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Allelopathy assessments for the environmental biosafety of the salt-tolerant transgenic *Eucalyptus camaldulensis*, genotypes *codA* 12-5B, *codA* 12-5C, and *codA* 20C

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Abstract Allelopathy tests were conducted on salt-tolerant transgenic eucalyptus trees conferring bacterial *codA* gene in the designated net-house conditions under Type II use (contained use) of the Japanese law on environmental biosafety aiming for Type I (field use) application. Three transgenic and corresponding nontransgenic genotypes were employed for four different tests: (1) sandwich bioassay; (2) soil germination method; (3) gas chromatography (GC) for volatile substances from the plants; and (4) high-performance liquid chromatography (HPLC) on phenolic compounds from fresh leaves, which are the primary allelopathic substances on the species. The simple approaches, the bioassays, indicated no significant difference between the transgenic and nongenetically modified genotypes. There was no qualitative difference between the transgenic and nontransgenic lines by GC or HPLC. Absence of any quantitative difference was suggested by repetitive examination and subsequent analysis of variance assessments with the chromatographic methods and bioassays. Moreover, it was also indicated that bioassays should be the primary assessment method for allelopathy in considering the simplicity, speed, low cost, and reproducibility of these methods. Overall, substantial equivalence was considered on the three transgenic genotypes with *codA* gene when compared with the nontransgenic *Eucalyptus camaldulensis* lines. The experiments supported the application to isolated field testing of the transgenic *Eucalyptus camaldulensis* genotypes as the first case and experience in Japanese regulatory approval processes Type I Use for the deliberate release to the environment.

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Introduction

Allelopathy tests on the host and transgenic plants are the key elements in environment biosafety assessments. Allelopathy tests aim to evaluate the negative interactions from genetically modified (GM) organisms to native organisms surrounding the transgenic entities. The evaluation targets could be interactions with plants, soil microorganisms, etc. The choice of the specific methodology for risk assessment is conditional and should be done case by case.[1,2] The employment of the specific technical method in individual cases is based on the factors of host genotype, transgene, and environment. It is also important to consider how the transgenic plants are deliberately released to the environment; for example, small-scale field testing or commercial mass production. Furthermore, components of the methodology of the risk assessment such as cost efficiency, time, simplicity, and precision and quantity of overall information should be considered.[1,3]

There are many reports on the risk assessments of annual plant species in many countries, but as yet are scarce in Japan. These reported cases are mainly for agricultural crops, which can be easily monitored for environmental effects.[1] There is also some information available on tree species such as eucalypts and poplar in databases such as the OECD Biosafety BioTrack (http://www.oecd.org/department/0,2688,en_2649_34385_1_1_1_1_1,00.html) and the Biosafety Clearing-House,[4] but as yet the cases of perennial plant species are meager in number compared with annual crops, and case building is an important challenge for perennial species. In the case of the use of transgenic trees, in addition to confined field testing for the seedling stage without flowering, more elements and their modality of measurement on the environmental effects should be considered, such as the allelopathic influence on the vegetation surrounding the transgenic entities.

The majority of eucalypts, of which there are more than 500 species,[5] are known to have allelopathic influence in nature,[6] and specific attention should be paid to the transgenic eucalypts that do not exceed the level of negative effects of nontransgenic forms.

Our group has worked on the development and testing of various eucalypts with transgenes conferring abiotic stress tolerances for future application to major field production.[7] One of the streamlines of such transgenic materials is with the *codA* gene derived from *Arthrobacter globiformis*, which induces salt tolerance by increasing the amount of the competitive solute, glycinebetaine, in the plant cell.[8] The *codA* gene is driven by a constitutive promoter CAMV35S, and the GM seedlings have shown relevant phenotypes associated with salt and drought tolerances in net-house evaluations.[9] By using the transgenic materials, we have tested and made recommendations on the allelopathic influence of the transgenic materials compared with the nontransgenic originals.

Four different testing methods for allelopathy examination were applied in this experiment to examine whether substantial differences exist between transgenic and nontransgenic *Eucalyptus camaldulensis* seedling trees. The methods were: (1) sandwich bioassay; (2) soil germination method; (3) gas chromatography for volatile substances from the plants; and (4) high-performance liquid chromatography (HPLC) on phenolic compounds from fresh leaves, which are the primary allelopathic substances on the plants. The four methods have been employed to provide risk information as an aid to decision making under the Japanese regulatory system, but the choice of the method depends on the characteristics of the transgenic organisms and the targeted environment. In addition, the choice of method depends on the consequence of primary assessment: no visible risk or potential risk, followed by more refined evaluation to examine further possible risks. We seek to provide recommendations for a primary choice of the four methods to allow a systematic approach to specific allelopathy testing.

In addition to the study of allelopathy evaluation, this research was conducted to support regulatory application for relevant environmental biosafety information required by the Japanese government for deliberate release of transgenes to the confined field as Type I Use (Field use, http://www.bch.biodic.go.jp/english/e_index.html),[4,9,10] especially by introducing the documents in Japanese.

