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Enhancing rice resistance to fungal pathogens by transformation with cell wall degrading enzyme genes from *Trichoderma atroviride*[*]

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Abstract: Three genes encoding for fungal cell wall degrading enzymes (CWDEs), *ech*42, *nag*70 and *gluc*78 from the biocontrol fungus *Trichoderma atroviride* were inserted into the binary vector pCAMBIA1305.2 singly and in all possible combinations and transformed to rice plants. More than 1800 independently regenerated plantlets in seven different populations (for each of the three genes and each of the four gene combinations) were obtained. The *ech*42 gene encoding for an endochitinase increased resistance to sheath blight caused by *Rhizoctonia solani*, while the exochitinase-encoding gene, *nag*70, had lesser effect. The expression level of endochitinase but exochitinase was correlated with disease resistance. Nevertheless, exochitinase enhanced the effect of endochitinase on disease resistance when the two genes co-expressed in transgenics. Resistance to *Magnaporthe grisea* was found in all kinds of regenerated plants including that with single *gluc*78. A few lines expressing either *ech*42 or *nag*70 gene were immune to the disease. Transgenic plants are being tested to further evaluate disease resistance at field level. This is the first report of multiple of expression of genes encoding CWDEs from *Trichoderma atroviride* that result in resistance to blast and sheath blight in rice.

Key words: *Agrobacterium*-mediated transformation, Rice sheath blight, Rice blast, *Trichoderma atroviride*, Chitinase, Glucanase
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INTRODUCTION

Rice sheath blight (*Rhizoctonia solani*) and rice blast (*Magnaporthe grisea*) are two serious plant diseases in China. Rice cultivars with high level and durable resistance to these two pathogens are not easy to obtain by traditional breeding methods due to the lack of germplasm resistant to *R. solani* and the high genetic diversity of *M. grisea*.

Cell wall degrading enzyme (CWDE) genes from biocontrol fungi belonging to the genus *Trichoderma* have been demonstrated to encode proteins with high antifungal activity against a wide range of plant pathogenic fungi.

In this work, the endochitinase *ech*42 (EC 3.2.14), exochitinase gene *nag*70 (EC 3.2.1.52) and

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the exo-1, 3-*β*-glucanase gene *gluc*78 (EC 3.2.1.58) from *Trichoderma atroviride* were used to prepare seven different plasmids containing all combinations of the three genes and transform rice via *Agrobacterium tumefaciens*. The transgenic rice plants constitutively over-expressing each gene alone, either two genes in combination, or three genes together were tested for their response to rice sheath blight and blast pathogens.

MATERIALS AND METHODS

Plasmid binary vectors

First the gene *ech*42 and gene *nag*70 were cloned in plasmid pBIN (Endo+Nag), while the gene *gluc*78 was cloned in plasmid pBS-*gluc*78. Each gene was then cloned behind the *Act*1 promoter carried by plasmid pCOR104. The entire *Act*1:*ech*42:*nosT*, *Act*1:*nag*70:*nosT* or *Act*1:*gluc*78:*nosT* cassettes were later cloned into binary vector pCAMBIA1305.2 producing the vectors named pCAMBIA (ech), pCAMBIA (nag) and pCAMBIA (gluc).

The *Act*1:*nag*70:*nosT* or *Act*1:*gluc*78:*nosT* cassette was then cloned downstream of *ech*42 gene of pCAMBIA (ech) to produce double gene constructs pCAMBIA (ech+nag) and pCAMBIA (ech+gluc). The *Act*1:*gluc*78:*nosT* cassette was placed behind *nag*70 of pCAMBIA (nag) and pCAMBIA (ech+nag), resulting in another double gene construct pCAMBIA (nag+gluc), as well as a plasmid with three genes named pCAMBIA (ech+nag+gluc). (Fig.1).

Plasmids pCAMBIA (ech), pCAMBIA (nag), pCAMBIA (gluc), pCAMBIA (ech+nag), pCAMBIA (ech+gluc), pCAMBIA (nag+gluc) and pCAMBIA (ech+nag+gluc) were transferred to *A. tumefaciens* strain EHA105. The orientation of the genes within the T-DNA in all the binary vectors was confirmed by PCR and by restriction enzyme analysis.

