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ORIGINAL PAPER

Evaluation of potential impacts on biodiversity of the salt-tolerant transgenic *Eucalyptus camaldulensis* harboring an RNA chaperonic *RNA-Binding-Protein* gene derived from common ice plant

Ngoc-Ha Thi Tran · Taichi Oguchi · Etsuko Matsunaga · Akiyoshi Kawaoka · Kazuo N. Watanabe · Akira Kikuchi

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Abstract We recently reported that a genetic transformation of the *RNA-Binding-Protein* (*McRBP*), an RNA chaperone gene derived from common ice plant (*Mesembryanthemum crystallinum*), alleviated injury and loss of biomass production by salt stress in *Eucalyptus camaldulensis* in a semi-confined screen house trial. In this study, we assessed the potential environmental impact of the transgenic *Eucalyptus* in a manner complying with Japanese biosafety regulatory framework required for getting permission for experimental confined field trials. Two kinds of bioassays for the effects of allelopathic activity on the growth of other plants, i.e., the sandwich assay and the succeeding crop assay, were performed for three transgenic lines and three non-transgenic lines. No significant differences were observed between transgenic and non-transgenic plants. No significant difference in the numbers of cultivable microorganisms analyzed by the spread plate method were observed among the six transgenic and non-transgenic lines. These results suggested that there is no significant difference in the potential impact on biodiversity between the transgenic *McRBP-E. camaldulensis* lines and their non-transgenic comparators.

Keywords RNA-binding protein · *Mesembryanthemum crystallinum* · Transgenic trees · Environmental risk assessment (ERA) · Biosafety

Ngoc-Ha Thi Tran and Taichi Oguchi have contributed equally to this work.

N.-H. T. Tran
Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8752, Japan

Present Address:
N.-H. T. Tran
Institute of Tropical Biology, Vietnam Academy of Science and Technology, Ho Chi Minh City, Vietnam

T. Oguchi (✉) · K. N. Watanabe · A. Kikuchi
Tsukuba Plant Innovation Research Center, University of Tsukuba, Gene Research Center Bldg., Ten-nodai, Tsukuba, Ibaraki 305-8572, Japan
e-mail: ta1oguchi@gene.tsukuba.ac.jp

T. Oguchi · K. N. Watanabe · A. Kikuchi
Faculty of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8752, Japan

E. Matsunaga · A. Kawaoka
Agri-Biotechnology Research Laboratory, Nippon Paper Industries Co., Ltd., 5-21-1 Oji, Kita Ward, Tokyo 114-0002, Japan

Introduction

Planting forests is an effective way to further the greening of our planet while also contributing to many related goals, such as ecological sustainability or applying biomass in the industrial sector (FAO 2015; UNDP 2016). Following their successful application in crop plants, genetically modified breeding techniques have been applied to woody plants to meet certain specific demands, such as overcoming the barrier of extreme conditions with high/low temperature, salinity or drought, or supplying high lignin/cellulose components to biomass (FAO 2010; Häggman et al. 2013; Osakabe et al. 2011). This has led to much work in this area, and to the approval of three transgenic tree events for commercialization—namely, two events of *Populus* in China and one event of *Eucalyptus* in Brazil (ISAAA 2019; USDA 2019). Before approvals were authorized, these genetically engineered trees containing the transgenic events underwent national regulatory review and risk assessments aligned with international standards (Biosafety-Clearing-House 2015; CBD-COP-MOP8 2016; ISAAA 2019), just as for transgenic crops developed over the last 20 years (ISAAA 2016, 2017; "National Academies of Sciences, Engineering, and Medicine" 2016; Parisi et al. 2016).

