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**Gene Note**

# Isolation of a drought-responsive alkaline α-galactosidase gene from New Zealand spinach

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**Abstract** We isolated a cDNA clone encoding a New Zealand spinach (*Tetragonia tetragonioides*) alkaline α-galactosidase that we designated *TtAGA1*. The clone was found by monitoring an up-regulated transcripts accumulation with the differential display reverse transcription–polymerase chain reaction method during a treatment of New Zealand spinach shoots with drought. Deduced amino acid sequence suggests that the gene belongs to a family of plant-specific alkaline α-galactosidases. When detached shoots of New Zealand spinach were treated with abiotic stresses such as drought, administration of mannitol (150 mM), wounding, salinity (200 mM and 400 mM sodium chloride), dark, and cold (4°C), drought most enhanced the accumulation of the *TtAGA1* transcripts. Treatments with mannitol, wounding, salinity, and dark increased the transcripts accumulation, but cold did not. It is supposed that the degradation of galactosyl saccharides may correlate with drought responses in New Zealand spinach growing in seashore areas.

**Key words:** Alkaline α-galactosidase, drought stress, New Zealand spinach, *Tetragonia tetragonioides*.

Water stresses, such as drought and high salinity, are major factors that decrease plant growth, and physiological and molecular alterations occur in many plants exposed to these stresses. Water-stress-responsive genes and their regulatory networks have been characterized by using Arabidopsis (Shinozaki et al. 2003; Bray 2004). At the same time, stress-tolerant plants such as resurrection plants have been studied, because they show remarkable tolerance for desiccation. Reports about resurrection plants, such as *Craterostigma plantagineum* and *Xerophyta viscosa*, suggest that they have characteristic mechanisms to survive severe desiccation (Vicré et al. 2004). Thus, it is significant to study genes of plants that can grow in environments where the growth of general plants is suppressed. Seashore plants may be useful targets for the study of water-stress-related genes, because these plants are exposed to complicated environmental factors, such as desiccation, high salinity, extreme temperature, intensive daylight, and gales. Although some seashore plants, such as *Bruguiera sexangula* and *Calystegia soldanella*, have been studied (Yamada et al. 2002; Hara et al. 2002), there is limited information published about stress-related genes of seashore plants.

New Zealand spinach (*Tetragonia tetragonioides*, Aizoaceae) is a perennial seashore plant commonly found in Japan (Figure 1A). The plant is widespread in littoral and estuary systems of the Pacific region from Japan and south-east China to South America, Australia, New Zealand, and Pacific islands (Gray 1997). In some regions, New Zealand spinach is used as a leafy vegetable (Haase 1990; Jaworska 2005). Anti-ulcerogenic and anti-inflammatory activities were indicated in compounds isolated from New Zealand spinach (Okuyama and Yamazaki 1983; Kato et al. 1985; Cambie and Ferguson 2003). In the Shizuoka area, Japan, New Zealand spinach grows in sandy fields near the seashore, especially as undergrowth to pine woods or near tetrapods close to the shore. Although it appears that New Zealand spinach can adapt to drought and high salinity, there is little information about the mechanism of its responses to such stresses. Here we report a stress-responsive gene in New Zealand spinach.

First, we searched for drought-stress-responsive genes in New Zealand spinach. In March 2006, shoots of New Zealand spinach were harvested from three different fields, i.e., the Abe River Estuary, the Ohya Seashore, and the Miho Seashore, all of which are in Shizuoka City (Figure 1B). For drought treatments, the shoots were put on dry filter paper and incubated at room temperature (15–25°C) for 48 h. Sampling periods were 0, 24, and 48 h after the treatments. During incubation, water contents of shoots changed from *ca*. 80% (0 h) to *ca*. 45% (48 h). Total RNA (1 μg per sample) extracted from the treated and the non-treated samples with an RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan) was subjected to

