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Overexpression of a gene for 26S proteasome subunit RPN10 confers enhanced resistance to canavanine, an analog of arginine, in transgenic rice (*Oryza sativa* L.)

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Abstract

The RPN10 subunit is a component of the 19S regulatory complex that is essential for the ubiquitin-dependent proteolytic activity of the 26S proteasome (EC 3.4.99.46). To address the functional role of RPN10, we examined phenotypic changes that occurred when the *rpn10* gene was overexpressed in rice plants (*Oryza sativa* L cv Nipponbare). These transgenic plants showed enhanced resistance to canavanine, suggesting that the overexpression of the *rpn10* gene accelerates the degradation of abnormal proteins containing canavanine instead of arginine.

Key words : *Oryza sativa*, Overexpression, Proteasome, RPN10.

Abbreviations

RPN10, regulatory particle non-ATPase 10; C2, major component 2 (alpha-type subunit of 20S proteasome)

The 26S proteasome (EC 3.4.99.46) is a highly conserved multicatalytic endopeptidase complex present in all eukaryotes and it catalyzes the ATP-dependent degradation of various cellular proteins that have been tagged with a multiubiquitin chain as a degradation signal (Coux *et al.*, 1996; Baumeister *et al.*, 1998; Hershko and Ciechanover, 1998; Rechsteiner, 1998; Tanaka, 1998). The ubiquitin/26S proteasome system controls numerous physiological and developmental events. In plants, molecular genetic analyses revealed that the individual components of this system are implicated in almost all aspects of plant biology (Vierstra, 2003).

The 26S proteasome consists of two particles, the 20S core protease (CP) and the 19S regulatory particle (RP). The CP is a barrel-shaped particle formed by the axial stacking of four rings (two α rings and two β rings), arranged in the order of $\alpha\ \beta\ \beta\ \alpha$. One RP binds to each end of the CP. Each RP consists of two particles, a lid and a base. The base contains six AAA-ATPase subunits (RPT1 to RPT6) and three non-ATPase subunits (RPN1, RPN2, and RPN10). The base faces the CP, and the lid binds to the outer end of the base and contains nine additional non-ATPase subunits (RPN3, RPN5 to RPN9, and RPN11 to RPN13) (Glickman *et al.*, 1998).

The RPN10 subunit was reported to play pleiotropic roles in the 26S proteasome. It was originally identified by its ability to bind polyubiquitin chains in vitro (van Nocker *et al.*, 1996a). Subsequently, it was found that RPN10 is not the sole ubiquitin receptor, because yeast *rpn10* knockout strains are viable (van Nocker *et al.*, 1996b). However, the knockout strains are more sensitive to the amino acid analog canavanine and had higher steady-state levels of ubiquitin-protein conjugates than wild type yeast. RPN10 may also have a role in connecting the lid to the base (Glickman *et al.*, 1998).

As in yeast, an *rpn10* disruption mutant of the moss *Physcomitrella patens* is viable but developmentally impaired and its growth hypersensitive to

amino acid analogs (Girod *et al.*, 1999). In *Arabidopsis*, mutant plants expressing an altered RPN10 subunit exhibit a pleiotropic phenotype, including a decreased seed germination and a low growth rate (Smalle *et al.*, 2003). The mutant plants are also more sensitive to abscisic acid, salt, sucrose stress, and DNA-damaging agents than the wild type.

To address the functional role of RPN10, we examined the phenotypes of rice plants in which the *rpn10* gene was overexpressed, because rice is both an economically important crop and an experimental monocot model. Our results revealed that transgenic rice plants that overexpress the *rpn10* gene exhibit enhanced resistance to canavanine.

