Theor Appl Genet (2000) 100:832–839 © Springer-Verlag 2000

K. Datta · Z. Koukolíková-Nicola · N. Baisakh
N. Oliva · S.K. Datta

Agrobacterium-mediated engineering for sheath blight resistance of indica rice cultivars from different ecosystems

Received: 26 July 1999 / Accepted: 27 August 1999

Abstract A concise T-DNA element was engineered containing the rice class-I *chitinase* gene expressed under the control of CaMV35S and the hygromycin phosphotransferase gene (*hph*) as a selectable marker. The binary plasmid vector pNO1 with the T-DNA element containing these genes of interest was mobilized to *Agrobacterium tumefaciens* strain LBA4404 to act as an efficient donor of T-DNA in the transformation of three different indica rice cultivars from different ecosystems. Many morphologically normal, fertile transgenic plants from these rice cultivars were generated after *Agrobacterium*-mediated transformation using 3-week-old scutella calli as initial explants. Stable integration, inheritance and expression of the chimeric *chitinase* gene were demonstrated by Southern blot and Western blot analysis of the transformants. Bioassay data showed that transgenic plants can restrict the growth of the sheath blight pathogen *Rhizoctonia solani*. Bioassay results were correlated with the molecular analysis. Although we obtained similar results upon DNA-mediated transformation, this report shows the potential of the cost-effective, simple *Agrobacterium* system for genetic manipulation of rice cultivars with a pathogenesis-related (PR) gene.

Key words *Agrobacterium* · Chitinase · T-DNA · Sheath blight · Rice

Communicated by G. Wenzel

K. Datta · Z. Koukolíková-Nicola · N. Baisakh · N. Oliva
S.K. Datta (✉)
Plant Breeding, Genetics and Biochemistry Division,
International Rice Research Institute,
MCPO Box 3127, 1271 Makati City, Philippines
Fax: (63-2) 8911292, 7612406
e-mail: SDATTA@CGIAR.ORG

Z. Koukolíková-Nicola
Botanisches Institut der Universität Basel,
Hebelstrasse 1, 4056 Basel, Switzerland

Introduction

The transfer of foreign genes into plants by the Gram-negative soil bacterium *Agrobacterium tumefaciens* is a standard and popular technique in the genetic engineering of dicotyledonous plants (Fraley et al. 1986; Hooykaas and Schilperoort 1992; Zambryski 1992), whereas monocotyledons were for a long time considered to be non-hosts of this bacterium. Since no tumors develop in the latter upon inoculation with *A. tumefaciens* (DeCleene 1985), only the development of suitable drug resistance and reporter marker genes eventually allowed the detection of successful transformation events of monocot cereals, such as maize (Ishida et al. 1996), barley (Tingay et al. 1997) and wheat (Mooney et al. 1991). In rice, the production of transgenic plants was first reported for the japonica cultivars (Chan et al. 1993; Hiei et al. 1994). The insertion of the reporter glucuronidase (*gus*) gene and the selectable marker hygromycin phospotransferase (*hph)* gene into more recalcitrant indica rice varieties using *Agrobacterium*-mediated transformation was also subsequently demonstrated. (Aldemita and Hodges 1996; Datta et al. 1996; Rashid et al. 1996). More recently, Cheng et al. (1998) used *Agrobacterium* to introduce the *cryIA(b)* and *cryIA(c)* genes into a japonica rice cultivar to control stem borer.

Agrobacterium-mediated gene integration is a precise event; that is, DNA between the two defined T-DNA border sequences is transferred exclusively. Several sophisticated plant transformation vectors based on this naturally occurring gene transfer system have been developed and are employed in genetic engineering (Fraley et al. 1986). The widely used binary system consists of a helper Ti plasmid providing the virulence functions needed for transfer and a small vector carrying an artificial T-DNA (Bevan 1984; An et al. 1988). The binary vectors replicate in *E. coli* as well as in *A. tumefaciens* and allow easy cloning of the genes of interest between the T-DNA borders. In the investigation reported here, we used the vector pCGN1589 (McBride and Summerfelt 1990), which is a precursor of improved transforma-

tion vectors that are highly stable in *A. tumefaciens*, are able to replicate (high copy) in *E. coli* and contain a gentamycin resistance gene, as a strong selectable marker for bacteria and the *LacZ'* gene segment from pUC18 (Yanisch-Parron et al. 1985) as a source of unique restriction sites in the T-DNA region for cloning genes of interest. In contrast to other binary vectors, its T-DNA region is very concise, containing only the minimal left and right border regions, and is very stable in *Agrobacterium* during growth without selection.

Chitinase may have a protective role against fungal pathogens (Boller 1985). It hydrolyzes the β–1,4 linkages of chitin, which is the major component of many fungal cell walls. Among the pathogenesis-related proteins (PR-proteins), chitinase has been studied extensively for its antifungal activity (Neuhaus 1999). Besides its ability to attack the fungal cell wall directly, chitinase releases oligo-*N*-acetylglucosamines that function as elicitors for the activation of defense-related responses in rice cells (Ren and West 1992). Constitutive expression of bean chitinase in transgenic tobacco plants was shown to result in a significant reduction of fungal growth and delay in the development of rice sheath blight disease caused by *Rhizoctonia solani* (Broglie et al. 1991). A similar type of antifungal activity has been observed with transgenic cucumber harboring the rice *chitinase* gene against gray mold *Botrytis cinerea* (Tabei et al. 1998) and in transgenic tobacco cells with bacterial *chitinase* gene against the sheath blight pathogen *R. solani* (Jach et al. 1992).

