Transgenic Rice Plants Expressing a Novel Antifreeze Glycopeptide Possess Resistance to Cold and Disease

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Freezing injury and disease are both restrictive factors in crop production. In order to improve the tolerance ability to these stresses, a better way is to carry out genetic engineering by transferring dualfunctional genes. A predicted rice antifreeze glycopeptide gene was purposefully selected from rice blast-induced cDNA library. Northern blot demonstrated that the gene is expressed not only in blast-infected rice leaves, but also in low temperature-treated rice. In addition, the expressed protein in *Escherichia coli* exhibits strong antifreeze activities. The gene was overexpressed in rice plants transformed via *Agrobacterium tumefacient* EHA105. Overall 112 T0 transformants were obtained in this research. Cold tolerance and disease resistance of T1 transformants were, respectively, investigated. The results showed that plants containing overexpressed transgene can withstand –1 °C for 24 h without severe chilling injury after thawed, and that disease symptoms of the parallel transformants are highly reduced in response to blast infection, when compared with controls. The relationship of the gene and several pathogenesis-related protein genes to be chosen was analyzed and discussed. All these results confirmed the dual role of the cloned gene, and implied that genetic engineering using this kind of gene is a promising method to reduce biotic and abiotic stresses.

Key words: Antifreeze Glycopeptides, Cold Tolerance, Disease Resistance

Introduction

Temperature is one of the most key factors influencing geographic distribution, seasonal growth and individual development of plants. With the adaptation and evolvement of plants, it is not surprising to see that plants living in polar regions can stand subfreezing temperature without any injury, but others in tropical or semitropical regions are very sensitive to low temperature. Cold acclimation (Levitt, 1980) is a main mechanism for cold-tolerant plants reducing low temperature injury. It is well known that during the period of cold acclimation, lots of low temperature responsive genes are involved in the tolerance process (Gong *et al*., 2002; Hsieh *et al*., 2002), and much accumulated polysaccharides, antifreeze glycopeptides (AFGPs) and antifreeze proteins (AFPs) contribute to the resulting tolerance (Guy, 1990; Hughes and Dunn, 1996; Browse and Xin, 2001). Because of the variability of weather, however, plants as immovable and poikilothermic organisms are not always so lucky to go through cold acclimation and escape damages, but frequently suffer chilling or freezing injury in "unpredictable" cold weather.

AFPs are very essential in conferring antifreeze ability for most organisms (reviewed by Thomashow, 1999; Xin and Browse, 2000; Kreps *et al*., 2002; Xiong *et al*., 2002; Atici and Nalbantoglu, 2003). It has been thought that some accumulated AFPs in most plants, animals and microbes can bind to the faces of ice crystals and possess the properties of recrystallization inhibition and thermal hysteresis during freezing and finally make plants to exhibit freezing tolerance (Duman and Olsen, 1993; Ewart *et al*., 1999; Griffith and Yaish, 2004; Bravo and Griffith, 2005). Several genes encoding AFPs from animals or plants have been cloned and functionally analyzed (Jia and Davies, 2002). Compared with animal AFPs, plant AFPs exhibit much lower levels of thermal hysteresis value. For example, the thermal hysteresis value

Abbreviations: AFGP, antifreeze glycopeptide; AFP, antifreeze protein; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid; TH, thermal hysteresis; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EB, ethidium bromide; BSA, bovine serum albumin.

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ranges from 1 to 2 °C in fishes and from 5 to 10 °C in insects (Jia and Davies, 2002). But in plants, the levels of the thermal hysteresis appear to range from 0.1 to 0.6 °C (Worrall *et al*., 1998). Even the heat-stable AFP that confers a very strong recrystallization inhibition, exhibits a rather lower value, 0.1 °C in water and 0.45 °C in 30% sucrose (Sidebottom *et al.*, 2000).

Different from the AFPs above, a series of proteins are very interesting for their dual role in both antifreezing and pathogenesis-relation. Griffith and Ewart (1995) first reported this set of proteins. From then on, further studies on AFPs associated with pathogenesis-related (PR) protein characteristics in winter rye were carried out in Griffith' group. Based on their studies, three main points at least were included: (1) certain AFPs produce during the low temperature accumulation process present similarity with PR proteins and appear to have certain specific enzyme activity (for example chitinase activity); (2) the PR proteins induced by pathogens in plants can confer antifreeze activity; (3) the antifreeze activity PR proteins translated from the specific homologous gene of PR without antifreeze activity do not need post-translational modification (Yeh *et al*., 2000). Recently, two novel genes named *TaIRI-1* and *TaIRI-2* conferring *in vitro* ice recrystallization inhibition were cloned from cereals (Tremblay *et al*., 2005). The genes are up-regulated during cold acclimation in freezing-tolerant species and one of them is induced by jasmonic acid and ethylene. These results suggest that the genes probably exhibit dual-functional properties.

