Accumulation of Glycinebetaine in Rice Plants that Overexpress Choline Monooxygenase from Spinach and Evaluation of their Tolerance to Abiotic Stress

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INTRODUCTION

Rice (Oryza sativa) is one of the most important cereals in the world and is a popular model plant for studies of monocots. Improvements in the tolerance of cereal plants to abiotic stress are important if the efficiency of food production is to be increased. Furthermore, information on the modification of rice plants would be applicable to other cereal crops, such as wheat (Triticum aestivum), barley (Hordeum vulgare) and maize (Zea mays), and the converse would also be true. Many loci for genes that control tolerance to abiotic stress in plants have been identified by genetic analysis (e.g. Lanceras et al., 2004; Saito et al., 2004). However, many genes that control agronomically important traits remain to be identified and modified to generate new varieties with desirable traits. There is evidence that transgenic plants in which the expression of a single gene has been modified have enhanced tolerance to abiotic stress (Bajaj and Mohanty, 2005). Ideally, modification of a single gene should confer tolerance to more than one form of abiotic stress. Alternation in the pattern of expression of the gene for DREB1A, a transcription factor, improves the tolerance to drought, salt and freezing of Arabidopsis thaliana (Kasuga et al., 1999). Introduction of genes that are involved in the synthesis of compatible solutes, such as betaines, polyols and proline, into plants also contributes to tolerance to multiple forms of abiotic stress (Rathinasabapathi, 2000; Chen and Murata, 2002).

Glycinebetaine (GB), a quaternary ammonium compound, is a very effective compatible solute (Rathinasabapathi, 2000; Chen and Murata, 2002) and is found in a wide range of foods (de Zwart et al., 2003). In plants that synthesize GB, which are known as GB-accumulators, e.g. spinach (Spinacia oleracea), maize and barley, this compatible solute accumulates in leaves in response to a water deficit and salt stress, as well as during acclimation to cold (McCue and Hanson, 1990; Rhodes and Hanson, 1993; Kishitani et al., 1994). Moreover, GB has been shown in vitro to stabilize membranes of the oxygen-evolving photosystem II complex (Murata et al., 1992; Papageorgiou and Murata, 1995). GB also stabilizes the activity of ribulose 1,5-bisphosphate carboxylase/oxygenase in a transgenic cyanobacterium in vivo (Nomura et al., 1998). In higher plants, GB is synthesized from choline (Cho) via betaine aldehyde (BA). The first and second steps in the biosynthesis of GB are catalysed by choline monooxygenase (CMO) and betaine aldehyde dehydrogenase (BADH), respectively (Rhodes and Hanson, 1993). In Arthrobacter globiformis, choline oxidase (COD), encoded by codA, catalyses the conversion of Cho to GB in a single step (Ikuta et al., 1977). Choline dehydrogenase (CDH) and BADH, encoded by both betA and betB,
catalyse the conversion of Cho to GB, via BA, in *Escherichia coli* (Landfald and Strom, 1986). Yet another pathway, namely, the three-step methylation of glycine, for the biosynthesis of GB is catalysed by glycine sarcosine methyltransferase and sarcosine dimethylglycine methyltransferase in *Aphanothece halophytica* and *Ectothiorhodospora halochloris* (Nyyssola et al., 2000).

Rice is the only important cereal crop that does not accumulate GB. The rice genome includes a non-functional gene for CMO (P0545E05-33 on chromosome 6), which is probably a pseudo-gene, as well as two copies of a gene for BADH (OSJNBa0060P14-8 on chromosome 4 and P0456B03-101 on chromosome 8), both of which encode a signal sequence for targeting of the gene product to peroxisomes (International Rice Genome Sequencing Project, 2005). Transgenic rice plants that express COD accumulate GB and exhibit enhanced tolerance to salt and/or cold stress (Sakamoto et al., 1998; Mohanty et al., 2002), even though the concentrations at which GB accumulates are lower than those in stressed GB-accumulating plants, such as maize (Yang et al., 1995). Rice transformants that overexpress barley BADH and even wild-type rice plants have been shown to accumulate a considerable amount of GB, as compared with rice plants that express COD, when they are supplied with exogenous BA, and such plants develop significant tolerance to salt, cold and heat stress (Kishitani et al., 2000). Enhancement of the synthesis of GB improves drought and chilling tolerance in maize, a crop plant that naturally accumulates GB (Quan et al., 2004a, b). Therefore, an attempt was made to enhance the accumulation of GB in rice by introducing the gene for the enzyme that catalyses the first step in the synthesis of GB. A gene for CMO from spinach was the first such gene to be isolated from a higher plant (Rathinasabapathi et al., 1997), and it has been expressed in tobacco and *Arabidopsis*, neither of which normally accumulates GB (Nuccio et al., 1998, Hibino et al., 2002). As far as is known, however, there have been no reports of rice plants that express CMO. In the present study, the effects of endogenously accumulated GB were evaluated in transgenic rice plants that expressed a gene for CMO from spinach and the tolerance to temperature stress and salt stress of transgenic seedlings and the productivity of mature transgenic plants was investigated.

