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Note

Environmental risk assessment of impacts of transgenic *Eucalyptus camaldulensis* events highly expressing bacterial *Choline Oxidase A* gene

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Abstract Under the Japanese biosafety regulatory framework for transgenic plants, data for assessing a transgenic plant's impact on biodiversity must be submitted in order to obtain approval for a confined field trial. We recently reported the development of four novel transgenic *Eucalyptus camaldulensis* clones expressing the bacterial *choline oxidase A* (*codA*) gene, i.e., codAH-1, codAH-2, codAN-1, and codAN-2, and evaluated their abiotic tolerance by semiconfined screen house trial cultivation. Here we evaluated the impacts of the transgenic *E. camaldulensis* clones on productivities of harmful substances from those clones to affect soil microorganisms and/or other plants in the environment. A comparison of the assessment data between the transgenic trees and non-transgenic comparators showed no significant difference in potential impacts on biodiversity. The results contribute to sound-science evidence ensuring substantial equivalence between transgenic and non-transgenic *E. camaldulensis*.

Key words: biosafety, *choline oxidase A* (*codA*), environmental risk assessment (ERA), *Eucalyptus camaldulensis*, transgenic trees.

Biotech crops have been used in commercial cultivation for more than 20 years and in 2016 were planted in 26 countries; they have demonstrated their advantages, with less clear evidence of a risk to the environment (ISAAA 2016, 2017; National Academies of Sciences, Engineering, and Medicine 2016; Parisi et al. 2016). This is because biotech events need to meet not only rules established by various countries but also all regulatory demands set forth in the Cartagena Protocol on Biosafety to the Convention on Biological Diversity (CBD) before being approved for release in commercial areas. In the case of USA and Canada, the large producers of biotech crops, although they have not ratified Cartagena Protocol on Biosafety, they have their own serious regulation framework for releasing biotechnology plants to commercial areas (McHughen and Smyth 2012; National Academies of Sciences, Engineering, and Medicine 2017; Smyth and McHughen 2012). In general, most of the concern of these regulations focus on the impacts of a biotech plant on biodiversity, the balance of the ecology of the receiving environment, and whether there is any potential impact of a biotech plant on its environment. The evaluation protocols for transgenic plants differ from country to country and depend case by case on the biotech event. With more than two decades of experience in biotech crops, it seems that there is a clear frame for evaluating the environmental safety of transgenic crops. In the case of biotech application on plantation trees, the basic concept of environmental risk assessment on biodiversity is the same, although some additional considerations are derived from the biological characteristics peculiar to plantation trees, such as long lifespan, large size, and wide ecological interaction, as well as from traits peculiar to plantation trees, such as modification of lignin content or quality (CBD-COP-MOP8 2016; Häggman et al. 2013).

Eucalyptus trees currently are the most important forestry plantation trees. The genus *Eucalyptus* consists

Abbreviations: codA, choline oxidase A; CBD, Convention on Biological Diversity; ERA, environmental risk assessment; ANOVA, analysis of variance.

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of more than 600 species of flowering trees and shrubs, most of which are native to Australia, with additional species found in Papua New Guinea, Indonesia, and on the island of Mindanao in the Philippines (Nishimura 1987). *Eucalyptus camaldulensis* Dehnh., with the common name River red gum, is one of nine species of fast-growing *Eucalyptus* trees that are found on plantations all over the world. Previously, the development of transgenic *E. camaldulensis* trees was reported (Chen et al. 2001; Harcourt et al. 2000; Ho et al. 1998; Kawaoka et al. 2006; Mendonça et al. 2013; Mullins et al. 1997; Valério et al. 2003). An environmental risk assessment of transgenic *E. camaldulensis* has also been reported (Kikuchi et al. 2006, 2009; Yu et al. 2013b). Moreover, field trials were conducted under type 1 use approvals by the Japanese Ministry of Education, Culture, Sports, Science, and Technology and by the Japanese Ministry of the Environment.