Rice transformation

The immature embryos of rice (*Oryza sativa* L ssp. *Japonica* cv Ishikari-shiroge) were explanted on NB medium containing N6 macro-elements, B5 micro-elements, B5 vitamins, MS Fe salts, 30 g/L sucrose, 2.0 mg/L 2,4-D, 500 mg/L proline, 500 mg /L glutamine, 300 mg/L casein hydrolysate, 2.6 g/L Gelrite, pH 5.8. The embryos were then incubated at 26 °C in the dark for 6 days. The primary embryogenic calli were excised and propagated on NB medium for another 3 days. Vigorously growing light-yellow fragile embryogenic calli were selected and placed into bacterial (*A. tumefaciens* strain EHA 105) suspension and immersed for 20 min with occasional shaking. Excess bacterial suspension was removed from calli by placing them on a pad of dry sterile paper. The inoculated calli were transferred onto NB medium with 100 μmol/L acetosyringone and 2 g/L inositol, and incubated at 25 °C in the dark for 3 days. For selection of transformed cells, the co-cultivated calli were then transferred onto NB medium with 300 mg/L cefatoxime, 50 mg/L hygromycin B. After 3 weeks, the vigorously growing hygromycin-resistant embryogenic calli were transferred from selection medium to regeneration medium (NB medium with 0.5 mg/L 6-BA, 0.5 mg/L NAA, 4.0 mg/L KT, 300 mg/L cefatoxime and 50 mg/L hygromycin B, and 2,4-D was omitted) and cultured at 25 °C with 12-hour dark/light photoperiod. About 4 weeks later, the regenerated hygromycin-resistant plantlets were transferred to rooting

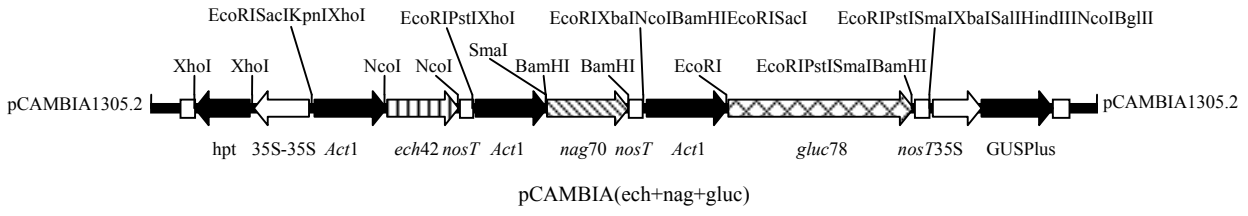

Fig.1 Structure of plasmid pCAMBIA (ech+nag+gluc) containing three genes of *ech*42, *nag*70 and *gluc*78
hpt, hygromycin phosphotransferase encoding gene; 35S-35S, enhanced cauliflower mosaic virus 35S promoter; *Act*1, *Act*1 promoter; *nosT*, nos terminator

medium containing 1/2N6 macro-elements, B5 micro-elements, B5 vitamins, MS Fe salts, 30 g/L sucrose, and 8 g/L agar for root elongation.

PCR analysis

Total rice DNA was extracted from young leaves as described by Lu and Zheng (1992). The primer pairs used to detect *ech*42, *nag*70 and *gluc*78 were echF: GCGCTGCAGGCCACTCTCATT, echR: AGTTGGGGTTGGACGGGTTGG; nagF: GGCAT TGACCGCGGTGTTGAGG, nagR: CACGGGGCC AGATGATGTTGTCCA; glucF: CCAGCCCGGTG CCATCATTGT, glucR: AAAGTGTTGCGGTTGT CCCTGTCG.

This treatment (echF:echR, nagF:nagR, and glucF:glucR) would amplify a specific 754 bp, 818 bp or 759 bp fragment specific for *ech*42, *nag*70 or *gluc*78, respectively. For all primers, the following PCR conditions were used: 5 min preheating at 94 °C; and 35 cycles with 1 min denaturing at 94 °C, 1 min annealing at 57 °C and 45S elongating at 72 °C.

Southern blot analysis

Genomic DNA of the transgenic rice and non-transformed rice was digested with *Xba*I. Total digested solutions of plants with multiple exogenous genes were divided to 2–3 parts according to gene numbers, and electrophoretically separated on 0.8% agarose gel. PCR products of each gene contained in the plasmids were used as probes. Probe labeling, hybridization and signal detaction were performed using standard procedures as described by the manufactors (ECL direct nucleic acid labeling and detection systems, Amersham).

