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. Morphophysiological 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 level of oryzacystatin I in transgenic lines. The results suggest that oryzacystatin I disrupts the normal activity of cysteine proteinases, which are involved in important physiological processes.

Cystatins are strong, natural inhibitors of cysteine proteinase activity. They have been identified in a wide variety of organisms ranging from bacteria to human cells (Turk and Bode, 1991). They occur in seeds (Fernandes et al., 1993), tubers (Waldron et al., 1993), and ripening fruit (Dopico et al., 1993). In contrast to the substantial progress achieved in characterizing the structure of plant cystatins, the physiological function of these proteins is not well understood. Nevertheless, at least two functions have been proposed: regulation of protein turnover and host plant defense against insects (Botella et al., 1996). Because a number of insects and nematode species contain cysteine proteinases in their intestines, where food is digested, overexpression of cystatins in transgenic plants may be an efficient way to control those plant pests (Ryan et al., 1990).

One of the best characterized plant cystatins, identified from rice (*Oryza sativa* L.) seeds, is oryzacystatin I (Abe et al., 1987), which has been overexpressed in transgenic plants and has insecticidal activity (Orr et al., 1994). The cysteine proteinases of higher plants may fulfill an important role in cell metabolism, and their inhibition by cystatin overexpression in

transgenic plants might cause undesirable phenotypic alterations. To address this issue, a study was undertaken to examine the phenotypic characteristics of transgenic tobacco plants overexpressing the oryzacystatin I gene.

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 the expression level of the inhibitor in transgenic lines. The quantification of inhibitor in leaves of parental lines was performed by a papain inhibition assay according to Masoud et al. (1993). Initially, crude extracts from leaves were prepared in phosphate buffer (0.13 M NaCl, 0.002 M KCl, 0.01 M Na₂HPO₄, 0.001 M KH₂PO₄, pH 7.0) and centrifuged at 10,000 × g_{av}. Protein concentration was determined in the supernatant by the Bradford (1976) method. Papain (1.5 µg) was added to 75 µg of total soluble protein plus L-cysteine as activator and the mixture was preincubated at 37 °C for 15 min. Subsequently, 2 mg of n-benzoyl L-arginine p-nitroanilide (BAPNA) (Sigma Chemical Co., St. Louis) were added and the mixture was incubated for a further 60 min at 37 °C. Enzyme activity was determined by absorbance at 410 nm. Values obtained were expressed as

a percentage of the activity of control extracts (no inhibitor present). The transgenic lines obtained were selfed and kept at 25 °C for seed production.

Selection of homozygous plants. Seeds from the R1 progeny from plants showing the highest (H3) and the lowest (E5) accumulation of inhibitor, and in which previous studies had indicated that the transgene had segregated in a 3:1 ratio, were germinated in MS medium containing 120 µg·L⁻¹ kanamycin (Sigma). Plants showing increased vigor, growth, and papain inhibition were considered homozygous; those plants not showing incorporation of the transgene (as indicated by PCR) were considered as control plants.

Phenotypic analysis of transgenic plants. The following phenotypic characters were examined in 18 individuals of the R1 progeny of the two selected transgenic lines: height, growth rate, biomass, number of leaves and axillary buds, time of flowering, and number and morphology of flowers and fruit. Data were tested by analysis of variance. When significant differences were found, the values were compared according to the Tukey–Kramer multiple comparison test ($P \leq 0.01$).

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 that of the controls (Table 2). Interestingly, the growth rate of plants from line H3 fell from 154% to 111% 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.

	Papain activity (%) ^a
Control	100.0
<i>Transgenic line</i>	
E5	94.6 ± 4.2
B4	93.9 ± 0.65
C3	62.4 ± 0.08
H3	56.8 ± 0.05

^aThe data are presented as the reduction in papain activity relative to control (not transformed) plants (= 100% papain activity) and represent the means of three replicates.

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Fig. 1. Growth rate of transgenic plants expressing the oryzacystatin I gene. The values shown represent the growth rate of transgenic plants as a percentage of that of control plants. The values represent the mean of 18 individual plants.

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.

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Fig. 2. A comparison of a control and a transgenic plant. Seeds from control and transgenic plants were germinated and seedlings maintained in a greenhouse at 25 °C. Plants were irrigated daily and were fertilized with one-half strength MS salts every 3 weeks. The plants shown are 50 d old. (left) Control plant; (right) transgenic plant (line H3).

Table 2. Effect of oryzacystatin I gene expression on phenotypic characters of tobacco plants.

Characteristic	Genotype		
	Control	E5	H3
Dry wt. (g)	58 a ²	60 a	77 b
Time to flower (d)	100 a	95 a	76 b
No. flowers/inflorescence	15 a	14 a	20 b
No. seeds/fruit	2378 c	2671 a	3076 b

²Mean separation within characteristics by Tukey-Kramer multiple comparison test, $P \leq 0.01$. Each value is the mean for 18 observations.

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