Plant protection is a major challenge to agriculture worldwide. The most desirable strategy is the incorporation into commercially acceptable cultivars of disease resistance genes which enhance the plant's own defense-related responses, such as the *chitinase* gene. One of the most devastating biotic stresses in rice is sheath blight disease caused by *R. solani*, which is widespread in all rice-growing countries. Constitutive expression of the rice class-I *chitinase* gene resulted in enhanced resistance to *R. solani* of transgenic rice plants produced by direct DNA-mediated transformation (Lin et al. 1995). We report here a successful and efficient transformation system of indica rice by *Agrobacterium* with the same *chitinase* gene to enhance sheath blight resistance. This model system indicates the great potential to engineer agronomically important rice cultivars.

Materials and methods

Plasmid construction and bacterial strains

The *Hin*dIII/*Pst*I fragment (1.9 kb) of the pmnus7–13a vector containing the hygromycin phosphotransferase (*hph*) gene under the control of the cauliflower mosaic virus (CaMV) 35 S constitutive promoter (unpublished) was cloned into the binary vector pCGN1589 (a precursor of the binary vectors described by McBride and Summerfelt 1990) and digested with the *Hin*dIII and *Pst*I restriction enzymes. The resulting binary vector pZ2–6 carries between the T-DNA borders the widely used *hph* gene as a selectable marker and a slightly modified pUC18 (Yanisch-Perron et al. 1985) polylinker useful for inserting genes of interest. The *Hin*dIII fragment (1.53 kb) of the pGL2 plasmid (Lin et al. 1995), containing the rice class-I *chitinase* gene linked to the constitutive CaMV35 S promoter (CaMV-Chi11), was excised and cloned into the unique *Hin*dIII site of pZ2–6, resulting in the binary vector pNO1 (see Fig. 1 for details of the construction). The plasmids were maintained in the DH5α strain of *E. coli* (Hanahan 1983).

The binary vector pNO1 was mobilized to two different *Agrobacterium* strains: LBA4404, containing a disarmed octopine Ti plasmid (Hoekema et al. 1983), and A281, the supervirulent strain

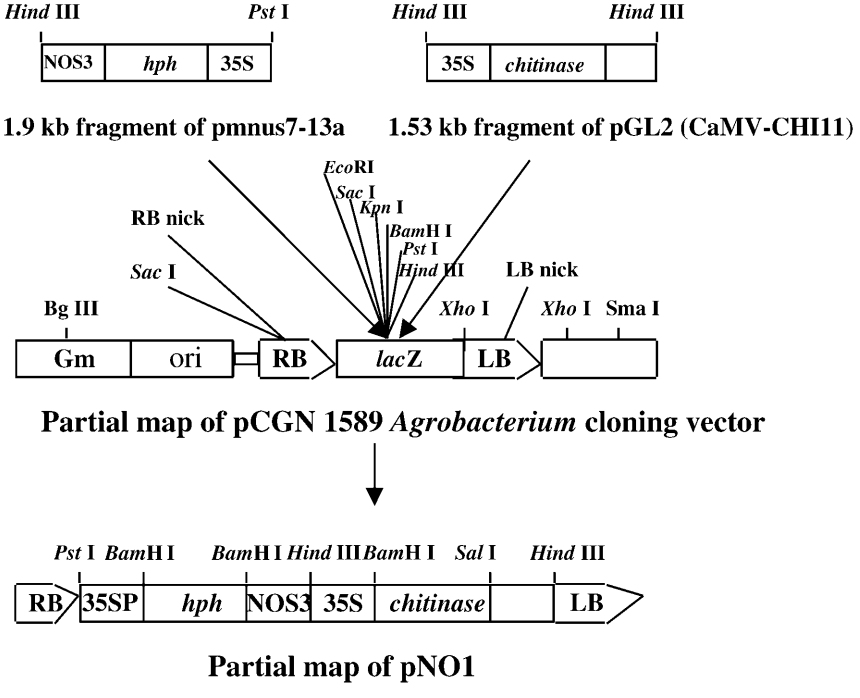


Fig. 1 Partial diagram showing the construction of the binary vector pNO1.The *Hin*dIII/*Pst*I fragment of pmnus7–13a containing the hygromycin phosphotransferase (*hph*) gene and *Hin*dIII fragment of pGL2 (CaMV-Chi11) containing the *chitinase* gene were cloned into the binary vector pCGN1589. The resulting binary vector is pNO1 (not drawn to scale)

(Guyon et al. 1980; Hood et al. 1986; Jin et al. 1987), carrying the wild-type agropine Ti plasmid pTiBo542. *E. coli* strain GJ23 was used as a helper strain for mobilization. The two *Agrobacterium* strains, designated LBA4404 (pNO1) and A281 (pNO1), carrying the binary vector pNO1 were used in our transformation experiments. These strains were maintained in LB medium supplemented with 20 µg/ml rifampicin (SIGMA) plus 40 µg/ml gentamycin (GIBCO-BRL).

Plant material and production of explants

Three different indica rice cultivars from different ecosystems were chosen. Basmati122, a commercial aromatic cultivar, Tulsi, a rainfed lowland cultivar, and Vaidehi, a deepwater rice cultivar, were used for transformation. Embryogenic calli were obtained from 9- to 14-day-old immature embryos (Datta et al. 1998) for Basmati122; for Tulsi and Vaidehi, embryogenic scutellar calli from mature seeds were used. MS medium (Murashige and Skoog 1962) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) 2 mg/l was used for callus induction and proliferation. Three- to four-week-old actively growing embryogenic calli were taken as explants for transformation experiments.

