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Short Communication

Environmental biosafety assessment on transgenic *Eucalyptus globulus* harboring the choline oxidase (*codA*) gene in semi-confined condition

Xiang Yu[1,a], Akira Kikuchi[1,*], Etsuko Matsunaga[2], Teruhisa Shimada[2,b], Kazuo N. Watanabe[1]

[1] Faculty of Life and Environmental Sciences, Gene Research Center, University of Tsukuba, Tsukuba, Ibaraki 305-8572, Japan;
[2] Agri-Biotechnology Research Laboratory, Nippon Paper Industries Co. Ltd., Tokyo 114-0002, Japan
* E-mail: kikuike@gene.tsukuba.ac.jp Tel & Fax: +81-29-853-7729

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Abstract Transgenic woody plants have been rapidly developed in recent years, and the commercial use of these transgenic plants has been recognized as an important approach to solving environmental and food problems. Concomitantly, the potential impact of transgenic woody plants on peripheral ecosystems should be considered before they are released for practical commercial use. In this study, we have used environmental biosafety assessments to evaluate three lines of transgenic *Eucalyptus globulus* that harbor the *choline oxidase* (*codA*) gene and have previously been proven to have different levels of salt tolerance. The assessments included two allelopathy bioassays and a survey on rhizosphere microbes, which have shown practicability in other transgenic plants. The two allelopathy bioassays were used for evaluating the potential impact of the transformants on the surrounding vegetation. The survey on rhizosphere microbes was performed to investigate the potential impact of transformants on the rhizosphere microbe community. The results indicated there was no significant difference between the transformants and non-transformants with respect to the impact on the surrounding vegetation and the rhizosphere microbe community. A combination of our biological evaluation of *E. globulus* was then used to successfully obtain approval for the plantation of transgenic *E. globulus* in a Type I field trial in Tsukuba.

Key words: Environmental biosafety assessment, salt stress tolerance, special netted-house., transgenic *Eucalyptus globulus*

Transgenic crops and microorganisms have been widely developed and used commercially (Clive 2011; Watanabe et al. 2005). Of these, transgenic plants have been recognized as an important tool in resolving environment- and food-related problems (Donegan and Ramon 1999; Kikuchi et al. 2006). Transgenic plants are produced artificially by using genetic engineering techniques that are different from traditional breeding processes. In addition, transgenes do not always confer only the objective trait. Transgenic plants may have an impact on peripheral ecosystems. Therefore, the potential impact on peripheral ecosystems should be considered before transgenic plants are released into commercial fields or environments. Some investigations and environmental biosafety assessments have been performed on transgenic plants such as rice (Aoki et al. 2004), tomato (Shiomi et al. 1992), and potato (Mimura et al. 2008); however, most of them have been focused on herbal plant species and crops. Currently, perennial plants such as woody plants have not been well studied. Over 200 field trials on transgenic trees have been reported (Kikuchi et al. 2008); however, only a few studies have been performed on environmental biosafety assessments on transgenic plants.

Eucalyptus grows in temperate and tropical climates (Eldridge 1993). The trees belonging to this genus are used to produce pulp and fuel (Turnbull and Pryor 1984). Transgenic *Eucalyptus* has been developed in recent years to increase its stress tolerance (Hibino 2009; Kikuchi et al. 2006; Matsunaga et al. 2012; Yu et al. 2009) and pulp production (Shimazaki et al. 2009). The abiotic stress-tolerant transgenic *Eucalyptus* is expected to have increased woody material and ameliorate the deteriorating natural environment. We are collaborating to develop transgenic *Eucalyptus* with abiotic stress tolerance (Kikuchi et al. 2006, 2009; Matsunaga et al. 2012; Yu et al. 2009, 2013a, 2013b). *E. globulus* is one of the most variable species for industrial surfaces. We developed salt-tolerant *E. globulus* harboring a choline oxidase gene (*codA*) driven by the *Eucalyptus ubiquitin*

Abbreviations: codA, choline oxidase.
[a] Present address: RIKEN, Yokohama, Japan.
[b] Present address: Japan Pulp and Paper Research Institute Inc., Ibaraki Japan.
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promoter (Yu et al. 2013a). The *codA* gene was isolated from the soil bacterium *A. globiformis* (Ikuta et al. 1997). The salt stress tolerance evaluation, which was performed in a special netted-house, yielded three transgenic *E. globulus* lines, that is, 107-1, 1-9-1, and 2-1-1, showing high, medium, and low salt tolerance, respectively, at the young plantlet stage (Yu et al. 2009). The salt tolerance of these three transgenic lines was also evaluated at some other growth stages. Only 107-1 showed salt-tolerance properties in all the stages investigated, suggesting that it might be a potential tolerant line for practical application.