Plant diseases, just like cold stress, are terrific threateners to plants growth and productivity, and frequently lead to more percentage yield losses (Baker, 1997). To date, focusing on the mechanisms of plant resistance, including studies on many resistant genes (R), hypersensitive response (HR), systemic acquired resistance (SAR) and non-host resistance have been intensively studied (Rathjen and Moffett, 2003; Ryals *et al*., 1996). Besides these, tests of disease resistance engineering have been performed widely, and indeed some transgenic plants acquiring resistance to disease have been reported. In the mean time, genetic modifications of freezing tolerance in plants were also performed with significant progress (Jaglo-Ottosen *et al.*, 1998; Haake *et al*., 2002; Liu *et al*., 1998; Saijo *et al*., 2000). However, little has been carried out in the dual resistance to disease and cold stress. Since some AFPs, as stated above, can exhibit dual roles in both antifreezing and PR resistance, a set of these genes may supply ideal tools for improving tolerance to more than one stress. The aims of this research are to clone this kind of gene and verify its dual function in transgenic plants.

Materials and Methods

Plant and fungal materials, growth conditions and treatments

Rice (*Oriza sativa* subsp. *japonica* cv. *Nipponbare*) plants and rice blast fungus (*Magnaporthe grisea*) were used in this research. Normally, rice plants were grown at 25 °C under16 h light/8 h dark in lightweight plastic pots filled with water-saturated soil. At 4-leaf-stage, rice was inoculated by spraying conidiospores of *M. grisea*. The spores were collected from TOM (tomato-oatmeal medium: 150 mL/L tomato extract, 30–50 g/L oatmeal, 20 g/L agar) inoculated with *M. grisea* and suspended in 0.01% (v/v) Tween-20 to a content of 100 spores per 100 times of viewing field and were evenly sprayed onto rice leaves. After inoculation, the plants were kept at 25 °C, with 60% humidity in the darkness for 48 h, and then in light for 24 h. During this period, the symptoms should gradually emerge. Mock-inoculated plants as control were sprayed with Tween-20 (0.01%) without spores and incubated under the similar conditions. The appropriate leaves of inoculated rice were, respectively, excised, frozen in liquid nitrogen and stored at −80 °C for total RNA extraction after treatment for 48 h and 72 h. For cold induction, rice plants grown to 2-leaf-stage were kept in a culture room at 15 °C under continuous light. After 3 d, the plants were transferred to a chilling chamber of 8 °C under continuous light for another 3 d. Then leaves of treated rice were collected for RNA extraction after 3 d and 6 d. Control samples were collected from the rice grown in similar conditions with the exception of 25 °C incubation temperature. Freezing tolerance identification was carried out as follows: Rice plants were first grown under normal conditions until 2-leaf-stage, and then exposed at 8 °C to continuous light for 12 h. Subsequently, they were transferred into a −1 °C freezer under continuous light for 24 h. Before being returned in normal growth conditions, these plants were thawed at 8 °C for 12 h

under darkness. Survival of the seedlings was investigated after 3 d.

Cloning and sequence analysis of target gene

A rice blast-induced rice cDNA library was previously constructed in our laboratory (Pan *et al.*, 2000, unpublished). Briefly, poly(A)-enriched mRNA from rice leaves infected by *M. grisea* were collected and purified using poly(A) quick mRNA isolation kit (Stratagene, USA) and then the cDNA library was constructed using a SMART^{MT}-cDNA library construction kit (CLONTECH) following the manufacturer's instruction. Two-round screening of cDNA library was essentially performed according to Sambrook and Russell (2001). The clones were first screened with p32-labeled cDNA probes from mock-inoculated rice. Clones with no hybridization signals were picked and regularly printed onto a LB plate. The printed sub-library was subjected to a second round screening by using p32-labeled cDNA probes from cold-treated rice plants as above. Then clones with positive hybridization signal were selected and sequenced. Based on the sequence information and comparing that with sequences in NCBI (http://www.ncbi.nlm.nih.gov/), target gene related to induction of low temperature was selected as dual-functional candidate.

Expression patterns of the candidate gene, associated with disease and cold responses, were analyzed using RNA blot with the corresponding p32-labeled cDNA as probes. Gel blot was analyzed as previously described (Sambrook and Russell, 2001) with the following modification. After denaturing agarose gel electrophoresis, RNA separated in gel was blotted on a nylon membrane (Hybond N, Amersham, USA) by capillary transfer. The membrane was then fixed by UV crosslinking before subjected to hybridization.