Growth of *Agrobacterium* strains for cocultivation experiments

Agrobacterium tumefaciens strains LBA4404 (pNO1) and A281 (pNO1) were precultured as described by Koukolíková-Nicola et al. (1993) and Shen et al. (1993). The strains were grown at 28°C for 48 h in LB liquid medium supplemented with gentamycin (40 µg/ml) and rifampicin (20 µg/ml) and then subcultured in the same medium following a 1:20 dilution and grown for an additional 20 h, reaching a final titer of about $1–2 \times 10^9$ cfu/ml (optical density: $OD_{600} \approx 2$). Bacteria cells were washed twice with $MgSO_4$ (10 m*M*) by centrifugation and the optical density (OD_{600}) of the bacterial cultures was adjusted to 2 by resuspending in the MS liquid medium supplemented with 200 µ*M* acetosyringone (Sigma-Aldrich).

Cocultivation and plant regeneration

Rice embryogenic calli (mature or immature embryo-derived) were grown on MS medium supplemented with 2,4-D (2 mg/l) as described by Datta et al. (1992). Three-to-four-week-old embryogenic calli were immersed in the bacterial suspension and placed into the vacuum chamber. Two cycles of vacuum infiltration (0.4–0.6 atm) with a duration of 5 min each were applied. The calli were transferred without rinsing to the cocultivation medium (solid MS medium with 2 mg/l 2,4-D plus 200 µ*M* acetosyringone) and incubated at 25°C in the dark for 3 days. After the cocultivation, the material was washed thoroughly in sterile water containing 250 mg/l cefotaxime (GIBCO-BRL) and placed on a selection medium – solid MS medium supplemented with 250 mg/l cefotaxime and 50 mg/l hygromycin (Calbiochem). Proliferating colonies of cells surviving four selection cycles (in fresh medium, 15 days each) were transferred to a regeneration medium [MS with 2 mg/l kinetin and 1 mg/l 1-naphthaleneacetic acid (NAA)] and incubated at 25°C under a 16-h photoperiod (Datta et al. 1992). Regenerated plants with well-developed roots were eventually transferred to soil in pots and grown to maturity in the greenhouse for transgenic plants.

Hygromycin phosphotransferase (HPT) assays and Southern blot analysis

HPT assays

Hygromycin phosphotransferase assays were performed following the procedure described by Datta et al. (1990).

Southern blot analysis

Total DNA was isolated from leaf tissues using a standard procedure. Ten micrograms of DNA, digested with restriction endonucleases, was separated by electrophoresis on a 1% (w/v) TAE agarose gel and transferred under alkaline denaturing conditions to Hybond N^+ nylon membrane (Amersham). The radioactive probe was prepared by the random primer method using α–[^{32}P] dCTP and the Rediprime labeling system (Amersham). Hybridization with the probe was conducted according to the manufacturer's instructions. Following hybridization, the membrane was washed and exposed to X-ray Hyperfilm™ MP (Amersham).

Analysis of chitinase protein production

Protein extraction and Western blot analysis were done following the method described by Datta et al. (1998). Proteins were extracted from fresh leaves with 0.05 *M* Tris-HCl (pH 7.0) containing 10% glycerol and 0.1 m*M* phenyl methyl sulfonyl fluoride (PMSF, Sigma). Fifty-microgram aliquots of the proteins separated on a 10% polyacrylamide gel in the presence of sodium dodecylsulfate (SDS-PAGE) were transferred to a nitrocellulose membrane that was probed with antiserum to bean chitinase, which cross-hybridizes to the rice class-I chitinase (Datta et al. 1999), using horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) as the second antibody (Bio-Rad). HRP staining was carried out with 30% H_2O_2 and HRP color reagent (Bio-Rad).

Bioassay

Seeds from selfed T_O transgenics of Basmati122 were germinated and grown to plants and screened for the *chitinase* gene by Southern blot analysis. Those Southern-positive T_1 plants were used for bioassays. Resistance to the sheath blight pathogen *R. solani* was assessed by the method described by Datta et al. (1999). The pathogen growing on potato dextrose agar (PDA) plates was transferred into a bottle containing a rice hull-grain mixture and incubated at 28°C for 10 days. Five grams of the pathogen-rice hull mixture were placed in the middle of the tillers of each plant at its maximum tillering stage. The symptoms (size and number of lesions) caused by fungal infection were scored after 14 days. The relative integrated infection index was calculated for each transgenic plant by the standard formula (INGER 1996).

Relative integrated infection index (RI)

$$\frac{\text{DI of transgenic plant}}{\text{DI of non-transformed control plant}} \times 100$$

Where:

$$\text{DI} = \frac{\Sigma \text{Xi Pi}}{\text{N}}$$

Xi = number of tillers showing same grade of infection
Pi = infection rate
N = total number of tillers inoculated

Results

Vector construction and rice transformation

Figure 1 describes the construction of the binary vector pNO1**,** containing an engineered *hph* gene and the *CaMV-Chi11* gene in between the T-DNA borders (see Materials and methods for details).