The guideline of study on transgenic plants, which was promulgated in the Cartagena Protocol (CBD), suggests that a step-by-step evaluation of transgenic plants should be done (e.g. a growth room, a special netted-house, and an isolated field) (Kikuchi et al. 2006; Schomberg 1998; Yu et al. 2009). A series of environmental biosafety assessments on the transgenic *Eucalyptus* should also be performed before they are planted in a commercial field so that the possible impact of these transgenic plants on the native ecosystem can be determined. The Japanese government also requires that an environmental biosafety assessment be performed to identify the risks to the rhizosphere microbe community as well as to the surrounding vegetation.

We focused on determining the impact of above three transgenic *E. globulus* harboring a choline oxidase (*codA*) gene (107-1, 1-9-1, and 2-1-1) on the peripheral ecosystem in a special netted-house. Envronmental biosafety assessment has been done for these three transgenic lines compared with non-transgenic lines (No. 8-8, No. 8-20 or No. 1). Although a transformation system has been established in *E. globulus* (Matsunaga et al. 2012), some difficulties are still experienced in rooting from shoot buds and in acclimatization of small plantlets. We tried to evaluate all the samples at the same time. However, it was difficult to prepare stably enough number of plantlets for that reason. Lines 107-1 and No. 8-8 were evaluated in Experiment 1. Lines 1-9-1, 2-1-1, and No. 1 were evaluated in Experiment 2. Because the germination rates were too low in the soil-mix method of the first series, additional evaluation was performed using lines 107-1 and No. 8-20 in Experiment 3. They were grown in a special netted-house for 14-month-old trees in Experiment 1 and 3, and 11-month-old trees in Experiment 2. Using the sandwich method (Fujii et al. 2003; Yu et al. 2013b), we found that the germination rate and sprout growth of lettuce seedlings were almost the same in both transgenic line 107-1 and non-transgenic line No. 8-8 (Figure 1A). Further analysis using the ANOVA showed that there was no significant difference between transgenic line 107-1 and non-transgenic line No. 8-8 with respect to root elongation, hypocotyl growth, and seed germination ($p>0.10$). Similarly, transgenic lines 1-9-1 and 2-1-1 did not significantly differ from non-transgenic line No. 1 with respect to both hypocotyl growth and root elongation ($p>0.10$) (Figure 1B).

In the soil-mix method (Shiomi et al. 1992; Yu et al. 2013b), the germination rate and sprout growth of lettuce seedlings seemed to be almost the same for the transgenic line 107-1 and non-transgenic line No. 8-20 (Figure 2A), except for the hypocotyl length. ANOVA showed that transgenic line 107-1 and non-transgenic line No. 8-20 did not significantly differ with respect to root elongation ($p>0.10$), hypocotyl growth, ($p>0.10$) and seed germination ($p>0.10$). The transgenic lines 1-9-1 and 2-1-1 did not significantly differ from non-transgenic line No. 1 with respect to hypocotyl growth and root elongation ($p>0.10$; Figure 2B).