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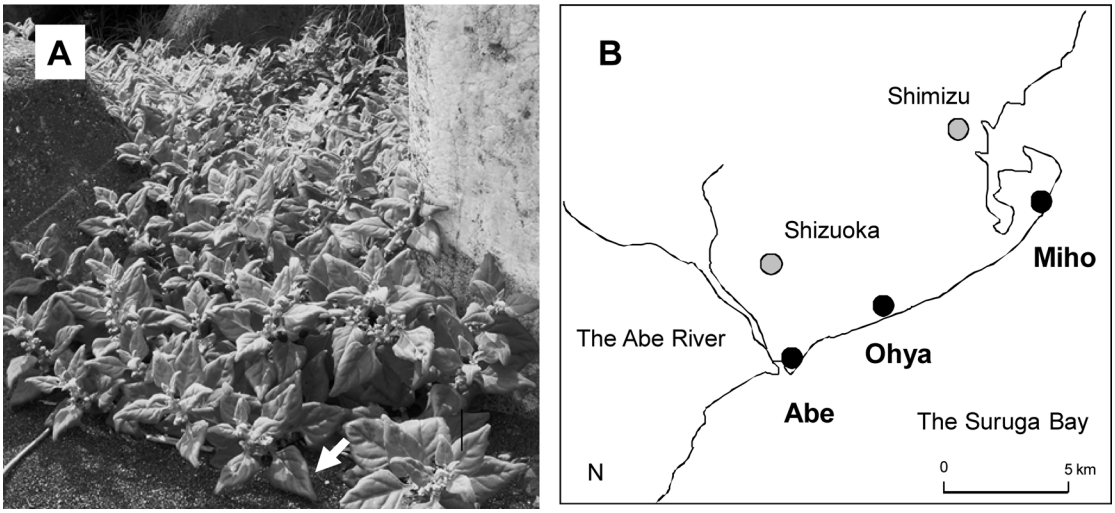


Figure 1. New Zealand spinach in the Shizuoka area, Japan. (A) New Zealand spinach growing at the Abe River Estuary. The length of a true leaf indicated by a white arrow is *ca*. 8 cm. (B) A map showing the Shizuoka area, Japan. Locations of the Abe River Estuary, the Ohya Seashore, and the Miho Seashore are indicated.

mRNA differential display reverse transcription–polymerase chain reaction (DDRT-PCR) according to the methods described previously (Yoshida et al. 1994; Sturtevant 2000). Ten kinds of arbitrary primers (AP-A-01 to AP-A-10, mRNA Fingerprinting Kit, Nippongene, Tokyo, Japan) and three kinds of 3′-anchor primers (3′-AnchorPrimer-1 to 3′-AnchorPrimer-3, Nippongene) were used. RT-PCR was performed with an RNA PCR Kit Ver. 3.0 (Takara, Tokyo, Japan). The annealing temperature of the PCR (28 cycles) was 40°C. The DDRT-PCRs with thirty primer combinations provided *ca*. 200 fingerprints per sample.

After checking the fingerprints, one band, whose intensity was enhanced by the drought treatment in all samples harvested at the three different fields, was obtained. The interesting band (*ca*. 1500 bp) formed when AP-A-02 and 3′-AnchorPrimer-1 were used for the DDRT-PCR. The PCR product was cloned into the pDrive Cloning vector of a PCR Cloning Kit (Qiagen). BLAST analysis showed that a partial sequence of the PCR product might be a portion of an open reading frame of galactosidase. To obtain an entire sequence of the cDNA, we performed rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA Amplification Kit (Takara). The full-length cDNA was amplified from a total RNA of the plant sampled at the Abe River Estuary. The length of the cDNA was 2630 bp (Accession number: AB434769). The open reading frame (2301 bp) encoded a polypeptide consisting of 767 amino acids. Since a BLAST search showed that the clone was similar to plant alkaline α-galactosidases (AGAs) that were formerly called seed imbibition proteins, we designated the clone *Tetragonia tetragonioides* alkaline α-galactosidase 1 (*TtAGA1*).