Activity assay for chitinase and glucanase

Leaf tissue (50 mg) was ground in 1 ml of the assay buffer described by Bolar *et al.*(2000). Chitinolytic enzyme assays were the same as those described by Bolar *et al.*(2001). Exo-1,3-*β*-glucanase activity was determined by measuring the amount of reducing sugar released from laminarin.

Evaluation of disease resistance

For sheath blight resistance assay, small pieces of toothpick (0.8–1 cm) were spread on the surface of PDA medium inoculated with *R. solani* AG-1-A. The toothpick pieces covered with hyphae of the pathogen were then put inside the rice sheath at maximum tillering stage. Three tillers of each transgenic plant were inoculated. The symptom caused by fungal infection was measured 5 days after inoculation up to 30 days after heading, by measuring the length of lesions.

For rice blast resistance assay, young leaves of 5- to 6-leaf stage were put on the surface of soaked filter paper and sprayed with a spore suspension of *M. grisea* strain A49 *in vitro*. The conidia concentration was adjusted to 5×10^5 conidia/ml. After inoculation, leaves were kept in the dark at 26 °C for 36 h and transferred to a light circumstance at 26 °C for further incubation. Disease reactions were scored 3 days after inoculation. The diameters of lesions were used to quantify disease severity.

RESULTS AND DISCUSSION

Rice transformation with CWDE genes from *Trichoderma atroviride*

The three CWDE genes from *Trichoderma atroviride,* were successfully transferred into rice in seven different combination, with obtained transformation rate ranging from 24.6% to 55.5%. A total of more than 1800 hygromycin resistant plants were obtained. The transformation and regeneration frequencies appeared to decrease with the increase of T-DNA size in the various vectors used.

Molecular analysis of regenerated plants

The individual hygromycin-resistant plants were tested for the presence of T-DNA using PCR with primers specific for *ech*42, *nag*70 and *gluc*78 coding regions. Among the 164 plants detected positive, 139 contained the intact exogenous gene. Most of the plants tested with *ech*42 or *nag*70 specific primers were positive, with a proportion of 96.0% and 97.9%, respectively, while the frequency of *gluc*78-positive plants was much lower (79.1%). Southern blot analysis of PCR-positive plants was conducted with probes specific for *ech*42, *nag*70 and *gluc*78 coding sequence. The results (data not shown) confirmed that the exogenous genes were intergraded into the genomes of transgenic rice

plants in the seven populations.

Evaluation of disease resistance to sheath blight

Transgenic rice plants of seven populations as vigorous as the controls were inoculated with *R. solani* at the maximum tillering stage. All the transgenic plants with high endochitinase (*ech*42) activity suffered less disease than the controls. There was significant negative correlation between endochitinase activity and lesion length with plants carrying the *ech*42 gene alone (R^2=93.0%, P=0.02). The line N73-9 with the *nag*70 was the only transgene that produced high level of exochitinase but showed mild resistance to sheath blight. In addition, there was no significant correlation between the level of exochitinase and resistance to *R. solani* (R^2=48.8%, P=0.122). The correlation between glucanase (*gluc*78) transgenic activity and disease resistance was not analyzed since the high glucanase activity resulted in reduced vigor. Several plants co-expressing the two chitinase genes were significantly less affected by fungal diseases than the controls. The expected lesion length of transgenic plants deduced from the linear regression analyses of disease resistance versus endochitinase activity were bigger than the observed values, indicating that fungal exochitinase can enhance the effect of endochitinase on disease resistance of rice as previously demonstrated in apple (Bolar *et al.*, 2001).

Evaluation of disease resistance to rice blast

Transgenic plants of seven populations and controls were inoculated with *M. grisea* strain A49 *in vitro*. All the populations showed increased resistance to blast and a higher then controls ratio of resistant plants, with a few of the lines appearing totally immune to this pathogen. Among the seven populations, the transgenic plants with *ech*42 and *nag*70 performed best in resistance to blast disease.

In conclusion, at a population level, all of the transgenic lines showed a significantly improved resistance to the sheath blight and blast fungus in the experiments. In fact, some of the transgenic plants are being tested to confirm the gene actual express by Northern analysis and to further evaluate disease resistance at field level. We expect that combination of this transgenic strategy based on the use of fungal genes and traditional breeding methods will provide fungal disease resistant rice lines with good agronomic phenotypes.

This is the first report of multiple of expression of transgens encoding CWDEs from *Trichoderma atroviride* that results in resistance to blast and sheath blight in rice.

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