Negrutiu, 1981) containing 25 mg/ml kanamycin (Sigma). After 3–4 weeks kanamycin-resistant colonies were isolated and cultured plants were regenerated as described by André *et al.* (1986).

N. plumbaginifolia and *N. tabacum* protoplasts transformation by electroporation

The method is mostly that of Fromm *et al.* (1985): 0.4-ml aliquots of 1×10^6 protoplasts resuspended in electroporation buffer (Fromm *et al.*, 1985) were incubated in ice with 10 µg supercoiled plasmid DNA for 5 minutes, then submitted to electroporation (100 V during 5 nsec), held 10 min on ice, diluted in 3 ml K3 medium (Nagy and Maliga, 1976) containing 0.1 mg/ml NAA, 0.2 mg/l BAP, 0.4 M sucrose, cultured at 26°C until harvested, frozen in liquid nitrogen before protein extraction.

NPT-II activity test

For transient expression assays, a frozen pellet of cells was sonicated 5 s in extraction buffer (Van den Broeck *et al.*, 1985) on ice. For stably transformed calli, or leaves, stems or roots of stably transformed plants, plant materials were homogenized with a glass rod in the extraction buffer. In both cases, protein contents were measured according to the Bio-Rad protein assay, and loaded on a non-denaturing polyacrylamide gel for *in situ* localization (Reiss *et al.*, 1984; Van den Broeck *et al.*, 1985).

Total DNA extraction

The same method (Dellaporta *et al.*, 1983) was used to extract total DNA from frozen cells (a few days after protoplast transformation) as well as from stably transformed calli, excepted for the way of breakage of the plant cells. Soon after transformation, breakage of the cells was achieved just by freezing them in liquid nitrogen. On the contrary, calli were broken first by grinding them in a mortar in liquid nitrogen, then by homogenizing them for 30 s with an ultra turax blender in the extraction buffer at 4°C.

RNA extraction

Total RNA was extracted from frozen transformed cells in a 8 M guanidium chloride buffer as described by Logemann *et al.* (1987).

Recombinant DNA techniques

Recombinant DNA procedures, DNA or RNA gel electrophoresis, Southern hybridizations and nick translations were carried out as described by Maniatis *et al.* (1982). Electroelution-purified insert 7 was digested by exonuclease II and nuclease S1 as described by Barnes *et al.* (1983), and the generated DNA fragments were cloned blunt-ended in pLGVneo-nos digested by *EcoRI*.

Acknowledgements

The authors wish to thank Allan Caplan and Jean-Pierre Hernalsteens for critical reading of the manuscript; Sylvie Burgeon, Stefaan Van Gijsegem, and Karel Spruyt for the preparation of the figures and Bénédicte Jadot and Martine De Cock for typing of the manuscript.

