PERFORMANCE OF TRANSGENIC *PETUNIA* X *HYBRIDA* PLANTS CONTAINING THE P_{SAG12}-*ipt* GENE GROWN UNDER NUTRITIONAL DEFICIENCY[1]

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ABSTRACT

Nutritional deficiency in plants can causes chlorosis, necrosis, defoliation, reduced growth, reduced yield, and premature senescence. Exogenous applications of cytokinin can alleviate these symptoms. This study was conducted to evaluate the effects of an auto regulated cytokinin production on the nutritional deficiency tolerance in *Petunia x hybrida* plants. A chimeric gene of the senescence-specific SAG12 promoter and the *ipt* gene coding for *isopentenyl transferase*, a rate-limiting enzyme in the cytokinin biosynthesis pathway, was introduced into *Petunia x hybrida* plants by *Agrobacterium*-mediated transformation. Two transgenic plants containing the *ipt* gene were selected and self-pollinated to obtain the lines evaluated for nutritional deficiency tolerance analysis. Both transgenic P_{SAG12}–*ipt* lines analyzed were more tolerant to nutritional deficiency than wild-type plants. The results indicated that endogenous cytokinin can counteract nutritional deficiency symptoms.

Key words: *Isopentenyl transferase* gene (*ipt*), cytokinin, transgenic, nutritional deficiency, *Petunia x hybrida*.

RESUMO

Avaliação de plantas transgênicas de *Petunia x hybrida* contendo o gene P_{SAG12}-*ipt* cultivadas sob deficiencia nutricional

Deficiência nutricional em plantas pode causar clorose, necrose, desfolha, redução do crescimento e da produtividade e senescência prematura. Aplicações exógenas de citocinina podem aliviar tais sintomas. Este estudo foi efetuado para avaliar os efeitos da produção auto-regulada de citocinina na tolerância de plantas de petúnia (*Petunia x hybrida*) à deficiência nutricional. Um gene quimérico contendo o promotor SAG12 unido ao gene *ipt*, o qual codifica a produção de *isopentenyl transferase*, uma enzima da rota metabólica da biossíntese de citocinina, foi introduzido em petúnia através de transformação mediada por *Agrobacterium*. Duas plantas contendo o gene *ipt* foram selecionadas e autopolinizadas para obtenção das linhas a serem submetidas à deficiência nutricional. Ambas as linhas P_{SAG12}–*ipt* avaliadas foram mais tolerantes à deficiência nutricional que as plantas do tipo selvagem. Os resultados indicam que a produção endógena de citocinina pode aumentar a tolerância das plantas à deficiência nutricional.

Palavras-chaves: *gene isopentenyl transferase (ipt)*, citocinina, transgênico, deficiência nutricional, *Petunia* x *hybrida*.

1. INTRODUCTION

Cytokinin is a class of plant hormones involved in several developmental and physiological processes (MOK & MOK, 1994). Cytokinin also acts controlling gene expression and repression to induce plants to physiological and morphological changes necessary to adapt them to the environment (HARE et al., 1997). Under stressing conditions, such as

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nutrient deficiency, which can lead to senescence processes, cytokinin level decreases as a plant response to the adverse conditions (HARE et al., 1997).

Cytokinin exogenous applications alleviate symptoms of restricted mineral supply (KUIPER et al., 1989; HARE et al., 1997). The plant gene that encodes the first enzyme involved in the cytokinin synthesis process has not been identified yet; however, an *Agrobacterium tumefaciens* gene that encodes the same enzyme was identified and isolated, the *isopentenyl transferase* (*ipt*) gene (GAN & AMASINO, 1996). Through genetic engineering using a chimeric gene containing the *ipt* gene linked with the promoter senescence-associated gene 12 (SAG12) the *ipt* gene expression occurs under senescence signals and then the IPT enzyme catalyzes cytokinin production, which in turn delays senescence (GAN & AMASINO, 1995; GAN & AMASINO, 1996; DERVINIS, 1999). Without senescence signals, *ipt* gene expression is attenuated, due to the promoter activity (GAN & AMASINO, 1995; GAN & AMASINO, 1996). Considering that exogenous cytokinin application alleviate symptoms of nutritional deficiency and the auto regulated cytokinin production observed in transgenic plants containing the P_{SAG12}–*ipt* gene, *Petunia x hybrida* plants containing the P_{SAG12}–*ipt* gene under nutritional deficiency might increase the cytokinin production, as a response to the stressing condition, showing better horticultural performance than wild-type plants.

