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Enhanced Germination under High-Salt Conditions of Seeds of Transgenic *Arabidopsis* with a Bacterial Gene (*codA*) for Choline Oxidase*

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Arabidopsis thaliana **was transformed previously with the *codA* gene from the soil bacterium *Arthrobacter globiformis*. This gene encodes choline oxidase, the enzyme that converts choline to glycinebetaine. Transformation with the *codA* gene significantly enhanced the tolerance of transgenic plants to low temperature and high-salt stress. We report here that seeds of transgenic plants that expressed the *codA* gene were also more tolerant to salt stress during germination than seeds of non-transformed wild-type plants. Seedlings of transgenic plants grew more rapidly than those of wild-type plants under salt-stress conditions. Furthermore, exogenously applied glycinebetaine was effective in alleviating the harmful effects of salt stress during germination of seeds and growth of young seedlings, a result that suggests that it was glycinebetaine that had enhanced the tolerance of the transgenic plants. These observations indicate that synthesis of glycinebetaine in transgenic plants *in vivo*, as a result of the expression of the *codA* gene, might be very useful in improving the ability of crop plants to tolerate salt stress.**

Key words: *Arabidopsis* — Choline oxidase — Germination — Glycinebetaine — Salt tolerance

Salinity is one of the major environmental constraints that limit the growth and productivity of plants (Boyer 1982, Epstein *et al.* 1980). Salinity caused, for example, by the accumulation of salt in fields as a consequence of repeated irrigation with salt-containing water, is a serious agricultural problem in many parts of the world (McKersie and Leshem 1994). The development of crops with enhanced tolerance to salt has been one of the major goals in agriculture throughout the world. There are some examples of successful development of salt-tolerant crops by traditional breeding methods. However, recent advances in genetic engineering have produced particularly remarkable results (Murata *et al.* 1992a, Kavi Kishor *et al.* 1995, Tarczynski *et al.* 1993, Xu *et al.* 1996). The application of the genetic engineering of plants to improvements in tolerance to salt is critical if we are to ensure a stable supply of agricultural products from the limited arable land on the earth's surface. Identification of genes that control the tolerance of plants to salt represents an important challenge to plant molecular biologists.

Glycinebetaine (hereafter betaine) is one of the so-called compatible solutes that is widely distributed in higher plants (Robinson and Jones 1986), animals (Garcia-Perez and Burg 1991, Lever *et al.* 1994) and bacteria (Csonka 1989). Some higher plants accumulate betaine in response to both salt stress and cold stress (Wyn Jones and Storey 1981). Several taxonomically distant plants have been identified as accumulators of betaine, whereas others, such as *Arabidopsis*, potato and tomato, do not accumulate betaine (Wyn Jones and Storey 1981).

It is generally accepted that betaine protects cells from salt stress by maintaining osmotic balance with the environment (Robinson and Jones 1986) and by stabilizing the quaternary structures of complex proteins (Bernard *et al.* 1988, Incharoenskdi *et al.* 1986, Papageorgiou and Murata 1995, Santoro *et al.* 1992, Winzor *et al.* 1992). In photosynthetic systems, betaine stabilizes both the oxygen-evolving photosystem II complex (Papageorgiou and Murata 1995, Murata *et al.* 1992b, Papageorgiou *et al.* 1991) and the enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (Incharoensakdi *et al.* 1986) at high concentrations of NaCl. The protective effects of betaine at low temperature have been demonstrated in bacteria, as well as in higher plants (Coughlan and Heber 1982, Kishitani *et al.* 1994, Ko *et al.* 1994, Nomura *et al.* 1995). Exogenously applied betaine increases the tolerance of plants to various stresses in a controlled environment, as well as under field conditions (Makela *et al.* 1996), with varying degrees of effectiveness.