We amplified the coding region of RPN10 from rice cDNA (Yanagawa *et al.*, 1998) by PCR using the primers 5′-ATAGGATCCGCCATGGTGCTCGAGGCG-3′ and 5′-ATAGAGCTCTTTCTTCTCATCTTCTGGCTTG-3′ in which *Bam*HI and *Sac*I sites were included respectively. A binary vector, the pMLH7133-GUS plasmid (Mochizuki *et al.*, 1999; Mitsuhara *et al.*, 1996), was digested with *Bam*HI and *Sac*I and ligated with the PCR fragment. *Agrobacterium tumefaciens* EHA101 (Hood *et al.*, 1986) was transformed with the binary vector, pMLH7133-GUS or pMLH7133-RPN10, using the Gene Pulsar apparatus (25 μF, 200 Ω, 2.50 kV). The transformation of rice (*Oryza sativa* cv Nipponbare) seeds was performed as described by Tanaka *et al.* (2001) or Hiei *et al.* (1994), with some modifications. Transgenic plantlets were selected on a hygromycin-containing medium. The pMLH7133-GUS vector was also introduced into rice plants that were used as the control. The transformants (any generations) were selected by PCR analysis using a set of primers: 5′-ATCTCCACTGACGTAAGGGATGACG-3′ (specific to the 35S pro moter) and 5′-ACAGGATTCAATCTTAAGAAACTTT-3′ (specific to the nos terminator). The seeds of T1 or T2 plants that had been selected were pooled and used to produce the T2 or T3 generation. The rates of plants that contained the transgene in ~10 plants of the T2 generation were 77%, 78%, 80%, 89%, and 92% in the control, S3, S4, S9, and S10 lines, respectively.

Total RNA was isolated from mature leaves by an SDS-phenol method or an aurin tricarboxylic acid method. Aliquots of 20 μg of total RNA were resolved by electrophoresis on 1.2% agarose gels containing 20% formaldehyde and transferred onto nylon membranes (Hybond-N, Amersham). After prehybridization, the membranes were incubated with ^{32}P-labeled DNA probes for 16 h and then washed twice with 2 x SSC containing 0.1% SDS at 42℃ for 5 min and twice with 0.1 x SSC containing 0.1% SDS at 42℃ for 15 min. To detect *rpn10* gene transcripts, full-length cDNA for rice RPN10 was used as the probe. Cloned rice C2 (Umeda *et al.*, 1997) and RPN3 (Yanagawa *et al.*, 2002) cDNAs were also used as probes.

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose filter (Millipore, Bedford, MA, USA) as previously described (Towbin *et al.*, 1979). Rice RPN10 was detected with a polyclonal antibody against rice RPN10 as described by Yanagawa *et al.* (1998).

Rice seeds (T2 generation) were sterilized and germinated in water for 3 days. The 3-day-old

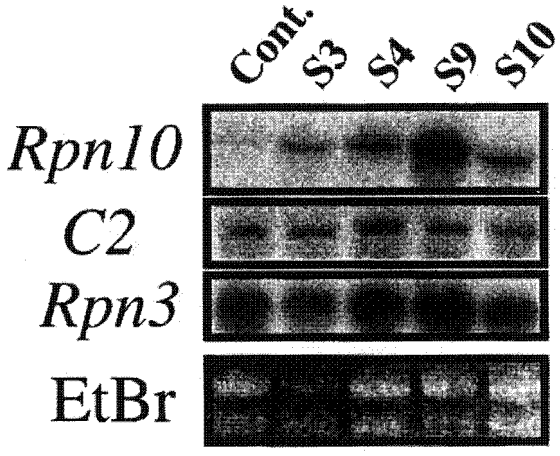

Fig. 1. Detection of RPN10, C2, and RPN3 transcripts in mature leaves of T2 transgenic lines. Total RNA was extracted from mature leaves, and samples (20 μg of RNA per lane) were subjected to RNA blot analysis using full-length cDNA for rice RPN10, C2, and RPN3 as probes. The amounts of RNA loaded are indicated by EtBr (ethidium bromide)-stained ribosomal bands. Cont: control plants transformed with the vector pMLH7133-GUS. S: sense plant.

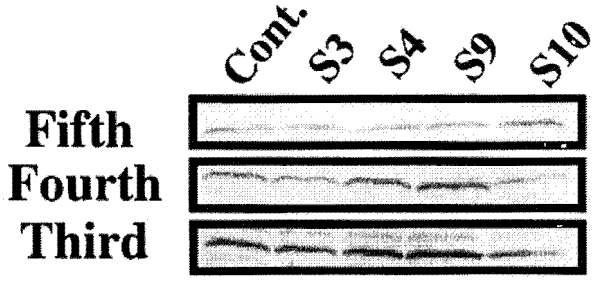

Fig. 2. Detection of RPN10 in young leaves of T2 transgenic lines. Total protein was extracted from the fifth, fourth, and third leaves at the five-leaf stage of plant growth, and samples (5 μg of protein per lane) were subjected to SDS-PAGE and immunoblot analysis using a polyclonal antibody against rice RPN10.

