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Expression of an engineered cysteine proteinase inhibitor (Oryzacystatin-IΔD86) for nematode resistance in transgenic rice plants

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Abstract We have used a genotype-independent transformation system involving particle gun bombardment of immature embryos to genetically engineer rice as part of a programme to develop resistance to nematodes. Efficient tissue culture, regeneration, DNA delivery and selection methodologies have been established for elite African varieties ('ITA212', 'IDSA6', 'LAC23', 'WAB56-104'). Twenty-five transformed clones containing genes coding for an engineered cysteine proteinase inhibitor (oryzacystatin-IΔD86, *OC-I*ΔD86), hygromycin resistance (*aphIV*) and β-glucuronidase (*gusA*) were recovered from the four varieties. Transformed plants were regenerated from all clones and analysed by PCR, Southern and western blot. Detectable levels of *OC-I*ΔD86 (up to 0.2% total soluble protein) in plant roots were measured in 12 out of 25 transformed rice lines. This level of expression resulted in a significant 55% reduction in egg production by *Meloidogyne incognita*.

Key words *Oryza sativa* · Transformation · Oryzacystatin-*I*ΔD86 · Nematode resistance

Introduction

It is estimated that *Meloidogyne* spp. (root-knot nematodes) account for the majority of the $100 billion (US) of annual loss attributed to nematode damage in crops (Sasser and Freckman 1987). In the tropics, average crop production is reduced by 11–25% as a result of damage by nematodes (Sasser 1979). *Meloidogyne* ssp. are an important component of the range of root parasitic and foliar nematodes that can cause severe crop losses in the various rice ecosystems (Bridge et al. 1990). The majority of *Oryza sativa* cultivars are susceptible to *Meloidogyne* spp. (Bridge et al. 1990). *M. graminicola* causes losses in both upland and lowland rice in Asia either before flooding or in intermittent cultivation systems (Prot 1994), while *M. incognita* is principally a problem of upland rice and can reduce plant height and yield by 60% in West Africa (Plowright and Hunt 1994; Babatola 1984). The wide host range of *Meloidogyne* spp. that attack rice also places other crops in rotation with rice at significant risk. Their control is therefore an important component in the development of sustainable agricultural production systems. Unfortunately, chemical control is unsuitable on economic and environmental grounds. Clearly, here is an important opportunity to use biotechnology to improve rice production by developing resistance to nematodes. However, to date, there is no report of transgenic resistance against any nematode by any means in cereals.

The introduction of pest-specific anti-nutritional factors into crop plants offers a promising approach for the control of a wide range of pests. Protease inhibitors have been used to engineer insect (Hilder et al. 1987; Johnson et al. 1989; Boulter et al. 1989; Duan et al. 1996) and nematode (Hepher and Atkinson 1992; Urwin et al. 1995; 1997) resistance. Cowpea trypsin inhibitor expressed in transgenic potato was shown to affect fecundity and the male/female ratio of *Meloidogyne* spp. and *Globodera* spp., respectively (Hepher and Atkinson 1992). Cysteine proteinase inhibitors (cystatins) represent an attractive option for a safe defence strategy against specific pests because they are the only class of proteinases not expressed in the digestive

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system of mammals (Atkinson et al. 1995). Rice Oryzacystatin-I (OC-I) or maize (CC-I) cystatins have already been introduced and constituively expressed in model rice varieties (Hosoyama et al. 1994; Irie et al. 1996), potato (Benchekroum et al. 1995) and tobacco (Masoud et al. 1993), but their effect on nematodes was not evaluated. A variant of Oryzacystatin-I (OC-IΔD86) produced by site-directed mutagenesis was shown to mediate nematode resistance when expressed into tomato hairy roots (Urwin et al. 1995) and *Arabidopsis* plants (Urwin et al. 1997). Despite these efforts there is no report to date of proteinase inhibitor-mediated transgenic resistance against any nematode in cereals.

One of the key elements in developing efficient genetic engineering of rice for pest, disease or herbicide resistance is the ability to genetically transform any given elite variety. In the work presented here we have used a genotype-independent transformation system involving particle gun bombardment of immature embryos (Christou et al. 1991) to genetically engineer a range of elite African rice varieties. We report the introduction and expression of a variant of Oryzacystatin-I (OC-IΔD86) into African elite rice cultivars resulting in a significant 55% reduction in egg production by *M. incognita*. This strategy seeks to provide a basis for concomitant control for *M. incognita* and other root parasites of rice.