The impact on the rhizosphere microbe community

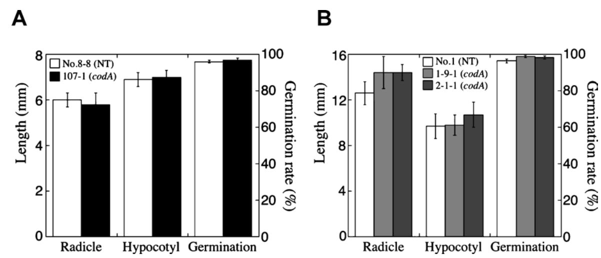

Figure 1. Allelopathy evaluation using the sandwich method for transgenic *E. globulus* expressing the *codA* gene. The white and black bars in the left graph (A) represent non-transgenic line No. 8-8 and transgenic line 107-1, respectively in Experiment 1. The white bars in the graph on the right (B) represent non-transgenic line No. 1. The light and dark gray bars represent transgenic lines 1-9-1 and 2-1-1, respectively in Experiment 2. The error bars indicate standard error. Four individuals from lines No. 8-8 and 1-9-1 and five individuals from lines No. 1, 107-1, and 2-1-1 were used as plant materials. Nine replications were tested per individual. ANOVA did not show any significant difference between non-transformants and transformants ($p>0.10$).

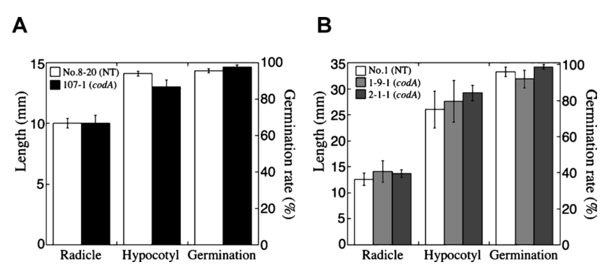

Figure 2. Allelopathy evaluation using the soil-mix method for transgenic *E. globulus* expressing the *codA* gene. The white and black bars in the left graph (A) represent non-transgenic line No. 8-20 and transgenic line 107-1, respectively in Experiment 3. The white bars in the graph on the right (B) represent non-transgenic line No. 1. The light and dark gray bars represent transgenic lines 1-9-1 and 2-1-1, respectively in Experiment 2. The error bars indicate standard error. Three individuals from line No. 8-20, four individuals from line 1-9-1, and five individuals from lines No. 1, 107-1, and 2-1-1 were used as plant materials. Six replications were tested per individual. ANOVA did not show any significant difference between non-transformants and transformants ($p>0.10$).

was evaluated in both transgenic and non-transgenic *E. globulus* by using a plate culture method (Shiomi et al. 1992; Yu et al. 2013b). The populations of fungi, actinomycetes, and bacteria were larger in the non-transformant line No. 8-8 than in the transgenic line 107-1 (Figure 3A). ANOVA showed no significant difference between the three rhizosphere microbe populations ($p>0.10$). The fungal population seemed to be smaller and the actinomycete and bacterial populations seemed to be larger in non-transformant line No. 1 than in transgenic lines 1-9-1 and 2-1-1 (Figure 3B). ANOVA showed that transgenic lines 1-9-1 and 2-1-1 also did not significantly differ from non-transgenic line No. 1 with respect to all the three rhizosphere microbe populations ($p>0.10$). There were no significant differences in the fungal, bacterial, and actinomycete colony numbers between transgenic and non-transgenic plants.

The growth of lettuce sprouts differed in allelopathy evaluation performed using both methods in each Experiment (Figures 1, 2). Furthermore, all three rhizosphere microbe populations were decuple higher in Experiment 2 than in Experiment 1 (Figure 3). These fluids might be derived from a difference in incubation temperature and seed lot in the allelopathy evaluation and from seasonal alterations or differences in the soil batch in the rhizosphere evaluation. Comparisons using a non-transformant line at the same age are necessary to evaluate the impact of each transformant.

The basic concept in environmental biosafety assessments of transformants is substantial equivalence with the host plant species. It is difficult to define the risk of transgenic plants because all plants have some impact on ecosystems. Therefore, the impact of the host plant is defined as "no risk". Only the difference in impact between non-transgenic and transgenic plants is evaluated. In this study, there was no significant difference between the non-transformant and the three transformant lines. From that point of view, compared with non-transformant *E. globulus*, the transformant lines 107-1, 1-9-1, and 2-1-1 had no impact on the surrounding vegetation and rhizosphere microbe community.

