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Short Communication

Mycorrhizal colonization of transgenic *Eucalyptus camaldulensis* carrying the *mangrin* gene for salt tolerance

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Abstract Transgenic products and the creation of new organisms with innovative transgenic traits generally raise risk assessment concerns because of potential risks to nontarget organisms. Cartagena Protocol on Biosafety and Japanese government regulations require scientific environmental risk assessments of living modified organisms prior to release to avoid adverse effects on the environment. Soil microorganisms, such as the arbuscular mycorrhizal fungi, aid in plant nutrient acquisition and protection from environmental stresses such as salt stress. In this study, we used a salt-tolerant transgenic *Eucalyptus camaldulensis* transformed with the *mangrin* gene from a mangrove plant and evaluated the interactions between environmental stress-tolerant transgenic plants and arbuscular mycorrhizal fungi. Our results indicated that these transformants were substantially equivalent to nontransformants in terms of arbuscular mycorrhizal fungi colonization under both saline and nonsaline conditions, and that the arbuscular mycorrhizal fungi colonization could potentially enhance the salt tolerance of the transgenic plant.

Key words: *Eucalyptus camaldulensis, mangrin*, mycorrhizal colonization, transgenic.

Soil salinity is a major adverse environmental constraint that hinders land productivity worldwide. A large portion (7%) of the global land surface is saline in nature (Szabolcs 1994). Up to 20% of the world's arable land and up to 50% of all irrigated land are adversely affected by salinity mainly due to unsuitable irrigation practices (FAO 2008; Miyake et al. 2006). In most saline environments, sodium chloride is the predominant salt that causes growth reduction in nonresistant plants.

Certain microorganisms form close associations with plant roots, which enriches the capacity of the plants to acquire nutrients and protects them against various stresses. Arbuscular mycorrhizal (AM) fungi are one of those microorganisms that can play an important role against stresses, and they colonize more than 80% of plant species under natural conditions (Read 1991; Rosendahl 2008). Most members of the Glomeromycota participate in this symbiosis (Berbee and Taylor 2000; Gehrig et al. 1996; Schüßler et al. 2001). The symbiosis develops in the cortical cells of the plant root, where the AM fungi form extensively branched hyphae called arbuscules. Additionally, extraradical hyphae extend into the rhizosphere as well. These hyphae acquire phosphates and nitrates from the soil and translocate them to the plant, and in exchange, the AM fungi receive carbohydrates from the plant. Thus, symbiosis is important to plant health and ecosystem functioning (Heijden et al. 1998; Heijden and Scheublin 2007; Smith and Read 1997). Changes in root biochemistry or morphology may severely disturb the formation or functioning of mycorrhizal symbiosis. AM fungi also exist in saline environments, where they enhance plant growth and tolerance to salinity (Aliasgharzadeh et al. 2001; Juniper and Abbott 1993). The use of AM fungi has gained importance recently as a practical way to alleviate soil stresses, including salinity, on plant growth (Al-Karaki 2000, 2006; Al-Karaki and Hammad 2001; Giri and Mukerji 2004; Miransari and Smith 2007; Miransari et al. 2008).

Currently, one means of decreasing the unfavorable effects of salinity on plant growth and making saline land cultivable is to produce transgenic plants that are highly tolerant to salinity stress. However, transgenic plants raise risk assessment concerns because the creation of new organisms with innovative transgenic traits can cause potential risks to nontarget organisms (Azevedo and Araujo 2003; Hilbeck and Schmidt 2006), including soil microorganisms. Both Cartagena Protocol

Abbreviations: AM, arbuscular mycorrhizal; EC, electrical conductivity; −M−S, without mycorrhiza without salt; +M−S, with mycorrhiza without salt; −M+S, without mycorrhiza with salt; +M+S, with mycorrhiza with salt.

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on Biosafety and Japanese government regulations require environmental risk assessments on living modified organisms prior to release to the environment to protect biological diversity. The effects of transgenic plants on soil and plant microbe interactions have been studied in several transgenic plants. The results varied, and some researchers attributed the effects to environmental and varietal differences (Baumgarte and Tebbe 2005; Powell et al. 2007; Wei et al. 2006; Zwahlen et al. 2003). The role of mycorrhizal fungi and their relationship with the host plant as a major aspect of plant ecology is often neglected in transformation research. Mycorrhizal associations between *E. camaldulensis* and AM fungi develop naturally in the field, where they improve plant growth (Pagano and Scotti 2008). The concern that transgenic technology could harm arbuscular mycorrhiza has been stated previously (Glandorf et al. 1997). Some transgenic cultivars of maize reportedly reduced mycorrhizal colonization potentials (Castaldini et al. 2005; Turrini et al. 2004). Differences in colonization levels due to varietal differences were also observed in transgenic herbicide-tolerant soybeans (Powell et al. 2007).