Materials and methods

Plant tissue preparation

Ten-to fifteen-day-old immature seeds from cvs 'ITA212', 'IDSA6', 'LAC23' and 'WAB56-104' (Provided by WARDA, Ivory Coast) were harvested from greenhouse-grown rice plants. Seeds were sterilized with 2.5% (w/v) sodium hypochlorite for 5 min and rinsed three times with sterile distilled water. Glumes were removed under a dissecting microscope, and the seeds were sterilized again for 10 min and rinsed as described above. The embryos were aseptically removed and plated on CCM3 medium for 2 days before bombardment. The CCM3 medium contains CC basal salts (Hartke and Lorz 1989), 20 g/l sucrose, 15 g/l mannitol, 15 g/l Sorbitol, 2 mg/l 2,4-D (2,4 dichlorophenoxyacetic acid), 1 g/l casein hydrolysate, 100 ml/l coconut water (SIGMA C-5915) and 6 g/l agarose type I (SIGMA A-6013). The final pH was adjusted to 5.7 with KOH before autoclaving. Filter-sterilized CC vitamins were added after autoclaving.

Plasmids and particle bombardment

Plasmids pHyg-OcIΔD86 (CaMV35Spromoter::AMVleader::*OC-I*ΔD86::NOSpolyA + CaMV35Spromoter::*aphIV*::SpolyA) and pJIC200 (ubi-5′ region::*gusA*::NOSpolyA) were used for transformation (*aphIV* provides hygromycin resistance; *gusA* codes for β-glucuronidase). The *OC-I*ΔD86 gene was designed by protein engineering to improve the Ki and efficacy of native rice OC-I protein against nematodes; *OC-I*ΔD86 differs from *OC-I* by only one amino acid (Asp86 deleted, Urwin et al. 1995). Rice immature embryos were osmotically conditioned for 4 h prior and 16 h after bombardment by transfer to CCM3 medium containing 55 g/l manitol, 55 g/l sorbitol (Vain et al. 1993). Particle bombardment was carried out as described previously (Christou et al. 1991).

Selection and regeneration of transformed plants

Two days after bombardment, immature embryos were subcultured onto CCM3 medium supplemented with 50 mg/l of hygromycin (CCM3H50). After 10 days, embryogenic calli initiated from the scutellum were dissected into small pieces and transferred to fresh hygromycin-containing medium, with subculture at 10-day intervals. After 6–8 weeks, plants were regenerated by transferring the hygromycin-resistant embryogenic calli onto CCM3H50 medium without 2,4-D (CCR2H50). Plantlets were first germinated on plates, then grown in tubes on MSR6H50 medium prior to transfer to the greenhouse. The MSR6H50 medium contains 40% (v/v) MS basal salts (Murashige and Skoog 1962), 10 g/l sucrose, 2 g/l phytagel (SIGMA P-8169), 4 g/l bactoagar (Difco), pH 5.7. Filter-sterilized B5 vitamins (Gamborg et al. 1968) and hygromycin (50 mg/l) were added after autoclaving.

Polymerase chain reaction (PCR)

DNA was prepared using a rapid extraction protocol. Leaf samples were ground in a 1.5-ml Ependorf tube using a hand pestle, sand and liquid nitrogen. Two hundred microliters of extraction buffer (500 m*M* NaCl, 100 m*M* TRIS-HCl pH 8 and 50 m*M* EDTA; Ellis 1994) and 20 µl 20% (w/v) SDS were added to the powder before further grinding. After centifugation (5 min at 12 000 rpm) the supernatent was removed and added to an equal volume of isopropanol for DNA precipitation. After centrifugation, the DNA pellet was washed with 70% ethanol and suspended in TE buffer. PCR was performed in a total volume of 25 µl containing 25 ng rice genomic DNA, 10 m*M* TRIS-HCl pH 8.3, 2.5 m*M* $MgCl_2$, 50 m*M* KCl_2, 0.05% Noridet p40, 200 µ*M* dNTPs, 0.4 µ*M* of each primer and 1 unit of *taq* polymerase. DNA was denatured at 95°C for 1 min followed by 30 cycles of amplification (30 s at 95°C, 30 s at 60°C, 1 min at 72°C) and by 10 min at 72°C.

GUS (β-glucuronidase) activity

Leaf tissue from 5-week-old rice plants was assayed fluorometrically for β-glucuronidase activity according to Jefferson (1987).