**MATERIALS AND METHODS**

**Transgenic plant materials**

For the construction of an expression vector for spinach CMO, a DNA fragment was isolated from the plasmid pPCMO (Hibino et al., 2002) that encoded CMO and signal peptides for targeting of proteins to chloroplasts (Rathinasabapathi et al., 1997) using *SacI*. This fragment was ligated into the corresponding site of the binary vector pBI101 (Ariizumi et al., 2002), which was constructed for overexpression of individual genes under the control of the promoter of a gene for ubiquitin from maize (Cornejo et al., 1993). This resultant construct was named

**Ubi::CMO** (Fig. 1A). This plasmid was introduced into the *japonica* rice cultivar ‘Sasanishiki’ by *Agrobacterium*-mediated transformation, as described previously (Yokoi et al., 1997). Hygromycin-resistant plants were selected on MS medium (Murashige and Skoog, 1962) that contained 50 mg l⁻¹ hygromycin and examined the synthesis of CMO in the resultant transformants by western blotting analysis.

**Western blotting analysis**

Young leaves of rice plants and of spinach, as a control, were ground in liquid nitrogen and extracted in 150 ml of...
soluble-protein buffer [100 mm Tris–HCl (pH 8.0), 1 mm EDTA and 2 mm DTT]. After centrifugation of these extracts (600 g, 10 min, 4°C), supernatants were subjected to western blotting analysis. Sixty micrograms of protein per extract, as estimated by Bradford’s assay (with a kit from BIO-RAD, USA) were fractionated on SDS–polyacrylamide gel (10% polyacrylamide), and bands of protein were electrophroded onto a polyvinylidene difluoride membrane. As the primary antibody, a CMO-specific antibody raised against CMO from spinach in rabbit was used (Hibino et al., 2002) at a dilution of 1:2000. Immunoreactive proteins were detected as previously described by Okada et al. (2003).

Southern blotting analysis

A digoxigenin-labelled probe was prepared by PCR with the binary vector Ubi::CMO and the primer pair Sp PCM-O-F and Sp PCM-O-R described by Hibino et al. (2002), using the PCR DIG-Labeling Mix from Roche Diagnostics (Switzerland). The thermal-cycling conditions were as follows: 1-min denaturation at 94°C; 30 cycles of 1-min denaturation at 94°C, 1-min annealing at 58°C, 1-min extension at 72°C and a final 3-min extension at 72°C. Genomic DNA was isolated with a DNeasy Plant Mini Kit (QIAGEN, Germany) from young leaves. Genomic DNA (2 μg) was digested with HindIII (TAKARA BIO, Japan) and subjected to electrophoresis on 1% agarose gel. Southern blotting analysis was performed as described previously by Shirasawa et al. (2004).

Quantification of glycinebetaine and choline

Levels of GB and Cho in the leaves were quantified as described previously by Arakawa et al. (1990) with minor modifications. Quaternary ammonium compounds were precipitated overnight as periodides (Wall et al., 1960) and analysed by 1H-NMR spectroscopy (Jones et al., 1986) in a Fourier-transform NMR spectrometer (JMN-600; JEOL, Japan). Cho was applied to T1 plants that were growing on MS-medium (Murashige and Skoog, 1962) by adding 5 mm choline chloride to the growth medium.

Examination of stress tolerance

Ten T2 plants per line were used for each stress test. All tests were simultaneously repeated three times. Plants were cultured in 750-mL containers (50 cm2 x 15 cm) filled with synthetic soil for rice seedlings (Kumiai Gousei Baido 3; Sanken Soil, Japan) in a greenhouse at 23/18°C (day/night) under natural light for 4 weeks as the control conditions. Since extreme differences in temperature between daytime and night-time are critically stressful to plants and sometimes occur under natural conditions, groups of plants were grown at 28/13°C for 5 weeks to subject them to temperature stress. For the salt-stress test, plants were treated by adding 25 mL of 100 mm NaCl daily to the soil after growth for 10 d at 23/18°C. Then 25 mL of 150 mm NaCl were added daily for 5 d and finally 50 mL of water were added daily for 3 d. After each treatment, plants were dried at 75°C for 2 d. The resultant dry weights were evaluated as a measure of stress tolerance. An allocation index (%) was calculated for each plant, as follows: [dry weight of shoot (mg)/{(dry weight of shoot (mg) + dry weight of root (mg))} x 100. Four plants per line were grown to maturity in 1/5000-a pots in a greenhouse for examination of plant height, sink and source size, panicle number and 1000-grain weight.