The Government of Japan signed the Cartagena Protocol on Biosafety on November 21, 2003, and the Cartagena Act, the domestic law to ensure the implementation of the Cartagena Protocol, entered into force on February 19, 2004 (Watanabe et al. 2004; Government of Japan 2003). Prior to the enactment of the Cartagena Act, regulations regarding the biosafety of genetically modified organisms had individual guidelines for each ministry and agency depending on the purposes and context—i.e., guidelines for research and industry, guidelines for environmental and laboratory safety—but all these separate guidelines were unified by the Cartagena Act (Watanabe et al. 2004). The Cartagena Act regulates organisms harboring extracellularly processed nucleic acids (living modified organisms; LMOs) and their uses, and aims to prevent their use from affecting biodiversity (Government of Japan 2003). The Cartagena Act divides the uses of LMOs into roughly two types: applications without measures to prevent dispersion into the environment ("Type 1 Use"), and applications with measures to prevent dispersion into the environment ("Type 2 Use") (Government of Japan 2003). All field trials of LM plants correspond to Type 1 Use regardless of the scales and purpose, and require risk assessments under the Cartagena Act and associated regulations (MoE and MAFF 2007). Because the protection goals of the environmental risk assessments are to conserve Japan's diversity of native organisms, the assessments seek to evaluate risk from three perspectives: the competitive impact, the crossing ability, and the production of harmful substances (MoE and MAFF 2007). The first perspective concerns the competitive impact of transgenic plants on surrounding plants (native species)—namely, whether they have greater invasiveness or weediness potential (MoE and MAFF 2007). The second is the crossing ability between transgenic plants and conventional plants, which could allow vertical gene flow into the next hybrid generation, potentially replacing the wild relatives (MoE and MAFF 2007). The third perspective seeks to address the risk of novel or enhanced toxin production, since the dying plant bodies or roots systems of transgenic plants may release various chemical components that potentially represent a greater risk of harmful effects to other species (MoE and MAFF 2007), i.e., plant species or soil microbes. In the case of requesting permission for Type 1 Use of LM plants in Japan, the applicant is requested to submit assessment data on the existence of native organisms affected by the LM plant, and level of impact if the affected organisms are existing, from each of the three perspectives (MoE and MAFF 2007). The Ministry of Agriculture, Forestry and Fisheries indicates some specific methods for these assessments (MoE and MAFF 2007). In addition, when submitting the assessment data for Type 1 Use in Japan, any data obtained in a specific netted room or isolated field in Japan are usually required, although there are some exceptions (MoE and MAFF 2007).

Eucalyptus spp. are currently the most important trees used for forestry plantation. This genus is native to Australia, Papua New Guinea, Indonesia and the island of Mindanao in the Philippines (Eldridge et al. 1994; Nishimura 1987). *Eucalyptus camaldulensis*, one of the economically important species of *Eucalyptus*, has been widely cultivated all over the world (Boland et al. 2006; CAB-International 2000; Doran and Brophy 1990; Nishimura 1987). In our previously reported work, three novel salt-tolerant transgenic *E. camaldulensis* lines overexpressing the *RNA-Binding-*

Protein (*McRBP*) gene from *M. crystallinum*, driven by a constitutive MC8 promoter were developed and evaluated in a semi-confined screen house (Tran et al. 2019). They showed clear tolerance to both acute salinity stress (400 mM NaCl within 6 weeks) and chronic salinity stress (70 mM NaCl within 24 weeks), but there was no difference in their phenotype under non-stress conditions (Tran et al. 2019). From these results, we concluded that these lines of transgenic *McRBP-E. camaldulensis* lines could be the candidates for practical use in plantations or afforestation in highly saline soil (Tran et al. 2019). However, these results come from the experiments conducted in the screen house and not been in an outdoor environment. In addition, before releasing the genetic engineering trees into the environment, the ERA based on the experimental field trial studies (Government of Japan 2003; MoE and MAFF 2007). In this current work, we assessed the potential effects on biodiversity of an experimental confined field trial of these three transgenic *McRBP-E. camaldulensis* lines in comparison with those of independent non-transgenic *E. camaldulensis* clonal lines in a manner complying with the Japanese biosafety regulatory flamework.

Materials and methods

Plant materials and cultivation conditions

This study was designed to examine the potential effects on biodiversity of three transgenic *McRBP-E. camaldulensis* lines, i.e., 2–5–4, 2–5–6 and 2–5–7 (Tran et al. 2019). Transgenic *E. camaldulensis* lines were generated by shoots induced from seedlings derived from industrially produced bulk seeds. *E. camaldulensis* seedlings were derived from different seeds from the cross-pollinated population. Thus, the transgenic *E. camaldulensis* lines differ not only with respect to transgenic events but also in terms of the host genetic background. All the transgenic lines used in this work were T0 generation and vegetative propagated progenies. Because it is difficult to prepare a near-isogenic non-transgenic *E. camaldulensis* line from transgenic lines, three independent non-transgenic clonal lines, i.e., cam2, cam6 and CML2, derived from plantation forest trees were used as comparators (Tran et al. 2019).

All plant materials were cultivated in 15-cm diameter pots for over 6 months in a semi-confined screen house at the University of Tsukuba, Tsukuba, Ibaraki prefecture, Japan under the stipulations of Japan's Ministry of Education, Culture, Sports, Science and Technology biosafety regulatory framework for Type 2 Use (Ministerial Ordinance No. 1, 2004), which were described in detail previously (Tran et al. 2018b, 2019; Yu et al. 2009). The semi-confined screen house was a special netted house with all windows (roof and side windows) covered by a mesh screen to inhibit pollen dispersal and the entry of insects, a front chamber in the entrance to prevent exposure of the plants to the outside environment, and two parallel channels to store drainage. In addition, the temperature was controlled by opening and closing the windows and by a gas heater (Tran et al. 2018b, 2019; Yu et al. 2009).