No scientific literature was found on the mycorrhizal colonization of transgenic *Eucalyptus* plants engineered for environmental stress tolerance; therefore, this study of the association between salt-tolerant transgenic *Eucalyptus* and AM fungi was intriguing. We used a salt-tolerant transgenic *E. camaldulensis* transformed with the *mangrin* gene (Yamada et al. 2002) from the mangrove plant *Bruguiera sexangula*. We evaluated the interactions between environmental stress-tolerant transgenic plants and AM fungi.

The plant material consisted of three nontransgenic *E. camaldulensis* lines (CML2, cam2 and cam6) and three transgenic salt-tolerant *E. camaldulensis* lines transformed with the *mangrin* gene (F15, F60 and F65). Among the nontransgenic control lines, CML2 showed high salt tolerance (>50% survival rate) compared with the other two lines (<40% survival rate) when irrigated with 600 mM NaCl (Yu et al. 2009). Among the transgenic lines, F15 and F65 were highly salt-tolerant with a relative survival rate of more than 1.5 compared with CML2, whereas F60 was removed from the high salt-tolerant category because relative survival rate was between 1.0 and 1.5 (Yu et al. unpublished data). The expression of transgenes in all lines was confirmed by RT-PCR. The three nontransgenic plant lines and three transgenic lines were selected mainly on the basis of their different levels of salt tolerance.

The AM fungi used in this study were *Glomus intraradices* (MAFF520059) and *Glomus etunicatum* (MAFF520053) obtained from Genebank, National Institute of Agrobiological Sciences (NIAS) in Japan. The inoculum was prepared by growing each strain separately in sorghum (*Sorghum bicolor* L Moench) for four months in a soil–sand mixture. When the colonization levels in the plant roots reached more than 80%, 5 g of the soil–sand mixture from each of the growing strains was mixed, for a total of 10 g. *Eucalyptus* plantlets were placed on the 10 g of inoculum, and their roots were covered with new soil–sand mixture.

The experiment had a 2×2×2 factorial design with the following factors: (1) irrigation (tap water or salt water), (2) inoculation (noninoculated or inoculated with a mixture of AM fungi), and (3) plant genotype (nontransgenic or transgenic). Each combination of the factors involved five replicates, each represented by one plant in a 500 ml plastic pot. The treatments were designated and arranged as follows: without mycorrhiza without salt (-M-S), with mycorrhiza without salt (+M-S), without mycorrhiza with salt (−M+S), and with mycorrhiza with salt (+M+S). At the start of the experiment, an inoculum was placed in the pot containing the soil–sand mix just before transplanting the plantlets. All the plant pots were watered with tap water for one month prior to the start of the salt treatments. Salt treatments began after four weeks when the mycorrhization had been established in the AM fungi treatments. On the basis of the results of a preliminary study, a salt condition of 200 mM NaCl was selected. At that treatment level, nontolerant plants showed salt stress effects but AM fungi still survived. The plants were irrigated continuously for four weeks, about 100 ml every other day, with an automatic system (EY4200, National, Tokyo, Japan). Electrical conductivity (EC) of the soil was monitored weekly just before the salt treatments to the end of the experiment. The EC of the substrate reached a maximum of $11.33 \pm 0.49\,\mathrm{mS\,cm^{-2}}$ for the salt treatments and $0.35 \pm 0.08\,\mathrm{mS\,cm^{-2}}$ for the tap water treatments.

Mycorrhizal colonization was assessed at the end of the experiment to determine the effects of both the transformation and salt treatment on mycorrhization. Percent root colonization was determined using the gridline intersect method (Giovannetti and Mosse 1980) after clearing and staining with 0.05% trypan blue in lactoglycerol (Koske and Gemma 1989; Philips and Hayman 1970). The AM fungi colonized both the nontransgenic and the transgenic plants to the same level (Figure 1). The mycorrhizal structures, including intracellular hyphae, arbuscules, and vesicles, were similarly observed in the nontransgenic and transgenic plants (Figure 2). An ANOVA of the arcsine-transformed mycorrhizal colonization data showed no significant differences between the nontransgenic and transgenic genotypes ($p=0.506$) (Table 1). In a comparison of treated plants that were inoculated and watered with tap water and those that were watered with salt water, the salt water treated plants reduced colonization levels to

