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Original Paper

Transcriptional enhancement of a bacterial *choline oxidase A* gene by an *HSP* terminator improves the glycine betaine production and salinity stress tolerance of *Eucalyptus camaldulensis* trees

Ngoc-Ha Thi Tran[1,†], Taichi Oguchi[2,3,*,†], Etsuko Matsunaga[4], Akiyoshi Kawaoka[4], Kazuo N. Watanabe[2,3], Akira Kikuchi[2,3]

[1] Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8752, Japan; [2] Tsukuba Plant Innovation Research Center, University of Tsukuba, Tsukuba, Ibaraki 305-8752, Japan; [3] Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8752, Japan; [4] Agri-Biotechnology Research Laboratory, Nippon Paper Industries Co., Ltd., 5-21-1 Oji, Kita Ward, Tokyo 114-0002, Japan
* E-mail: ta1oguchi@gene.tsukuba.ac.jp Tel & Fax: +81-29-853-7726

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Abstract Novel transgenic *Eucalyptus camaldulensis* trees expressing the bacterial *choline oxidase A* (*codA*) gene by the Cauliflower mosaic virus (CaMV) 35S promoter and the *Arabidopsis thaliana heat shock protein* (*HSP*) terminator was developed. To evaluate the *codA* transcription level and the metabolic products and abiotic stress tolerance of the transgenic trees, a six-month semi-confined screen house cultivation trial was conducted under a moderate-stringency salt-stress condition. The transcription level of the CaMV 35S promoter driven-*codA* was more than fourfold higher, and the content of glycine betaine, the metabolic product of codA, was twofold higher, with the *HSP* terminator than with the *nopaline synthase* (*NOS*) terminator. Moreover, the screen house cultivation revealed that the growth of transgenic trees under the salt stress condition was alleviated in correlation with the glycine betaine concentration. These results suggest that the enhancement of *codA* transcription by the *HSP* terminator increased the abiotic stress tolerance of *Eucalyptus* plantation trees.

Key words: *choline oxidase A* (*codA*), *Eucalyptus camaldulensis*, glycine betaine, *Heat Shock Protein* terminator, salt tolerance.

Introduction

The atmospheric concentration of CO_2 has been increasing over the last century, rising from 280 ppm before the Industrial Revolution to over 400 ppm in 2016 (NIES 2016). Atmospheric CO_2 is a potential greenhouse gas, and is considered to promote global warming (UNFCCC 1992). Responding to climate change is one of the Sustainable Development Goals of the United Nations, and it is thus urgent that we research and develop technologies to improve the global atmospheric CO_2 balance. Forests play an important role in the terrestrial carbon dioxide sink, and in habitats for biodiversity conservation. However, human activities for industry and food production have broken this balance through the excessive use of fossil fuels and deforestation. Therefore, forest plantations play an important role in the effort to compensate for the loss of natural forest to address carbon dioxide fixation (Brockerhoff et al. 2013; Keenan et al. 2015).

The *Eucalyptus* genus is currently one of the most important trees for industrial plantation. It has been reported that over 600 species of this genus are native to the Australian continent and neighboring islands, such as Papua New Guinea, Indonesia, and the island of Mindanao in the Philippines (Nishimura 1987). Globally, a group of species known as the "big nine", which include *E. camaldulensis* and *E. globulus*, make up approximately 90% of the tree species in industrial *Eucalyptus* plantation forests (Booth 2013; Eldridge et al. 1994; Nishimura 1987; OECD 2016).

Global *Eucalyptus* plantation forests are mainly distributed in the tropical and subtropical regions of more than 90 countries, and totaled about 19.6 million

Abbreviations: ANOVA, analysis of variance; CaMV, Cauliflower mosaic virus; codA, choline oxidase A; EC, electric conductivity; FW, fresh weight; HSP, heat shock protein; NOS, nopaline synthase; RGR, relative growth rate; QY, quantum yield of photosynthesis.

† These authors contributed equally to this work.

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hectares in 2008, accounting for about 7% of the total industrial plantation forest area (Trabado 2008). Their biomass resources are used for pulpwood, firewood, timber, shelterbelts, and essential oils (Boland et al. 1984; CAB International 2000; Doran and Brophy 1990; Nishimura 1987). On the other hand, afforestation has also played a part in the restoration of forests degraded by human activities, and *Eucalyptus* plantation trees have been used for this purpose in southwest Africa and South Asia (Ashton et al. 2014; Boulmane et al. 2017; Holl 2017; Lamb et al 2005).

It is considered that abiotic stress-tolerance breeding of *Eucalyptus* forest trees could expand potential plantation areas. Molecular breeding using genetic transformation technology makes it possible to utilize genetic resources beyond species barriers. We have been researching and developing abiotic stress-tolerant *Eucalyptus* trees using genes related to salt tolerance and osmotic-stress tolerance derived from microorganisms and halophyte plants. The *choline oxidase A* gene, *codA*, which is derived from the soil bacterium *Arthrobacter globiformis*, is an abiotic stress-tolerance gene, and has been used for salt-tolerant breeding of *E. camaldulensis* and *E. globulus* (Kikuchi et al. 2009; Matsunaga et al. 2012; Oguchi et al. 2014; Yu et al. 2009, 2013a, 2013b).