Assessment of the allelopathic impact of plant bodies on peripheral plants by sandwich assay

To estimate the potential risks posed by dying plant bodies on the peripheral plant population, we determined the allelopathic activity of leachates released from dried leaves by the sandwich assay as described in previous reports (Fujii et al. 2003; Tran et al. 2018a). The leaves were collected from three plants each of the transgenic and non-transgenic lines cultivated for more than 6 months in the screen house and dried at 60 °C for 24 h. Two doses of dried leaf tissue (10 mg and 50 mg) were placed in the middle of two 5-mL low-melting-point agar layers in a 6-well plate (34 mm in diameter and 16 mm in depth). In this study, we chose Lettuce (*Lactuca sativa* var. *capitata*) as the indicator plant. Lettuce was often chosen as the indicator species for evaluating allelopathic activities because of its properties as simultaneous and rapid germination, reliable germination and homogeneity, high sensitivity to bioactive substances and easiness of purchase with low cost (Fujii et al. 2003). This species was generally used to assess the allelopathic impacts of many plant species (Fujii et al. 2003; Itani et al. 2013; Mardani et al. 2015; Morikawa et al. 2012) as well as to evaluate bioassays used in the ERA of transgenic plants (Kikuchi et al. 2009; Ko et al. 2019; Oguchi et al. 2014; Tran et al. 2018a; Yu et al. 2013a, b). Five lettuce seeds (cv. Gokuwase Cisco; Takii Seed, Kyoto, Japan) were sowed into each well.

After incubating at 25 °C for 3 days in the dark, the radicle and hypocotyl lengths were measured. For each dose of dry leaf tissue, nine wells were used for each evaluation. Six wells were used for blank agar media (not containing dry leaf tissue).

Assessment of the allelopathic impact of roots on peripheral plants by succeeding crop assay

To evaluate the potential risk conferred by the allelopathic activity of roots onto the peripheral wild plant population, the succeeding crop assay (Atosaku test) was performed among the transgenic and non-transgenic plants (Asakawa et al. 1992). Approximately 100 g of soil without *E. camaldulensis* roots was collected from three cultivated pots for each of the transgenic lines and non-transgenic lines and was distributed into 6 plastic cell trays (4-cm square, 4-cm deep). Five lettuce seeds were sown into each soil cell, and were incubated at 25 °C under a dark condition. The growth of lettuce seedlings was measured after sowing 5 days (Asakawa et al. 1992; Tran et al. 2018a). Eighteen cells were used for each evaluation and six cells were used for blank new soil.

Assessment of potential impact on the population of soil microorganisms by the spread plate method

In order to estimate the potential impact of harmful substances secreted from the roots on the soil microorganism community, the numbers of culturable aerobic soil microorganisms were compared between transgenic *McRBP-Eucalyptus* and the non-transgenic plants. Approximately 60 g of soil without *E. camaldulensis* roots was collected from three cultivated pots for each of the transgenic and non-transgenic lines. Evaluation of the numbers of culturable aerobic soil microorganisms were performed according to our previous report (Ko et al. 2019; Oguchi et al. 2014; Tran et al. 2018a; Yu et al. 2013b). Two kinds of plate culture media were used: the oxytetracycline-glucose-yeast extract (OGYE) medium to assess soil fungi and the peptone-tryptone-yeast extract-glucose (PTYG) medium to evaluate soil actinomycetes and bacteria other than actinomycetes. 30 g of soil was dried at 80 °C for 24 h as the dry weight sample. The remaining 30 g soil was mixed with 270 mL of sterile 15 mM phosphate buffer (pH 7.0). The diluted soil suspension was spread on petri dishes (9 cm in diameter) containing OGYE/PTYG media and was incubated in the dark at room temperature. Fungal colonies were counted after 3 days of incubation, and actinomycete and bacterial colonies were counted after 7 days of incubation (Oguchi et al. 2014; Tran et al. 2018a).

Statistical analysis

The data were subjected to a statistical analysis using either one-way/two-way analysis of variance (ANOVA) or the split plot analysis of variance (Perry et al. 2009). Each ANOVA was performed using R ver. 3.6.0 software (2019-04-26) and/or Microsoft Excel 2016 MSO (16.0.9001.2102) (Microsoft, Redmond, WA). The Tukey–Kramer multiple comparisons test was used as necessary, with R.