less than half (Figure 1). The ANOVA revealed that the percentage of colonization was significantly lower in the salt treatment ($p<0.001$) (Table 1), which indicates that the influence on colonization was stronger in the environment (saline or nonsaline) than in the transformation with the *mangrin* gene. Some researchers reported that high salt concentration significantly lowered the levels of root colonization by AM fungi (Aliasgharzadeh et al. 2001; He et al. 2007; Juniper and Abbott 1993). Results from this study corroborate those of other reports, which found no influence on mycorrhizal colonization in transgenic plants (Knox et al. 2008; Powell et al. 2007). Several other published studies describe the effect of transgenes on mycorrhizae. Vierheilig et al. (1995) showed that transgenic tobacco, expressing pathogenesis-related proteins or chitinases, did not affect mycorrhizae. Symbioses with mycorrhizal *Glomus mosseae* were not significantly affected in aubergine (*Solanum melongena*) constitutively expressing a natural antimicrobial peptide isolated from dahlia and resistant to pathogenic *Botrytis* and *Verticillium* (Turrini et al. 2004). One report on mycorrhiza in field-planted transgenic aspen trees found that observed mycorrhizal fungi, associated with most transgenic trees, showed the same colonization levels and diversity as those on nontransgenic trees (Kaldorf et al. 2002). However, more recent studies showed significantly reduced root colonization rates by AM fungi in transgenic apple plants constitutively expressing chitinases *nag70* and *ech42* (Schäfer et al. 2008). This underscores the need for a case-by-case evaluation since the potential effects of transformation may be influenced by both the transgene and the plant species.

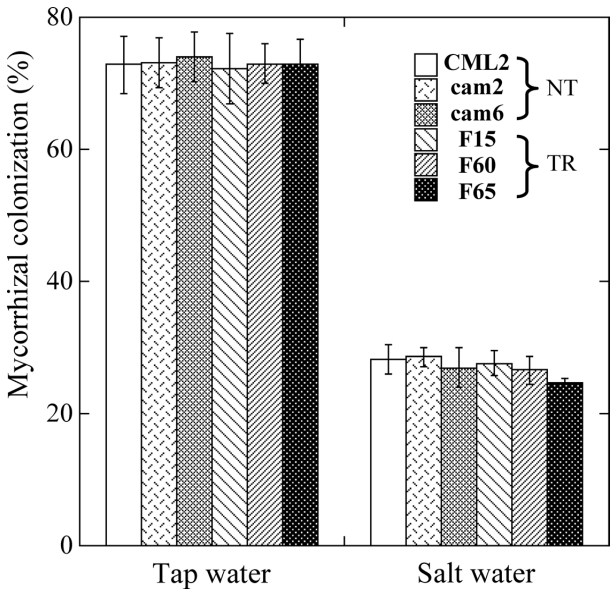

Figure 1. Mycorrhizal colonization in *mangrin* transgenic *E. camaldulensis*. Three nontransgenic lines (NT: CML2, cam2, and cam6) and three *mangrin* transgenic lines (TR: F15, F60, and F65) were used in the experiment. The plants were inoculated with arbuscular mycorrhiza (*Glomus intraradices* and *Glomus etunicatum*) divided into two groups each consisting of five plants per line. One group was watered with tap water and the other group was watered with 200 mM NaCl. The error bars indicate SE.

To determine the association between the transgene and AM fungi in protecting the plant from salt stress, chlorophyll contents were measured as an index of salt