Expression and purification of recombinant protein in E. coli

Based on the sequence and the predicted open reading frame (ORF, amino acids 1–420), the ORF fragments of the cloned gene were amplified using PCR with a pair of specific primers (OF, 5′-TTCGGATCCGATGAAATACCTGCTG-3′; OD, 5′-GTGCTCGAGGTGCATCATCTGGCC-3′; the underlined letters are additional restriction enzyme site and shield bases). The PCR product was completely double-restricted with *BamHI* and *XhoI* and in frame ligated into expression vector pET22b(+) (Invitrogen) restricted with the same enzymes. Then the ligation product was electroporated in *E. coli* JM109 competent cells. For prokaryotic expression, *E. coli* JM109 was grown at 37 °C until the culture reached an OD_{600} value of 0.4. Incubation was continued for 4 h after adding IPTG. Then total proteins from the collected cells were dissolved in solution I [20 mM Na_2HPO_4 (pH 7.2), 0.5 mM NaCl]. A fraction of soluble proteins was obtained after centrifugation at 4 °C for 30 min at 14,000 × *g* and loading onto a Ni^{2+}-NTA-agarose column (3 mL bed volume). The column was successively washed with binding buffers containing 100 mM imidazole, and then the immobilized proteins were eluted with 500 mM imidazole. His-tagged protein was dialyzed against solution I and stored at 4 °C for further use. Protein concentrations after purification were determined by using bovine serum albumin (BSA) as the standard (Bradford, 1976). The efficiency of this purification procedure was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). According to Xu *et al.* (1998), periodic acid-Schiff (PAS) staining approach was used to identify carbohydrate-modified recombinant proteins separated by SDS-PAGE. The staining reagent was bought from Sigma (St. Louis, MO, USA).

Antifreezing activity assays

Both different methods were, respectively, used to test the antifreezing activity. Because AFPs have the ability to alter the morphology of ice crystals in solution, the shape of ice crystals in fusion protein solution (0.1 or 1 mg/mL) and BSA solution (5 mg/mL, as control) was observed as described by Kobashigawa *et al.* (2005). To observe the ice crystal morphology, we used a photomicroscope equipped with an LTS120 temperature controller (THMSE600). A droplet aliquot of the sample solution was once frozen and subsequently heated until a single ice crystal was observed. The morphological change of the crystal was then observed with a very small cooling rate (0.01 °C/min). Thermal hysteresis as a non-colligative depression of the freezing point that differs from the melting point was measured using differential scanning calorimetry (DSC, Du Pont 910) and following Hansen and Baust (1988) and Zhang *et al.* (2004). Briefly, sample (0.1, 0.5, 1, 1.5 mg/mL) was

quickly frozen to −25 °C, held for 5 min, and warmed at 0.5 °C/min to an annealing temperature near the sample melting temperature. When the sample was in the state of partially melting, it should be slowly cooled to −10 °C at 1 °C/min. BSA solution (5 mg/mL) as control was subjected to a similar cycle. The maximum difference between annealing temperature and onset of the freezing exotherm during the slow cooling steps was used as an approximation to the samples freezing point depression activity. All measurements were repeated 3 times.

Overexpression of the cloned gene in transgenic rice

To determine the biofunction of the gene in rice plants, an overexpression vector was constructed based on the binary vector pCAMBIA1301 (CAMBIA). The gene was amplified from the screened clone using the following primers: a forward primer, 5-GGTAGATCTATGGCTCGTGGCCTCG-3, and a reverse primer, 5-GCTGGTCACCTTAGTGCATCATCTG-3. The PCR product double restricted with *BglII* and *BstEII* was ligated into the pCAMBIA1301 vector to replace the GUS gene under control of the 35S cauliflower mosaic virus promoter. Rice callus was prepared from immature embryos, and the generation of the transgenic rice lines (*Oriza sativa* subsp. *japonica* cv. *Nipponbare*) was carried out via *Agrobacterium tumefacient*-mediated transformation procedures according to Hiei *et al.* (1994). The T1 transgenic rice plants were grown to 2-leaf-stage for freezing tolerance identification and to 4-leaf-stage for the disease resistance assay. In this part, cold tolerance assay, blast fungal inoculation and RNA blot analysis were all performed according to methods described above. Control plants were generated from rice transformation following the same procedures with the exception of using empty pCAMBIA1301 without the cloned gene.

Expression analysis of PR protein genes in transgenic rice

Expression of three PR genes in transgenic rice was analyzed based on RNA blot. Hybridization was carried out using p32-labeled *Gns1* (Simmons *et al.*, 1992) to detect the expression of the *β*-glucanase gene, *RC24* (Xu *et al.*, 1996) to detect the expression of a basic chitinase gene and *RBBI2–3* (Qu *et al.*, 2003) to detect the expression of a Bowman-Birk proteinase inhibitor gene. Total RNAs extracted from 4-leaf-stage transgenic rice were analyzed as described above.