The *codA* gene encodes an enzyme catalyst for the biosynthesis of glycine betaine, which is an *N*-methyl-substituted derivative of glycine (Rhodes and Hanson 1993). It also functions as an osmoprotectant in bacteria, yeast, and plant cells (Ikuta et al. 1977). In transgenic plants harboring the *codA* gene, the codA protein played an important role in enhancing abiotic stress tolerance to stressors such as drought, salinity, high temperature, UV radiation, and heavy metals (Ashraf and Foolad 2007; Chen and Murata 2008, 2011; Giri 2011; Kurepin et al. 2015; Sakamoto and Murata 2002). In previous studies, we reported the developments of transgenic *E. camaldulensis* and *E. globulus* harboring *codA*, and evaluated their salt tolerance in contained culture room cultivations, semi-confined screen house trials, and a field trial (Kikuchi et al. 2009; Matsunaga et al. 2012; Oguchi et al. 2014; Yu et al. 2009, 2013a, 2013b).

The *heat shock protein* (*HSP*) terminator derived from *Arabidopsis thaliana* has been demonstrated to increase both the transcription and translation of target transgenes in *Arabidopsis*, tobacco, rice, tomato, and lettuce (Hirai et al. 2011; Kurokawa et al. 2013; Matsui et al. 2011; Nagaya et al. 2010). Given this, we expected that the *HSP* terminator would enhance the transcription of exogenous abiotic stress-tolerance gene(s), and abiotic stress tolerance in *Eucalyptus* plantation trees. We previously reported that the *HSP* terminator enhanced CaMV 35S promoter-driven *codA* transcription and the production of glycine betaine compared to the *NOS* terminator *in vitro* transgenic *E. camaldulensis* shoots (NEDO 2013). In this study, we carried out a semi-confined screen house evaluation of young trees regenerated from these transgenic clones, and confirmed their enhanced *codA* transcriptional levels, glycine betaine production, and abiotic stress tolerance.

Materials and methods

Plant materials and cultivation conditions

Transgenic *E. camaldulensis* trees were generated by the Agrobacterium-mediated method, with pBI121 derived from Ti-plasmid vectors whose T-DNA regions were replaced with the constructs shown in Figure 1A. In this study, we used two clones of transgenic *E. camaldulensis* harboring codAH T-DNA, codAH-1 and codAH-2, and two clones of transgenic *E. camaldulensis* harboring codAN T-DNA, codAN-1 and codAN-2. Three non-transgenic *E. camaldulensis* clones—cam2, cam6, and CML2—were used as control plants (Yu et al. 2009).

Plantlets were propagated by stem cutting. In the spring and summer of 2016, stems with two nodes were trimmed from young branches of trees cultivated in the screen house, then placed in 4-cm square, 4-cm deep plastic cell trays filled with Kanuma trass. After rooting, each plantlet was transferred into a 4.5-cm square, 12.5-cm deep pot filled with

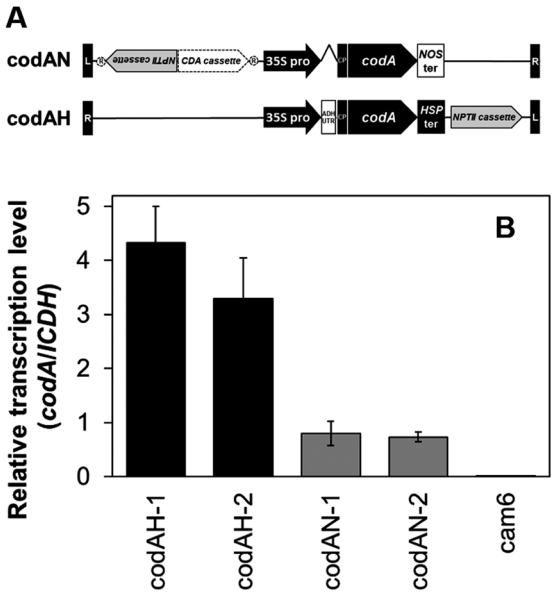

Figure 1. T-DNA constructs and transcriptional levels of transgenes. (A) Schematic diagrams of the T-DNA region of codAH and codAN. codA, a *choline oxidase* gene derived from *A. globiformis*; 35S pro, CaMV 35S promoter; NOS ter, *nopaline synthase* terminator; HSP ter, *heat-shock protein 18.2* terminator; ADH UTR, *A. thaliana ADH* transcriptional enhancer; CP, *N. tabacum rbcS* chloroplast transit peptide; NPTII cassette, expression cassette of *neomycin transferase II*; CDA cassette, expression cassette of *cytidine deaminase*; R, specific recombination site of *Zygosaccharomyces rouxii recombinase R*; RB and LB, right and left border. (B) Transcriptional level of *codA* estimated by qRT-PCR. Error bars in B: Standard error of three biological replications.

a mixture of pumice, Akadama (red granular) soil, and Kanuma trass (1 : 1 : 1), and was cultivated in the screen house for approximately 9 months. The young trees were then transferred into vinyl pots with high slits (15 cm diameter, 10 cm deep) and cultivated until May 2017, when they were used for the trials.