Compared with the non-transformants, the three transformant lines of *E. globulus* did not have any impact on the surrounding vegetation and rhizosphere microbe community. Additionally, the three transformant lines of *E. camaldulensis* expressing *codA* did not show any impact on the surrounding vegetation and rhizosphere microbe community compared with the non-transformants (Kikuchi et al. 2006, 2008, 2009). It indicated that this transgene might be a useful gene that enhances the salt tolerance of various tree species without affecting the ecosystem. The evaluation performed in the special netted-house (semi-contained conditions) showed that the transformant line 107-1 had strong salt tolerance in small plantlets (Yu et al. 2009) and in mature trees (Yu et al. 2013a).

The main item required for application for Type I use (field trial and practical use) in Japan is environmental biosafety. The possible impact of leakage of the transformants on the native ecosystem should also be evaluated. The biological property of *E. globulus* was evaluated in an isolated field in Tsukuba in parallel with an evaluation of the three transformant lines in a special netted-house. From this evaluation, the competitiveness against the natural vegetation and the overwintering

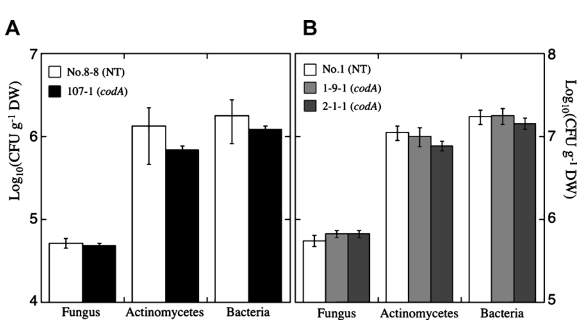

Figure 3. Survey of microorganisms with cultivable colonies. The white and black bars in the left graph (A) represent non-transgenic line No. 8-8 and transgenic line 107-1, respectively in Experiment 1. The white bars in the graph on the right graph (B) indicate non-transgenic line No. 1. The light and dark gray bars represent transgenic lines 1-9-1 and 2-1-1, respectively in Experiment 2. The error bars indicate standard error. Four individuals in from lines No. 8-8 and 1-9-1, and five individuals from lines No. 1, 107-1, and 2-1-1 were used as plant materials. ANOVA did not show any significant difference between non-transformants and transformants ($p>0.10$).

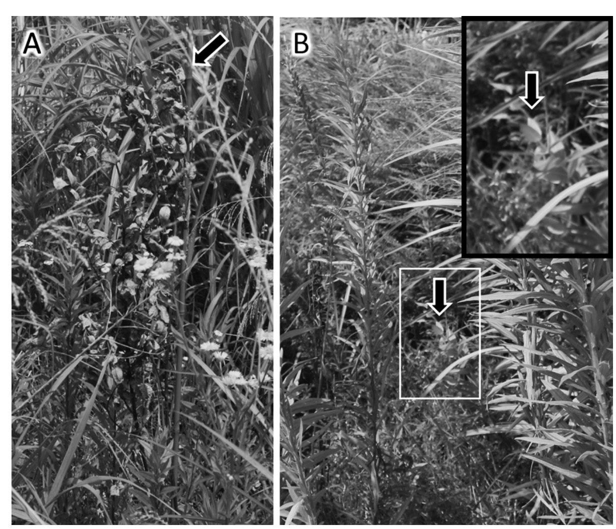

Figure 4. Evaluation of the competitiveness against natural vegetation, overwintering ability, and weediness of non-transformant *E. globulus*. Half of the planted trees grew around one meter in height and did not overwinter. (A) Some wilted trees sprouted a bud flush, but the growth was dominant and surrounded by weeds. (B) The arrow indicates non-transformant *E. globulus*. Inside image of black rectangle is close-up image corresponds to the white rectangle. They did not show high competitiveness or overwintering ability in Tsukuba.

ability of *E. globulus* were found to be weak in Tsukuba (Figure 4). Based on these evaluation data, application documents for an isolated field trial (Type I use in Japan) using these transformants were submitted to the Minister of Education, Culture, Sports, Science and Technology of Japan and the Japanese Environment Minister on July 30th, 2007. These field trials were approved on February 8th, 2008 (J-BCH 2008).

Hence, the practicability of use of transgenic *E. globulus* could be expected even in open field conditions. Moreover, the results of this study provide valuable information that may ultimately lead to the practical use of transgenic plants in addressing food, natural resource, and environmental issues.

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