Rice 1Cys-peroxiredoxin over-expressed in transgenic tobacco does not maintain dormancy but enhances antioxidant activity

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Abstract Possible functions that have been proposed for the plant 1Cys-peroxiredoxin, include activity as a dormancy regulator and as an antioxidant. The transcript level of rice 1Cys-peroxiredoxin (*R1C-Prx*) rapidly decreased after imbibition of rice seeds, but the protein was detected for 15 days after imbibition. To investigate the function of this protein, we generated transgenic tobacco plants constitutively expressing the *R1C-Prx* gene. The transgenic R1C-Prx plants showed a germination frequency similar to control plants. However, the transgenic lines exhibited higher resistance against oxidative stress, suggesting that antioxidant activity may be its primary function.

Key words: Antioxidant activity; Rice 1Cys-peroxiredoxin; Seed dormancy; Transgenic rice 1Cys-peroxiredoxin tobacco

1. Introduction

It is well known that the generation of reactive oxygen species (ROS) is an inevitable process in all aerobic organisms, especially when they encounter pathological and physiological stress conditions [1,2]. The ROS generated by oxidative stress can cause serious damage to biological macromolecules such as protein, lipid, and nucleic acid and eventually lead to cell death [3]. To cope with the deleterious effects of ROS, aerobic organisms express various antioxidant proteins including superoxide dismutase, catalase, ascorbate-dependent peroxidase [4], and others. Recently, a new type of thiol-specific antioxidant (TSA) protein, now renamed peroxiredoxin (Prx), that functions as peroxidase only when coupled to a sulfhydryl reducing system [5] had been identified. Based on the amino acid sequence and immunological properties [6], the Prx proteins can be divided into six distinct groups (Prx I–VI), all of which contain one or two conserved cysteine residues, called 1Cys- and 2Cys-Prx. The multiple isotypes of 2Cys-Prx in a wide variety of organisms have diverse cellular functions, such as an antioxidant, an endogenous regulator of apoptosis [7], and as an intracellular signaling molecule [8,9]. In addition, the 2Cys-Prx proteins in plants contain an N-terminal transit peptide that was shown to function as a radical scavenger coupled to the photosynthetic machinery in chloroplast [10,11].

Although the protein structure of the 1Cys-Prx had been elucidated and several cDNAs encoding the protein have been isolated and characterized from yeast, animal, and plant cells [12–15], the cellular function of 1Cys-Prx remains ill-defined, and its physiological electron donor, except for the yeast mitochondrial 1Cys-Prx [16], is still the subject of much controversy [17]. In plants, the *1Cys-Prx* is specifically expressed in the nucleus of immature embryos and the aleurone layers of the seed. The expression level is significantly increased late in seed development and maintained in mature seeds during storage [18]. However, when the non-dormant seed is imbibed, the transcript level becomes dramatically reduced and completely disappears after seed germination. These observations suggested that the functional role of the 1Cys-Prx in plants might be related to the maintenance of seed dormancy [13], rather than its antioxidant activity reported in other organisms such as yeast and mammalian cells. However, recently, Haslekas et al. [18] reported that the transcript level of *1Cys-Prx* did not correlate with the abscisic acid (ABA) level. ABA was shown to be required for the induction of seed dormancy [19] in ABA-deficient or insensitive mutants of *Arabidopsis* [13]. Therefore, to elucidate the authentic in vivo role of the 1Cys-Prx in a plant, we generated transgenic tobacco plants constitutively expressing rice 1Cys-peroxiredoxin (R1C-Prx). We then compared the germination frequency and antioxidant activity of the transgenic R1C-Prx plants with those of wild type plants.

2. Materials and methods

2.1. cDNA clone and plant materials

The clone of *R1C-Prx* cDNA used in this experiment was kindly donated from the MAFF DNA Bank, Japan (GenBank accession number C19186). For Northern and Western blot analyses, rice seeds (*Oryza sativa* L. cv. Dong-jin) were used after surface-sterilization with 70% ethanol and 4% (w/v) sodium hypochlorite. The sterilized seeds were imbibed at either 4 or 25°C for 24 h and transferred to sterile vermiculite. They were grown in a growth chamber maintained at 25°C during the day and 20°C at night, a 16-h photoperiod, and 65% relative humidity.