Results

Target gene encodes a predicted antifreeze glycopeptide

Through two rounds of screening of the rice cDNA library, 60 single clones with relatively strong hybridization signal were randomly picked. cDNA insertion from each of the 60 single clones was sequenced. According to the partial sequence and that compared with sequence information in GenBank, one gene predicted as AFGP gene (accession number XM_483093) was selected as candidate for further study (here we denominated it

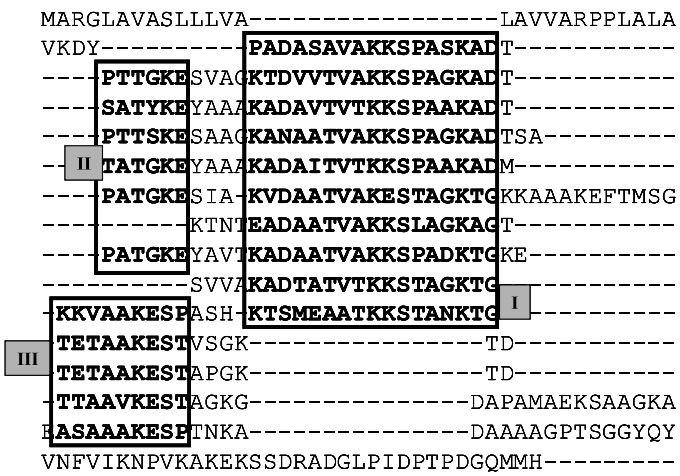

Fig. 1. Antifreezing traits in the amino acid sequence of AFGP. The amino acid sequence of AFGP is rich in hydrophilic amino acids (24.29% alanine; 15.71% lysine; 12.86% threonine), and more than one half of the amino acids is composed of three groups: a 17-amino acid is repeated ten times in part I, a 6-amino acid sequence is repeated 6 times in part II, and a 9-amino acid sequence is repeated 5 times in part III.

as rice *afgp*, *rafgp*). The sequence of *rafgp* contains an ORF of 1260 base pairs (bp) and encodes a protein with 420 amino acids. The predicted gene product has a molecular mass of 41.5 kDa. The amino acid composition of the deduced product is enriched in alanine (24.29 mol%), lysine (15.71 mol%) and threonine (12.86 mol%) residues; and more than one half of the amino acids is composed of a 17-amino acid sequence that is repeated ten times (part I), a 6-amino acid sequence that is repeated 6 times (part II) and a 9-amino acid sequence that is repeated 5 times (part III) (Fig. 1). All these traits are shown to be important in many AFPs (reviewed by Thomashow, 1999). Thus, they probably supply the primary evidence for its AFP nature. As noted in Materials and Methods, because the cDNA library was oriented to disease resistance, genes screened from the library may be functionally associated with rice disease resistance. However, no obvious motifs similar to other known resistance or related genes exist in the polypeptide sequence through widely searching in Genbank. Therefore, we assess that it should be a novel gene.

Expression patterns of rafgp in rice

In order to confirm the relationship between the expression profile of *rafgp* and induction of cold or blast, Northern analysis was carried out using p32-labeled *rafgp* as probes. The results indicated that both blast fungus infection (72 h) and cold treatment (3 d at 15 °C and subsequently 3 d at 8 °C) induced the expression of *rafgp* (Fig. 2). Although the level of expression was very low and only a weak hybridization signal could be detected at normal loading amount of RNA (20 μg/well), when we increased the amount of loading RNA to 50 μg/well, the hybridization signal could be clearly demonstrated (Fig. 2).

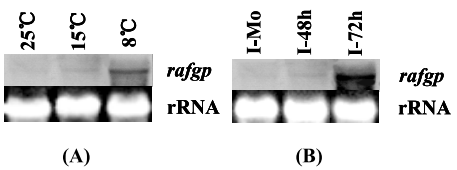

Fig. 2. Expression of afgp in rice treated by rice blast or low temperature RNA analysis of rafgp expression in wild-type rice: (A) with the treatment of cold (15 °C) for 3 d and subsequent 8 °C for another 3 d, rice grown in normal condition (25 °C) as control; and (B) with the treatment of rice blast fungal infection, *i. e.* after inoculation as noted in Materials and Methods, samples were collected at 48 h (I-48 h) and 72 h (I-72 h), mock-inoculated as control (I-Mo). 18S ribosomal RNA staining with EB was used as a loading control.

Expression of the fusion protein in E. coli

Because of the low level of expression of *rafgp* in rice plants, it was difficult for us to obtain enough novel proteins for further research. Prokaryotic expression system was set up based on pET22b(+) vector and recombinant protein was successfully produced in *E. coli.* The fusion protein has been purified to SDS-PAGE homogenous by Coomassie brilliant blue R-250 staining, and 21.1 mg pure protein has been obtained per liter medium. The molecular mass of the fusion protein is approximately 45 kDa. In addition, a red band corresponding to the 45 kDa position was visualized in the SDS-PAGE gel while stained by periodic acid-Schiff reagent (Fig. 3). This experiment verified the protein to be a kind of glycopeptide. On the other hand, this experiment also suggested that *E. coli* supports the same modification system as eukaryotic cells to process glycopeptide precursor.