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We have cloned the *codA* gene for choline oxidase from the soil bacterium *Arthrobacter globiformis* (Deshnium *et al.* 1995). Under high-salt conditions, cells of this bacterium are capable of synthesizing choline oxidase (choline: oxygen 1-oxidoreductase; E.C. 1.1.3.17), which is the enzyme that catalyzes the conversion of choline to betaine, and of accumulating a high level of betaine (Ikuta *et al.* 1977). We transformed the cyanobacterium *Synechococcus* sp. PCC 7942 with this gene. The transformed cells of the cyanobacterium acquired the ability to convert choline to betaine and accumulated betaine at levels as high as 60-80 mM. Moreover, these transformed cells were tolerant both to salt stress and to low-temperature stress (Deshnium *et al.* 1995, Deshnium *et al.* 1997). Recently, we introduced the ability to synthesize betaine into *Arabidopsis thaliana*, a plant that normally does not accumulate betaine, by transforming it with the *codA* gene. The transformed plants synthesized active choline oxidase, accumulated high levels of betaine and had a significantly elevated capacity to tolerate stress (Hayashi *et al.* 1997, Alia *et al.* 1998).

In the present study, we examined the effects of betaine that had accumulated *in vivo* and that had been applied exogenously on the tolerance to salt stress of wild-type and transformed *A. thaliana* during germination of seeds, as well as during the growth of young seedlings. Our results clearly demonstrate that transformation with the *codA* gene is an effective approach for improving the salt tolerance of a higher plant.

Materials and Methods

Seeds from three independent lines of transformed *Arabidopsis thaliana* that harbored the *codA* gene were obtained as described previously (Hayashi *et al.* 1997). The standard procedure for germination of seeds was as follows. Seeds were surface-sterilized in a solution of sodium hypochlorate (20%, w/v) that contained Triton X-100 (0.02%, w/v) for 5 min at 22 C. After washing with sterile water, seeds were placed in petri dishes that contained MS medium (Murashige and Skoog 1962) supplemented with various amounts of NaCl to give final concentrations from 0 to 400 mM and solidified with 0.5% Gelan gum (Wako Pure Chemical Industries, Osaka, Japan). The plates were incubated at 4 C for 48 hr in darkness for synchronization of germination. Then they were incubated at 22 C in growth chambers, with daily illumination for 16 hr at 70 μmol photon m^{-2} sec^{-1} and a dark period of 8 hr.

Levels of betaine in leaves and seeds were determined by NMR spectrometric analysis of quaternary ammonium compounds (Robinson and Jones 1986). 100 mg of dry seeds or about 300 mg of dried leaves (after incubation in an oven at 80 C for 72 hr) were powdered in a ceramic mortar in liquid nitrogen. The powder was suspended in 25 ml of 1.0 M H_2SO_4 and incubated at 25 C for 2 hr. Cell debris was removed by centrifugation at 3,000×*g* for 10 min, and betaine was recovered from the supernatant by the periodide precipitation method (Wall *et al.* 1960). The resultant periodide adduct of betaine was collected by centrifugation at 1,000×*g* for 30 min and dissolved in 0.5 ml of CD_3OD (Wako Pure Chemical Industries), which contained 0.5 mM of 2-methyl-2-propanol (Wako Pure Chemical Industries) as an internal standard. The ^{1}H-NMR spectrum was recorded at 25 C with an NMR spectrometer (AMX 360 Wb; Bruker, Karlsruhe, Germany) with a pulse time of 5.0 msec and an acquisition time of approximately 4 sec. A dominant peak assignable to betaine was detected, and integrated intensities of peaks were used to quantify concentrations of betaine.

To determine the effects of salt stress on seedling growth, five seeds from wild-type plants and five T3 seeds from each of three independent transgenic lines were allowed to germinate on plates of MS medium that had been prepared with various concentrations of NaCl and solidified with Gellan gum. After incubation at 0 C for 2 days, the plates were incubated at 22 C under 16 hr of light (75 μmol m^{-2} sec^{-1}) and 8 hr of darkness daily for completion of germination and growth of seedlings.

Results and Discussion

Accumulation of betaine in seeds and leaves of transgenic Arabidopsis

Figure 1 shows the levels of betaine in seeds of the three transgenic lines. On the basis of dry weight, levels of betaine in the leaves were about the same as those in the seeds of the three transgenic lines. There was no direct correlation between the levels of betaine in the leaf tissues and the seeds in the individual lines of transgenic plants (Fig. 1). Differences in levels of expression of the *codA* gene or in the stability of choline oxidase in the different tissues might explain differences in levels of betaine in leaves and seeds among the individual transgenic lines.