References

- André, D., Colau, D., Schell, J., Van Montagu, M. and Hernalsteens, J.-P. (1986) *Mol. Gen. Genet.*, **204**, 512–518.
- Banerji, J., Olson, L. and Schaffner, W. (1983) *Cell*, **33**, 729–740.
- Barnes, W.M., Bevan, M. and Son, P.H. (1983) In Wu, R., Grossman, L. and Moldave, K. (eds), *Recombinant DNA, Part C (Methods in Enzymology, Vol. 101)*. Academic Press, New York, pp. 98–122.
- Budar, F., Thia-Toong, L., Van Montagu, M. and Hernalsteens, J.-P. (1986) *Genetics*, **114**, 303–313.
- Dean, C., van den Elzen, P., Tamaki, S., Dunsmuir, P. and Bedbrook, J. (1985) *EMBO J.*, **4**, 3055–3061.
- Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) *Plant Mol. Biol. Rep.*, **1**, 19–21.
- Fleck, J., Durr, A., Fritsch, C., Lett, M.C. and Hirth, L. (1980) *Planta*, **148**, 453–454.
- Fleck, J., Durr, A. and Hirth, L. (1983) In Potrykus, I., Harms, C.T., Hinnen, A., Hütter, R., King, P.J. and Shillito, R.D. (eds), *Protoplasts 1983, Poster Proceedings* (Experientia Supplementum, Vol. 46). Birkhäuser Verlag, Basel, pp. 240–241.
- Fromm, M., Taylor, L.P. and Walbot, V. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 5824–5828.
- Graham, F.L. and Van Der Eb, A.J. (1973) *Virology*, **52**, 456–467.
- Hain, R., Stabel, P., Czernilofsky, A.P., Steinbiss, H.H., Herrera-Estrella, L. and Schell, J. (1985) *Mol. Gen. Genet.*, **199**, 161–168.
- Herrera-Estrella, L., De Block, M., Messens, E., Hernalsteens, J.-P., Van Montagu, M. and Schell, J. (1983) *EMBO J.*, **2**, 987–995.
- Herrera-Estrella, L., Van den Broeck, G., Maenhaut, R., Van Montagu, M., Schell, J., Timko, M. and Cashmore, A. (1984) *Nature*, **310**, 115–120.
- Kaulen, H., Schell, J. and Kreuzaler, F. (1986) *EMBO J.*, **5**, 1–8.
- Koncz, C. and Schell, J. (1986) *Mol. Gen. Genet.*, **204**, 383–396.
- Krens, F.A., Molendijk, L., Wullems, G.J. and Schilperoord, R.A. (1982) *Nature*, **296**, 72–74.
- Logemann, J., Schell, J. and Willmitzer, L. (1987) *Anal. Biochem.*, in press.
- Lörz, H., Baker, B. and Schell, J. (1985) *Mol. Gen. Genet.*, **199**, 178–182.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor Laboratory, New York.
- Morelli, G., Nagy, F., Fraley, R.T., Rogers, S.G. and Chua, N.-H. (1985) *Nature*, **315**, 200–204.
- Nagy, J.I. and Maliga, P. (1976) *Z. Pflanzenphysiol.*, **78**, 453–455.
- Negrutiu, I. (1981) *Z. Pflanzenphysiol.*, **104**, 431–442.
- Pazkowski, J., Shillito, R.D., Saul, M., Mandák, V., Hohn, T., Hohn, B. and Potrykus, I. (1984) *EMBO J.*, **3**, 2717–2722.
- Potrykus, I., Saul, M.W., Petruska, J., Paszkowski, J. and Shillito, R.D. (1985) *Mol. Gen. Genet.*, **199**, 183–188.
- Reiss, B., Sprengel, R., Will, H. and Schaller, H. (1984) *Gene*, **30**, 211–218.
- Simpson, J., Timko, M.P., Cashmore, A.R., Schell, J., Van Montagu, M. and Herrera-Estrella, L. (1985) *EMBO J.*, **4**, 2723–2729.
- Simpson, J., Schell, J., Van Montagu, M. and Herrera-Estrella, L. (1986) *Nature*, **323**, 551–554.
- Spena, A., Hain, R., Ziervogel, U., Saedler, H. and Schell, J. (1985) *EMBO J.*, **4**, 2739–2743.
- Timko, M.P., Kausch, A.P., Castresana, C., Fassler, J., Herrera-Estrella, L., Van den Broeck, G., Van Montagu, M., Schell, J. and Cashmore, A.R. (1985) *Nature*, **318**, 579–582.
- Van den Broeck, G., Timko, M.P., Kausch, A.P., Cashmore, A.R., Van Montagu, M. and Herrera-Estrella, L. (1985) *Nature*, **313**, 358–363.
- Velten, J., Velten, L., Hain, R. and Schell, J. (1984) *EMBO J.*, **3**, 2723–2730.
- Weier, H., König, M. and Gruss, P. (1983) *Science*, **219**, 626–631.
- Werr, W. and Lörz, H. (1986) *Mol. Gen. Genet.*, **202**, 471–475.

Received on May 18, 1987; revised on June 16, 1987