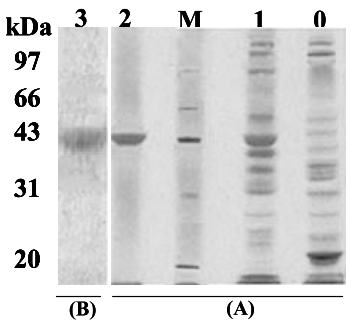

Fig. 3. SDS-PAGE of fusion AFGP in *E. coli*.: (A) Coomassie brilliant blue R-250 staining; (B) periodic acid-Schiff (PAS) staining. Lanes: M, standard marker proteins; 0, lysate of induced culture of *E. coli* containing empty pET22b(+) vector; 1, cell lysate from *E. coli* containing pET22(b) with rafgp; 2 and 3, purified fusion AFGP.

Antifreezing activity

In general, the shape of an ice crystal appears to be bipyramidal in the presence of AFP. In the absence of AFP, however, it is simply round (DeVries, 1983; Bravo and Griffith, 2005). Therefore, levels of antifreeze activity can be defined using the complex degree of the ice crystal: the bipyramidal shape stands for high antifreezing activity and

Table I. Thermal hysteresis activity of the fusion AFGP. Different resulting concentrations of AFGP solution (0.1, 0.5, 1, 1.5 mg/mL) and BSA (5 mg/mL) as samples subjected to a similar measurement cycle were performed. All measurements were repeated 3 times and the maximum difference between annealing temperature and onset of the freezing exotherm during the slow cooling steps was used as an approximation to the samples freezing point depression activity. T_{hd}, hold temperature; $T_{0,}$ temperature at the onset of the freezing exotherm during the slow cooling; T_H, thermal hysteresis temperature, equal to ($T_{hd} - T_0$).

Sample	Concentration [mg/mL]	T_{hd} [°C]	T_0 [°C]	T_H [°C]
BAS	5.0	−0.85	−0.85	0.00
AFGP	0.1	−0.74	−0.92	0.18
AFGP	0.5	−1.20	−1.55	0.35
AFGP	1.0	−0.96	−1.41	0.45
AFGP	1.5	−1.83	−2.43	0.60

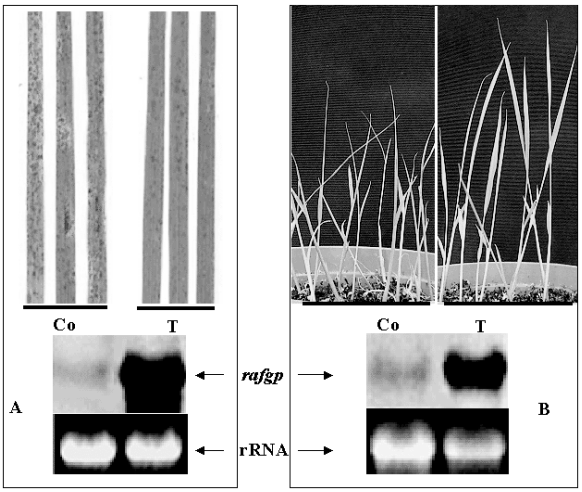

Fig. 4. Enhanced tolerance to disease and cold in transgenic rice. (A) 4-Leaf-stage transformants and controls (with empty vector) were inoculated with conidiospores of *M. grisea*. Disease investigation and Northern analysis were performed after treated for 72 h. (B) 2-Leaf-stage transformants were exposed at 8 °C for 12 h, then subjected to −1 °C for 24 h (RNA was extracted at this time for Northern analysis), subsequently thawed at 8 °C for 12 h. Survival of the seedlings was investigated after grown at normal conditions for 3 d. p32-Labeled rafgp was made as probe, and 18S ribosomal RNA staining with EB was used as a loading control. Co, control transformant; T, transformant.

the hexagonal or flat shape stands for low or no activity. In this study, 0.1 and 1 mg/mL resulting concentrations were, respectively, used to observe the shape of crystals. Hexagonal shape of a single ice crystal emerged in 0.1 mg/mL AFGP solution with the drop of temperature. When the concentration of fusion protein solution was increased to 1 mg/mL, bipyramidal shape of crystals was formed. In contrast, only flat shape of crystals could be observed in BSA solution though the concentration of protein solution was 5 mg/mL. Thus, at this point, the fusion protein possesses high levels of antifreeze ability. The antifreeze ability has also been confirmed through measuring the thermal hysteresis. In general, unique AFP in different concentrations of solution correspondingly has different thermal hysteresis, so 0.1, 0.5, 1 and 1.5 mg/mL resulting solutions of AFGP were, respectively, assayed in this research. From the result (Table I), our fusion protein exhibits a high thermal hysteresis of 0.6 at a concentration of 1.5 mg/mL.