Western blot hybridization for OC-I activity

Total protein was isolated from roots of rice plants. Ten microgams of total root protein and standard quantities of OC-I protein were loaded on SDS PAGE gels using the BioRad mini system. The OC-I protein was recovered from an *E-coli* expression system (Urwin et al. 1995). The gel was blotted according to BioRad protocols and probed with a polyclonal antibody raised against OC-I (Urwin et al. 1995). The bands were visualized using an image analyser system consisting of a monochrome CCD camera (Sony) plus a frame grabber and appropriate software (Quantimet 500, Leica).

Bioassays with nematodes

Four-week-old rice plants were potted into a sand/loam mix infected with *Meloidogyne incognita* (an African population from the West Africa Rice Development Association). Plants were harvested 42 days after exposure to nematode-infested soil, and the root mass

was cleaned thoroughly in water and blended for 10 s in a Waring blender with sodium hypochlorite diluted to give 1% available chlorine. Nematode eggs were washed from the blended roots into a large volume of water using a 1-mm sieve. The eggs were collected on an open sieve, resuspended and counted in 1-ml aliquots using a Peter's counting slide (Southey 1986).

Results and discussion

Selection and regeneration of transformed plants

The methodology described in this report allowed the production of highly embryogenic rice tissue and the subsequent transformation of more than 30 African, South American and Asian elite varieties (Christou et al. 1991; Vain et al. 1997). Six to eight weeks following particle bombardment, clear differential growth was observed between transformed (hyg+) and non-transformed (hyg−) calli during proliferation, regeneration and germination. Twenty-five independently transformed hyg+ clones were selected from the four African genotypes tested in this study (Table 1). Transformation of the hyg+ clones was further confirmed by GUS, PCR, Southern and western blot analyses of regenerated plants. Transformation frequencies of up to 8% were obtained from all of the elite African genotypes tested.

Analysis of transformed plants

The regenerated plants (Fig. 1) were first screened by PCR using two primers (ACTCACCGCGACGTCTGTCG forward 5′ and GATCTCCAATCTGCGGGATC reverse 3′) amplifying a 1.3-kb fragment containing the *aphIV* gene. All the plants regenerated from the 25 independently transformed hyg+ clones were PCR+ for this set of primers. Southern blot analyses of regenerated plants confirmed the integration of the pHyg-OcIΔD86 plasmid into the rice genomic DNA (Vain et al. 1997).

When both pHyg-OcIΔD86 and pJIC200 (the *gusA*-containing plasmid) were used for transformation, the regenerated plants were also screened by PCR using

Table 1 Presence and expression of *aphIV*, *gusA* and *OC-I*ΔD86 genes in transgenic rice plants

Clone no.	Plasmid used[a]		*aphIV*		*gusA*		*OC-I*ΔD86
	pHyg-OcIΔD86 (*aphIV* and *OC-I*ΔD86)	pJIC200 (*gusA*)	PCR	Resistance to hygromycin	PCR	Expression[b]	Expression[c]
ITA212 X1	+	−	+	+	−	−	ND
ITA212 15	+	−	+	+	−	−	ND
ITA212 X5	+	−	+	+	−	−	ND or <0.1%
ITA212 X4	+	−	+	+	−	−	0.014%
ITA212 X3	+	−	+	+	−	−	0.1–0.2%
ITA212 4	+	−	+	+	−	−	0.1–0.2%
ITA212 17	+	−	+	+	−	−	0.02–0.03%
ITA212 45	+	−	+	+	−	−	ND
ITA212 1	+	+	+	+	+	28 958	<0.1%
IDSA6 Y1	+	−	+	+	−	−	ND or <0.1%
IDSA6 20	+	+	+	+	+	119	ND or <0.1%
IDSA6 9	+	+	+	+	−	−	<0.1%
IDSA6 Y2	+	+	+	+	+	11 474	<0.25%
IDSA6 2	+	+	+	+	+	0	ND or <0.1%
IDSA6 3	+	+	+	+	+	255	ND or <0.1%
IDSA6 4	+	+	+	+	+	54 767	ND or <0.1%
LAC23 6	+	−	+	+	−	−	0.012%
LAC23 3	+	+	+	+	+	0	<0.25%
LAC23 1	+	+	+	+	+	0	ND or <0.1%
LAC23 2	+	+	+	+	+	0	<0.1%
LAC23 3b	+	+	+	+	+	22 202	<0.1%
WAB56-104 1	+	−	+	+	−	−	<0.25%
WAB56-104 24	+	+	+	+	+	7 820	ND or <0.2%
WAB56-104 26	+	+	+	+	−	−	ND or <0.1%
WAB56-104 38	+	+	+	+	+	26	ND or <0.1%