Results

Transgenic *RBP-E. camaldulensis* and the Japanese biosafety regulation framework for transgenic plants in Type 2 Use

In this study, the host plant was *E. camaldulensis* Dehnh, commonly known as River red gum, which is an evergreen angiosperm tree belonging to the family *Myrtaceae*, genus *Eucalyptus* and section *Exsertaria* (OECD 2016). It is native to Australia, and distributed over a wide area with latitudes ranging from 2° 48′ S along the Mary River in the tropical Northern territory, to 38° 15′ S in the cool, temperate, southwestern Victoria region, and at altitudes ranging from 20 to 70 m, and in areas with rainfalls of 200–1100 mm annually (CAB-International 2018; Eldridge et al. 1994). *Eucalyptus* is an exotic genus in most regions in the northern hemisphere. This genus is a poor invader, because pollen dispersal and pollination rarely occur by wind; in most cases they are performed by specific birds that can only be found in Australia (Cremer 1977; Griffin 1980; OECD 2016; Ruthrof et al. 2003). Furthermore, seeds and seedlings of *Eucalyptus* have high mortality when exposed to inconvenient conditions (OECD 2016).

In Japan, *E. camaldulensis* is an exotic species, and is currently not used for plantations, but only as an ornamental tree (Nishimura 1987). The invasion risk assessment of *E. camaldulensis* was performed in an

isolated field in Tsukuba city, Ibaraki prefecture, Japan in 2006. The results illustrated that *E. camaldulensis* is less invasive than wild weeds (Kikuchi et al. 2006). Taken together, these characteristics indicate that *E. camaldulensis* trees are unlikely to compete with native species, which is a point of concern in the Japanese regulation framework (MoE and MAFF, 2007). Moreover, in regard to the third issue, the productivity of harmful substances, allelopathic assessment experiments of several transgenic *Eucalyptus* events were performed in both a semi-confined screen house (Type 2 Use) and confined field (Type 1 Use) and revealed no adverse impacts, as reported in our previous studies (Kikuchi et al. 2006, 2009; Oguchi et al. 2014; Tran et al. 2018a; Yu et al. 2013a, b).

In this study, each transgenic *E. camaldulensis* line harbored one copy of the T-DNA construct (Tran et al. 2019). The stability of transgene integration in the host plant genome and the expression of transgenes in three *RBP-E. camaldulensis* lines were demonstrated in our previous report (Tran et al. 2019). This T-DNA construct includes two transgenes—*McRBP* and *NPTII*—that can encode functional proteins. *McRBP* is a novel *RNA-Binding-Protein* gene from *M. crystallinum*. This gene encodes a protein sequence of 306 amino acid residues that contains two RNA-recognition motifs (RRM_1; Pfam identifier PF00076) and that acts as an RNA chaperone in vivo (Tran et al. 2019). By means of its RNA chaperone activities, McRBP improves the abiotic stress tolerance of the three above-mentioned transgenic *E. camaldulensis*

Table 1 Analyses of variance of measurements of the sandwich assay

Testing object	Source	*Df*[a]	Sum Sq[b]	Mean Sq[c]	F value[d]	Pr(> F)[e]	
Hypocotyl length	Line[f]	5	0.44	0.09	0.034	0.999	ns[k]
	Tg[g]	1	0.16	0.16	0.021	0.887	ns[k]
	Error[h]	4	0.28	0.07	0.027	0.998	ns[k]
	Dosage[i]	1	190.44	190.44	74.159	3.16E−09	***[l]
	Replication[j]	2	3.01	1.51	0.587	0.563	ns[k]
	Residuals	27	69.34	2.57			
	Total	35	263.23				
Radicle length	Line[f]	5	86	17	2.349	0.0681	ns[k]
	Tg[g]	1	54	54	0.426	0.5180	ns[k]
	Error[h]	4	32	8	1.142	0.3580	ns[k]
	Dosage[i]	1	4073	4073	553.924	< 2E−16	***[l]
	Replication[j]	2	7	4	0.501	0.6117	ns[k]
	Residuals	27	199	7			
	Total	35	4365				

[a]Degrees of freedom

[b]Sum of squares

[c]Mean of squares

[d]Variance ratio against error

[e]Probability of F-value

[f] ariance derived from difference of lines

[g]Component of variance derived from between transgenic and non-transgenic, in variance among the six line

[h]Component of variance not derived from between transgenic and non-transgenic, in variance among the six line