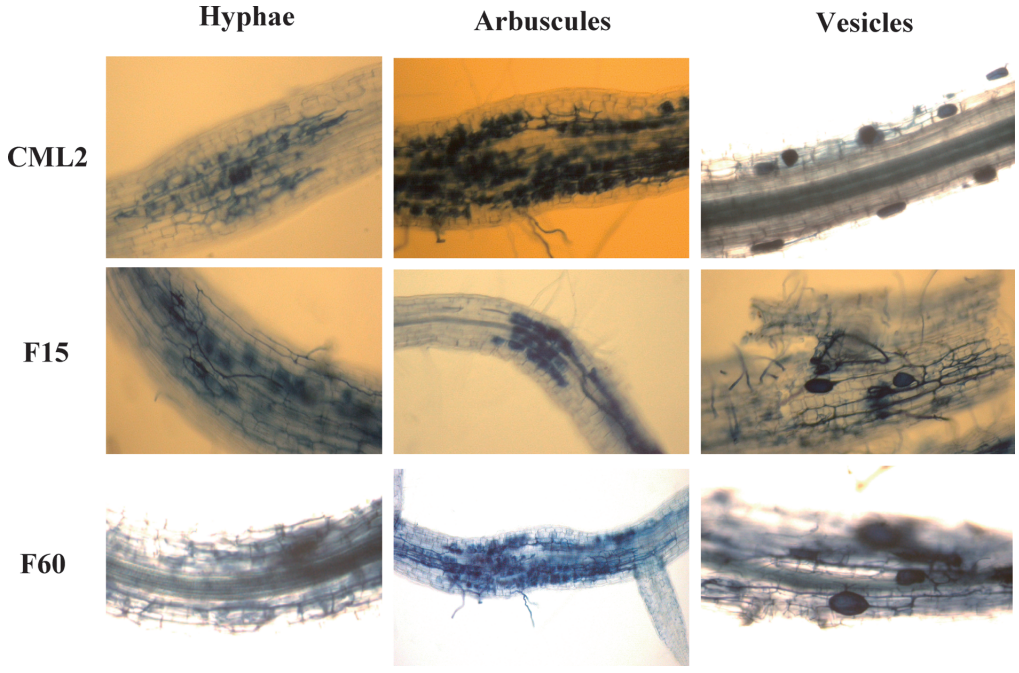

Figure 2. Representative photographs of mycorrhizal root structures showing hyphae, arbuscules, and vesicles. Line CML2 represents the nontransgenic line, and lines F15 and F60 represent the *mangrin* transgenic lines; all were from the water treatment.

Table 1. ANOVA of percent AM fungi colonization

Source	df	Sum of squares	Mean square	F-value	*P*	Significance
Genotypes (G)	1	20.417	20.417	0.448	0.506	ns
Treatment (T)	1	31602.150	31602.150	693.319	0.000	***
G×T	1	3.750	3.750	0.082	0.775	ns
Error	56	2552.533	45.581			

Effects of plant genotype (G: nontransgenic or *mangrin* transgenic), treatment (T: tap water or 200 mM NaCl), and both interactions. ns and *** indicate no significance and significant difference ($p<0.001$), respectively.

Table 2. Chlorophyll contents in leaves

Genotype	Line	−M−S	−M+S	+M−S	+M+S
Nontransformant	CML2	37.8 ± 3.1	32.3 ± 2.5	37.1 ± 3.7	36.8 ± 1.3
	cam2	35.0 ± 2.8	27.2 ± 1.4	40.1 ± 2.1	37.9 ± 7.3
	cam6	35.6 ± 2.1	36.8 ± 1.4	41.3 ± 2.8	35.9 ± 4.1
Transformant	F15	34.9 ± 8.2	38.0 ± 4.6	40.7 ± 3.7	38.7 ± 1.8
	F60	38.3 ± 2.4	36.4 ± 2.8	38.6 ± 2.5	37.9 ± 7.3
	F65	37.8 ± 2.3	37.2 ± 1.7	40.7 ± 1.6	38.3 ± 2.5

Plant material consisted of three nontransgenic (CML2, cam2, and cam6) and three transgenic (F15, F60, and F65) plant lines. Treatments were as follows: −M−S, without mycorrhiza without salt; +M−S, with mycorrhiza without salt; −M+S, without mycorrhiza with salt; and +M+S, with mycorrhiza with salt. The water treatments were irrigated with tap water, and the salt treatments were irrigated with 200 mM NaCl. Chlorophyll contents were measured at four weeks using a SPAD chlorophyll meter. Error bars are SE.

Table 3. ANOVA on chlorophyll content

Source	df	Sum of squares	Mean square	*F*-value	*P*	Significance
Lines (L)	5	128.42	25.68	0.77	0.580	ns
Inoculation (I)	1	266.42	266.42	7.95	0.007	**
Treatment (T)	1	115.77	155.77	3.46	0.666	ns
Treatment (T)	5	92.61	18.52	0.55	0.737	ns
L×T	5	49.49	9.89	0.30	0.913	ns
I×T	1	18.50	18.50	0.55	0.467	ns
L×I×T	5	171.31	34.26	1.02	0.415	ns
Error	48	1607.94	33.49			

Effects of the plant line (L: CML2, cam2, cam6, F15, F60, or F65), mycorrhizal inoculation (I: inoculated or noninoculated), salt treatment (T: tap water or 200 mM NaCl), and their interaction. ns and ** indicate no significance and significant difference ($p<0.01$), respectively.

stress tolerance. The chlorophyll contents were determined as a SPAD value using the SPAD chlorophyll meter (SPAD 502, Konica Minolta, Tokyo, Japan). The process was repeated three times: before the start of salt treatments, two weeks after salt treatments, and four weeks before the end of the experiment (Table 2). We conducted an ANOVA to evaluate which factors influence chlorophyll contents among lines, AM fungi inoculation, and salt treatment. No significant difference was found without AM fungi inoculation ($p<0.01$) (Table 3). Inoculation of *E. camaldulensis* with AM fungi also seemed to enhance plant growth and maintain higher chlorophyll contents (Abdel-Fattah and Mohamedin 2000). The ANOVA results showed that both the salt treatment and transgene had no effect on chlorophyll contents.