RESULTS

Characterization of transgenic rice plants

Eighty-six independent transgenic rice plants were generated by Agrobacterium-mediated transformation. In western blotting analysis of extracts of leaves, 12 of the 86 T0 transformants that expressed greater amounts of CMO than that of spinach (Fig. 1B) were selected. Four transformants, each of which carried a single transgene by Southern blotting analysis, were selected, and the T1 progeny of three (CMO80, CMO98 and CMO100) of the four lines chosen. The T1 plants of the three lines were tested for resistance to hygromycin. Each T1 line (CMO80-3, CMO98-102 and CMO100–102) that was selected for further analysis was homozygous for Ubi::CMO (all 20 tested progeny survived on MS medium that contained 50 mg L−1 hygromycin). A T1 plant derived from a CMO80 T0 plant that lacked the transgene as the control plant, namely, -CMO was used. The appropriate presence or absence of the transgene and its expression was confirmed by Southern and western blotting analysis, respectively (Fig. 1C) and GB and Cho were quantified in leaves of three T2 plants per line. The mean concentration of GB was 0.42, 0.29 and 0.43 μmol g−1 d. wt in leaves from the CMO80-3, CMO98-102 and CMO100–102 plants, respectively, and no GB was found in wild-type and -CMO plants (Fig. 1D). The concentrations of GB were determined after supplying exogenous Cho to transgenic plants. Upon application of Cho, the level of GB that accumulated in transformants reached approximately ten times that in plants to which Cho was not supplied (data not shown), suggesting a shortage of Cho in transformants.

Stress tolerance of seedlings that accumulated glycinebetaine

Under control conditions, the averages of growth parameters (dry weight of shoots and roots) of three transgenic lines, namely, CMO100–102, CMO80-3 and CMO98-102, were more vigorous than those of wild type. The allocation index, namely, the shoot dry weight as a percentage of the total dry weight of the three lines, increased (Table 1), while the index for -CMO was similar to that for the wild type. In subsequent analyses, -CMO plants were omitted because there was little difference between them and the wild type under control conditions. The shoot dry weight of transformants tended to increase, as compared with that of the wild type, under salt stress. Conversely, the root dry weight of transformants decreased, respectively, compared with that for the wild type. As the result, the allocation indices of three CMO lines under salt stress were significantly elevated (Table 1).
Glycinebetaine in CMO-rice

Table 1. Various parameters related to the growth of seedling after exposure to salt stress and temperature stress*

<table>
<thead>
<tr>
<th>Line</th>
<th>Dry weight (mg plant⁻¹)</th>
<th>Allocation (%)</th>
<th>No. of tillers (mg plant⁻¹)</th>
<th>Allocation (%)</th>
<th>No. of tillers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem length (cm)</td>
<td>Sink size (g d. wt)</td>
<td>Source size (g d. wt)</td>
<td>No. of panicles</td>
<td>1000-grain weight (g)</td>
</tr>
<tr>
<td>wt</td>
<td>Shoot 149 ± 5b</td>
<td>76-7.6 ± 0.5c</td>
<td>2.0 ± 0.4b</td>
<td>8.0-9.0 ± 0.1c</td>
<td>1.5 ± 0.0b</td>
</tr>
<tr>
<td></td>
<td>Root 45 ± 1b</td>
<td>30 ± 1b</td>
<td>2.0 ± 0.4b</td>
<td>35 ± 1b</td>
<td>35 ± 1b</td>
</tr>
<tr>
<td>CMO80-3</td>
<td>Shoot 195 ± 2ab</td>
<td>78.4 ± 0.3b</td>
<td>2.3 ± 0.1ab</td>
<td>82.7 ± 0.3b</td>
<td>1.3 ± 0.1b</td>
</tr>
<tr>
<td></td>
<td>Root 54 ± 1c</td>
<td>26 ± 1c</td>
<td>3.0 ± 2b</td>
<td>30 ± 2b</td>
<td></td>
</tr>
<tr>
<td>CMO98-102</td>
<td>Shoot 193 ± 9b</td>
<td>77.6 ± 0.4b</td>
<td>2.1 ± 0.1b</td>
<td>85.0 ± 0.5a</td>
<td>1.5 ± 0.1ab</td>
</tr>
<tr>
<td></td>
<td>Root 56 ± 2</td>
<td>24 ± 2</td>
<td>2.9 ± 3</td>
<td>35 ± 1c</td>
<td></td>
</tr>
<tr>
<td>CMO100-102</td>
<td>Shoot 211 ± 6b</td>
<td>78.9 ± 0.4a</td>
<td>2.4 ± 0.1a</td>
<td>84.3 ± 0.8ab</td>
<td>1.7 ± 0.1a</td>
</tr>
<tr>
<td></td>
<td>Root 57 ± 3</td>
<td>27 ± 2a</td>
<td>3.2 ± 4</td>
<td>36 ± 2b</td>
<td></td>
</tr>
<tr>
<td>-CMO</td>
<td>Shoot 181 ± 11b</td>
<td>75-6 ± 0.1c</td>
<td>2.0 ± 0.1b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Root 58 ± 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is given as the mean ± standard error (n = 30). Within columns, means followed by the same letter are not significantly different at P < 0.05 (LSD test).