Improvement of tolerance to freezing and disease resistance

In order to identify the *in vivo* dual functions of AFGP, we investigated the effects of rice plants under pathogen and cold stress. Based on the results, control plants subjected to inoculation of the blast fungus were severely infected, emerging typical symptoms of lesion spots on the leaves (Fig. 4A). However, the transgenic rice overexpressing *rafgp* gained improved resistance, with reduced spots on leaves after inoculation. RNA extracted from the treated transgenic rice was analyzed. As expected, a higher level of expression was detected in transgenic rice leaves. Correspondingly, the expression of control plants subjected to the similar treatment appeared to be very low (Fig. 4A). Cold stress assays also demonstrated the improved cold tolerance in transgenic rice. After 0 °C cold treatment for 24 h, the growth of most transgenic rice tested could be basically recovered to normal state except for slight leaf wilt, but the controls were severely damaged. Likewise, the different extent of cold tolerance between rice plants with and without transgene was consistent with the different expression levels (Fig. 4B).

The altered expression of PR proteins in transgenic rice

It is well known, that resulting resistance comprises a series of complex processes in which most

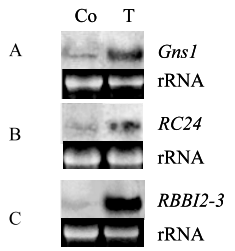

Fig. 5. PR protein genes expression patterns in transgenic rice. 4-Leaf-stage transformants and non-transformants were inoculated with conidiospores of *M. grisea*. Northern analysis was performed after treated for 72 h; P32-labeled *Gns1*, *RC24* and *RBBI2-3* were, respectively, used as probe; 18S ribosomal RNA staining with EB was used as a loading control. Co, control transformant; T, transformant. Expression patterns of (A) Gns1; (B) Rc24; and (C) RBBI2-3 are shown, respectively.

pathogenesis-related proteins are frequently involved. Because *rafgp* possesses a novel gene sequence containing no homologous motifs or conserve domains compared with sequences of disease resistance genes and related, its mechanisms involved in the disease resistance is not clear. In order to confirm its direct or indirect effect on conferring resistance to disease, three PR genes were chosen as markers to identify whether or not these genes were activated in transgenic rice. Interestingly, expression of the marker genes chosen was all enhanced when compared with control plants (Fig. 5). Particularly, a rice Bowman-Birk inhibitor gene (*RBBI2-3*), among genes tested, was strongly expressed (Fig. 5C). *RBBI2-3* had been reported to possess high resistance activity to the blast fungus (Qu *et al*., 2003). Therefore we think that more than one PR protein may be cooperatively involved in the case of resistance.

Discussion

Before our research, the cDNA sequence of *rafgp* had been accessioned in GenBank and predicted as a kind of AFGP gene (http://www.ncbi.nlm.nih.gov/). Excepting this, according to our knowledge, there are not any more studies about the gene to be published to date. Here, we obtained this gene in the rice cDNA library from blast-infected leaves. Activation of the gene is correlated with both pathogen infection and low temperature induction. Fusion protein expressed in *E. coli* exhibits strong antifreeze activity. In addition, the gene overexpressed in rice makes transformants to acquire enhanced resistance to not only disease but also freezing (0 °C). Northern analysis showed that the increased tolerance correlates with high levels of expression of the transgene. All these results indicated that this gene acts in a dual role and should be responsible for tolerance to disease and freezing.

Concerning the ability of antifreezing, we think it is very easy to understand. In general, AFGPs and AFPs are very hydrophilic, containing much more amino acids like asparagine, alanine, lysine, threonine, valine and serine in the protein sequence (Sidebottom *et al*., 2000) and lacking amino acids with hydrophobic side chains, for example arginine or phenylalanine. Above all, conserved repeat composed of certain amino acids is also a significant trait of AFPs in the primary structure (Thomashow, 1999). All these traits can be easily recognized in our sequence, and the repeats of amino acids are particularly obvious (Fig. 1). As mentioned above, proteins are defined as antifreezing according to two qualifications, the complex ice crystal shape and the thermal hysteresis. Besides the two qualifications, if adding the enhancement of cold tolerance in transformants, we own enough reasons to believe that *rafgp* is in fact a gene of antifreezing. Concerning the disease resistance, no doubt, the gene was resistance-related, which can be deduced from the increased resistance to the rice blast and the production of several chosen PR protein genes in transgenic rice. However, we puzzle the limited homologous motifs correlated with the known disease resistance or resistance-related genes. Surprisingly but interestingly, through searching in GenBank amino acid identity appears with the membrane glycoprotein from *equine herpesvirus* (identities = 24%, 71/292, gi|2114321|dbj|BAA20037.1|), the cell wall surface anchor family protein from *Streptococcus pneumoniae* (identities = 24%, 83/344, gi|25389341|pir||E95206) and a predicted mucin-associated surface protein (MASP) from *Trypanosoma cruzi* (identities = 29%, 84/281, XP_819558). Since all these three proteins were deduced from microbes or insects, this suggested that the overexpressed AFGP might act as a heterogeneous elicitor to induce rice resistance. If this is true, the expression of the checked PR proteins in transgenic rice should be rational.

