Pleiotropic Effects in Transgenic Tobacco Plants Expressing the Oryzacystatin I Gene

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Abstract. A study was undertaken to evaluate possible phenotypic alterations in transgenic tobacco (Nicotiana tabacum L. cv. Xanthi) plants expressing the oryzacystatin I gene. Morphophysiologic parameters, such as growth rate, biomass, and leaf, flower, and fruit characteristics were analyzed. Transgenic plants overexpressing the cysteine proteinase inhibitor showed increased growth rate and dry weight, earlier flowering, and increased numbers of flowers and seeds. These pleiotropic effects were correlated with expression of the transgene (as indicated by PCR) were considered as control plants.

Materials and Methods

Plant material. Transgenic Nicotiana tabacum plants (cv. Xanthi) used in this study were obtained as described previously (Gutiérrez-Campos et al., 1999) and contained the oryzacystatin I cDNA under the control of the 35S CaMV promoter. The transgenic plants were maintained in a greenhouse at 25°C with daily irrigation and were fertilized with one-half strength Murashige-Skoog (MS) salts every 3 weeks.

Analysis of papain inhibition in crude extracts from R0 transgenic plants. Based on inhibition assays, two lines showing the highest and the lowest accumulation of the inhibitor were selected from all the R0 transgenic lines. Crude extracts from line H3 inhibited papain activity 43%, whereas crude extracts from line E5 inhibited it only 5% (Table 1).

Results and Discussion

Analysis of papain inhibition in crude extracts from R0 transgenic plants. Based on inhibition assays, two lines showing the highest and the lowest accumulation of the inhibitor were selected from all the R0 transgenic lines. Crude extracts from line H3 inhibited papain activity 43%, whereas crude extracts from line E5 inhibited it only 5% (Table 1).

Phenotypic analysis of transgenic plants. Growth. A direct relation was evident between the expression level of the inhibitor and growth rate (Fig. 1). In all determinations, all plants from line H3 were taller than control plants, whereas plants from line E5 were comparable to control plants in height (Fig. 2). These results are supported by biomass determinations, where the dry weight of plants from line H3 was 34% greater than the controls (Table 2). Interestingly, the growth rate of plants from line H3 fell from 15.4% to 11.1% of the control plants between 85 and 90 d after germination (Fig. 1). By this time, plants showed signs of advanced senescence. These results suggest that constitutive expression of the oryzacystatin I not only accelerates the growth rate but also shortens the life cycle.

Table 1. Inhibition of the activity of papain by crude extracts from transgenic plants expressing the oryzacystatin I gene.

<table>
<thead>
<tr>
<th>Control</th>
<th>100.0</th>
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</thead>
<tbody>
<tr>
<td>E5</td>
<td>94.6 ± 4.2</td>
</tr>
<tr>
<td>B4</td>
<td>93.9 ± 0.65</td>
</tr>
<tr>
<td>C3</td>
<td>62.4 ± 0.08</td>
</tr>
<tr>
<td>H3</td>
<td>56.8 ± 0.05</td>
</tr>
</tbody>
</table>

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The data are presented as the reduction in papain activity relative to control (not transformed) plants (100% papain activity) and represents the means of three replicates.
**Effect on leaves.** No significant differences were found in the number of leaves or axillary buds in any transgenic line examined (data not shown). However, leaves from transgenic plants were larger and darker (Fig. 2).

**Effect on flowering.** An inverse relation was found between the expression level of the inhibitor and time of appearance of floral buds. Flowering occurred 25 d earlier in line H3 plants than in control plants (Table 2). These results support the earlier suggestion that constitutive expression of the inhibitor shortens the life cycle of transgenic tobacco plants.

Plants with a higher level of inhibitor (H3) produced more flowers than did control plants (Table 2). However, some aspects of flower morphology, such as size, and width, or weight of the pistil, were not affected (data not shown).

**Effect on fruit.** In agreement with the results on flowering, plants from line H3 produced more fruit per inflorescence than did control plants, although the differences were nonsignificant (18.8 vs. 16.0). Size and weight of the fruits was similar in transgenic and control plants (data not shown).

**Effect on number of seeds and seed germination.** Fruit in all transgenic plants contained more seeds than did fruit of control plants, including those with a low level of inhibitor. Even lines with low levels of the inhibitor, such as E5, produced more seeds than did the control (Table 2). However, germination percentage was not affected (Table 2).

These results demonstrate unexpected phenotypic effects induced by constitutive expression of the oryzacystatin I in transgenic tobacco lines. Because higher plants contain endogenous cysteine proteinases involved in different processes (Kato et al., 1999; Yonezawa et al., 1998), we originally had hypothesized that constitutive expression of the inhibitor might be detrimental. However, the changes observed in growth rate, biomass, flowering time and number, and morphology of flowers and seeds are beneficial traits for many agricultural crops. Cysteine proteinases have recently emerged as key enzymes in the regulation of programmed cell death of animals and plants (Solomon et al., 1999), and have an important role in plant senescence (Guerrero et al., 1998). It is tempting to speculate that constitutive expression of the inhibitor disrupts the normal activity of the cysteine proteinases involved in these last processes, resulting in an accelerated growth rate and altered programmed cell death.

We obtained very similar results to those presented here using a different tobacco cultivar Vena Amarilla (data not shown). Currently, we are evaluating the effect of constitutive expression of inhibitors from different sources in transgenic plants of tomato (Lycopersicon esculentum Mill.) and Physalis ixocarpa Brot. ex Hornem.

Constitutive expression of the oryzacystatin I gene induces pleiotropic effects in transgenic tobacco plants. Clear alterations were induced in growth rate, biomass, flowering time, and number of flowers and seeds. If these effects can be demonstrated in other crops, the expression of cystatin genes may be utilized in a controlled manner to enhance desirable, specific, agronomic characters.

### Literature Cited