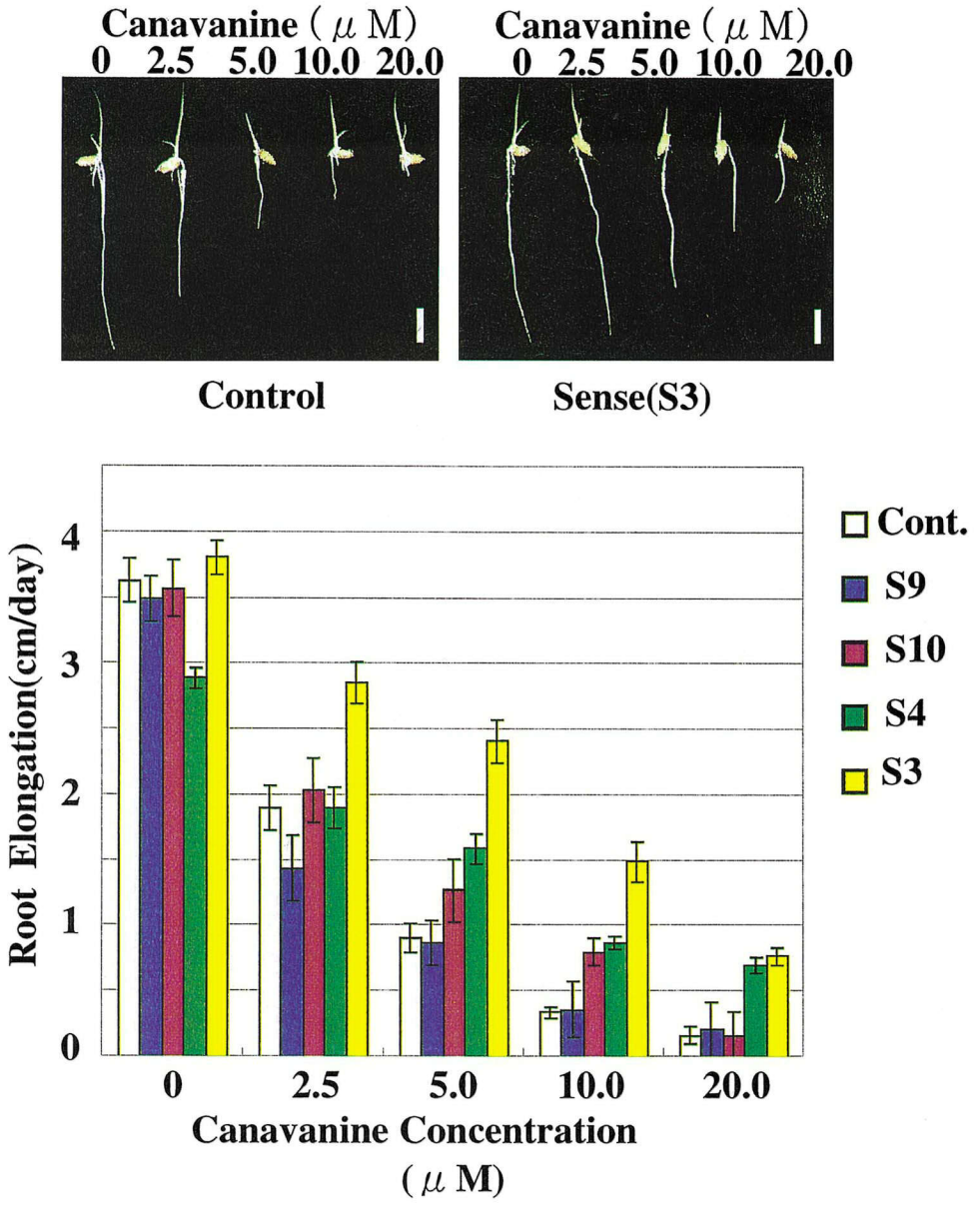

Fig. 3 Enhanced resistance to canavanine in T2 transgenic plants. Three-day-old seedlings were transferred to 0.8% agar containing various concentrations of canavanine (an arginine analog) and incubated at 27°C. Top : Photographs of control and transformed plants 3 days after growing on agar containing the indicated concentrations of canavanine. Bars = 5 mm. Bottom : Root elongation velocity values are means for 8-10 seedlings (±SE). Cont. : control plants transformed with the vector pMLH 7133-GUS, S : sense plant.

plants were placed on 0.8% agar (8 mL) containing canavanine, an analog of arginine, in glass tubes. The plants in the glass tubes were grown for 3 days at 27℃ under a 12 h light: 12 h dark regime. Root length was measured on the second and third days after canavanine treatment and used to calculate root elongation velocity.