Effects of salt stress on the germination of seeds

To evaluate the effects of various concentrations of NaCl on the germination of seeds of the wild type and the three independent lines of transgenic *Arabidopsis*, seeds were

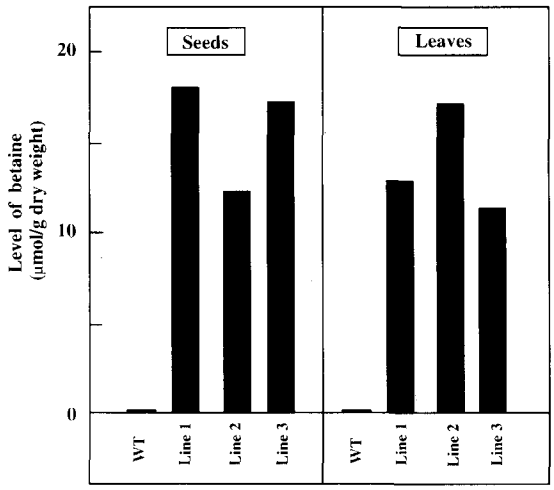

Fig. 1. Levels of betaine in wild-type seeds and leaves and in seeds and leaves of three transgenic lines of *Arabidopsis*.

surface-sterilized and transferred to medium that contained 0, 100, 200, or 300 mM NaCl. In the absence of NaCl, wild-type seeds and seeds from the three independent lines of transgenic plants germinated rapidly. Thus, the expression of the *codA* gene appeared to have no detrimental effects on germination. The rate of germination within two days was 100% in all lines tested (Fig. 2).

The germination of seeds of wild-type plants was severely depressed under high-salt conditions. There was a delay in germination in 100 and 200 mM NaCl, and germination was completely inhibited in 300 mM NaCl (Fig. 2). By contrast, the seeds of transgenic lines gave much higher rates of germination. At 200 and 300 mM NaCl, seeds of line 1 plants germinated faster and at a higher rate than the wild-type seeds. Furthermore, there were differences among the seeds of the three transgenic lines tested. The order of tolerance to salt was line 1 > line 3 > line 2, and it reflected the levels of betaine in the seeds.

Effects of exogenous betaine on the germination of seeds during salt stress

If the accumulation of betaine in the seeds of transgenic plants was responsible for their accelerated and enhanced germination under salt-stress conditions, we would expect that betaine, when applied exogenously, would also enhance the tolerance of seeds to NaCl. We added betaine at 5 mM to germination medium that contained 0, 100 or 200 mM NaCl. Wild-type seeds and seeds of line 1 transgenic plants were sown onto the various media after surface sterilization as described above. As shown in Fig. 3 when the medium did not contain betaine, seeds of line 1 plants germinated and seedlings grew well in 0 and in 100 mM NaCl. In 200 mM NaCl, germination still occurred, but the growth of seedlings was significantly reduced. Wild-type seeds germinated and wild-type seedlings grew well in NaCl-free medium, as did the seeds from the transgenic plants. In 100 mM NaCl, all wild-type seeds germinated but growth of seedlings was markedly depressed. In 200 mM NaCl, some seeds were able to germinate but no seedlings were able to grow.

Figure 3 shows that exogenously applied betaine stimulated the germination of seeds and the growth of seedlings of both types of plant. However, the effects of betaine were more dramatic in the wild-type plants than in the transgenic plants. In NaCl-free medium, exogenous betaine had no significant effects. In the presence of 100 mM NaCl, exogenous betaine markedly stimulated the growth of the wild-type seedlings, whereas the germination and growth of transformed plants were hardly affected. In 200 mM NaCl, the germination of wild-type seeds and the growth of wild-type seedlings were significantly enhanced by betaine at 5 mM. At the same concentration of NaCl, the growth of transgenic seedlings was also enhanced by betaine at 5 mM. These observations indicate that exogenous betaine effec-