[a] +, plasmid used; −, plasmid not used
[b] In pmoles MU/min per mg protein after substraction of background level (37 pmoles MU/min per mg protein)
[c] In percentage of total soluble proteins. Each western analysis was repeated twice. ND = non-detected, <0.1% = detected as inferior to the lower standard (here 0.1%) but not quantified

Fig. 1 IDSA6 (IDB21) and ITA212 (ITC66) regenerated fertile transgenic rice plants containing the engineered *OC-I*ΔD86 oryzacystatin gene

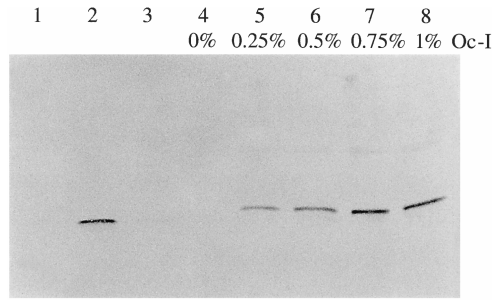

Fig. 2 Western blot of an SDS PAGE gel probed with a polyclonal antibody raised against Oryzacystatin OC-I. *Lanes 1–3* were loaded with 2 μg total protein from T_0 transformed rice plants regenerated from three independent lines (ITA212 nos. 45, 4 and 15, respectively). *Lanes 4–8* were loaded with 2 μg total protein from an untransformed rice plant to which 0, 0.25, 0.5, 0.75, or 1% OC-IΔD86 protein with a 6xHis tag (Urwin et al. 1995) was added

two primers (CAACCAGATCTCCCCCAAATC forward 5′ and TTCGGCGTGGTGTAGAGC reverse 3′) amplifying a 1.6-kb fragment of the ubi5′ region::*gusA* gene. A 92% co-transformation frequency of pHyg-OcIΔD86 and pJIC200 was observed (23 out of 25 clones, Table 1). Such high co-transformation frequencies have already been reported using direct transfer of DNA (Weising et al. 1988; Gordon Kamm et al. 1990).

The expression of unselected *gusA* and *OC-I*ΔD86 genes was confirmed by enzymatic assays and western blot analysis, respectively. Detectable levels of GUS expression in regenerated plants was measured in 62% of the clones tested (8 out of 13 clones). Large variation among the transgenic lines for expression of the *gusA* gene in the transformed plants was observed (Table 1). Detectable levels of OC-IΔD86 in plant roots were measured in 48% of the transformed rice lines (12 out of 25 clones, Fig. 2). Expression up to 0.2% of total soluble protein (tsp) was observed in rice roots (Table 1). However, most of the transformed plants expressed *OC-I*ΔD86 at lower levels (<0.1% tsp). Several factors might be responsible for this low level of *OC-I*ΔD86 expression. Firstly, the CaMV 35S promoter does not generally lead to high levels of transgene expression in monocotyledonous species. In addition, the short length (380 bp) of the CaMV 35S promoter used in this study, when compared to longer CaMV 35S promoter regions (880 bp) used in other vectors (e.g. pBI121, Clonetech) might have affected levels of transgene expression. However, the CaMV 35S promoter has been reported in the past to potentially produce up to 2% of tsp of transgene in rice (Irie et al. 1996; Christou, unpublished). Secondly, the high level of homology (99.4% at the nucleotide level) between *OC-I*ΔD86 and *OC-I* might also be responsible for the low level of *OC-I*ΔD86 expression observed in the transgenic rice plants. Homology-dependent gene silencing between transgenes and endogenous genes has been reported in many plant species (Napoli et al. 1990). We are currently designing new constructs containing tissue-specific and highly-expressed constitutive promoters associated with heterologous cystatins to test these hypotheses.

No significant correlation between the level of expression of *OC-I*ΔD86 (in pHyg-OcIΔD86) and *gusA* (in pJIC200) genes was observed (Table 1). Transgenic plants exhibiting high levels of *gusA* expression (clones ITA212 no. 1, IDSA6 no. Y2 and no. 4, LAC23 no. 3, WAB56-104 no. 24) did not express *OC-I*ΔD86 at a high level despite the presence of both pHyg-OcIΔD86 (containing *OC-I*ΔD86) and pJIC200 (containing *gusA*) plasmids in the genome of the transformed rice plants (Table 1). In this particular case, co-transformation with a marker gene could not be used to predict the expression level of co-transformed genes and could not be used as a substitute for protein fusion constructs (Hosoyama et al. 1995).