To compare the protein sequences of the alkaline α-galactosidases, raffinose family oligosaccharide (RFO) synthases, and acid α-galactosidases, we produced an unrooted phylogenetic tree using ClustalW (the DDBJ homepage) with a Phylodendron application (http://iubio.bio.indiana.edu/treeapp/treeprint-sample2.html). A phylogenetic tree based on amino acid sequences indicated that TtAGA1 belongs in the cluster where alkaline α-galactosidases are classified (Figure 2, number 6). Since *Cucumis melo* Aga1 (Figure 2, number

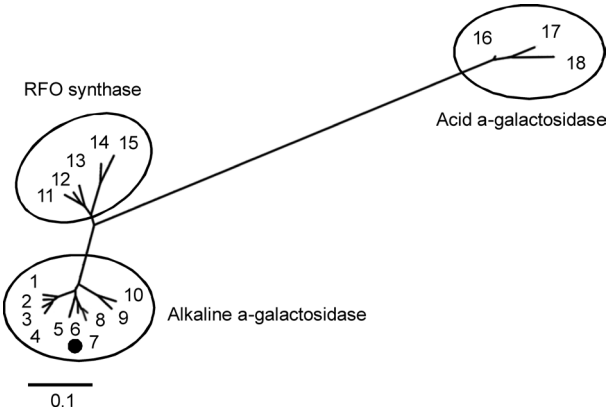


Figure 2. Phylogenetic tree based on deduced amino acid sequences showing relationships between alkaline α-galactosidases, raffinose family oligosaccharide (RFO) synthases, and acid α-galactosidases. Accession numbers or locus codes for the clones are as follows: (1) *Oryza sativa* AAL65392 (*Osh69*), (2) *Cucumis melo* AAM75139, (3) *Lycopersicon esculentum* AAN32954 (*Aga*), (4) *Arabidopsis thaliana* At1g55740, (5) *Zea mays* AAQ07251 (*ZmAGA1*), (6) *Tetragonia tetragonioides* AB434769 (*TtAGA1*, present study, closed circle), (7) *Pisum sativum* EF433424 (*PsAGA1*), (8) *C. melo* AAM75140 (*Aga2*), (9) *Z. mays* AAQ07252, (10) *A. thaliana* At5g20250, (11) *C. sativus* AAD02832, (12) *A. thaliana* At5g40390, (13) *P. sativum* CAD20127, (14) *C. sativus* ABV44498, (15) *A. thaliana* At4g01970, (16) *C. sativus* ABC55266, (17) *P. sativum* CAF34023, and (18) *L. esculentum* AAF04591. The unrooted phylogenetic tree was produced by ClustalW and Phylodendron software.

2), *Cucumis melo* Aga2 (number 8), and *Lycopersicon esculentum* Aga (number 3) were demonstrated to show alkaline $\alpha$-galactosidase activity (Carmi et al. 2003), we suspect that TtAGA1 may have the same activity.

To investigate the alteration of *TtAGA1* expression by abiotic stresses, we analyzed the effects of stresses such as drought, administration of mannitol (150 mM), wounding, administration of sodium chloride (200 mM and 400 mM), dark, and cold (4°C) on transcripts accumulation of *TtAGA1*. For our analysis, shoots of New Zealand spinach (length: *ca*. 15 cm from the apex) with eight true leaves were harvested at the Miho Seashore in October 2007, brought to the laboratory, and pre-incubated at 18°C for 24 h under continuous light and with a water supply. Then, the shoots were cut at the position ca. 3 cm from the apex. Each detached shoot containing an apex and three true leaves was immediately subjected to the stress treatment. Administration of mannitol (150 mM) or sodium chloride (200 mM or 400 mM) was performed by setting the shoots in plastic cups (diameter: 5.5 cm, height: 8 cm) containing the corresponding solution. The cups were incubated at 18°C in the light. For treatments of wounding, dark, and cold, the shoots were put in cups with deionized water. Wounds were applied by rubbing whole true leaves with a spatula. The cups were incubated at 18°C in the dark (dark treatment). Cold treatment was done at 6°C in the dark. For exposure to drought, the shoots were put in empty cups and kept at 18°C in the light. Control shoots were put in cups containing deionized water and kept at 18°C in the light. The shoots were incubated for 48 h. Sampling periods were 0, 12, 24, and 48 h after the treatments.