Glucanase, chitinase and Bowman-Birk proteinase inhibitor are three very important members in the superfamily of PR proteins that confer signifi-

cant resistance to plant disease. At the molecular level, for example, the overexpressed β-glucanase (EC 3. 2.1.73), which is encoded by the rice *Gns1* gene and hydrolyzes 1,3;1,4-β-glucosidic linkages to 1,3;1,4-β-glucan, exhibits enhanced resistance to *M. grisea* (Nishizawa *et al*., 2003). Endo-chitinase (EC 3. 2.1.14) is an enzyme that endolytically hydrolyzes chitin, a β-1,4-linked polymer of *N*-acetylglucosamine. *RC24* (Xu *et al*., 1996) used as probe in this research encodes a rice endo-chitinase. It can be rapidly induced by fungal elicitor and confer rice disease resistance. Bowman-Birk inhibitor (BBI) consists of a series of serine protease inhibitors that have repetitive cysteine-rich domains with reactive sites for the trypsin or chymotrypsin family. Recently, one of the rice BBI genes, *RBBI2-3* has been cloned and transferred into rice (Qu *et al*., 2003). The result that transgenic lines overexpressing *RBBI2-3* appear to confer strong resistance to the fungal pathogen *Pyricularia oryzae* confirms its disease resistance function. In our research, it is very coincident that the three PR proteins were all expressed. Therefore, we assess some PR proteins; at least the three proteins mentioned above contribute to certain resistance in this case. On the other hand, these PR proteins are frequently associated with cold response and tolerance. For example, PR-2, PR-3, and PR-5 proteins isolated from different plants are induced by low temperature or pathogens infection (reviewed by Nishizawa *et al*., 2003). In addition, AFPs sometimes possess cooperative effects (Nishimiya *et al*., 2005). Therefore, although the fusion AFGP in our report exhibits high freeze activity, we have no reasons to expel the three PR proteins having participated in the antifreezing case in transgenic rice. In summary, we obtained transgenic rice plants that acquired both freezing tolerance and disease resistance, suggesting that overexpressed AFGP of rice may prove useful in generating dual role engineering plants.

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Atici O. and Nalbantoglu B. (2003), Antifreeze proteins in plants. Phytochemistry **64**, 1187–1196.

Baker B., Zambryski P., Staskawicz B., and Dinesh-Kumar S. P. (1997), Signaling in plant-microbe interaction. Science **276**, 726–733.

Bradford M. M. (1976), A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem. **72**, 1105–1112.

Bravo L. A. and Griffith M. (2005), Characterization of antifreeze activity in Antarctic plants. J. Exp. Bot. **56**, 1189–1196.

Browse J. and Xin Z. (2001), Temperature sensing and cold acclimation. Curr. Opin. Plant Biol. **4**, 241–246.

DeVries A. L. (1983), Antifreeze peptides and glycopeptides in cold-water fishes. Ann. Rev. Physiol. **45**, 245–260.

Duman J. G. and Olsen T. M. (1993), Thermal hysteresis protein activity in bacteria, fungi, and phylogenetically diverse plants. Cryobiology **30**, 322–328.

Ewart K. V., Lin Q., and Hew C. L. (1999), Structure, function and evolution of antifreeze proteins. Cell. Mol. Life Sci. **55**, 271–283.

Gong Z., Lee H., Xiong L., Jagendorf A., Stevenson B., and Zhu J. K. (2002), RNA helicase-like protein as an early regulator of transcription factors for plant chilling and freezing tolerance. Proc. Natl. Acad. Sci. **99**, 11507–11512.

Griffith M. and Ewart K. (1995), Antifreeze proteins and their potential use in frozen foods. Biotechnol. Adv. **13**, 375–402.

Griffith M. and Yaish M. W. F. (2004), Antifreeze proteins in overwintering plants: a tale of two activities. Trend Plant Sci. **9**, 399–405.

Guy C. L. (1990), Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41**, 187–223.

Haake V., Cook D., Riechmann J. L., Pineda O., Thomashow M. F., and Zhang J. Z. (2002), Transcription factor CBF4 is a regulator of drought adaptation in *Arabidopsis*. Plant Physiol. **130**, 639–648.

Hansen T. N. and Baust J. G. (1988), Differential scanning calorimetric analysis of antifreeze protein activity in the common mealworm, *Tenebrio molitor*. Biochim. Biophys. Acta **957**, 217–221.

Hiei Y., Ohta S., Komari T., and 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.

Hsieh T. H., Lee J. T., Yang P. T., Chiu L. H., Charng Y. Y., Wang Y. C., and Chan M. T. (2002), Heterology expression of the *Arabidopsis* C-repeat/dehydration response element binding factor 1 gene confers elevated tolerance to chilling and oxidative stresses in transgenic tomato. Plant Physiol. **129**, 1086–1094.

Hughes M. and Dunn M. (1996), The molecular biology of plant acclimation to low temperature. J. Exp. Bot. **47**, 291–305.