2.2. Preparation of a polyclonal antibody for the R1C-Prx and Western blotting

The GST gene fusion system (Pharmacia) was used to generate the

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Abbreviations: ABA, abscisic acid; CaMV, cauliflower mosaic virus; DNP-hydrazone, 2,4-dinitrophenylhydrazone; MS, Murashige and Skoog; Prx, peroxiredoxin; R1C-Prx, rice 1Cys-peroxiredoxin; ROS, reactive oxygen species

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fusion protein in *Escherichia coli* strain BL21 (pLysS). The GST–R1C-Prx fusion protein was purified with the help of a glutathione-agarose affinity gel, and the native R1C-Prx was cleaved off by thrombin treatment. Using the purified R1C-Prx, a polyclonal antibody was raised in a rabbit as described previously [20]. Rabbit serum was diluted 1:50 000 in 5% skim milk solution for immunoreactions. Horseradish peroxidase-conjugated goat IgG was used as the secondary antibody, and the antigen was detected by chemiluminescence using an ECL detection kit (Amersham).

2.3. Northern analysis

Total RNA was extracted from the whole plant by the guanidium thiocyanate/phenol/chloroform extraction method and additionally purified by ultracentrifugation [21]. 20 μg of total RNA from each sample was denatured, separated in a 1.2% agarose gel and transferred onto a Hybond N^+-nylon membrane (Amersham). Hybridization was carried out in a solution containing 0.1% BSA, 1 mM EDTA, 0.5 M NaH_2PO_4, pH 7.2, 7% SDS at 65°C for 18 h using a ^{32}P-labeled *R1C-Prx* cDNA as probe. To show the loading amount of RNA in each lane, a ^{32}P-labeled rice β-*tubulin* gene probe was included.

2.4. Preparation of transgenic R1C-Prx tobacco plants

For construction of the transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi-nc), the *R1C-Prx* cDNA was ligated into a plant binary vector, *pBI121* linker derived from Ti-plasmid, in which the *R1C-Prx* was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in a sense orientation. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain LBA 4404, which was used to transform the tobacco plants by the standard leaf-disc transformation method [22]. R1 progeny of transgenic plants expressing a high level of R1C-Prx were used for the experiments and maintained in a growth chamber as described above.

2.5. Detection of oxidized proteins

Since the carbonyl derivatives of proteins generated from oxidative modification react with 2,4-dinitrophenylhydrazine, resulting in the formation of 2,4-dinitrophenylhydrazone (DNP-hydrazone) [23], an immuno-detection kit (Intergen) containing a polyclonal antibody specific to the DNP moiety on proteins was used to measure the carbonyl contents of oxidized proteins. Before Western blotting, the protein samples were separated by 10% SDS–PAGE.

3. Results and discussion

3.1. Expression of the R1C-Prx during germination of rice seeds

Since the expression of plant *1Cys-Prx* genes had been reported to be closely related to seed dormancy [13,24], we analyzed the expression of R1C-Prx both at the mRNA and protein level in rice seeds to determine whether the R1C-Prx expression would show a dormancy-related expression pattern. In independent triplicate experiments, transcripts of the *R1C-Prx* in dormancy-broken rice seeds were highly expressed in the seeds, but the mRNA level became significantly diminished immediately after seed germination. However, a small amount of the transcripts was still maintained up to 15 days after seed germination (Fig. 1A). Control mRNA of the rice β-*tubulin* gene was significantly increased at the first 5 days and the transcript level was slightly increased and maintained during the experiments. Furthermore, the protein level of R1C-Prx as measured by immunoblot analysis was relatively stable during the same time period as the mRNA (Fig. 1B). A significant decrease was observed only after 15–20 days post-germination [14]. These results suggest that the R1C-Prx may not play a role in the maintenance of seed dormancy.

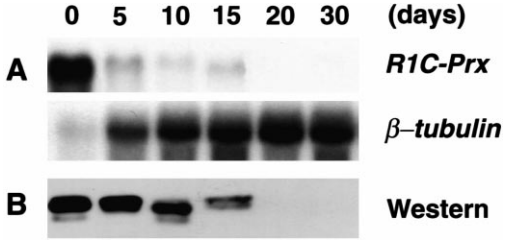

Fig. 1. Expression of the R1C-Prx at the mRNA and protein level during the germination of rice seeds. A: 20 μg of total RNA isolated from whole rice seedlings at the indicated time-points were analyzed by Northern blot using a α-^{32}P-labeled *R1C-Prx* probe and a rice β-*tubulin* probe (as loading control). B: 20 μg of protein isolated from the same samples as used in A were separated by SDS–PAGE, and the Western blot was probed with anti-R1C-Prx antibody.