Table 1 summarizes the results of the different rice transformation experiments. A total of 222 plants were

Table 1 Summary of *Agrobacterium*-mediated transformation with different rice cultivars

Cultivar	Explant[a]	Agro strain used	Number of explants cocultivated	Number of putative transformed calli after selection[b]	Number. of plants in greenhouse	Number of positive plants	
						HPT^+	S^+
Basmati122	SC	LBA4404 (pNO1)	275	52 (12)	45	ND	15/27
Basmati122	SC	A281 (pNO1)	175	40 (7)	0	–	–
Tulsi	SCM	LBA 4404 (pNO1)	400	69 (22)	113	11/16	45/46
Tulsi	SCM	A281 (pNO1)	300	114 (48)	0	–	–
Vaidehi	SCM	LBA 4404 (pNO1)	290	41 (15)	64	16/17	27/30

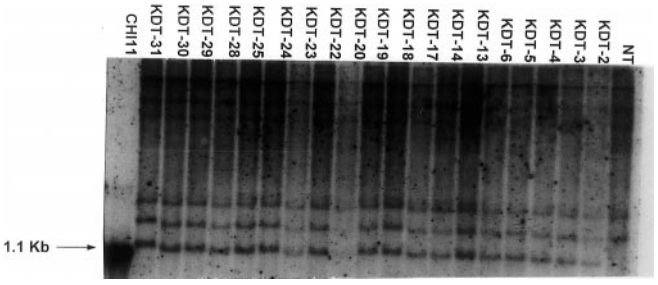
[a] SC = scutellar calli from immature embryo; SCM = scutellar calli from mature seeds
[b] Numbers in parentheses represent the no. of primary (independent) calli selected
[c] S^+ = Southern-positive for *chitinase* gene; HPT^+ = hygromycin phosphotransferase assay-positive

regenerated using the three different cultivars. Although putative transformed calli were obtained from calli cocultivated with both strains, LBA4404 (pNO1) and A281 (pNO1), green plants were regenerated only when LBA4404 (pNO1) was used. In the case of Basmati122, 45 green plants were regenerated from 52 hygromycin-resistant calli derived from 12 independently grown primary calli. For Tulsi, 113 plants were obtained from 69 Hg^R calli derived from 22 independently grown primary ones. In the case of Vaidehi, 64 plants were obtained from 41 Hg^R calli resulting from 15 independent primary ones.

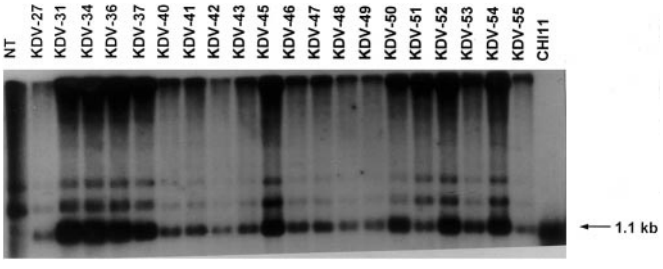
For Tulsi and Vaidehi, 80–90% of the plants grown in the greenhouse were healthy and fertile, but this was the case for only 27% for Basmati 122 plants. Some of the selected T_0 lines of three cultivars were grown for the next generation.

Molecular analysis and HPT assays

Putative T_0 plants were assayed by Southern analysis to detect the presence of the *chitinase* gene. Out of 27 putative transformants of Basmati122, 15 showed hybridization to a 1.1-kb (*Sac*I/*Sal*I) fragment of the coding sequence of the *chitinase* gene. One band of the expected size (1.1 kb) was detected in addition to the high-molecular-weight signals. For the Tulsi and Vaidehi cultivars, the HPT assay was done prior to molecular analysis to screen the putative T_0 transgenic plants. Leaf extracts from 11 out of 16 Tulsi plants and 16 out of 17 T_0 Vaidehi plants showed clear hygromycin phosphotransferase activity, suggesting the expression of the foreign *hph* gene in those plants. Further evidence came from Southern analysis of the *hph*-positive plants in which the presence of the introduced *chitinase* gene was detected. DNA from 46 T_0 Tulsi plants and 30 T_0 Vaidehi plants was digested with *Sac*I/*Sal*I to release the *chitinase* gene (1.1 kb). Of the 46 Tulsi plants, 45 showed the expected band of 1.1 kb (Fig. 2), whereas 27 out of 30 Vaidehi plants showed the expected band of the foreign *chitinase* gene (Fig. 3). Fragments larger than the expected size hybridizing to the *chitinase* probe, detected in both the transformants and the control plants of all the cultivars,



2

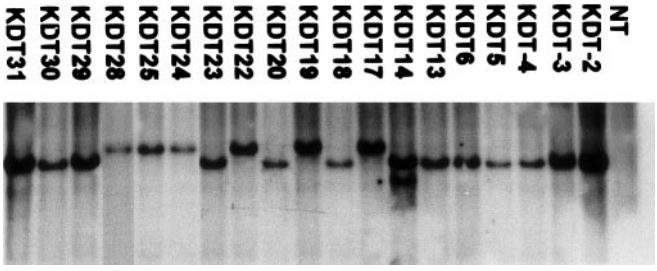


3

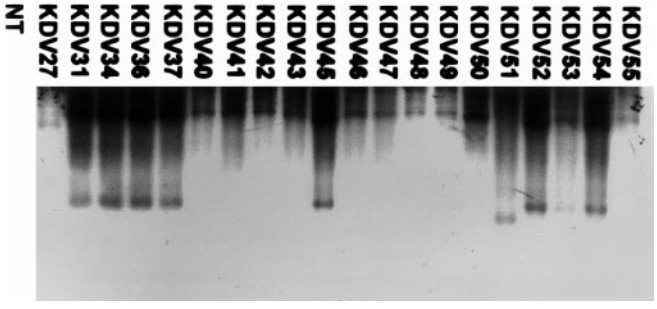
Figs. 2, 3 Southern blot analysis of transformed T_0 plants of rice cvs. Tulsi (KDT, **Fig. 2**) and Vaidehi (KDV, **Fig. 3**). The genomic DNA was restricted with *Sac*I/*Sal*I endonucleases. *NT* Non-transformed control rice plant. The Chi11 lane represents the 1.1-kb (*Sac*I/*Sal*I) fragment of plasmid DNA. The same 1.1-kb open reading frame of the *chitinase* gene was used as the probe

correspond to the endogeneous *chitinase* genes, as has been demonstrated in Lin et al. (1995).