The transcripts level was quantified by semi-quantitative RT-PCR (Goidin et al. 2001) using the QuantumRNA 18S Internal Standards Kit (Ambion, TX, USA). In this assay, the target RNA can be quantified as a relative amount using endogenous rRNA as an internal standard. After RT reaction, PCR proceeded through 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s. Specific primers for the *TtAGA1* were 5′-ATATTGACCCCAGGCTCA-3′ (from position 123 to position 140 of the cDNA clone) and 5′-GACAAGCTTCTGAGGCCT-3′ (from position 695 to position 712 of the cDNA clone). The ratio of 18S primer to competimer was 2 : 8. Amounts of PCR products at 23, 25, 27, and 30 cycles were monitored by 1.5% agarose gel electrophoresis with ethidium bromide staining. The PCR products for the target and rRNA were *ca*. 600 bp and *ca*. 300 bp long, respectively. After determining band intensity using NIH-Image software, we deduced the relative mRNA contents from the intensities of the target PCR products and the rRNA PCR products according to the instruction manual for the QuantumRNA Kit.

Effects of stresses on the transcripts accumulations of *TtAGA1* are show in Figure 3. During the incubation for 48 h, the relative transcripts accumulation of *TtAGA1* did

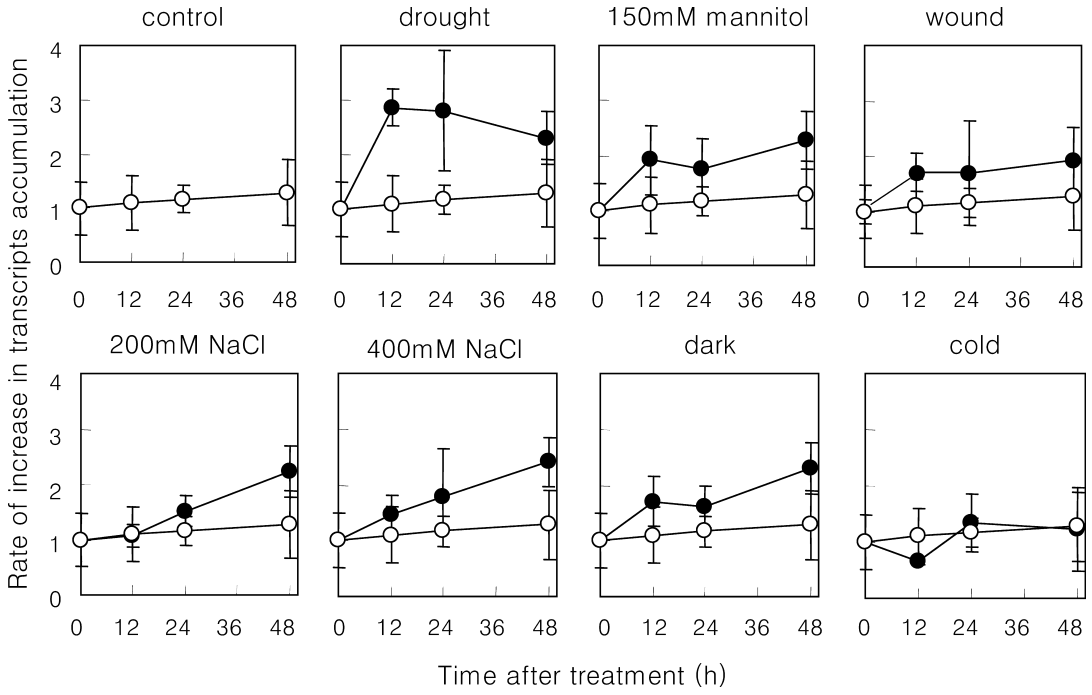


Figure 3. Effects of abiotic stresses on *TtAGA1* transcripts accumulation in New Zealand spinach shoots. The magnitude of transcripts accumulation was quantified by semiquantitative RT-PCR as a relative amount using endogenous rRNA as an internal standard. The mean value of the zero time samples (n=4) in the control is standardized. Values are shown as rates of increase. Open circles: control; closed circles: stress treatment. Values and bars represent means±S.D. of 4 samples.