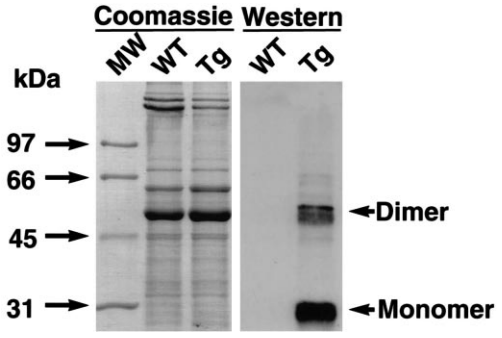

Fig. 2. SDS–PAGE and Western blot analysis of the proteins isolated from wild type (WT) and transgenic R1C-Prx (Tg) plants. Proteins isolated from WT and Tg plants grown for 3 weeks were subjected to SDS–PAGE and either stained with Coomassie blue or blotted and probed with anti-R1C-Prx antibody.

3.2. Generation of transgenic tobacco constitutively expressing the R1C-Prx

To better discern an in vivo function of the 1Cys-Prx in plants, we constructed transgenic tobacco plants that constitutively expressed R1C-Prx under the control of the CaMV 35S promoter. Transgenic plant lines over-expressing R1C-Prx were selected after Northern analysis and verified by immunoblot analysis. Since the 18 transgenic tobacco plants over-expressing R1C-Prx gave nearly the same results (data not shown), we describe here the data obtained from transgenic line #3. A high level of the R1C-Prx, whose molecular weight was estimated to be 27 kDa, was detected by immunoblot analysis in transgenic R1C-Prx plants grown for 3 weeks, whereas the control plants harboring only the empty vector did not express the protein at all (Fig. 2). Furthermore, small amounts of a dimeric form of R1C-Prx, in addition to the monomer, were also detected in the Western blot analysis. These dimers did not separate under reducing SDS–PAGE conditions, which is unlike the yeast 2Cys-Prx [25]. When we analyzed the protein under non-reducing SDS–PAGE [25] conditions, most of the R1C-Prx protein actually existed in dimeric form (data not shown).

3.3. Comparison of germination frequency between wild type and transgenic R1C-Prx plants

Using the transgenic R1C-Prx plants, we asked whether the continued expression of R1C-Prx would lead to dormancy. The R1 progeny of the transgenic tobacco and wild type seeds after imbibition at 4°C for 24 h were plated on a water-soaked

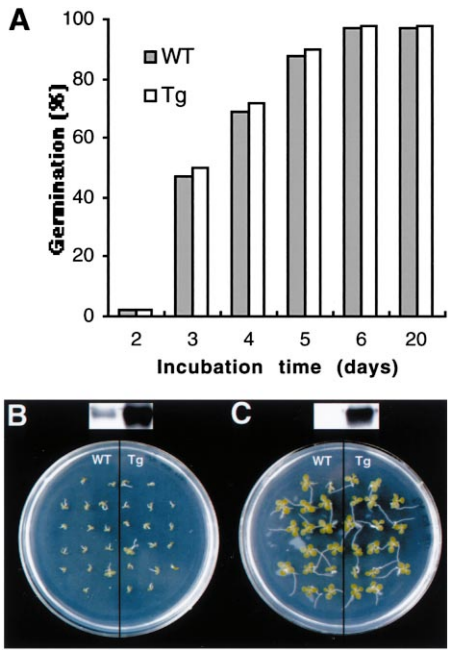

Fig. 3. Comparison of the germination frequency of wild type (WT) and transgenic R1C-Prx (Tg) plants. A: Germination efficiencies of WT and Tg plant seeds were compared after imbibition of the seeds at 4°C for 24 h. The imbibed seeds were plated on an agar plate containing MS medium and transferred to a 26°C growth chamber. Germination was scored at the time points indicated by radical extrusion. B and C: Photographs of tobacco seedlings of WT and Tg plants grown for 10 days (B) or 3 weeks (C) in a growth chamber. The insets show the immunoblots of the proteins of WT and Tg plants grown for 10 days or 3 weeks on agar plates containing MS medium probed for R1C-Prx.