The same blots (Figs. 2, 3) were reprobed with a 1.1-kb (*Bam*HI) hygromycin fragment to detect the cointegration of the *hph* gene in the genome of *Chi11*-positive transgenics (Figs. 4, 5). Hybridization with the *hph* gene as the probe showed differences among the Tulsi T_0 plants (Fig. 4) and Vaidehi T_0 plants (Fig. 5) with respect to the banding pattern of the *hph* gene. In Tulsi T_0 plants, although signals were observed in all the plants analyzed (except KDT 14 with two bands of different sizes), the bands were of two different molecular sizes. The case was similar for Vaidehi, where *hph* signals of different molecular sizes were observed, with the number of signals ranging from two to four.



4



5

Figs. 4, 5 Southern blot analysis for the *hph* gene (Southern blots of **Figs. 2** and **3** reprobed with the *hph* gene) in T_0 transformants of rice cvs. Tulsi (KDT, **Fig. 4**) and Vaidehi (KDV, **Fig. 5**). The genomic DNA was restricted with *Sac*I/*Sal*I endonucleases. NT Non-transformed control rice plant. A 1.1-kb (*Bam*HI) *hph* fragment of plasmid DNA was used as the probe

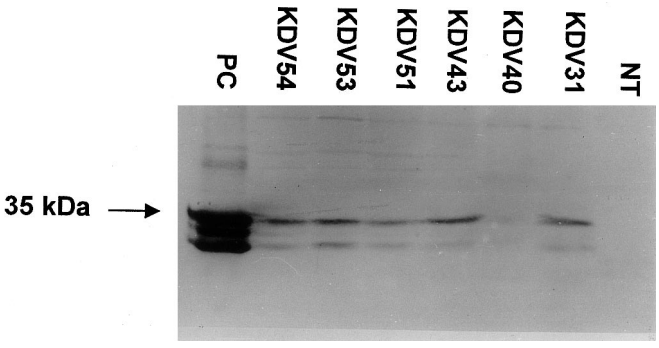
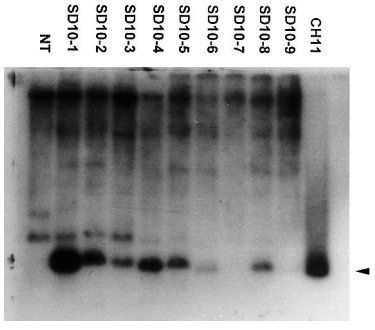
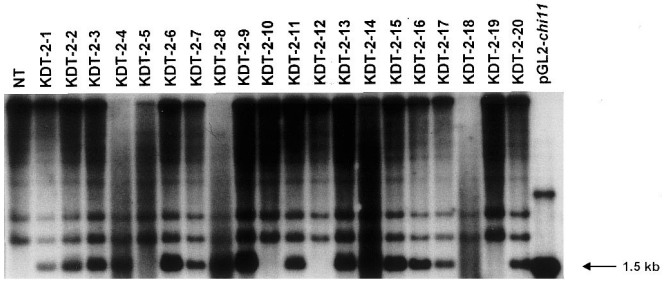


Fig. 6 Western blot analysis showing chitinase protein levels in T_0 plants of Vaidehi. The positive control (*PC*, transgenic CBII with *chi11* gene) is shown in the *extreme left lane*. *NT* Non-transformed negative control. *Arrow* indicates the expected 35-kDa chitinase protein for transgenics



7



8

Fig. 7 Southern blot analysis of 9 T_1 descendants of one transgenic line of Basmati122 (SD10). *NT* Non-transformed control DNA, *Chi11* positive control consisting of a 1.1-kb fragment (*Sac*I/*Sal*I) of pGL2Chi11 (*arrow marker*). The same 1.1-kb *chitinase* open reading frame was used as the probe

Fig. 8 Southern blot analysis of 20 T_1 descendants of one transgenic line of Tulsi (KDT2). *NT* Non-transformed control DNA, and *Chi11* positive control consisting of a 1.5-kb fragment (*Hind*III) of pGL2Chi11 (*arrowhead marker*). The 1.1-kb (*Sac*I/*Sal*I) *chitinase* open reading frame was used as the probe

Expression of chitinase

We examined production of the foreign chitinase protein in transgenic plants by Western blot analysis using a polyclonal antibody against rice chitinase. T_1 progenies of Basmati122 plants and T_0 plants of Vaidehi and Tulsi, positive in screening by Southern analysis for the presence of the *chitinase* gene, were analyzed. The extracted leaf proteins were separated electrophoretically on an SDS gel. A major band (approx. 35 kDa), corresponding to the chitinase protein detected in the positive control (leaf extract from known chitinase-positive plants from earlier DNA-mediated transformation experiments, Lin et al. 1995), was observed in all transformed plants. In addition to the 35-kDa chitinase protein band, one to two fast migrating bands were also seen (Fig. 6). These additional bands represent the endogenous chitinase protein (28 kDa) and a 30-kDa protein resulting from proteolytic degradation during sample preparation. All plants found to be positive by Southern analysis were also positive in Western analysis.

Transmission of transgenes to the next generation

Inheritance of the transgenes was studied in the T_1 generation. Segregation for the *chitinase* gene, detected by Southern blot analysis, was observed in progenies of 4 primary transformants (at least 20 progenies from each transformant analyzed) of the three cultivars. All T_1 progenies analyzed conformed approximately to a 3:1 segregation of Basmati line 10 and Tulsi line 2 (Figs. 7, 8). Data was not shown for Vaidehi.