The screen house used in this study met the requirements of the special netted house described by the Japanese Regulatory Framework for Transgenic Research; the details have been reported previously (Yu et al. 2009). The temperature in the screen house was controlled by opening and closing the windows and by using a gas heater; side windows were opened in the daytime during spring and throughout the day and night during summer except in the case of strong winds. A thermostat controlled the opening of the roof window when the room temperature was 25°C or higher, and turned on a gas heating system when the room temperature reached 15°C or lower (Yu et al. 2013c). The 3-year average temperatures of the screen house are shown in Supplementary Figure S1.

Expression analysis of the transgene

Total RNA was extracted from young leaves with an RNeasy® Plant Mini Kit (Qiagen). Reverse transcription (RT) reactions were performed using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo). For the qRT-PCR, the reaction volume of 10 μl contained 5 μl of Thunderbird™ SYBR® qPCR Mix, 0.5 μmol/L each of the primer pair, 1X ROX, and an aliquot of template. The real-time PCR reactions were performed with an ABI PRISM 7900 (Applied Biosystems, Carlsbad, CA, USA) (Iiyoshi et al. 2017). The real-time PCR reaction occurred with the following step-cycle program: pre-incubation at 95°C for 1 min, follow by 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 10 s, and extension at 72°C for 30 s each cycle. The primer pairs for qRT-PCR were as follows: 5′-CAA TGA AGT CGT GCT CTC CA-3′ and 5′-GAG TCC ACC AGG ACC TCG AT-3′ for *codA*, and 5′-TTG CTG CTC TTG ATG GAC AG-3′ and 5′-AAA CGC ACG TCT TGG TCT TT-3′ for the *Eucalyptus isocitrate dehydrogenase* (*ICDH*) gene used as an endogenous reference gene for expression analysis (Boava et al. 2010). The plasmid subclones of both the *codA* and *ICDH* amplicon fragments were used to calibrate the quantitation. The expression of the *codA* transgene was calculated based on the ratio of the quantity of transgene to that of the *ICDH* gene.

Salt tolerance assay

Five individual plants per a clonal line were subjected to the salt stress treatment. These young trees were transferred to slit pots; this style of pot allows water to rise quickly from the slits at the bottom of the pot to the soil surface. Three times per week, the pots were placed on a plastic container filled with water containing 70 mM NaCl until the water rose from the bottom slits to the surface of the pot soil. As the control, five individual plants per a clonal line were treated in the same manner but using plain tap water.

To evaluate the level of accumulated salt in soil, we measured the soil electric conductivity (EC) with a FieldScout® Direct Soil EC Probe (Spectrum Technologies, Aurora, IL, USA) on the day after the day of the watering treatment. During treatment, we measured the plant height and basal diameter monthly. As the volume of woody biomass, the D^2H index was calculated as follows:

$$D^2H = (\text{Basal diameter})^2 \times (\text{Plant height})$$

The average biomass growth rate between date1 and date2 ($GR_{1:2}$) was calculated from D^2H on date1 and D^2H on date2 as follows:

$$GR_{1:2} = \frac{D^2H_2 - D^2H_1}{\text{Date}_2 - \text{Date}_1}$$

The relative growth rate (RGR) was calculated as the ratio of GR under the salt stress condition to GR in the control treatment.

We also measured the quantum yield of photosynthesis (QY) of the leaves every 8 weeks using a FluorPen FP100 instrument (Photon Systems Instruments, Drasov, Czech Republic). Two leaves of each plant were measured (three spots per leaf), and the mean of these six QY measurements was calculated for each plant.

Glycine betaine content in leaves

Leaves of 18-month-old transgenic clone trees and a non-transgenic clone, cam6, were analyzed for their glycine betaine contents. Fifty grams of mature leaves were collected from multiple trees of a respective clone and sent to the MASIS Laboratory (Hirosaki, Aomori, Japan). The leaves were homogenized and sonicated in 80% (v/v) ethanol, and then the aqueous layer was analyzed by high-performance liquid chromatography (HPLC) using a Prominence HPLC System (Shimazu Corporation, Kyoto, Japan) and an AB Sciex API 3200 MS/MS (Danaher Corporation, Washington D.C., USA).

Statistical analysis

The data were subjected to statistical analysis using either a two-way analysis of variance (ANOVA) or a split plot analysis of variance (Perry et al. 2009). Each ANOVA was performed using R ver. 3.4.2 software (2017-09-28) and/or Microsoft Excel 2016 MSO (16.0.9001.2102) (Microsoft, Redmond, WA, USA). The Tukey–Kramer multiple comparisons test was used as necessary, with R.

Results

Transcript levels of codA in transgenic E. camaldulensis trees

The *HSP* terminator from *Arabidopsis thaliana* has been demonstrated to increase the transcript of target transgenes in *Arabidopsis*, tobacco, rice, tomato, and lettuce (Hirai et al. 2011; Kurokawa et al. 2013; Matsui et al. 2011; Nagaya et al. 2010). In this study, we prepared two kinds of transgenic *E. camaldulensis* trees harboring