The *choline oxidase A* (*codA*) gene, derived from the soil bacterium *Arthrobacter globiformis*, codes an enzyme catalyzing the reaction to synthesize glycine betaine, which functions as an osmotic protectant in organelles and cytosol (Ashraf and Foolad 2007; Chen and Murata 2008, 2011; Giri 2011; Kurepin et al. 2015; Sakamoto and Murata 2002). We have developed the transgenic *E. camaldulensis* and *E. globulus* harboring the *codA* gene. We also conducted environmental risk assessments of these transgenic *Eucalyptus* trees on productivities of harmful substances to affect soil microorganisms and/or other plants by special-netted house cultivation and isolated field trials. The results showed no significant differences between non-transgenic and transgenic *Eucalyptus* trees regarding potential impacts on the biodiversity of both soil microorganisms and other plants (Kikuchi et al. 2006, 2009; Oguchi et al. 2014; Yu et al. 2013a, 2013b). In this study, we assessed the potential impact on biodiversity of four novel transgenic *E. camaldulensis* harboring the *codA* gene, i.e., codAH-1, codAH-2, codAN-1, and codAN-2, which we reported recently (Tran et al. 2018). The codAH and codAN transgenic trees were different in terminator sequences for *codA* transcription, i.e., *HSP* terminator and *NOS* terminator, respectively (Figure 1). The transcriptional levels of *codA* in codAHs were more than fourfold higher than codANs (Tran et al. 2018). The accumulation levels of glycine betaine in codAHs were more than 1.7- and 40-fold higher than codANs non-transgenic lines, respectively (Tran et al. 2018).

The biodiversity impact of transgenic plants is basically evaluated on the substantial equivalence concept and by the familiarity of the plant species. The Japanese government defines an impact on biodiversity as an "adverse effect that could pose an unacceptable risk of impairment to the preservation of species or populations of wild fauna or flora or any other Adverse Effect on Biological Diversity" (MAFF 2013; MoE 2013). Specifically, evaluation data on the potential risks regarding the following three points must be submitted in order to obtain approval for a Type 1 Use (field trial): "competition with native species", "hybridization with native wild species", and "producing harmful substances". Moreover, regarding the potential damage caused by harmful substances, it is necessary to submit experimental data as instructed in Notifications from the Ministry of Agriculture, Forestry, and Fisheries, and from the Ministry of the Environment of Japan (MAFF 2013; MoE 2013); i.e., "Productivity of harmful substances (secretion from roots to affect the other plants)", "Productivity of harmful substances (secretion from roots to affect microorganisms in soil)", and "Productivity of harmful substances (substances in the plant body to affect the other plants after dying out)".

In this study, we evaluated the substantial equivalence of four clonal lines of transgenic *E. camaldulensis* harboring the *codA* gene, i.e., codAH-1, codAH-2, codAN-1, and codAN-2, which are described in previous reports, to the conventional *E. camaldulensis* in the possible damage caused by harmful substances. We cultivated the four transgenic clonal lines and three independent non-transgenic clonal lines, i.e., cam2, cam6, and CML2, in 15-cm-diameter pots in a special netted house located in Tsukuba, Japan, for 6 months or more. Bioassay methods were used to evaluate the damage caused by harmful substances. The plant materials and cultivation conditions were described in detail previously (Tran et al. 2018).

The potential impacts on the ability of harmful substances secreting from the roots to affect other plants were evaluated by the succeeding crop assay (Atosaku assay) as described in previous reports (Asakawa et al. 1992) Soil samples were collected from pot-cultivated transgenic or non-transgenic *E. camaldulensis* trees for

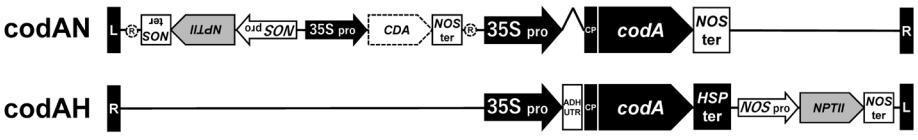

Figure 1. Schematic diagrams of the T-DNA region of codAH and codAN. codA, a *choline oxidase* gene derived from *A. globiformis*; 35S pro, CaMV 35S promoter; NOS ter, *nopaline synthase* terminator; HSP ter, *heat-shock protein 18.2* terminator; ADH UTR, *A. thaliana ADH* transcriptional enhancer; CP, *N. tabacum rbcS* chloroplast transit peptide; *NPTII*, *neomycin transferase II*; CDA, *cytosine deaminase A*; NOS pro, *nopaline synthase* promoter; R, specific recombination site of *Zygosaccharomyces rouxii recombinase R*; RB and LB, right and left border.