[i]Variance derived from difference of dosage

[j]Variance derived from difference of biological replication

[k]Not significant at an alpha level of 0.05

[l]Significant differences at an alpha level of 0.001

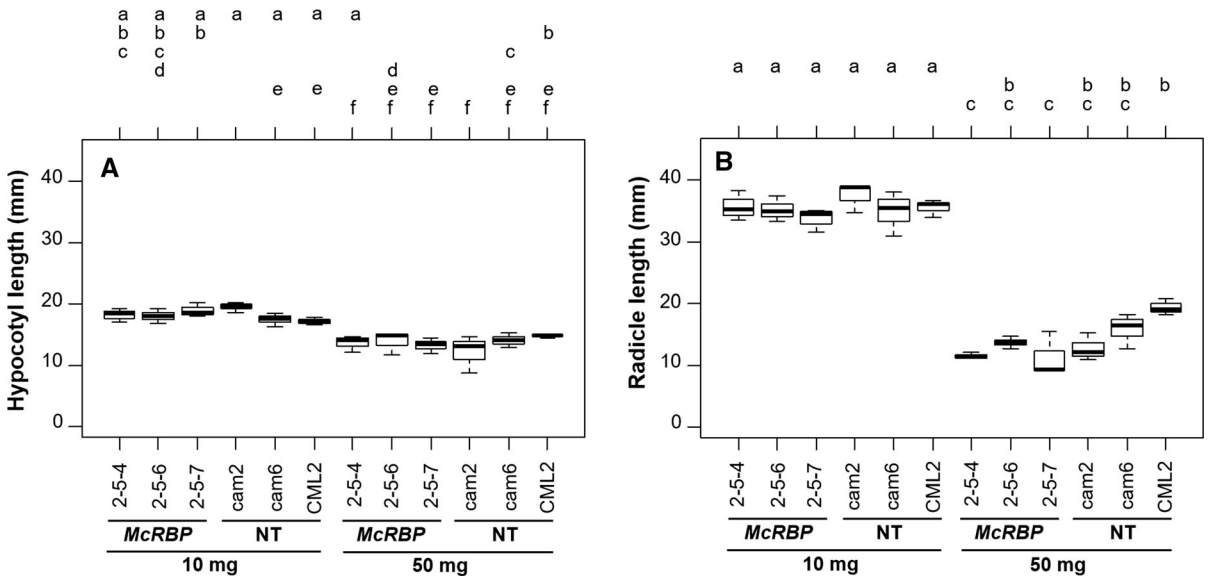

Fig. 1 Assessment of allelopathic activities by sandwich assay. Allelopathic activities of leaves of the *McRBP*-transgenic and non-transgenic control *E. camaldulensis* lines were evaluated by the growth of the recipient lettuce seedlings sowed on agar media containing 10 mg or 50 mg of dried *E. camaldulensis* leaves. The relative growth of hypocotyls and radicles compared to that on the blank agar media are shown in **a**, **b**, respectively. The letters on the upper edge of panels indicate significant differences by the Tukey-HSD test ($\alpha = 0.05$). Error bars indicate standard error (n = 3)

lines but with no enzymatic activity (Tran et al. 2019). Moreover, in other reports, it was confirmed by a database (Allergen Database for Food Safety by National Institute of Health Science, Japan) search that McRBP does not contain any suspected allergenic sequences (Nakamura et al. 2005, 2014). In the case of the selectable marker gene *NPTII*, risk assessments have already been conducted, and it has been internationally agreed that expression of *NPTII* causes no considerable risk to human health or the environment (EFSA 2007; Fuchs et al. 1993; OGTR 2017). On the other hand, the T-DNA construct includes other DNA fragments, which are functional sequences such as promoters (MC8 promoter and *NOS* promoter), terminators (*HSP* terminator and *NOS* terminator) and transcriptional enhancer (5′ untranslated region of *AtADH* gene; *AtADH*-5′UTR), but they do not encode any protein or peptide fragment.

Familiarity of the organisms serving as donors of the nucleic acids is also important as a biosafety evaluation point for LMOs. *M. crystallinum* is the donor organism of the *McRBP* gene (Nakamura et al. 2005, 2014), and *Arabidopsis thaliana* is the donor organism of two DNA fragments, the *HSP* terminator (Nagaya et al. 2010) and AtADH-5′UTR (Sugio et al. 2008). The donor organism of *NPTII* is *Escherichia coli* (Fuchs et al. 1993; Rothstein et al. 1981). *Escherichia coli* is a gram-negative bacterium, a harmless member of the normal microbiota of the human intestinal tract (Percival, and Williams 2014). Agrobacterium (*R. radiobactor*) is a gram-negative soil bacterium that is able to transfer genes in the T-DNA region to plant cells and cause diseases in plants (Nonaka et al. 2017), and it is the donor organism of the *NOS* promoter (An et al. 1990; Shaw et al. 1984), *NOS* terminator (Bevan et al. 1983; Depicker et al. 1982) and the right and left border of T-DNA (Barker et al. 1983). In addition, the milk vetch dwarf virus is dependent on plants, and it is the donor organism of the MC8 promoter (Shirasawa-Seo et al. 2005). As a result of the assessment based on the family-friendliness of the nucleic acid donors thus far, the unintended biosafety risks arising from the nucleic acid-donating organisms would be limited.