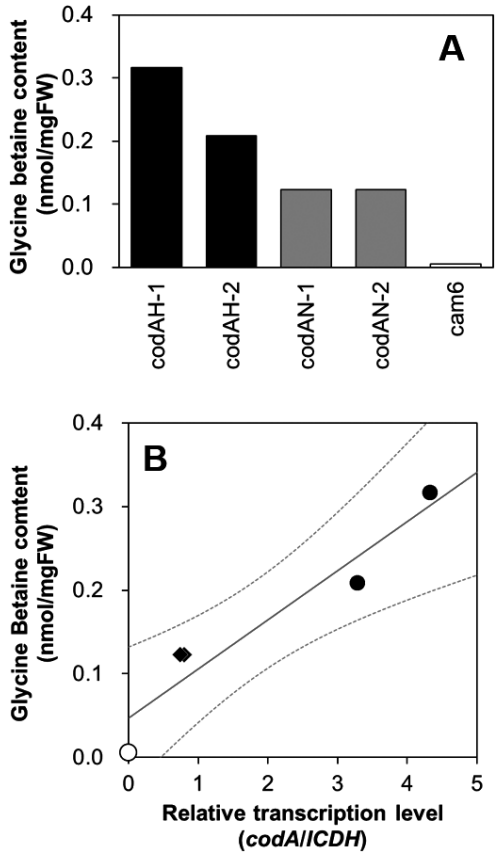

Figure 2. Glycine betaine content. (A) Glycine betaine content in mature leaves of *E. camaldulensis* trees grown for about one year in the screen house. (B) Correlation analysis between relative transcription levels of *codA* and glycine betaine contents. Filled circles, filled diamonds and open circles indicate codAH-, codAN-transgenic and non-transgenic trees, respectively. The solid line indicates the fit curve (adjusted R^2=0.8903, p<0.05). Dashed lines indicate upper and lower limits of the 95% confidential interval of the fit curve.

CaMV 35S promoter-driven *codA* expression cassettes with different transcriptional terminators, i.e., the *HSP* terminator and *NOS* terminator (Figure 1A) (NEDO 2013). Transcription levels of *codA* were evaluated by quantitative reverse transcription PCR (qRT-PCR) (Figure 1B). The transcription levels of *codA* in clones codAH-1 and codAH-2 were four- and fivefold higher than those in codAN-1, respectively (Figure 1B).

Glycine betaine content of transgenic and non-transgenic E. camaldulensis trees

The bacterial *codA* gene encodes an enzyme related to glycine betaine biosynthesis, and thus the exogenous expression of *codA* in plant cells was expected to increase the production and cellular accumulation of glycine betaine. We therefore evaluated the glycine betaine contents of leaves from transgenic and non-transgenic *E. camaldulensis* trees cultivated in a semi-confined screen house (Figure 2A). The glycine betaine content in leaves of non-transgenic *E. camaldulensis* trees was 0.06 mg/100 g fresh weight (FW) (i.e., 0.005 nmol/mg FW; Figure 2A), which almost similar to that in the mature leaves of non-transgenic *E. globulus* (Yu et al. 2013a). In contrast, the glycine betaine contents of codAH and codAN transgenic trees were in the range of 0.21–0.32 nmol/mg FW and 0.12 nmol/mg FW, respectively (Figure 2A). Correlation analysis among transgenic clones revealed a positive correlation between the transcript levels of *codA* and glycine betaine content (adjusted R^2=0.890, p<0.05) (Figure 2B). These results indicated that the exogenous transcription of the bacterial *codA* gene produced glycine betaine in mature leaves of transgenic *E. camaldulensis* trees, and that the content level was correlated to the transcript level of *codA*.

Salt stress tolerance of transgenic E. camaldulensis trees

To evaluate salt tolerance, semi-confined screen house trial cultivation of transgenic and non-transgenic *E. camaldulensis* trees with or without salt stress treatment was conducted from the end of May to November 2017 in Tsukuba, Japan (Figure 3A–G). In order to assess salt tolerance under simulated conditions that reflect the salinity of potential plantation and/or afforestation sites, we conducted cultivation trial of transgenic and non-transgenic *Eucalyptus* in soil treated with 70 mM NaCl, i.e., moderate salinity. In the first 8 weeks of the treatment, the soil EC in the pots slowly increased to an average of 9.0 mS/cm, then fluctuated slightly in the range of 8.2–9.1 mS/cm over the next 16 weeks until the treatment was terminated (Figure 3H). Pots treated with fresh tap water in the same manner as those in the salt-stress treatment were used as controls; the soil EC in these pots was kept in the range of 0.3–0.7 mS/cm throughout the experiment (Figure 3H).

The growth profiles of the control treatment were diverse among trees of non-transgenic clones, and it was suspected that this diversity was caused by genetic inequivalence (Figure 3E–G). Therefore, the comparison of the effect of stress on growth in transgenic and non-transgenic clones was performed using the relative growth rate normalized to the respective growth rate in the control treatment (Figure 3I). During the first two months, the relative growth rate of the treatment groups to the control groups (RGRs) in each clone was in the range of 43–61% and showed no remarkable difference between transgenic and non-transgenic clones (Figure 3I). Soil EC levels increased during the first two months, and rose above 9 by the end of the period, which is considered a severe condition for general salt-tolerant crops (Goodin et al. 1990; Sakadevan and Nguyen 2010). Thus, the RGR of non-transgenic clones during the subsequent two months was decreased to less than 10%, in the range of 1.7–6.5% (Figure 3I). In contrast, the RGRs of the codAH- and codAN-transgenic clones