* See text for details.

Table 2. Productivity of mature plants

<table>
<thead>
<tr>
<th>Line</th>
<th>Stem length (cm)</th>
<th>Sink size (g d. wt)</th>
<th>Source size (g d. wt)</th>
<th>No. of panicles</th>
<th>1000-grain weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>96.5 ± 1.3b</td>
<td>45 ± 0.6b</td>
<td>38.8 ± 1.6a</td>
<td>22.5 ± 1.2a</td>
<td>20.3 ± 0.3ab</td>
</tr>
<tr>
<td>CMO80-3</td>
<td>93.0 ± 2.4b</td>
<td>39.1 ± 1.8c</td>
<td>35.7 ± 1.5a</td>
<td>22.0 ± 0.7a</td>
<td>19.4 ± 0.2b</td>
</tr>
<tr>
<td>CMO98-102</td>
<td>86.8 ± 1.2b</td>
<td>40.4 ± 4.6a</td>
<td>32.2 ± 4.7a</td>
<td>22.5 ± 2.2a</td>
<td>20.9 ± 0.4b</td>
</tr>
<tr>
<td>CMO100-102</td>
<td>91.8 ± 1.1b</td>
<td>41.3 ± 5.2b</td>
<td>36.4 ± 3.7a</td>
<td>23.5 ± 2.3a</td>
<td>20.4 ± 0.4ab</td>
</tr>
<tr>
<td>-CMO</td>
<td>102.3 ± 1.5a</td>
<td>39.0 ± 2.9c</td>
<td>38.7 ± 3.2a</td>
<td>20.0 ± 1.1a</td>
<td>20.9 ± 0.1a</td>
</tr>
</tbody>
</table>

Each value is given as the mean ± standard error (n = 4). Within columns, means followed by the same letter are not significantly different at P < 0.05 (LSD test).

Similarly, shoot dry weight and allocation indices were elevated in the CMO lines after exposure to temperature stress (Table 1). The numbers of tillers on the three transgenic lines were elevated under both control and stress conditions, in particular, under temperature stress (Table 1). The aerial parts of transformant seedlings that accumulated GB grew more vigorously and the underground parts grew less vigorously than those of wild-type plants, in which no GB accumulated.

Productivity of mature transgenic plants

The agronomical traits, such as biomass, length of stems, weight of spikelets as sink size, weight of leaves and stems as source size, number of panicles and weight of 1000 grains, of control and transgenic plants, were examined to evaluate the effects of the accumulation of GB in mature plants. While stems of CMO98-102 plants were shorter than those of wild-type plants, the other traits did not differ significantly among the five lines examined (Table 2). Thus, GB in rice plants did not affect grain production but did have a negative effect on stem length.