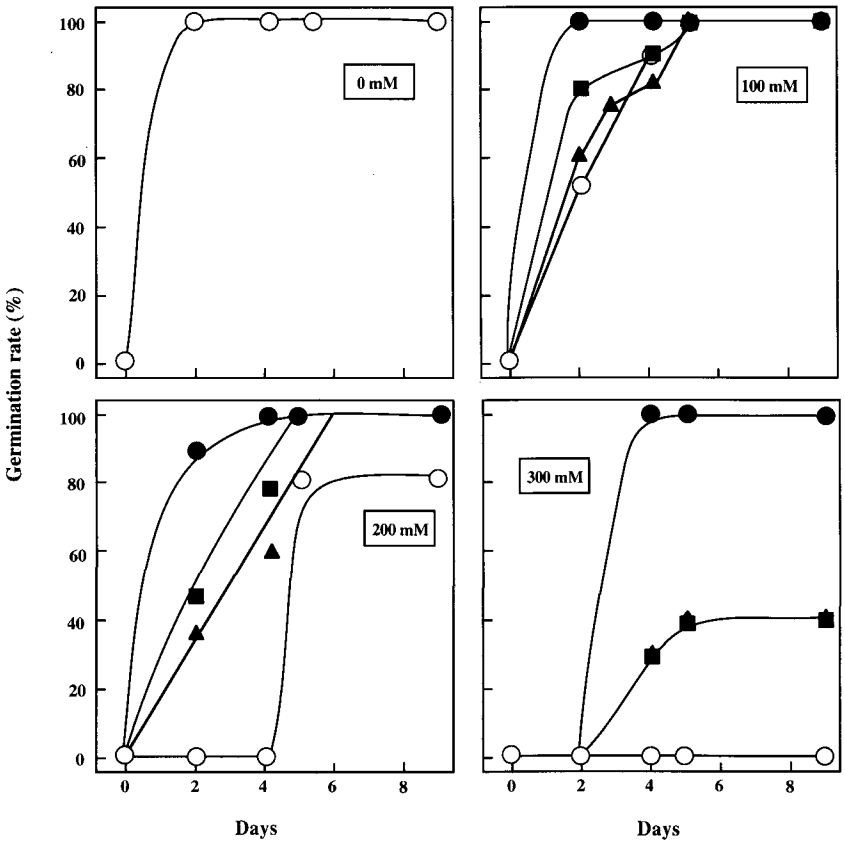

Fig. 2. Effects of salt stress on germination of wild-type seeds and of seeds from transgenic *Arabidopsis*. ○—○, Wild type; ●—●, line 1; ▲—▲, line 2; ■—■, line 3. Concentrations of NaCl are given in boxes.