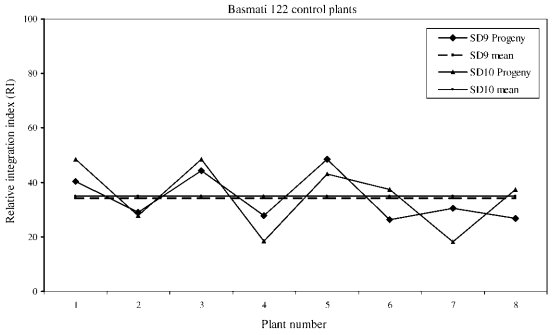


Fig. 9 Bioassay of Basmati122 T_1 progeny from two different independent lines (SD9 and SD10). The relative integrated infection index (RI) is shown for 8 selected representative Southern-positive T_1. The mean was calculated taking all the plants together

Bioassays

From the T_0 transgenic plants (Basmati122) that were positive for the functional *chitinase* transgene in Southern blot analysis and Western blot analysis, we selected several lines for bioassays in the T_1 generation. These assays were performed with the sheath blight pathogen *R. solani* using a tissue culture-derived non-transformed Basmati122 as the negative control and T_1 progenies from Southern- and Western-positive selected lines (SD1, SD3, SD9, SD10) at their maximum tillering stage (50 days after sowing), and the disease symptoms were scored as percentage sheath area infected. Different levels of disease progression (the number and size of the lesions) were observed 2 week after inoculation. The lesion areas in the transgenic plants were smaller than those of the control plants. Figure 9 shows the bioassay results of some of the T_1 progenies of two transgenic lines (SD9 and SD10) that were positive in Southern analysis. The infection level was significantly lower in the transgenic lines than in the controls, although variation in the relative integrated infection index was observed.

Discussion

In this study, a large number of rice plants carrying the *chitinase* (*Chi11)* gene have been produced in three different elite indica rice cultivars using the *Agrobacterium* transformation system. These results represent a convincing confirmation of *Agrobacterium*-mediated transformation of rice and show the potential of this technique for genetic engineering of indica rice. The frequencies of transformation obtained in this study seem to be higher than our other systems of transformation (protoplast and biolistic) used to obtain transgenic rice (Lin et al. 1995; Datta et al. 1999), although genotypic differences were observed.

In our experiment, we used two different *Agrobacterium* strains: LBA4404, which contains an octopine Ti-plasmid whose T-DNA was deleted, and A281, which carries the wild-type supervirulent agropine Ti-plasmid pTiBo542, which contains all the T-DNA genes responsible for tumor formation in dicotyledonous plants (the so-called *onc* genes). A281 induces large, early appearing tumors on a wider range of plants than do other *Agrobacterium* strains, and it has been shown that some of the virulence genes, located outside of the T-DNA, are responsible for the high transformation frequency of pTiBo542 (Hood et al. 1986; Jin et al. 1987). We suspect that the true reason, calli transformed by the A281 (pNO1) did not produce any regenerated plants is due to the presence of the *onc* genes. Although no tumor formation has been observed in monocotyledonous plants except for some swelling in *Narcissus* (Hooykaas-van Slogteren et al. 1984) and isolation of crown gall tissue in *Asparagus*, expression of the *onc* genes within the transformed cells might nevertheless interfere with their regeneration ability. Because a high proportion of the transformed cells contains both T-DNAs, the "artificial" one from the binary vector and the "natural" one from the Ti-plasmid, most of the Hg^R cells thus probably also contain the *onc* genes (DeFramond et al. 1986). Results of Cheng et al. (1998), showing that regenerated plants were obtained using a T-DNA-less derivative of the pTiBo542, support our hypothesis. We further confirm the results of Cheng et al. (1998) showing that it is not necessary to use the supervirulent strain for rice transformation. For Basmati122, 27% of the transgenic plants were fertile, whereas for Tulsi and Vaidehi, 80–90% of the transgenic plants were fertile. This observation also suggests a genotypic effect on plant regeneration and seed-setting ability.

It is evident from the HPT assay and Southern analysis that the two genes, *hph* and *chitinase*, carried by pNO1 are reproducibly transferred via *Agrobacterium* and stably integrated into the rice genome. The two genes are tightly linked in the majority of the transformed plants except in some T_0 transgenics where rearrangements and/or multiple copies of the *hph* gene were observed (Figs. 4, 5). Southern analysis using *Sac*I/*Sal*I-digested DNA hybridized with *Chi11* revealed that in all of the transformed plants, the expected hybridization signals for *Chi11* remained intact when integrated into the rice genome. Inheritance studies of the Basmati122 T_1 progeny showed that the *chitinase* gene is transmitted to the T_1 progeny, demonstrating stable incorporation of T-DNA into the rice nuclear DNA, and the 3:1 segregation ratio suggested that the *chitinase* gene was integrated at a single locus. However, the different patterns of *hph* integration observed in the T_0 transgenics of Tulsi and Vaidehi lead us to the assumption that there might have been some multiple T-DNA integration events. The *chitinase* gene remained intact without any rearrangement, whereas rearrangements occurred in the *hph* gene (Figs. 4, 5) possibly because of the short, truncated T-DNA generated during or after integration of a full-length T-DNA into the plant genome (Matsumoto et al. 1990). Although there are reports on transgenic cell lines