DISCUSSION

In transgenic rice plants that expressed cDNA for spinach CMO with a transit peptide for targeting of the product to chloroplasts, the levels of GB were very low, ranging from 0.29 to 0.43 μmol g⁻¹ d. wt, in spite of the expression of CMO. However, there was a 10-fold increase in levels of GB when Cho was supplied to the rice plants that expressed CMO (abbreviated as CMO-rice, the format used for other combinations of enzyme and plant name, hereafter). Similar results, with limited accumulation of GB, have been reported in CMO-<i>Arabidopsis</i>, with levels increasing upon the addition of exogenous Cho (Hibino et al., 2002), as well as in CMO-<i>tobacco</i> (Nuccio et al., 1998). Levels of GB in CMO-<i>tobacco</i> increased upon enhancement of the synthesis of Cho (McNeil et al., 2001), showing that endogenous Cho is a limiting factor in this non-GB-accumulator. However, GB accumulated at levels of approx. 1–5 μmol g⁻¹ f. wt and 1 μmol g⁻¹ d. wt in COD-rice (Sakamoto et al., 1998; Mohanty et al., 2002) without a supply of exogenous Cho. Moreover, COD-<i>Arabidopsis</i> accumulated much more GB than did CMO-<i>Arabidopsis</i> (Hayashi et al., 1997; Hibino et al., 2002), and COD-<i>tobacco</i> also accumulated much more GB than did CMO-<i>tobacco</i> (Nuccio et al., 1998; Huang et al., 2000). Cho can be transported into chloroplasts, but the levels of GB in CMO-expressing plants were still lower than those in COD- or CDH-expressing plants. There are at least two possible explanations for the observation that the amounts of GB that accumulate in plants differ so much between plants that express enzymes derived from higher plants and those that express enzymes derived from bacteria. The first possible explanation is that the localization of spinach CMO and that of endogenous BADHs differ in CMO-rice.
The cDNA for spinach CMO that was used in the present study encoded the precursor to mature CMO and included a transit peptide, namely, chloroplast stromal targeting peptide (Rathinasabapathi et al., 1997). Thus, it was predicted that the mature CMO would be localized in the chloroplasts. By contrast, each BADH of rice contains SKL as the carboxy-terminal tripeptide, which delivers proteins to peroxisomes. It is likely that CMO and both BADHs were almost completely localized in chloroplasts and peroxisomes, respectively, in CMO-rice. In spinach, both CMO and BADH are targeted to chloroplasts (Nakamura et al., 1997; Rathinasabapathi et al., 1997). In barley, the two types of BADH, namely, BBD1 and BBD2, are localized in peroxisomes and the cytosol, respectively, with different patterns of expression (Nakamura et al., 2001). The compartment in which BA is converted to GB in monocotyledonous GB-accumulators has not been identified. Thus, at present, it is advantageous to use enzymes derived from bacteria that do not need to co-operate with BADH-like enzymes in specific cellular compartments because such enzymes can catalyse the conversion of Cho to GB in a single step. The second possible explanation for the above-mentioned observations is that the catalytic activity of CMO is lower than that of COD and CDH. The activity of purified CMO is extremely low, 393 pkat mg\(^{-1}\) (Burnet et al., 1995). Even in E. coli, cells that express spinach CMO have been found to accumulate about one-third as much betaine as E. coli that express CDH encoded by betA from E. coli under salt stress (Hibino et al., 2002). Certain trans-acting factors, modifiers or post-translational regulators, that are absent from non-GB-accumulators, such as rice, Arabidopsis and tobacco, might activate CMO in plants that do accumulate GB.

In the present study, the accumulation of GB in transgenic rice seedlings enhanced their tolerance to salt stress and temperature stress (Table 1). The aerial mass (shoot weight and tiller number) was greater than that of wild-type plants, while the underground mass (root weight) was slightly lower after salt stress and temperature stress (Table 1). The aerial mass of GB-accumulators, even if both belong to Poaceae and express active BADHs. One of the rice isozymes for BADH, P0456B03-101, has been reported to be a candidate gene for fragrance (Bradbury et al., 2005). Certainly, BADH has been shown to have broad substrate-specificity with respect to amino acids and related compounds (Trossat et al., 1997).

In the present study, transgenic CMO-rice plants accumulated GB at lower levels than those reported previously (Sakamoto et al., 1998, Mohanty et al., 2002). Although, in the present study, the transformants also exhibited tolerance to salt stress and temperature stress in the seedling stage, not enough GB is accumulated in the plants to improve their productivity. The CMO from a higher plant, spinach, has proved to be less effective for the accumulation of GB in non-GB-accumulating rice plants than bacterial COD and CDH. Recently, Waditte et al. (2005) have reported that Arabidopsis plants expressing genes for N-methyltransferase from A. halopityica accumulated a higher level of GB into roots, stems, leaves and flowers than COD-plants, and showed improved seed yield under stress conditions. This, rather than the introduction of CMO, may be more effective for non-GB-accumulating plants to produce GB. Even though a shortage of substrates has been observed in the plants expressing N-methyltransferase (Waditte et al., 2005), this is a much less serious problem than that posed by Cho of COD- and CDH-plants. By introduction of the gene for N-methyltransferase into rice plants to allow accumulation of a high level of GB, it is expected that resistance to abiotic stresses and hence productivity can be enhanced. It was concluded that this approach for accumulation of GB in rice can be expected to be useful in efforts to improve abiotic stress tolerance and productivity, though CMO-plants were less effective for accumulation of GB and improvement of productivity.

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