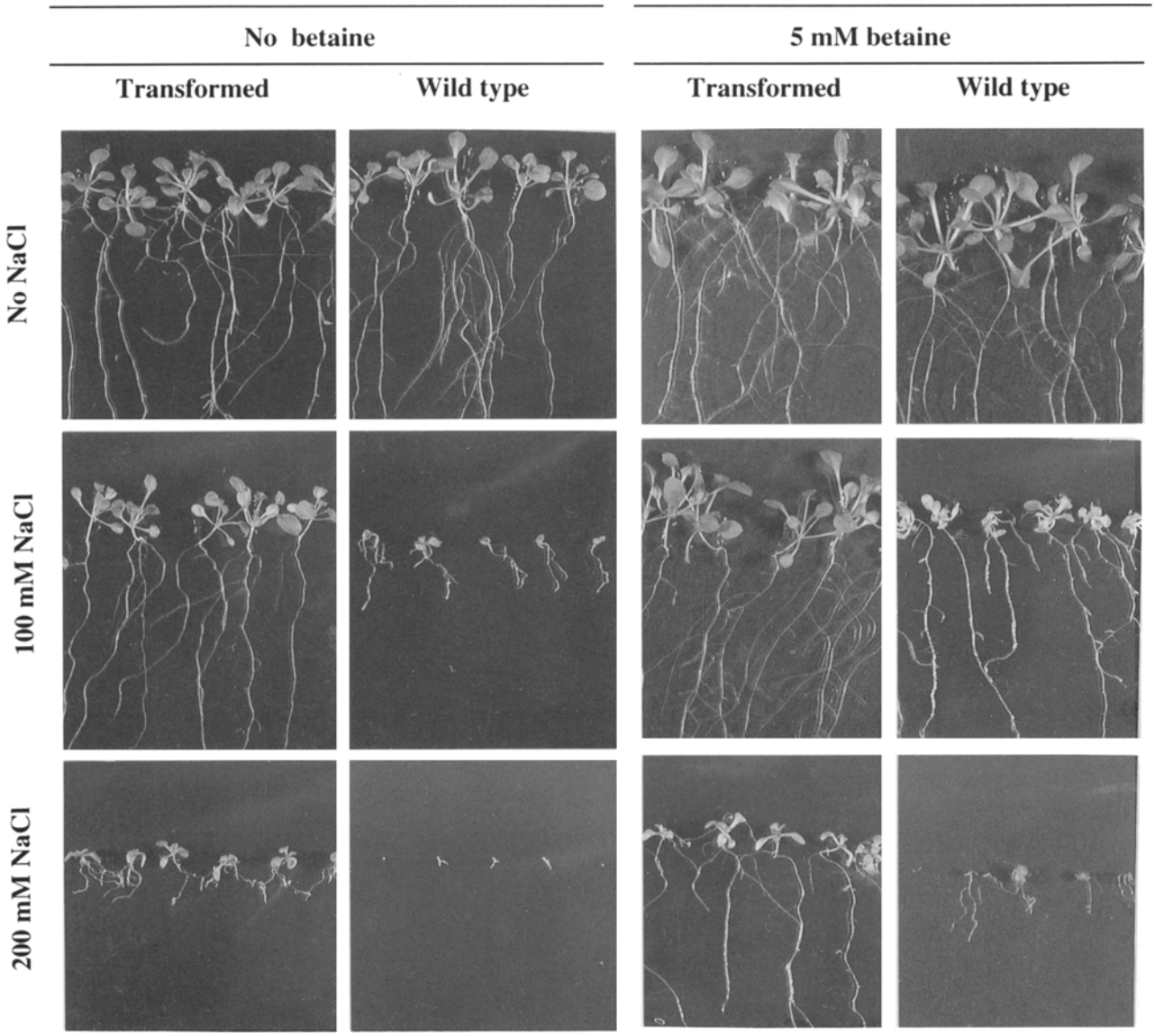

Fig. 3. Effects of exogenously applied betaine on the germination of seeds and on the growth of young seedlings of wild-type and line-1 transgenic plants under salt-stress conditions. Seeds were inoculated on solid medium that contained 0, 100, or 200 mM NaCl in the presence of 5 mM betaine or in its absence. Seeds and seedlings were allowed to germinate and grow, respectively, at 22 C for 7 days with 16 hr of illumination daily.

tively improved the germination of seeds and the growth of seedlings of both wild-type and transgenic plants under saline conditions. These results also suggest that the synthesis of betaine, catalyzed by choline oxidase, in the transgenic plants might have been the primary reason for the enhanced tolerance of these plants.

The accumulation of betaine was effective in enhancing the tolerance to salt from a very early stage in the life cycle of *Arabidopsis*, namely, from the germination stage to the seedling stage. Since most reports on improvements in the tolerance of transgenic plants to salt stress have dealt with the seedling stage, our finding that the *codA* gene can improve the tolerance to salt stress of transgenic plants from the early to the late phases of plant development is significant.

The information reported here and in the literature (Hayashi *et al.* 1997, Alia *et al.* 1998) allows two conclusions to be drawn, as follows. (1) The accumulation of betaine *in vivo*, as a consequence of the expression of the *codA* gene, can effectively protect plants from salt stress. (2) The tolerance to salt stress conferred by the *codA* gene extends over various stages of a plant's life cycle, from the germination of seeds to the growth of the mature plant. The use of the *codA* gene to improve the tolerance to salt of major food crops is currently being investigated in our laboratory.

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