6 months or more, and the growth of germinated lettuce seedlings sown on the soil was examined (Asakawa et al. 1992). The measurements were compared between the codAH lines and non-transgenic control lines and between the codAN lines and non-transgenic control lines, by analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test. The results of static testing by ANOVA revealed no significant difference in impacts on tested lettuce seedlings between each combination of transgenic and non-transgenic groups or among the lines (α=0.05; Table 1). The results of Tukey's HSD test agreed with these results (Supplementary Figure S1). Thus, there was no significant difference between the transgenic and non-transgenic lines in potential impacts on productivities of harmful substances secreting from the roots to affect other plants.

To evaluate potential impacts on productivities of harmful substances secreting from the roots to affect microorganisms in soil, the culturable aerobic microorganisms were counted by using two kinds of culture media; oxytetracycline-glucose-yeast extract (OGYE) medium and peptone-tryptone-yeast extract-glucose (PTYG) medium were used to count fungi and bacteria, respectively. The collected soil samples were suspended in 15 mM phosphate buffer (pH 7.0), and the diluted supernatants were spread onto OGYE or PTYG agar medium plates. After 3 or 7 days' incubation in the dark at 25°C for OGYE or PTYG, the formatted colonies

Table 1. Analyses of variance of measurements of the succeeding crop assay.

Comparison[a]	Testing object	Source	Df[b]	Sum Sq[c]	F value[d]	Pr(>F)[e]	
codAH×NT	Hypocotyle length	TG[f]	1	0.00375	1.6972	0.2219	ns[h]
		L[g]	3	0.0045589	0.6878	0.5798	ns[h]
		Residuals	10	0.0220953			
	Radicle length	TG[f]	1	0.019953	3.8268	0.07893	ns[h]
		L[g]	3	0.01501	0.9596	0.44898	ns[h]
		Residuals	10	0.052139			
codAN×NT	Hypocotyle length	TG[f]	1	0.0020167	0.7878	0.3956	ns[h]
		L[g]	3	0.0117056	1.5242	0.2680	ns[h]
		Residuals	10	0.0256			
	Radicle length	TG[f]	1	0.0006	0.0781	0.7856	ns[h]
		L[g]	3	0.007106	0.3081	0.8191	ns[h]
		Residuals	10	0.076867			

[a]Analyses were performed by the ANOVA function in the CAR package (ver.2.1-6) of R software (ver. 3.4.2). [b]Degrees of freedom. [c]Sum of squares. [d]Variance ratio against error. [e]Probability of F-value. [f]Tested difference between the transgenic and non-transgenic groups. [g]Tested difference among the five lines. [h]Not significant (α=0.05).

Table 2. Analyses of variance of numbers of culturable soil microorganism.

Comparison[a]	Testing object	Source	Df[b]	Sum Sq[c]	F value[d]	Pr(>F)[e]	
codAH×NT	Actinomyces	TG[f]	1	0.019267	1.3275	0.276	ns[h]
		L[g]	3	0.100689	2.3126	0.138	ns[h]
		Residuals	10	0.145133			
	Bacteria (exc. Actinomyces)	TG[f]	1	0.05415	4.8406	0.05243	ns[h]
		L[g]	3	0.10096	3.0082	0.08126	ns[h]
		Residuals	10	0.11187			
	Fungus	TG[f]	1	0.0024	0.3237	0.5819	ns[h]
		L[g]	3	0.024172	1.0869	0.3987	ns[h]
		Residuals	10	0.074133			
codAN×NT	Actinomyces	TG[f]	1	0.045067	2.7934	0.1256	ns[h]
		L[g]	3	0.042172	0.8713	0.4878	ns[h]
		Residuals	10	0.161333			
	Bacteria (exc. Actinomyces)	TG[f]	1	0.05415	2.851	0.1222	ns[h]
		L[g]	3	0.016222	0.2847	0.8354	ns[h]
		Residuals	10	0.189933			
	Fungus	TG[f]	1	0.004817	0.4807	0.5039	ns[h]
		L[g]	3	0.054972	1.8287	0.2056	ns[h]
		Residuals	10	0.1002			

[a]Analyses were performed by the ANOVA function in the CAR package (ver.2.1-6) of R software (ver. 3.4.2). [b]Degrees of freedom. [c]Sum of squares. [d]Variance ratio against error. [e]Probability of F-value. [f]Tested difference between the transgenic and non-transgenic groups. [g]Tested difference among the five lines. [h]Not significant (α=0.05).