Materials and methods

The transgenic materials confer *codA* gene is driven by the CaMV 35S constitutive promoter. Transgenic and original nontransgenic genotypes of *Eucalyptus camaldulensis* were grown in a net-house designated as Type II Use under the Japanese law (http://www.bch.biodic.go.jp/english/e_index.html). The 18-month-old seedlings were used for the assessments; these were grown in 30-l soil pots. All materials were exposed to salinity stress with 400 ml of 200 mM NaCl solution applied to the pot every second day for 4 weeks.

Sandwich method

Growth measurements of hypocotyls and roots were made on germinated lettuce seed cv. Great Lakes 366, with sandwich testing[11] containing the leaves from the transgenic *Eucalyptus camaldulensis* conferring *codA* and the nontransgenic counterpart trees as a biological assay. A dried leaf was placed in each well of a six-well multidish plastic plate (diameter 35 mm), and 5 ml of a low-melting agar (0.5% w/v) solution was poured on top. After solidification, another 5 ml of low-melting agar (0.5% w/v) was added on top of the first layer. After solidification, five lettuce seeds (*Lactuca sativa* L. var. *capitata*: Great Lakes 366 variety; Takii Seed, Kyoto, Japan) were placed in each well. Each plate was sealed with parafilm and incubated for 72 h at 25°C in darkness. The plates were then frozen at –20°C for 1 day to stop growth. After defrosting the agar, the germination rate and the lengths of roots and hypocotyls were recorded. Ten seeds per replication were used in a sandwich medium with four replications.

Soil germination

Soil used in growing transgenic and nontransgenic eucalypts was used in seeding lettuce cv. Great Lakes 366. One hundred milliliters of soil was placed in a plastic pot with a diameter of 8 cm as one replication. The pots were placed in a growth chamber at 25°C in darkness for 72 h. Irrigation was conducted daily. Thirty seeds per replication were used and four replications were tested.

Gas chromatography

Gas chromatography was conducted on the volatile substances sampled from the plants according to the National Institute of Agro-Environmental Sciences.[12,13] A 6.5-l plastic container was used to cover and seal off a branch of each tree in a growth room to accurately sample the volatile substances. The container was left in place for 1 h before the air inside the vessel was withdrawn by a suction pump into a glass sample vial via a teflon tube. The glass vial was heated to give an internal temperature of 200°C, helium gas was flushed at a rate of 30 ml/ min for 1 min, and the sample was introduced to the gas chromatograph (GC). The GC used a Thermon-1500 glass column (3.2 mm diameter × 2.1 mm). Helium was used as carrier gas, the column temperature was raised from 50°C to 210°C at a rate of 4°C/ min, and detection was made with a flame ionization detector (FID).

High-performance liquid chromatography

HPLC was conducted on the phenolic compounds from the leaves according to the methods described by Shiomi et al.[13]

Twenty grams of fresh leaves was crushed in eight volumes of 80% ethanol, and filtered after overnight incubation at room temperature. The extract was dried with a drier, 100 ml of distilled water was added, and pH was adjusted to 2.8 with 6 N HCl. This solution was placed in a separating funnel and mixed with an equal volume of ethyl acetate. The mixture was shaken for 30 min and then allowed to sit for overnight incubation. Then ordinary ethyl acetate extraction was conducted and the extract was dried and dissolved in 5 ml of methanol for HPLC. A Shimpak ODS column (6.0 mm diameter × 150 mm, Shimadzu) was used with a sample amount of 10 µl. The sample flow rate was 1.8 ml/min by 2 % acetic acid solution with concentration of 15 to 40 % methanol. Detection was achieved by monitoring the ultraviolet response at 254 nm.

Results

Sandwich method

The replications did not show significant difference and it seems that the reproducibility was adequate. (Table 1). There was no substantial variation between nontransgenic and transgenic lines with respect to the hypocotyl growth and root enlargement, and consequently there is no substantial allelopathic effect from the transgenic materials The results corresponded well with the previous report.[9]

Soil germination

The germination rate was around 70% for all soil samples obtained from the individual eucalyptus genotypes. Table 2 shows that the germination rate of the lettuce seeds was not altered.

Gas chromatography

Gas chromatography analysis did not show qualitative variation in the peak pattern as reported by Kikuchi et al.[9] However, it is possible that quantitative differences occurred for the of the individual peaks. Thus, quantitative assessment was made on the major peak to have difference as indicated in the previous study.[9] The peak was analyzed for three different samples per day and its average was used as one repetition. Five repetitions sampled on alternative days were used for analysis of variance (ANOVA). The results indicated that there was no substantial difference between transgenic and nontransgenic subjects (Table 3). While a

Table 1. Analyses of variance on growth measurements of hypocotyls and roots on germinated lettuce seed cv. Great Lakes 366, with sandwich testing containing leaves from transgenic *Eucalyptus camaldulensis* conferring *codA* and nontransgenic counterpart trees