containing full-length T-DNA in the chromosomes, cases of short truncated T-DNA integration have also been shown in several instances. This might result from the deletion of long stretches from both termini of the T-DNA before integration into the plant genome (Howard et al. 1989) or after synthesis of a normal T-DNA intermediate during transfer and/or the integration process (Herman et al. 1990). These observations are in conformity with our results where deletions or insertions in T-DNA might have occurred, resulting in *hph* fragments of different molecular sizes. Also, the experiment with agroinfection by Bakkeren et al. (1989) shows that both deletions and insertions can occur around the border sequences during transfer of T-DNA. Such rearranged or truncated *hph* probably resulted in the weaker activity or expression of the hygromycin phosphotransferase enzyme we observed in some of our T_0 transgenics. The expression of marker and/or chitinase transgenes in most of the transformants indicates preferential T-DNA insertion in transcriptionally active regions of the rice genome. We further presume with our ongoing molecular analysis (data not shown) that whole T-DNA as well as the total vector in some of the transformants was integrated into the rice genome when the genomic DNA was restricted with *Pst1* endonuclease having a unique restriction site in the vector. Our genetic analysis in the segregating T_1 generation for Tulsi and Vaidehi confirmed the locus-specific integration of the transgene(s).

Various other factors may affect the transformation efficiency of rice by *Agrobacterium*-mediated T-DNA transfer. Optimization of conditions for vacuum infiltration, cocultivation and selection of a suitable explant may play a vital role in transformation. Scutellum-derived (immature or mature) calli were shown to be an excellent starting explant in our transformation experiments with indica rice, as was also reported by Hiei et al. (1994) for japonica cultivars. The pre-treatment of tissues by wounding or enzymatic digestion of cell walls as reported by other studies (Chan et al. 1993) was not necessary in our case. Because wounded cells exude phenolic compounds such as acetosyringone that activate the *vir* genes that are responsible for the transfer of T-DNA from *A. tumefaciens* to the host cells (Stachel et al. 1985), we added acetosyringone to the cocultivation medium as an external inducer.

Chitinase protein production was transmitted through sexual generation to the T_1 plants of the T_0 chitinase-positive plants (Basmati122). The bioassay of the T_1 Southern-positive plants with sheath blight pathogen *R. solani* showed different levels of disease progression in individual plants. However, the infection level was significantly lower in transgenic progenies having a higher expression of PR-proteins than in the non-transgenic control plants, indicating that enhanced resistance to sheath blight disease can be achieved. Variation between individual T_1 plants is most probably due to different amounts of chitinase production. We have observed differences in the amount of the chitinase enzyme produced in the T_0 transgenic plants of Tulsi and Vaidehi (data not shown) from the radiometric estimation of the chitinase protein (Boller 1991). The enzyme level was higher in transgenics than in the control plants. This study highlights a simple, low-cost transformation system to develop transgenic indica rice with agronomically important genes.

Acknowledgments Zdena Koukolíková-Nicola thanks Dr. T. Boller for the opportunity to conduct part of the experiments in his laboratory at the Botanical Institute, Basel. The authors thank Dr. P. Hooykaas for the gift of LBA4404, Dr. K. McBride for the gift of pCGN1589, and Dr. E. Nester for the gift of A281. We also thank Dr. T. Mew and Isabelita Ona of the Entomology and Plant Pathology Division for helping with the bioassay. Thanks are due to Bill Hardy for comments and editorial assistance. Financial support from the BMZ (Germany) and the Rockefeller Foundation are gratefully acknowledged.

References

Aldemita RR, Hodges TK (1996) *Agrobacterium tumefaciens*-mediated transformation of indica and japonica rice varieties. Planta 199:612–617

An G, Evert PR, Mitra A, Ha SB (1988) Binary vectors. In: Gelvin SB, Schilperoort RA (eds) *Plant molecular biology manual A3*. Kluwer Academic Press, Dordrecht, the Netherlands, pp. 1–19

Bakkeren G, Koukoliková-Nicola Z, Hala B (1989) Recovery of *Agrobacterium tumefaciens* T-DNA molecules from whole plants early after transfer. Cell 57:847–857

Bevan M (1984) Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Res 12:8711–8721

Boller T (1985) Induction of hydrolase as a defense reaction against pathogens. In: Key, JL, Kosuge T (eds) *Cellular and molecular biology of plant stress* Alan R. Liss, New York, pp 247–262

Boller T (1991) Biochemical analysis of chitinases and β–1,3-glucanases. In: Gurr SJ, McPherson MJ, Bowles DJ (eds) *Molecular plant pathology. A practical approach, vol 2.* IRL Press, Oxford, pp 23–30

Broglie K, Chet I, Holliday M, Cressman R, Biddle P, Knowlton S, Mauvais CJ, Broglie R (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194–1197

Bytebier B, Deboeck F, De Greve H, Van Montagu M (1987) T-DNA organization in tumor cultures and transgenic plants of the monocotyledon *Asparagus officinalis*. Proc Natl Acad Sci USA 84:5345–5349

Chan MT, Chang HH, Ho SL, Tong WF, Yu SM (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α–amylase promoter/ β-glucuronidase gene. Plant Mol Biol 22:491–506

Cheng X, Sardana R, Kaplan H, Altosaar I (1998) *Agrobacterium*-transformed rice plants expressing synthetic *cryIA(b)* and *cryIA(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci USA 95: 2767–2772

Datta K, Oliva N, Torrizo L, Abrigo E, Khush GS, Datta SK (1996). Genetic transformation of indica and japonica rice by *Agrobacterium tumefaciens.* Rice Genet Newsl 13:136–139

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998) Constitutive and tissue-specific differential expression of *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest. Theor Appl Genet 7:20–30

Datta K, Velazhahan R, Oliva N, Ona I, Mew T, Khush GS, Muthukrishnan S, Datta SK (1999) Overexpression of cloned rice thaumatin-like protein (PR-5) in transgenic rice plants enhances environmental-friendly resistance to *Rhizoctonia solani* causing sheath blight disease. Theor Appl Genet 98:1138–1145