Allelopathic impact of *E. camaldulensis* bodies on peripheral plants

The Japanese biosafety regulation framework requires multiple assessments of allelopathic activities as an

Table 2 Analyses of variance of measurements of the succeeding crop assay

Testing object	Source	*Df*[a]	Sum Sq[b]	Mean Sq[c]	F value[d]	Pr(> F)[e]	
Hypocotyl length	Line[f]	5	54.55	10.911	8.183	0.00262	**[j]
	Tg[g]	1	5.78	5.780	1.464	0.2440	ns[k]
	Error[h]	4	48.77	12.193	9.147	0.0022	**[j]
	Replication[i]	2	1.05	0.527	0.395	0.6837	ns[k]
	Residuals	10	13.33	1.333			
	Total	17	68.93				
Radicle length	Line[f]	5	18.62	3.725	0.798	0.575	ns[k]
	Tg[g]	1	13.87	13.869	4.297	0.055	ns[k]
	Error[h]	4	4.75	1.188	0.255	0.900	ns[k]
	Replication[i]	2	0.23	0.117	0.025	0.975	ns[k]
	Residuals	10	46.65	4.665			
	Total	17	65.50				

[a]Degrees of freedom

[b]Sum of squares

[c]Mean of squares

[d]Variance ratio against error

[e]Probability of F-value

[f]Variance derived from difference of lines

[g]Component of variance derived from between transgenic and non-transgenic, in variance among the six line

[h]Component of variance not derived from between transgenic and non-transgenic, in variance among the six line

[i]Variance derived from difference of biological replication

[j]Significant differences at an alpha level of 0.01

[k]Not significant at an alpha level of 0.05

evaluation of the potential harmful impacts on native plants (MoE and MAFF 2007). The sandwich method is a bioassay method in which the allelopathic activities evaluated by monitoring the germination and growth of the plant on agar (MoE and MAFF 2007; Fujii et al. 2003; Tran et al. 2018a). In this work, three transgenic and three non-transgenic eucalyptus leaves were assayed by the sandwich method and the potential effect of substances released from dying plant bodies on the surrounding plants was evaluated by sandwich assay (Table 1, Fig. 1). The hypocotyl and radicle growth of lettuce seedlings at 10 mg and 50 mg leaf supplementation compared to that of the blank control are shown in Fig. 1. Table 1 shows the analysis of variance (ANOVA) table comparing the allelopathic activities among three transgenic and three non-transgenic lines of *E. camaldulensis*. Significant differences were observed between the two doses tested (10 mg and 50 mg; $\alpha < 0.001$), but no significant differences in the hypocotyl and radicle growth of the monitor plant were detected among the 6 lines ($\alpha = 0.05$) (Table 1). Tukey's honestly significant difference (HSD) test revealed a remarkable difference between the two doses (10 mg and 50 mg), but no significant difference among the six lines ($\alpha = 0.05$; Fig. 1). These results suggest that the allelopathic productivity of the transgenic *E. camaldulensis* leaves is not significantly different from that of the non-transgenic *Eucalyptus*.

Allelopathic impact of adjacent soil on peripheral plants

In case of release the transgenic plants to the environment, the possibility that substances produced by transgenic plants and infiltrate roots and their residues affect the surrounding vegetation through soil should be considered. To assess the potential allelopathic activity of soil adjacent the transgenic plants and their roots, the succeeding crop test was conducted