filter paper at 25°C, and their germination efficiencies were compared. Surprisingly, the wild type and the transgenic R1C-Prx plants did not differ in their germination frequency, and both seeds exhibited more than 95% germination at 6 days after imbibition (Fig. 3A). Furthermore, when the seeds were grown on an agar plate containing Murashige and Skoog (MS) medium for 10 days or 3 weeks, the presence of the transgene protein did not suppress the germination or the growth rate of the plants (Fig. 3B). Thus, no functional relationship to dormancy could be detected. To eliminate the cold effect on seed germination, we carried out the same experiment again with the transgenic and wild type seeds that were stored at 25°C for 3 months after they were imbibed at 25°C, instead of 4°C, for 24 h. In this study, nearly the same result was obtained from the two plant seeds with or without the cold treatment (data not shown), confirming the result of Fig. 3. However, considering the data of Graffin et al. [26], the possibility can not be completely excluded that the result may result from the after-ripening effect, which will be studied further in detail.

3.4. Enhanced resistance of the transgenic R1C-Prx tobacco plants against oxidative stress

The resistance of transgenic R1C-Prx plants to radical stress was measured after infiltrating whole wild type and transgenic R1C-Prx plants with 5 mM H_2O_2 solution. After the plants were taken out of the H_2O_2 solution, they were incubated in a growth chamber for 72 h to allow them to recover from the stress. Usually tobacco plants grown for 6 weeks under the conditions of our experiment had 18–20 leaves in total. Whereas the wild type tobacco plants showed serious lesions up to the 12th or 13th leaf from the bottom, the transgenic R1C-Prx plants showed similar damage only up to the 4th or 5th leaf. In general for both types of plants the older the leaves, the more damage they showed from the stress. The degree of damage for leaves 10, 12 and 14 is shown in Fig. 4A. The data strongly suggest that the transgenic R1C-Prx plants exhibited enhanced resistance against radical stress.

In another approach to evaluate resistance against oxidative stress, we employed an OxiBlot reagent kit, which immunologically detects the carbonyl group on oxidized proteins. The DNP-hydrazone generated from the reaction of protein carbonyl groups with 2,4-dinitrophenylhydrazine [23], can be detected by an antibody specific to the DNP moiety on the proteins. Water infiltrated leaves of mock and H_2O_2 treatment were compared. No difference between the wild type and transgenic R1C-Prx plants, in terms of leaf morphology (data not shown) or carbonyl contents in protein extracts was detected in control leaves (Fig. 4B). On the other hand, the protein carbonyl contents present in the 10th leaf from the bottom of the transgenic R1C-Prx plants treated with 5 mM H_2O_2 were much less than those of wild type plants. From these results, it can be concluded that the in vivo function of

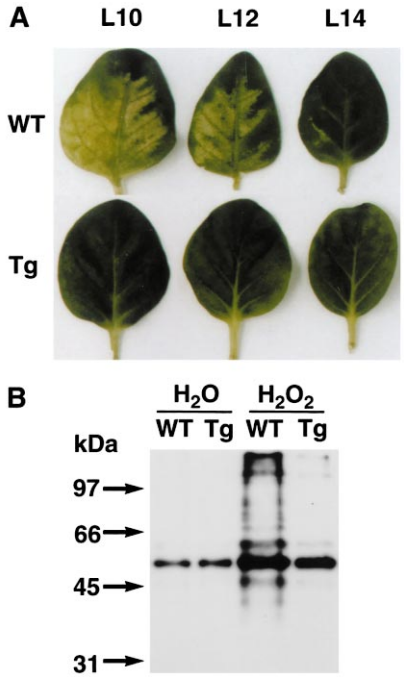

Fig. 4. Resistance of transgenic R1C-Prx plants against oxidative stress. A: Whole plants of wild type (WT) and transgenic R1C-Prx (Tg) plants grown for 6 weeks were immersed in 5 mM H_2O_2 solution and H_2O_2 was infiltrated into the plants using a pressure-controlled vacuum chamber. After treatment for 5 min, the plants were transferred to a growth chamber to recover from the stress under optimal growth conditions. Oxidative damage to the treated leaves was compared. L10, L12, and L14 indicate the number that the detached leaf occupied on the tobacco plant counting from the bottom. B: Western blot analysis of protein carbonyl contents in L10 of WT and Tg plants after treatment with H_2O or H_2O_2 using the same procedures as in A. The DNP-derivatized proteins prepared from both the WT and Tg plants were separated by 10% SDS–PAGE and the immunoblot was analyzed with a primary antibody specific to the DNP moiety on the oxidized proteins.

1Cys-Prx in plants may not be related to the maintenance of seed dormancy, but rather to protective activity against oxidative stress.

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