Table 3. Analyses of variance of measurements of the sandwich assay.

Testing object[a]	Source	Df[b]	Sum Sq[c]	F value[d]	Pr(>F)[e]	
Hypocotyl length	G[f]	2	0.03006	1.1072	0.3445	ns[i]
	L[g]	4	0.05321	0.9798	0.4344	ns[i]
	D[h]	1	1.36368	100.4409	9.168e-11	***[j]
	L:D	6	0.01274	0.1564	0.9861	
	Residuals	28	0.38015			
Radicle length	G[f]	2	0.0178	1.0363	0.3680	ns[i]
	L[g]	4	0.1079	3.1362	0.0299	*[k]
	D[h]	1	4.1838	486.5543	<2e-16	***[j]
	L:D	6	0.1291	2.5023	0.0458	*[k]
	Residuals	28	0.2408			

[a]Analyses were performed by the ANOVA function in the CAR package (ver.2.1-6) of R software (ver. 3.4.2). [b]Degrees of freedom. [c]Sum of squares. [d]Variance ratio against error. [e]Probability of F-value. [f]Tested difference among codAHs, codANs, and non-transgenic groups. [g]Tested difference among the seven lines. [h]Tested difference between the dosages of input leaf tissues. [i]Not significant at an alpha level of 0.05. [j]Significant differences at an alpha level of 0.001. [k]Significant differences at an alpha level of 0.05.

were counted. The colony number on PTYG medium after wiping the surface with 70% ethanol were taken as actinomycetes. The measurements were compared between the codAH lines and non-transgenic control lines and between the codAN lines and non-transgenic control lines by ANOVA and the HSD test, respectively. Static testing by ANOVA revealed no significant difference in impacts on the populations of culturable fungi, actinomycetes, and other bacteria between each combination of transgenic and non-transgenic groups or among the lines (α=0.05; Table 2). The Tukey's HSD results agreed with those results (Supplementary Figure S2). Thus, there was no significant difference between the transgenic and non-transgenic lines in potential impacts on productivities of harmful substances secreting from the roots to affect adjacent soil microorganisms.

The potential impacts on productivities of harmful substances in the plant body to affect other plants after dying out were evaluated by the sandwich assay. Fresh leaves collected from all four transgenic and non-transgenic *E. camaldulensis* trees for 6 months cultivation or more were dried at 60°C for 24 h. Then, 10 mg or 50 mg of dried leaf tissue was embedded in low-melting-point agar (0.5% w/v) and the growth of germinated lettuce seedlings sown on the agar was examined (Fujii et al. 1992). The measurements were compared among the four transgenic lines and non-transgenic control lines by ANOVA and Tukey's HSD test. ANOVA indicated a significant difference between the dosage (10 and 50 mg) of the leaf tissue, but no significant difference was observed in impacts on tested lettuce seedlings between the respective codAH- or codAN-transgenic groups and the non-transgenic groups (α=0.05; Table 3). Among the seven lines, a significant difference was observed in radicle growth (α=0.05; Table 3). However, this difference was not due to a difference in transgenes but due to errors, as supported by the result of Tukey's HSD test (Table 3 and Supplementary Figure S3). These results indicated that there was no significant difference in potential impacts on productivities of harmful substances in the plant body to affect other plants after dying out between the transgenic and non-transgenic lines.

In this study, we confirmed there was no significant difference between transgenic *E. camaldulensis* harboring *codA* and non-transgenic *E. camaldulensis* in their potential impacts on productivities of harmful substances to affect other plants and soil microorganisms. From these results, we concluded that the four transgenic *E. camaldulensis* clonal lines were substantially equivalent to non-transgenic *E. camaldulensis* regarding damage done to other plants and soil microorganisms by harmful substances. We have already confirmed that other transgenic *Eucalyptus* lines harboring the *codA* gene (*E. camaldulensis* and *E. globulus*) are also substantially equivalent to nonrecombinant in the damage potential of harmful substances (Kikuchi et al. 2006, 2009; Oguchi et al. 2014; Yu et al. 2013a). These results would suggest that improving the salt tolerance of the *Eucalyptus* genus by transformation of the *codA* gene does not affect productivities of harmful substances to affect biodiversity.

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