The data for each line in the nontransgenic and transgenic genotypes were then combined (Figure 3). Results showed that the chlorophyll contents in the nontransgenic genotypes were significantly decreased ($p<0.05$) by the salt treatment but were not altered in the transgenic genotypes (Figure 3). Because the nontransgenic genotypes were less salt-tolerant, salinity damage was easily detectable by the treatment with salt water (200 mM NaCl). The transgenic genotypes were salt-tolerant and, therefore, they might not have been affected by such a weak salt stress. In the inoculated treatment, the chlorophyll contents of both genotypes were decreased by the salt treatment, although not significantly (Figure 3). The salt treatment reduced the AM fungi colonization level in all lines (Figure 1), and this reduction might have caused the decrease in chlorophyll contents.

Our results indicated that the effect of salt on chlorophyll content was alleviated by inoculation with AM fungi in the nontransgenic genotype (Figure 3). This finding might also be due to the positive effects of AM fungi on plant growth and nutrient acquisition as reported in other AM fungi-inoculated plants grown under saline stress (Giri and Mukerjii 2004; Rabie 2005). In another study, higher chlorophyll contents were reported in mycorrhizal zucchini plants grown under

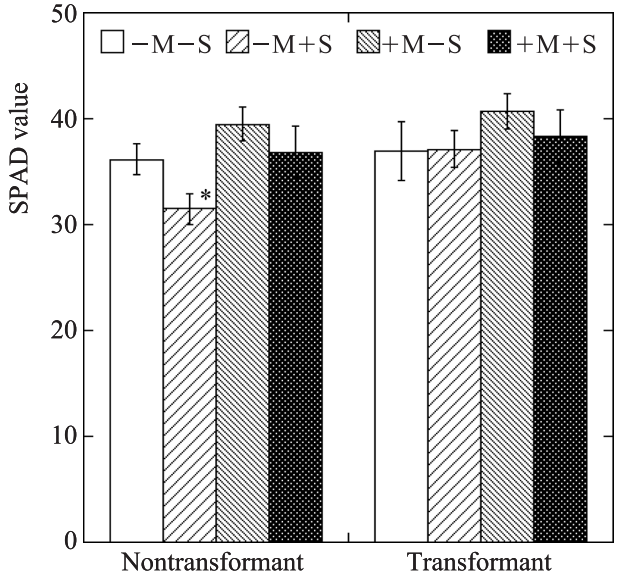

Figure 3. Change in chlorophyll contents. Treatments were −M−S, without mycorrhiza without salt; +M−S, with mycorrhiza without salt; −M+S, without mycorrhiza with salt; and +M+S, with mycorrhiza with salt. Salt treatment was watered with 200 mM NaCl. The water treatments (tap water) salt treatments (200 mM NaCl). The values were measured at four weeks using the SPAD chlorophyll meter. The error bars indicate SE. Asterisk denotes a significant difference from −M−S ($p<0.05$).

saline conditions (Colla et al. 2008). Although the exact mechanisms by which AM fungi alleviate salt stress remains unresolved, the mediation of metabolic processes by phosphorus and the compartmentalization of sodium within some tissues, including AM hyphae in the roots, could be contributing factors (Cantrell and Linderman 2001).

AM fungi colonized these transformants as well as nontransformants (Figures 1, 2). Studies indicate that transgenic *E. camaldulensis* with the *mangrin* gene is substantially equivalent to nontransgenic *E. camaldulensis* in terms of AM fungi colonization. However, AM fungi colonization conferred salt tolerance to the nontransformants at 200 mM NaCl (Figure 3). Inoculation did not assist significantly in the enforcement of salt tolerance on transformants. The treated condition is thought to be a weak stress for the transformants, as mentioned above. The application of 200 mM NaCl decreased the colonization rate of AM fungi to less than half (Figure 1) and may be the application limit for those AM fungi strains. Inoculation of salt-tolerant AM fungi may lead to enhanced performance of salt-tolerant transgenic plants such as the transgenic *E. camaldulensis* with the *mangrin* gene used in this study.

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