Datta SK, Peterhans A, Datta K, Potrykus I (1990) Genetically engineered fertile Indica -rice recovered from protoplasts. Bio/Technology 8:736–740

Datta SK, Datta K, Soltanifar N, Donn G, Potrykus I (1992) Herbicide-resistant indica rice plants from IRRI breeding line IR72 after PEG-mediated transformation of protoplasts. Plant Mol Biol 20:619–629

DeCleene M (1985) The susceptibility of monocotyledons to *Agrobacterium tumefaciens*. Phytopathol Z 113:81–89

DeFramond AJ, Back EW, Chilton WS, Kayers L, Chilton MD (1986) Two unlinked T-DNA can transform the same tobacco plant cell and segregate in the F_1 generation. Mol Gen Genet 202:125–131

Fraley RT, Roger SG, Horsch RB (1986) Genetic transformation in higher plants. CRC Crit Rev Plant Sci 4:1–46

Guyon P, Chilton MD, Petit A, Tempe J (1980) Agropine in "null-type" crown gall tumors: evidence for generality of the opine concept. Proc Natl Acad Sci USA 77:2693–2697

Hanahan D (1983) Studies on transformation of *E. coli* with plasmids. J Mol Biol 166:557–580

Herman L, Jacobs A, Van Montagu M, Depicker A (1990) Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. Mol Gen Genet 224: 248–256

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary vector strategy based on separation of *vir* and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179–180

Hood EE, Helmer GL, Fraley RT, Chilton MD (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168:1291–1301

Hooykaas PJJ, Schilperoort RA (1992) *Agrobacterium* and plant genetic engineering. Plant Mol Biol 19:15–38

Hooykaas-van Slogteren GMS, Hooykaas PJJ, Schilperoort RA (1984) Expression of Ti plasmid genes in monocotyledonous plants infected with *Agrobacterium tumefaciens.* Nature 311: 763–764

Howard EA, Winsor BA, De Vos G, Zambryski P (1989) Activation of T-DNA transfer process in *Agrobacterium* results in the generation of a T-strand-proteins complex: tight association of Vir D2 with the 5' ends of T-strands. Proc Natl Acad Sci USA 86:4017–4021

INGER (1996) Standard evaluation system for rice. International Rice Research Institute, Los Baños, Philippines

Ishida Y, Saito H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat Bio/Technol 14:745–750

Jach G, Logemann S, Wolf G, Oppenheim A, Chet I, Schell J, Logemann J (1992) Expression of a bacterial chitinase leads to improved resistance of transgenic tobacco plants against fungal infection. Biopractice 1:1–10

Jin S, Komari T, Gordon MP, Nester EW (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A281. J Bacteriol 169:4417–4425

Koukoliková-Nicola Z, Raineri D, Stephens K, Ramos C, Tinland B, Nester EW, Hohn B (1993) Genetic analysis of the virD operon of *Agrobacterium tumefaciens*: a search for functions involved in transport of T-DNA into the plant cell nucleus and in T-DNA integration. J Bacteriol 175:723–731

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta SK (1995) Genetic engineering of rice for resistance to sheath blight. Bio/Technology 13:686–691

Matsumoto S, Ito Y, Hosoi T, Takahashi Y, Machida Y (1990) Integration of *Agrobacterium* T-DNA into a tobacco chromosome: possible involvement of DNA homology between T-DNA and plant DNA. Mol Gen Genet. 224:309–316

McBride KE, Summerfelt KR (1990) Improved binary vectors for *Agrobacterium*-mediated plant transformation. Plant Mol Biol 14:269–276

Mooney PA, Goodwin PB, Dennis ES, Llewellyn DJ (1991) *Agrobacterium tumefaciens* gene transfer into wheat tissues. Plant Cell Tissue Organ Cult 25:209–218

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473–497

Neuhaus JM (1999) Plant chitinases (PR-3, PR-4, PR-8, PR-11). In: Datta SK, Muthukrishnan S (eds) *Pathogenesis-related proteins in plants.* CRC Press, Boca Raton, Florida, pp 77–105

Raineri DM, Bottino P, Gordon MP, Nester EW (1990) *Agrobacterium*-mediated transformation of rice (*Oryza sativa* L.). Bio/Technology 8:33–38

Rashid H, Yokoi S, Toriyama K, Hinata K (1996) Transgenic plant production mediated by *Agrobacterium* in Indica rice. Plant Cell Rep 15:727–730

Ren Y, West CA (1992) Elicitation of diterpene biosynthesis in rice (*Oryza sativa* L.) by chitin. Plant Physiol 99:1169–1178

Shen WH, Escudero J, Schlaeppi M, Ramos C, Hohn B, Koukolikova-Nicola Z (1993) T-DNA transfer to maize cells: histochemical investigation of β-glucuronidase activity in maize tissues. Proc Natl Acad Sci USA 90:1488–1492

Stachel SE, Messens E, Van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature 318:624–629

Tabei Y, Kitade S, Nishizawa Y, Kikuchi N, Kayano T, Hibi T, Akutsu K (1998) Transgenic cucumber plants harbouring a rice *chitinase* gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*). Plant Cell Rep 17:159–164

Tingay S, McElroy D, Kalla R, Fieg S, Wang M, Thornton S, Brettell R (1997) *Agrobacterium tumefaciens*-mediated barley transformation. Plant J 11:1369–1376

Yanisch-Parron C, Vieira J, Messing J (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp'18 and pUC19 vectors. Gene 33:103–119

Zambryski PC (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu Rev Plant Physiol Plant Mol Biol 43:465–490