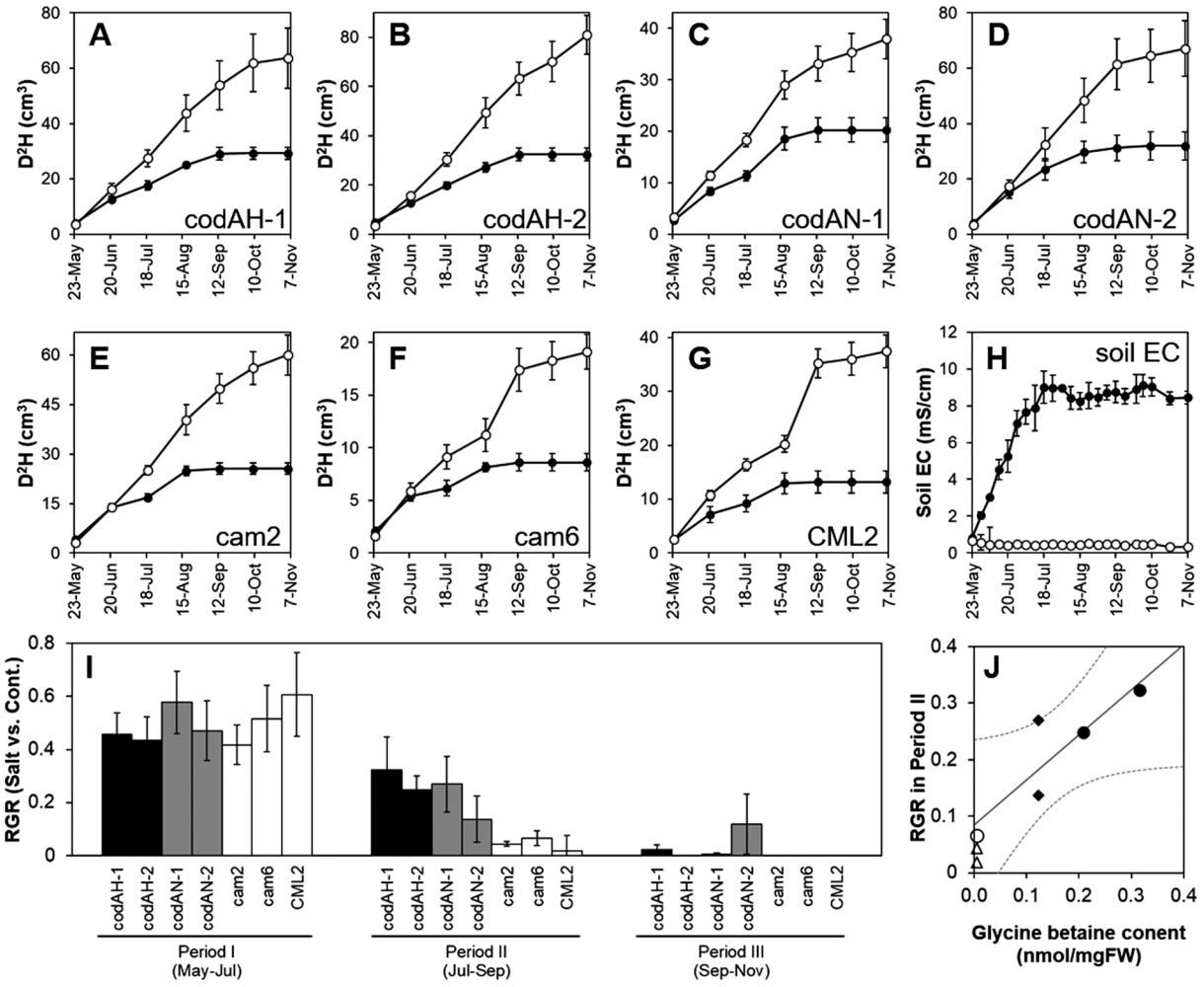

Figure 3. Biomass growth. (A–G) Fluctuation of the average D^2H index under the stress treatment (filled plots) and control condition (open plots) for codAH-1, codAH-2, codAN-1, codAN-2, cam2, cam6, and CML2, respectively. Error bars: Standard error (S.E.) of five biological replications. (I) Growth rates under stress treatment relative to those of the control during each two-month period. Black, gray, and white bars indicate RGRs of codAH-, codAN-transgenic, and non-transgenic trees, respectively. Error bars: S.E. estimated as the S.E. of the average D^2H index. (J) Correlation analysis between glycine betaine content and RGR in period II. Filled circles, filled diamonds and open circles indicate codAH-, codAN-transgenic and non-transgenic trees, respectively. Triangular plots indicate other non-transgenic trees with glycine betaine content extrapolated from that of cam6. The solid line indicates the fit curve calculated from measurements of four transgenic and one non-transgenic cam6 clones (adjusted $R^2=0.6875$, $p=0.05$). The dashed lines indicate upper and lower limits of the 95% confidential interval of the fit curve.