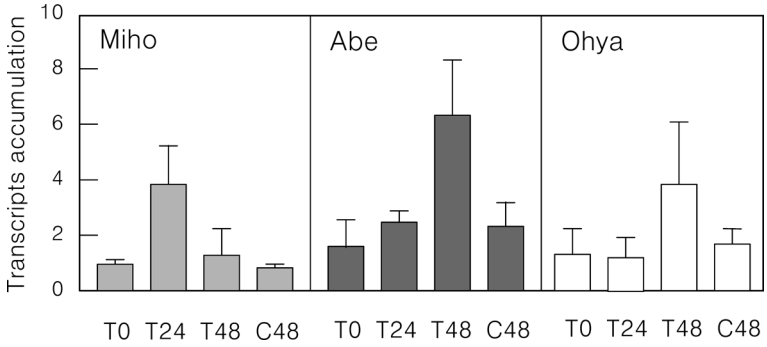


Figure 4. Effects of drought stress on *TtAGA1* transcripts accumulation in New Zealand spinach shoots. Plant samples were harvested at the Miho Seashore, the Abe River Estuary, and the Ohya Seashore. The magnitude of transcripts accumulation was quantified by semiquantitative RT-PCR as a relative amount using endogenous rRNA as an internal standard. The mean value of a sample "T0" in Miho (n=3) is standardized. T0: zero time control; T24: drought treatment for 24 h; T48: drought treatment for 48 h; C48: control for 48 h. Values and bars represent means±S.D. of 3 samples.

not change in the controls. Drought apparently stimulated the transcripts accumulation after 12 h of the treatment. Mannitol, wounding, sodium chloride, and dark enhanced the transcripts accumulation, but the enhancement degrees were lower than that under drought treatment. The level of transcripts accumulation was constant under the cold treatment during the period tested. These results suggest that the *TtAGA1* gene expression is affected by various abiotic stresses and that drought is an especially effective enhancer for gene expression. Further measurements of the transcripts accumulation indicated that the drought-responsive *TtAGA1* expression was observed not only in the Miho Seashore-derived New Zealand spinach but also the Abe River Estuary- and the Ohya Seashore-derived plants (Figure 4). In addition, the accumulation magnitude of *TtAGA1* transcripts reached maximum at 24 h after the start of the drought treatment in the Miho Seashore-derived plants, but the peak of accumulation occurred at 48 h in the Abe River Estuary- and the Ohya Seashore-derived plants. The Miho Seashore-derived plants were undergrowth in the pine woods adjacent to the sandy area of the seashore. On the other hand, both the Abe River Estuary- and the Ohya Seashore-derived plants grew in colonies beside tetrapods isolated in the sandy areas of the respective seashores. These different growth conditions may influence the sensitivity of the drought-responsive *TtAGA1* expression.

The stress responses in the expression of alkaline α-galactosidase genes have been reported in monocots. The expression of *ZmAGA1* (Figure 2, number 5) in maize seedlings was enhanced by cold and drought, but not by sodium chloride (Zhao et al. 2006). Rice *Osh69* (Figure 2, number 1) expression was up-regulated by dark and wounding (Lee et al. 2004). The mode of stress responses in *TtAGA1* was similar to the expression patterns of alkaline α-galactosidase genes in maize and rice, except that the *TtAGA1* expression was enhanced by high salinity. This means that both monocots and dicots have stress-responsive capability in the alkaline α-galactosidase genes.

The significance of stress response in plant alkaline α-galactosidase genes is not well understood. The rice *Osh69* is proposed to have a role in the chloroplast galactolipids degradation during leaf senescence. New Zealand spinach shoots, however, maintained their green color even when they were exposed to drought for 3 days, suggesting that the drought-responsive *TtAGA1* gene expression may not be correlated to the senescence process. Pea *PsAGA1* (Figure 2, number 7) is highly expressed in germinating seeds for breakdown of RFOs, which are storage carbohydrates in the seed. In addition, a maize *ZmAGA1* transcript accumulated when seed germination was interrupted by abiotic stresses. It is likely that the germinating seeds may need to degrade more RFOs to enhance carbohydrates utility under stresses. In this context, TtAGA1 may degrade galactosyl saccharides when growing conditions change. Since neither raffinose nor stachyose was detected in the non-stressed New Zealand spinach shoots (data not shown), other kinds of galactosyl saccharides may be utilized for substrates in the plant. Breakdown of galactosyl saccharides may be related to stress responses in New Zealand spinach that enable the plants to survive at the seashore. Further studies on the substrates of TtAGA1 in New Zealand spinach will proceed.

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