Measurement	Degree of freedom	Sum of square	Mean square	*F* value	Probability	Significance
Hypocotyl	Line	3	28.7213	9.5738	1.5513	ns
	Replication	3	18.3412	6.1137	0.9907	ns
	Line × rep	9	6.5438	0.7271	0.1178	ns
	Error	144	888.6754	6.1714		
	Total	159	942.2817			
Root	Line	3	5.872	1.9573	0.9243	ns
	Replication	3	18.477	6.159	2.9086	ns
	Line × rep	9	2.247	0.2497	0.1179	ns
	Error	144	304.914	2.1175		
	Total	159	331.51			

ns, Not significant

Table 2. Analysis of variance on seed germination testing with soil used for growing transgenic *Eucalyptus camaldulensis* conferring *codA* and their nontransgenic counterpart trees

Degree of freedom	Sum of square	Mean square	*F* value	Probability	Significance
Line	3	0.0075	0.0025	0.2308	ns
Error	12	0.13	0.0108		
Total	15	0.1375			

Table 3. Gas chromatography peak variation assess ment between transgenic and nontransgenic *Eucalyptus camaldulensis* trees

Degree of freedom	Sum of square	Mean square	*F* value	Probability	Significance
Line	3	3.3687	1.1229	0.5467	ns
Repetition	4	212.2623	53.066	25.838	*
Error	52	106.7963	2.0538		
Total	59	322.4273			

From Kikuchi et al.[9]
*Significant at the 1% level

Table 4. Testing of peak variation in high-performance liquid chromatography among transgenic and nontransgenic *Eucalyptus camaldulensis* trees

Degree of freedom	Sum of square	Mean square	F value	Probability	Significance
Line	3	2.41E–05	8.02E–06	0.2471	ns
Repetition	4	0.002392	0.000059	18.4285	*
Error	52	0.001687	3.24E–05		
Total	59	0.004103			

From Kikuchi et al.[9]
*Significant at the 1% level

sampling difference was observed, the genotype difference was not significant.

High-performance liquid chromatography

No qualitative difference was detected in the presence of the HPLC peaks, as reported by Kikuchi et al.[9] However, as in GC analysis, it is possible that individual peaks showed quantitative changes. Thus, quantitative assessment was made on the major peak to have difference as indicated in the previous study.[9] Three measurements from the same sample were regarded as a repetition, and five samples were taken each alternative day. The results indicated that there is no substantial difference between transgenic and nontransgenic lines, but a difference in repetition was observed (Table 4).

Discussion

In the present study, four different tests were conducted for the assessment of allelopathy in transgenic eucalypts conferring a salt tolerance gene: (1) sandwich bioassay; (2) soil mixing test; (3) gas chromatography; and (4) HPLC. There was no significant difference between transgenic and nontransgenic trees over the four experiments related to the allelopathic examination. With respect to the risk assessment, it is clear that no environmental risk is expected in terms of allelopathy.

Comparing each assessment method, there may be a primary choice for further prompt evaluation of allelopathy. For both GC and HPLC, there was no qualitative difference in the presence of the peaks. However, as to the reproducibility and stability of the assessment methods and the consequences for the judgment of the allelopathic effects, both results indicated significant variation in sampling that was revealed by repetition difference in the corresponding ANOVAs in Tables 3 and 4. Chromatographic analyses have more fluctuation of data variation due to nongenetic factors.[14] On the other hand, the biological assays by the sandwich method for growth inhibition and soil mixing testing for germination indicated constant results in the repeated trials. For environmental biosafety assessments, the priority is for biological evaluation rather than chemical evaluation to ensure a wide scope of evaluation on the integral effect of the subject, GM plants.

Having considered the logistics required to conduct the environmental risk assessment, it is important to have a simple system that allows a primary evaluation. Chromatography requires specific sampling, extraction, and facility setup. In considering the cost of the evaluation, it is clear that biological assays should be used as the primary test, while chromatography is optional. Nontechnological factors such as public perception[3,15] may influence the interpretation and decision making surrounding scientific risk assessment of deliberate release to the environment of transgenic plants. As such, the reproducibility and stability of the scientific data must be well accepted by diverse stakeholders in order to avoid the misinterpretation or misuse of the scientific results.

Overall, the biological assays with suitable repetitions are simple, provide useful data, and are easy to comprehend for the primary assessment of allelopathic risks. However, the use of GC and HPLC can also be considered in cases where the bioassays indicate the possibility of higher allelopathic effects. However, sufficient care must be taken in sampling to ensure uniformity and reproducibility of the results and to avoid misinterpretation of the data.

Four methods were examined for investigation of the environmental effects associated with allelopathy in transgenic *Eucalyptus camaldulensis* for governmental inspection and to aid in decisions on the deliberate release of the transgenic trees. For future assessment of allelopathy of transgenic materials, biological assays should be conducted initially and followed by other more detailed methods if the biological assay reveals the likelihood of a significant effect.

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