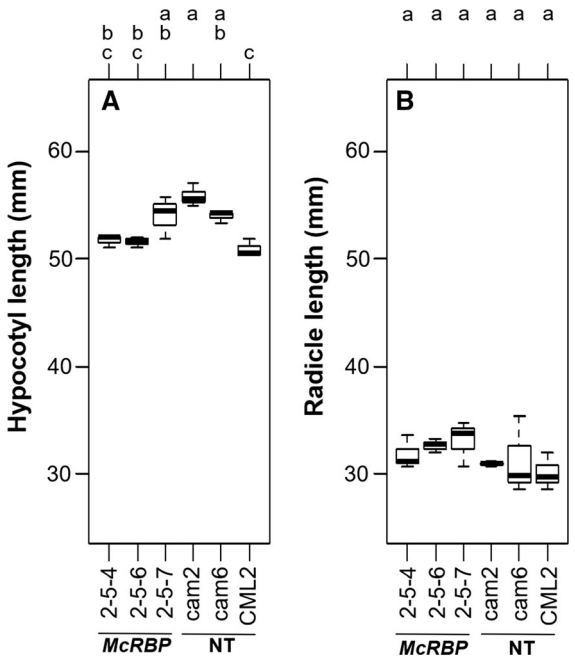

Fig. 2 Assessment of allelopathic activities by succeeding crop assay. Allelopathic activities of the secretory component from *McRBP*-transgenic and non-transgenic control *E. camaldulensis* lines were evaluated by the growth of the recipient lettuce seedlings sowed on soils collected from pots cultivated with *E. camaldulensis* for more than 6 months. The relative growth of hypocotyls and radicles compared to that on the fresh soil is shown in **a**, **b**, respectively. The letters on the upper edge of panels indicate significant differences by the Tukey-HSD test ($\alpha = 0.05$). Error bars indicate standard error (n = 3)

among *RBP-E. camaldulensis* and their conventional plants (Table 2, Fig. 2). The hypocotyl and radicle growths of recipient lettuce seedlings in cultivated soils are shown in Fig. 2. An ANOVA test indicated that there was no significant difference observed between RBP group and non-transgenic group ($\alpha = 0.05$; Table 2). Among the six lines, a difference was found in hypocotyl length ($\alpha = 0.05$; Table 2). However, this difference was not due to an effect of the transgene but due to error, and it was supported by the results from Tukey-HSD test (Table 2, Fig. 2). Consequently, no significant difference was observed in the potential effects on other plants of the productivity of harmful substances by *Eucalyptus* roots between the transgenic *Eucalyptus* and the non-transgenic *Eucalyptus*.

Impact on the population of soil microorganisms on adjacent soil

In case of release the transgenic plants to the environment, the possibility that substances produced by transgenic plants and infiltrate roots and their residues affect the soil microbioal population through soil should also be considered. The microbial population on soil adjacent to transgenic and non-transgenic reference plants was monitored by the spread plate method (Table 3, Fig. 3). The colony forming units (CFUs) of culturable soil microorganisms are shown in Fig. 3. Tukey-HSD tests indicated that there were no significant differences in the numbers of actinomycetes, bacteria (except actinomycetes), or fungi among the six lines consisting of three transgenic and three non-transgenic lines (Fig. 3). On the other hand, the ANOVA test indicated that the bacterial populations in the soil of the transgenic pots were significantly higher than those in the soil of non-transgenic pots (Table 3). Although the difference was significant, it appeared to have no adverse effect on the population of soil microorganisms on adjacent soil. We therefore tentatively propose that transgenic *RBP-E. camaldulensis* does not negatively impact the soil microbial biodiversity compared to the non-transgenic *E. camaldulensis*. A future evaluation on the confined field trial would be expected to provide additional detailed information on the impact of these transgenic *Eucalyptus* lines on soil microorganisms.

In our previous reports, ERA of transgenic *Eucalyptus* lines harboring *codA*/*Mangrin* genes were performed in a semi-confined screen house (Type 2 Use) (Kikuchi et al. 2009; Tran et al. 2018a; Yu et al. 2013a, b). Their effects on biodiversity were not significantly different from those of the non-transgenic *Eucalyptus*, so they were granted approval for Type 1 Use. An isolated field trial (Type 1 Use) for some of these transgenic *Eucalyptus* lines was carried out with ERA. The results indicated that transgenic *Eucalyptus* did not have an adverse impact on biodiversity (Oguchi et al. 2014). The results from this study are similar to our previous results on Type 2 Use, in as much as there were no adverse allelopathic effects on peripheral plants or microbial populations (Kikuchi et al. 2009; Tran et al. 2018a; Yu et al. 2013a, b).