Transgene expression in progeny (T_1 plants)

Fertile transformed plants were obtained from most of the transformed clones. Segregation analyses of the *aphIV*, *gusA* and *OC-I*ΔD86 genes were performed. Screening of the T_1 plants by PCR confirmed Mendelian inheritance of the *aphIV*, *gusA* and *OC-I*ΔD86 genes in all plants tested (Fig. 3). For the *OC-I*ΔD86 gene, two primers (TACAGTCTCAGAAGACCAAA forward 5′ and GTGAAATAGTACAAAGTGCC reverse 3′) amplifying a 0.6-kb fragment of the CaMV35Spromoter::AMVleader::*OC-I*ΔD86 gene

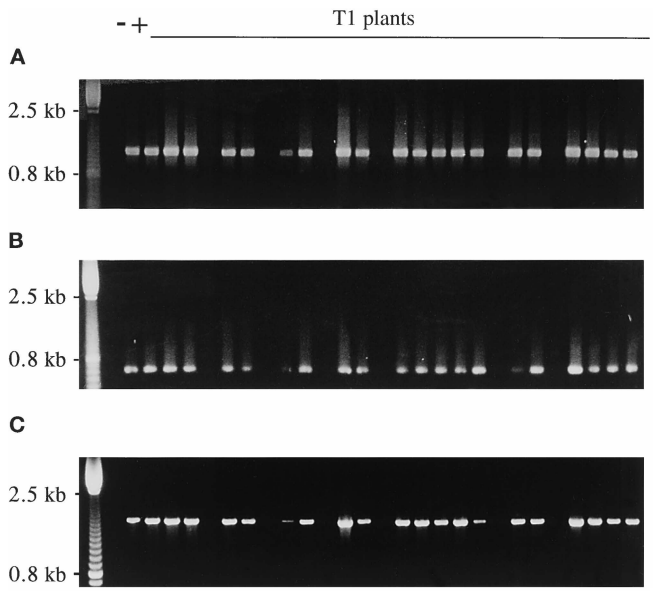

Fig. 3A–C PCR analysis of T_1 plant progeny of clone LAC23 no. 3b containing plasmids pHyg-OcIΔD86 (CaMV35Spromoter::AMV leader::*OC-I*ΔD86::NOSpolyA + CaMV35Spromoter::*aphIV*::SpolyA) and pJIC200 (ubi-5′ region::*gusA*::NOSpolyA). **A** PCR analysis using two primers amplifying a 1.3-kb fragment containing the *aphIV* gene, **B** PCR analysis using two primers amplifying a 0.6-kb fragment of the CaMV35Spromoter::AMVleader::*OC-I*ΔD86, **C** PCR analysis using two primers amplifying a 1.6-kb fragment of the ubi5′ region::*gusA* gene. Non-transformed plants (−) and plasmid DNA (+) were used as negative and positive controls, respectively

were used. Segregation frequencies indicated that transgenes were generally integrated at one locus (3:1 ratio after self pollination). However, segregation analysis for transgene expression showed both Mendelian (37%) and non-Mendelian (63%) inheritance. In approximately 50% of the lines, at least one of the transgenes was totally silenced in the progeny (T_1 plants).

Nematode challenge

A subset of regenerated transgenic T_0 plants were challenged with *M. incognita*. The experiment was based on three harvests totalling 25 western-negative and 27 western-positive plants (Table 1). All the positive plants expressed OC-IDD86 at a low level (<0.2% tsp). The challenge density of nematodes did not statistically affect rice plant development in terms of height and weight of green tissue (western positive $\log_{10}$ 2.21 ± 0.034 g; western negative $\log_{10}$ 2.11 ± 0.029 g) or weight of root tissue (western positive $\log_{10}$ 1.59 ± 0.094 g; western negative $\log_{10}$ 1.67 ± 0.058 g). After a 42 day exposure of the rice plants to nematode-infested soil, established females of *M. incognita* on the western-negative plants had produced an overall mean for the three harvests of 3631 ± 1381 eggs/g root mass. The corresponding overall mean for plants expressing a low level of OC-IΔD86 was 1995 ± 596 eggs/g root mass. The reduction in eggs on the latter plants to 55% of the controls was statistically significant ($P < 0.05$; two-way ANOVA for harvest date and OC-IΔD86 expression). This result is encouraging considering the low level of OC-IΔD86 protein in the transformed rice plants tested. We believe that by achieving a higher level of transgene expression (*OC-I*ΔD86 or heterologous cystatins) we will be able to generate transgenic rice plants exhibiting agronomically useful levels of resistance to nematodes.

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