Jaglo-Ottosen K. R., Gilmour S. J., Zarka D. G., Schabenberger O., and Thomashow M. F. (1998), *Arabid-*

opsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science **280**, 104–106.

Jia Z. and Davies P. L. (2002), Antifreeze proteins: an unusual receptor ligand interaction. Trend Biochem. Sci. **27**, 101–106.

Kobashigawa Y., Nishimiya Y., Miura K., Ohgiyab S., Miura A., and Tsuda S. (2005), A part of ice nucleation protein exhibits the ice-binding ability. FEBS Lett. **579**, 1493–1497.

Kreps J. A., Wu Y. J., Chang H. S., Zhu T., Wang X., and Harper J. F. (2002), Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol. **130**, 2129–2141.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277**, 680–685.

Levitt J. (1980), Responses of Plants to Environmental Stresses, 2nd ed. Academic Press, New York.

Liu Q., Kasuga M., Sakuma Y., Abe H., Miura S., Yamaguchi-Shinozaki K., and Shinozaki K. (1998), Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought and low-temperature responsive gene expression, respectively, in *Arabidopsis*. Plant Cell **10**, 1391–1406.

Nishimiya Y., Sato R., Takamichi M., Miura A., and Tsuda S. (2005), Co-operative effect of the isoforms of type III antifreeze protein expressed in Notched-fin eelpout, *Zoarces elongatus* Kner. FEBS J. **272**, 482–492.

Nishizawa Y., Saruta M., Nakazono K., Nishio Z., Soma M., Yoshida T., Nakajima E., and Hibi T. (2003), Characterization of transgenic rice plants over-expressing the stress-inducible β-glucanase gene Gns1. Plant Mol. Biol. **51**, 143–152.

Qu L., Chen J., Liu M., Pan N., Okamoto H., Lin Z., Li C., Li D., Wang J., Zhu G., Zhao X., Chen X., Gu H., and Chen Z. (2003), Molecular cloning and functional analysis of a novel type of Bowman-Birk inhibitor gene family in rice. Plant Physiol. **133**, 560–570.

Rathjen J. P. and Moffett P. (2003), Early signal transduction events in specific plant disease resistance. Curr. Opin. Plant Biol. **6**, 300–306.

Ryals J. A., Neuenschwander U. H., Willits M. G., Molina A., Steiner H. Y., and Hunt M. D. (1996), Systemic acquired resistance. Plant Cell **8**, 1809–1819.

Saijo Y., Hata S., Kyozuka J., Shimamoto K., and Izui K. (2000), Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J. **23**, 319–327.

Sambrook J. and Russell D. W. (2001), Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, New York.

Sidebottom C., Buckley S., Pudney P., Twigg S., Jarman C., Holt C., Telford J., McArthur A., Worrall D., Hubbard R., and Lillford P. (2000), Heat-stable antifreeze protein from grass. Nature **406**, 256.

Simmons C. R., Litts J. C., Huang N., and Rodriguez R. L. (1992), Structure of a rice β-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. Plant Mol. Biol. **18**, 33–45.

Thomashow M. F. (1999), Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50**, 571–599.

Tremblay K., Ouellet F., Fournier J., Danyluk J., and Sarhan F. (2005), Molecular characterization and origin of novel bipartite cold-regulated ice recrystallization inhibition proteins from cereals. Plant Cell Physiol. **46**, 884–891.

Worrall D., Elias L., Ashford D., Smallwood M., Sidebottom C., Lilford P., Telford J., Holt C., and Bowles D. (1998), A carrot leucine-rich repeat protein that inhibits ice recrystallization. Science **282**, 115–117.

Xin Z. and Browse J. (2000), Cold comfort farm: The acclimation of plants to freezing temperatures. Plant Cell Environ. **23**, 893–902.

Xiong L., Schumaker K. S., and Zhu J. K. (2002), Cell signaling during cold, drought, and salt stress. Plant Cell **14**, S165–183.

Xu Y., Zhu Q., Panbangred W., Shirasu K., and Lamb C. (1996), Regulation expression and function of a new basic chitinase gene in rice (*Oryza sativa* L.). Plant Mol. Biol. **30**, 387–401.

Xu H., Griffith M., Patten C. L., and Glick B. R. (1998), Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Can. J. Microbiol. **44**, 64–73.

Yeh S., Moffatt B., Griffith M., Xiong F., Yang D. S. C., Wiseman S. B., Sarhan F., Danyluk J., Xue Y. Q., Hew C. L., Doherty-Kirby A., and Lajoie G. (2000), Chitinase genes responsive to cold encode antifreeze proteins in winter cereals. Plant Physiol. **124**, 1251–1265.

Zhang D. Q., Liu B., Feng D. R., He Y. M., and Wang J. F. (2004), Expression, purification, and antifreeze activity of carrot antifreeze protein and its mutants. Protein Expr. Purif. **35**, 257–263.