were in the range of 25–32% and 14–27%, respectively (Figure 3I). Correlation analysis revealed a positive correlation between glycine betaine content and RGR in period II (adjusted $R^2=0.69$, $p=0.05$) (Figure 3J). During the last two months, almost no growth was observed in any of the transgenic and non-transgenic trees under the stress condition (Figure 3A–G, I). During the approx. 6 months of treatment, all transgenic and non-transgenic plants survived. The appearance of the plants post-treatment is shown in Figure 4. Non-transgenic plants, but not transgenic plants, showed clear damage that we attributed to salt stress (Figure 4). For a quantitative comparison of leaf injury, the quantum yield of photosynthesis (QY) in leaves was measured every 8 weeks throughout the experiment (Figure 5). No significant difference in QY was found between the salt treatment group and the control group until 8 weeks after treatment, i.e., the end of period I (Figure 5B). At 16 weeks after the salt treatment, i.e., the end of period II, significant decreases in QY were observed in two of the three non-transgenic lines (Figure 5C). Decreases in QY were also observed in two of the transgenic clones, codAH-2 and codAN-2, at the end of the 6-month salt treatment, but the decreases were not significantly different between these two transgenic lines (Figure 5D). These results suggested that exogenous *codA* expression also contributed to the alleviation of photosynthetic disorder under the abiotic stress condition (Figure 5). We also confirmed that the control treatment yielded no clear changes in QY (Figure 5). These results confirmed that accumulation of glycine betaine caused by exogenous expression of *codA* contributed to improved stress tolerance in transgenic *Eucalyptus* under a chronic non-lethal stress condition.

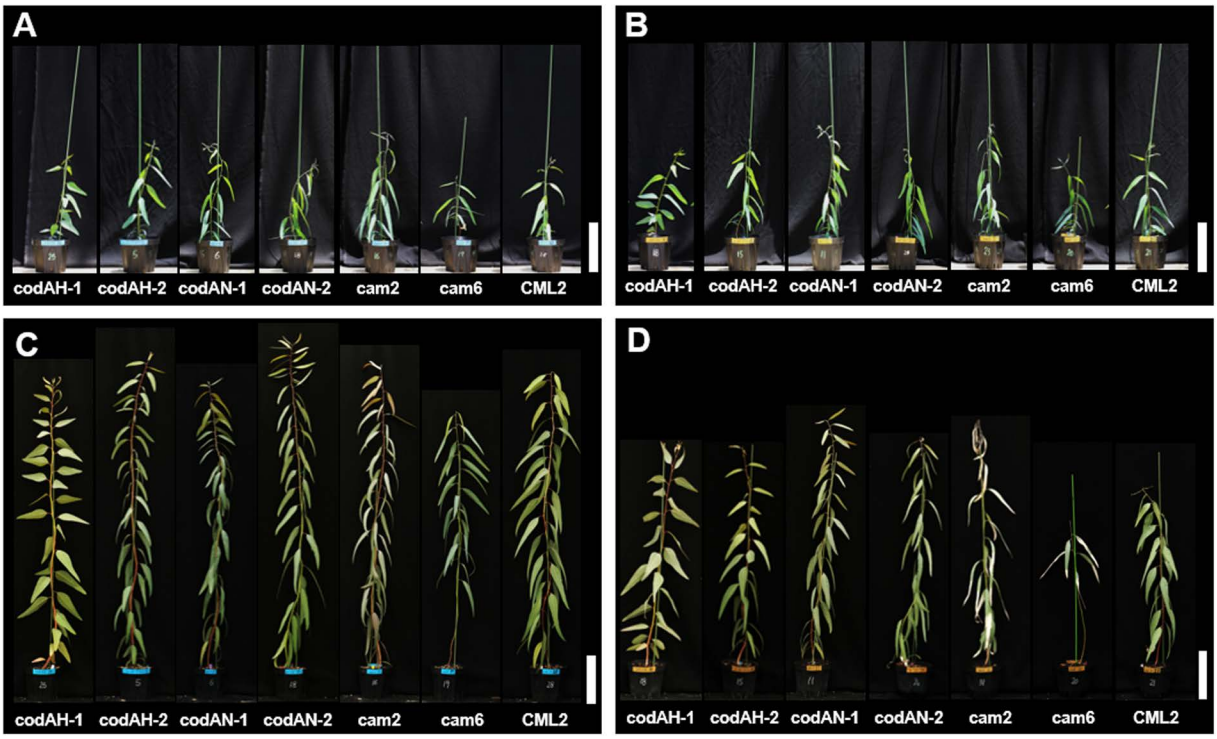

Figure 4. Appearance of trees before and after the salt stress treatment. Typical trees before and after the six-month salt stress treatment for the respective transgenic and non-transgenic clones were shown in B and D, respectively. Typical trees before and after the six-month control treatment were shown in A and C, respectively. Scale bar: 20 cm.

Discussion

HSP terminator enhancement of CaMV 35S promoter-driven codA transcription and glycine betaine content in E. camaldulensis

In previous studies, we developed transgenic lines of two *Eucalyptus* species, *E. camaldulensis* and *E. globulus*, harboring a bacterial *choline oxidase A* (*codA*) gene derived from the halobacterium *Arthrobacter globiformis*, and evaluated whether their salt tolerance was improved by the *codA* transcription (Kikuchi et al. 2006, 2009; Oguchi et al. 2014; Yu et al. 2013a, 2013b). After confirming the improvement of salt tolerance in both species, we then attempted a further augmentation of the salt tolerance in the transgenic *E. camaldulensis* by replacing the terminator of the *codA* expression cassette to the *HSP* terminator. In this study, we showed that the transcription levels of *codA* were four- to fivefold higher with the *HSP* terminator than with the *NOS* terminator (Figure 1B). It was confirmed that the copy number of transgene integration in codAH-1, codAH-2, and codAN-1 genome were one, respectively (Supplementary Figure S2). These results agreed with previous reports: CaMV 35S promoter-derived reporter gene transcription under control of an *HSP* terminator was reported to be 1.7- to 2.7-fold higher than that under the control of an *NOS* terminator in *Arabidopsis* protoplast cells (Nagaya et al. 2010). And miraculin protein accumulation in fruits of transgenic tomatoes expressing the *miraculin* transgene under control of an *HSP* terminator was 2.5- to 10-fold higher than that in fruits of transgenic tomatoes expressing this transgene under the control of an *NOS* terminator (Hirai et al. 2011). The consistency of these results suggests that transcriptional enhancement by *HSP* terminator technology was also applicable in the stable transgenic trees of *E. camaldulensis*.