Table 3 Analyses of variance of numbers of culturable soil microorganism

Testing	Source	*Df*[a]	Sum Sq[b]	Mean Sq[c]	F value[d]	Pr(> F)[e]	Significance
Actinomycetes	Line[f]	5	0.2138	0.04276	1.221	0.367	ns[j]
	Tg[g]	1	0.0886	0.08862	2.928	0.106	ns[j]
	Error[h]	4	0.1252	0.03130	0.894	0.503	ns[j]
	Replication[i]	2	0.0089	0.00444	0.127	0.882	ns[j]
	Residuals	10	0.3502	0.03502			
	Total	17	0.5729				
Bacteria	Line[f]	5	0.3826	0.07652	2.404	0.111	ns[j]
(exc. Actinomycetes)	Tg[g]	1	0.3351	0.33510	14.540	0.002	** [k]
	Error[h]	4	0.0475	0.01188	0.373	0.823	ns[j]
	Replication[i]	2	0.0031	0.00156	0.049	0.952	ns[j]
	Residuals	10	0.3182	0.03182			
	Total	17	0.7039				
Fungus	Line[f]	5	0.2440	0.04880	1.682	0.226	ns[j]
	Tg[g]	1	0.0014	0.00137	0.036	0.853	ns[j]
	Error[h]	4	0.2426	0.06065	2.091	0.157	ns[j]
	Replication[i]	2	0.0818	0.04090	1.410	0.289	ns[j]
	Residuals	10	0.2901	0.02901			
	Total	17	0.6159				

[a]Degrees of freedom

[b]Sum of squares

[c]Mean of squares

[d]Variance ratio against error

[e]Probability of F-value

[f]Variance derived from difference of lines

[g]Component of variance derived from between transgenic and non-transgenic, in variance among the six line

[h]Component of variance not derived from between transgenic and non-transgenic, in variance among the six line

[i]Variance derived from difference of biological replication

[j]Not significant at an alpha level of 0.05

[k]Significant differences at an alpha level of 0.01

Discussion

Although the protection goals of the environmental risk assessment of transgenic plants differ by nation and region, ERA is basically performed by comparing the possible impacts on biodiversity between transgenic plants and the appropriate comparators. In this study, according to the Japanese regulatory framework on prior to the experimental confined field trial stipulated by the Minister of Agriculture, Forestry and Fisheries (MAFF 2013), we have confirmed that there is no significant difference between transgenic *E. camaldulensis* lines harboring the *McRBP* gene and the non-transgenic *E. camaldulensis* lines in terms of the potential impacts of the productivity of harmful substances by *E. camaldulensis* on the peripheral plants and soil microorganisms. From the above results, the three transgenic *McRBP-E. camaldulensis* lines did not confer additional risk to the receiving environment in the comparison to non-transgenic *E. camaldulensis*. Therefore, we conclude that using a genetic transformation technique to create *McRBP* recombination in *E. camaldulensis* improved the salinity stress tolerance without adversely affecting the biodiversity. Although, the evaluation of the potential impact on biodiversity by the step-by-step

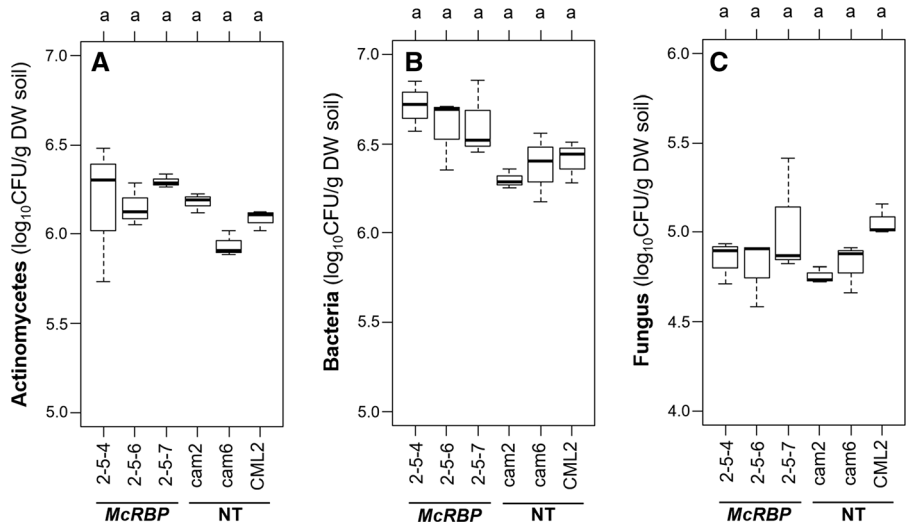

Fig. 3 Assessment of potential impact on soil microorganisms. The microorganisms were extracted from soils collected from pots cultivated with *E. camaldulensis* for more than 6 months. The diluted soil extracts were spread on an OGYE plate to test for bacteria and actinomycetes or a PTYG plate to test for fungi. The CFU of actinomycetes, bacteria (except actinomycetes), and fungi are shown in **a**–**c**, respectively. The letters on the upper edge of panels indicate significant differences by the Tukey-HSD test ($\alpha = 0.05$). Error bars indicate standard error (n = 3)

field trial would be required for the practical use of *McRBP-E. camaldulensis*.

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