Effect of Antimicrobial Peptides (AMPS) on Micorrhizal Associations

James McD. Stewart, Camila Nader, and Kanniah Rajasekaran

RESEARCH PROBLEM

The purpose of this research project is to provide information with which regulatory agencies can make informed decisions. The present study assessed the ability of genetically modified tobacco plants expressing antimicrobial genes to establish mycorrhizal associations. Since the mycorrhizal associations are fungal in nature, antimicrobial peptides (AMPs) are expected to be detrimental to the associations. The effects of these AMPs on mycorrhizal fungi are accepted as good indicators of their effect on soil microbiota in general. The objective of the current research is to evaluate the effect of expression of the antimicrobial peptides MSI-99 and D4E1 on the formation of mycorrhizal associations in roots of tobacco plants transformed with the respective genes.

BACKGROUND INFORMATION

AMPs expressed in genetically modified organisms (GMOs) have the potential to affect non-target organisms. From the perspective of environmental risk-assessment, the effect of each new integrated gene on soil-borne microbiota must be evaluated. Due to the difficulty of conducting full-scale field trials and the potential of (unknown) environmental consequences of field releases, laboratory-scale methodologies provide a good alternative for the initial evaluation (Kowalchuk et al., 2003). Tobacco, a species that is easy to regenerate and prone to develop mycorrhizal associations (Harley and Harley, 1987; Wang and Qiu, 2006), provides a valuable tool for relatively rapid assessment of risks associated with microbe-transgenic plant interactions before more recalcitrant crop species, such as cotton, are genetically engineered to express the AMPs. The hypothesis upon which this work is based is that expression of AMPs in the roots of transgenic tobacco plants will interfere with recognition events and establishment of symbiotic mycorrhizal associations because of their deleterious effect on mycorrhizal fungi.
RESEARCH DESCRIPTION

The mycorrhizal fungal species *Glomus mosseae* and *Gigaspora rosea* were tested for their ability to form mycorrhizal associations in two transgenic tobacco lines and a control wild-type plant. Transgenic *Nicotiana tabacum* L. cv. ‘Petit Havana’ and ‘Petit Havana’ seeds expressing DE1, an analog of cecropin under the control of an enhanced 35 S promoter (Cary et al., 2000), and MSI-99, an analog of Magainin 2 expressed in the plastids (DeGray et al., 2001), under the control of the 16S promoter, respectively, were tested. Wild-type *Nicotiana tabacum* L. cv. Xanthi plants, which have the same genetic background as the other two cultivars, were used as controls.

Tobacco seeds were sterilized by placing them in a microwave oven on high for 7 minutes. After being cooled for two minutes and microwaved once again for 8 minutes (Franco, non-published), transgenic and control seeds were germinated on 1% agar–0.25 MS medium containing 50 μg/mL of kanamycin or 500 μg/mL of spectinomycin for DE1 and MSI-99 lines, respectively. Wild-type plants were germinated without antibiotic. After five weeks, seedlings were transplanted to culture tubes filled one-third with sterile vermiculite and watered with 8 mL of one-half strength Hoagland’s solution. The tops of the tubes were covered with plastic wrap, and the seedlings grown at 25°C and 16-hr photoperiod.

The ‘sandwich system’ described by Giovannetti et al. (1993) was used. Sterile plants including roots were washed to remove vermiculite particles, and then the root systems were carefully spread on a cellulose nitrate filter (Whatman®, Kent, U.K.). Ten to 12 fungal spores were collected with forceps under a dissecting microscope and placed in direct contact with the root where lateral roots emerged. The roots were then covered with a second membrane, forming a “sandwich.” Two absorbent pads were added to ensure constant moisture and to provide support in the planting medium. The inoculated seedlings were planted in vermiculite in 36 cm² plastic pots, watered, and the pot covered with a plastic bag to avoid dehydration. Five replicates of each line/fungus were inoculated. The plants were grown at 25°C and 16-hr photoperiod and watered as needed. No nutrients were applied during this time.

Four weeks after inoculation, the roots were cleared and stained with 0.05% trypan blue in lacto-glycerol (Phillip and Hayman, 1970), then evenly distributed in a 88 mm diameter Petri dish. A grid of 20 lines (10 vertical and 10 horizontal; 9 x 9-mm squares) was placed on the bottom of the dish. Vertical and horizontal gridlines were scanned and the presence or absence of infection by mycorrhizal fungus was recorded at each point where the roots intersected a line. The total root length and mycorrhizal length were calculated by multiplying the number of root gridline intersects by 1.4141 (Giovannetti and Mosse, 1980). Data from each were collected and submitted to one-way ANOVA using JMP (version 6.0) after confirming equal variances and normal distribution of the samples. Means were compared using Student’s t LSD and Tukey-Kramer HSD test.

RESULTS AND DISCUSSION

The mean percentages of colonization by *G. mosseae* and *G. rosea* in the expressing lines and wild type are given in Tables 1 and 2, respectively. Statistical analysis
Summaries of Arkansas Cotton Research 2007

showed no significant differences in the percentage of colonization between D4E1 and WT plants by either *G. mosseae* or *Gi. rosea*. Surprisingly, transplastomic lines with MSI-99 showed a higher percentage of mycorrhizal colonization than control plants. Non-significant effects on mycorrhizal associations were observed previously in lines over-expressing pathogenesis-related genes (Vierheilig et al., 1995). Additionally, the expression of a defensin affected pathogenic fungus, but not AMFs (Turrini et al., 2004).

Since the transgene did not inhibit development of mycorrhizal associations, the original thesis (inhibition of mycorrhizal associations by AMPs) is false under the conditions and plant materials used in these experiments.

**PRACTICAL APPLICATION**

Considerable economic loss occurs in crops due to microbial pests. The tendency is to genetically engineer the crop plant to express AMPs without fully understanding the beneficial role certain microbes play in crop production. This is especially true for fungi that are involved in the formation of mycorrhizal associations. The results from the research reported here provide information about the ability of the transgenic plants to establish mycorrhizal associations in the presence of endogenous AMP concentrations that inhibit fungal pathogens.

**LITERATURE CITED**


protein from *Dahlia merckii* expressed in *Solanum melongena* is released in root exudates and differentially affects pathogenic fungi and mycorrhizal symbiosis. The New Phytologist 163:393-403.


### Table 1. Percentage of colonization by *Glomus mosseae*. Means from one-way anova.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of colonization&lt;sup&gt;z&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4E1</td>
<td>64.23 ± 2.53</td>
</tr>
<tr>
<td>MSI-99</td>
<td>81.64 ± 2.53&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT</td>
<td>58.65 ± 2.53</td>
</tr>
</tbody>
</table>

<sup>y</sup> Mean significantly different (P = 0.05) from wild-type plants.

### Table 2. Percentage of tobacco root colonization by *Gigaspora rosea*. Means from one-way anova.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% of colonization&lt;sup&gt;z&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4E1</td>
<td>55.70 ± 3.81</td>
</tr>
<tr>
<td>MSI-99</td>
<td>74.27 ± 3.31&lt;sup&gt;y&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT</td>
<td>52.00 ± 3.81</td>
</tr>
</tbody>
</table>

<sup>y</sup> Mean significantly different (P = 0.05) from wild-type plants.