The *codA* gene encodes an enzyme that catalyzes the biosynthesis of glycine betaine from choline. In higher plants, it is known that the biosynthesis pathways of glycine betaine from choline include two oxidation steps with catalysis of two different enzymes, choline monooxygenase (CMO) and NAD^+-dependent betaine aldehyde dehydrogenase (BADH), which are suggested to occur in the chloroplast (Chen and Murata 2002, 2011; McNeil et al. 2001; Nuccio et al. 2000). On the other hand, the bacterial codA enzyme requires no other enzyme for glycine betaine biosynthesis from choline, because it can catalyze both enzymatic reactions (Ikuta et al. 1977).

Eucalyptus species may have a natural glycine betaine deficiency. The glycine betaine contents of the transgenic *E. camaldulensis* lines evaluated in this study were 24- to 62-fold higher than that of non-transgenic *E. camaldulensis* (Figure 2A). However, glycine betaine values have been reported to be substantially higher in other plant species known as glycine betaine producers:

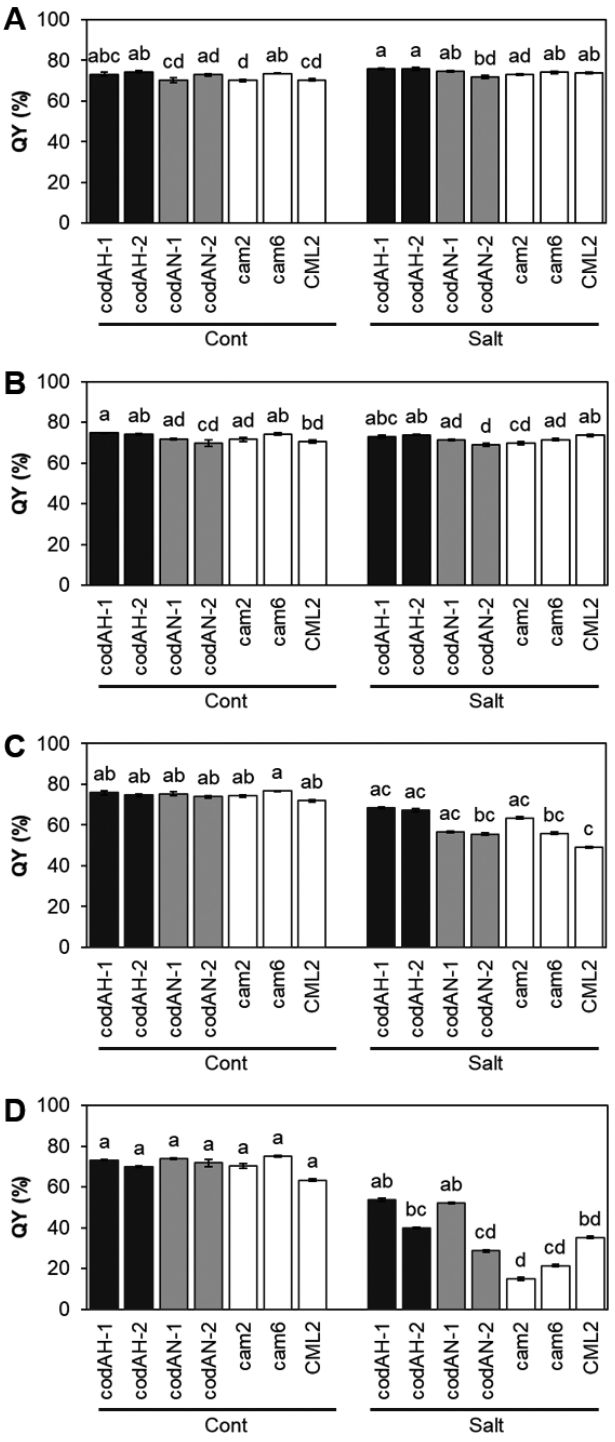

Figure 5. The changes in quantum yield of photosynthesis (QY). The mean QY values at 0-, 8-, 16-, and 24-week after the treatment were shown in A–D, respectively. Left 7 bars and right 7 bars show the measurements for control and salt treatment, respectively. The different letters at the top of the plot areas represent significant differences among clones by the Tukey-HSD test (α=0.05).

glycine betaine contents in the leaves of *Atriplex spongiosa*, wheat (*Triticum aestivum* L.), spinach (*Spinacia oleracea*), *Sorghum bicolor* L., and cotton (*Gossypium hirsutum* L.) were reported to be about 15, 6.5, 5.0, 4.0, and 3.5 μmol/g FW, respectively (Chen and Murata 2011). On the other hand, we confirmed the correlation between the transcription level of *codA* and the glycine betaine content in transgenic *E. camaldulensis*. Our results suggest that further transcriptional and/or translational enhancement of exogenous expression of *codA* might increase the glycine betaine production in *Eucalyptus* trees.