The *rpn10* gene from rice was inserted into a plant expression binary vector, pMLH7133-GUS, in the sense orientation. The inserted genes in this vector are expressed under the control of a strong promoter, which confers a higher constitutive expression level than the 35S CaMV promoter in rice protoplasts (Mitsuhara *et al.*, 1996). The *rpn10* constructs were introduced into rice plants via *Agrobacterium*-mediated transformation. PCR analysis showed that we obtained nine transgenic plants from approximately 1000 rice seeds. RNA blot analysis indicated that the *rpn10* gene was considerably overexpressed in the mature leaves of four independent lines established from the nine transgenic plants (**Fig. 1**).

To determine the influence of *rpn10* overexpression on the expression of genes for other subunits of the 26S proteasome, RNA blot analysis of the *rpn3* (an RP subunit) and *c2* (a CP subunit) genes was also carried out and it was revealed that the expression levels of these genes were not affected by the ectopic expression of the *rpn10* gene, at least in the mature leaves of the four transgenic plants tested (**Fig. 1**). The results of RNA blot analysis were the same irrespective of whether we used an SDS-phenol method or an aurin tricarboxylic acid method to extract and purify RNA.

In the young leaves of transgenic plants, no significant difference in the amount of RPN10 was detected between *rpn10*-overexpressing and control lines (**Fig. 2**). The amounts of RPN10 in the mature leaves and roots of the transformed and control plants also did not significantly differ (data not shown).

The translational incorporation of canavanine, an analog of arginine, generates aberrant proteins that require the ubiquitin/proteasome pathway for their removal (van Nocker *et al.*, 1996b). The *rpn10* gene disruption mutant from the moss *Physcomitrella patens* was sensitive to canavanine (Girod *et al.*, 1999). Therefore, we examined the resistance of the *rpn10* transgenic rice plants to canavanine and found a significantly enhanced resistance to canavanine in the S3, S4, and S10 lines (**Fig. 3**). Such enhanced resistance to canavanine was not observed in the control (**Fig. 3**) or in other rice (cv Chiyohonami) transgenic lines in which pMLH-GUS was introduced by Iwai *et al.* (2002) (data not shown). Our results thus indicate that the *rpn10*-overexpressing lines have an enhanced ability to eliminate these aberrant proteins.

However, the S9 line exhibited no resistance to canavanine (**Fig. 3**), in spite of having the highest level of RPN10 mRNA expression in the T2 generation. This finding suggests that an appropriate level of expression is necessary for resistance to canavanine or, alternatively, that canavanine resistance in the S9 plants was compromised by somatic mutations caused by the transformation and regeneration steps.

The RPN10 level did not change markedly in the three transgenic lines that exhibited enhanced resistance to canavanine (**Fig. 2**). The cause is not clear, but it is possible that only a slight increase in the level of RPN10 by the overexpression of the rpn10 gene may be sufficient for resistance or that the level of RPN10 may actually increase only in certain cells of rice plants.

A morphological change was observed in the S3 and S4 lines: in both lines, the development of tiller buds at the heading stage was delayed compared with the control plants (data not shown). The RPN10 subunit was reported to play pleiotropic roles in the 26S proteasome, as mentioned above. RPN10 not only binds to ubiquitin or multiubiquinated proteins but also binds to the ubiquitin-like domain of proteins (Hiyama *et al.*, 1999); in addition, it affects the structure of target proteins, as recently demonstrated in the DNA-repair protein hHR23a (Walters *et al.*, 2003). Thus, the phenotypes observed in our S3 and S4 lines may have resulted from the effects on these roles of the RPN10 subunit of the overexpression or ectopic expression of the *rpn10* gene.

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