The transgenic *Eucalyptus* lines expressing *codA* previously examined showed no morphological difference from non-transgenic *Eucalyptus*. In our experiments, however, the leaf angles of transgenic codAH-1 tended to be slightly different from those of the other transgenic and non-transgenic clones (Figure 4). In addition, the leaves of codAH-1 tended to be shorter and broader than those of the other clones (Supplementary Figure S3). The codAH-2 plants did not exhibit these morphological changes (Figure 4, Supplementary Figure S3). On the other hand, trees with these characteristics were observed in some individuals of non-transgenic *E. camaldulensis* population. The genetic backgrounds of the transgenic *E. camaldulensis* generated by the method used in this study were not homozygotic but heterozygotic, because hypocotyls of the seedlings derived from the outcrossing bulk seeds were used as the explants in the transformation experiments. From these facts, we considered that the morphological characteristics of codAH-1 were not caused by *codA* expression and/or glycine betaine accumulation but rather by polymorphism within the bulk seeds.

Moderate-salinity stress is suitable to evaluate salt tolerance required for assumed salinity lands

A common method for evaluating salt tolerance in a greenhouse is to determine the survival rate after short-term high-concentration saltwater treatment, and we have previously evaluated some transgenic *Eucalyptus* lines by this method (Yu et al. 2009, 2013c). This methodology is quick and the influence of other environmental factors is small. On the other hand, it is difficult to identify differences in the growth and/or QY by means of high-stringency/short-term tests, and thus evaluations of these parameters have generally been based on the proportion of surviving individuals. However, it can be difficult to prepare a sufficient number of cultivation trials for statistical detection based on the binomial distribution when using mature woody plants. In addition, it has been suggested that survival under severe stress conditions and growth performance under the moderate stress conditions more often encountered in the environment are not equivalent (Skirycz et al. 2011). The method we have described herein enabled maintenance of the soil EC value within a targeted range depending on the concentration of saltwater used for the treatment—e.g., around 8–9 with 70 mM NaCl treatment (Figure 3H). Under this condition, all trees survived but

their growth clearly differed between *codA*-expressing transgenic trees and non-transgenic trees (Figure 3). According to a classification of salt-damaged soil, soil treated with 70 mM NaCl mimics the soil (Grigore and Toma 2017; Richards 1954) comprising approx. 60% (i.e., >650,000 hectares) of the total global area of salt-affected soil (Wicke et al. 2011).

The alleviation of growth and photosynthetic disorders under the stress conditions in *codA*-expressing transgenic trees confirmed that the accumulation of glycine betaine caused by exogenous expression of *codA* contributed to the improvement of salt stress tolerance in transgenic *Eucalyptus* under chronic nonlethal stress conditions. Moreover, the positive correlation of RGR with glycine betaine content suggested that higher transcriptional enhancement of the exogenous *codA* gene might provide *Eucalyptus* trees with higher stress tolerance in the future. Because of the decreased growth rates of trees under the control treatment in period III, we should consider that other cultivation and environmental conditions might affect tree growth—e.g., seasonal fluctuations in temperature (Supplementary Figure S1). The alleviation of damage by salt treatment in the transgenic plants was also confirmed by QY measurement. A future field trial under the salinity condition would be required to evaluate stress tolerance for long-term cultivation.

Alleviation of the impact of salt-stress on growth of E. camaldulensis trees

In this study, we confirmed that addition of an *HSP* terminator resulted in higher levels of *codA* expression and higher contents of glycine betaine in transgenic *E. camaldulensis* trees. However, the salt tolerance of codAH-transgenic *E. camaldulensis* trees was not clearly increased compared to that of codAN-transgenic trees. We considered that the transcription level of *codA* even in codAH-transgenic trees would not generate enough glycine betaine to improve salt tolerance. In this study, it was suggested that the transcription level of *codA* was correlated with the glycine betaine content, and that the growth rate under the salt stress condition was also correlated with glycine betaine content. On the other hand, the *codA* transcription levels in the codAH clones were increased only 5-fold over those in the codAN plants, and the growth rate of the codAH plants under salt stress conditions was improved only 1.2-fold over that of codAN. To maintain 80% growth under normal conditions with salt stress treatment, we calculated that *codA* expression levels 8- and 10-fold higher than those in codAH-1 and codAH-2, respectively, would be required as a rough working target. Although the *codA* expression in the plants with the CaMV35S promoter was clearly increased by addition of an *HSP* terminator, we considered that the augmentation of *codA* expression in the codAH plants was not as high as that realized by overexpression of the transgene. For example, the reported transcription levels of *mangrin* expressed by using the Milk vetch dwarf virus MC8 promoter in transgenic *E. camaldulensis* were at least 10 times higher than the expression levels of *codA* in our codAH-1 and codAH-2 transgenic *E. camaldulensis* trees (Yu et al, 2013c). Future development of transgenic *E. camaldulensis* expressing *codA* by the MC8 promoter and *HSP* terminator would enable us to obtain higher tolerance by *codA* expression.

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