Petunia is an annual plant with a variety of colors of flowers, such as pink, red, pale, yellow, violet-blue, or white. Commercially petunias are propagated by seeds and used as bedding plant, or in various container types (porches, boxes, tubs, and hanging baskets) (DOLE, 1999). Petunia is a model plant system for genetic and cytogenetics research due to its genetic and physiological characteristics. *P. hybrida* is a diploid plant containing seven pairs of chromosomes and the cytological analysis allows distinguishing easily each chromosome, thus genetic analysis is not complicated (MAIZONNIER, 1984). Its life cycle is short, which decreases the time necessary to get experimental results and its flowering can be expected 8 weeks to 13 weeks after sowing (DOLE, 1999).

Few studies have evaluated petunia nutrition. It has been recommended to apply from 150 to 200 mg/L of 15N: 5P: 15K or 20N: 20P: 20K every time plants are watered (JOHNSON & SKELTON, 1972; BOODLEY, 1981; FRETT et al., 1985). The commercial nutritional recommendation for bedding plants, at continuous feeding, is 50 to 150 mg/L of 15N: 5P: 15K (Scotts - Sierra Horticultural Products Company, Marysville, OH, USA).

2. MATERIAL AND METHODS

The bacterial *ipt* gene involved in cytokinin biosynthesis was fused with the senescence-specific SAG12 promoter and with the marker gene for *neomycin phosphotransferase II* (*NPT II*) gene (GAN & AMASINO, 1995; DERVINIS et al., 1998). This genetic construct was introduced into petunia (*Petunia x hybrida* Hort. (Vilm.) cv. “V26”) via *Agrobacterium tumefaciens*-mediated transformation by DERVINIS et al. (1998). Several primary plants had the P_{SAG12}-*ipt* transgene presence confirmed (DERVINIS, 1999). Two of those transgenic plants containing the P_{SAG12}-*ipt* transgene were selected, named plant 1 and plant 2, and self-pollinated to obtain two lines of seeds for further analysis (DERVINIS, 1999).

Wild type seeds of commercial *Petunia x hybrida* Hort. (Vilm.) cv. V26, and seeds obtained from self-pollinated plant 1 and self-pollinated plant 2, were grown in a pad and fan cooled greenhouse (programmed to vent during the day/night at 27 °C/ 21°C). Before transplant plants were fertilized by hand every other irrigation with 150 mg/L of 15N: 5P: 15K 15K Cal-Mag, Water Soluble Fertilizer Miracle-Gro® - Excel Fertilizer (Scotts - Sierra Horticultural Products Company, Marysville, OH, US). The gene insertion after transformation frequently occurs just in one single locus, heterozygous, so was necessary to verify in the progeny obtained by self-pollination which plants contained this gene. It has been demonstrated that the *NPT II* gene, as a marker, co-segregates with the transgene it is physically linked with (DERVINIS, 1999). In this way, the presence of NPT II protein indicates the presence of *NPT II* gene and P_{SAG12}-*ipt* gene in the screened plant. In this period line 1 and line 2 plants were ELISA (enzyme-linked

immunossorbent assay) tested using the "PathoScreen Kit for Neomycin Phosphotransferase II System" (Agdia Inc., Elkhart, IN, USA). Six weeks after seeds sowing forty-eight ELISA positive plants from line 1, forty-eight ELISA positive plants from line 2, and forty-eight wild-type plants were potted in 1.5 L pots. These pots were arranged in a factorial scheme 3 x 4 (three genotypes and four nutritional solutions) in three complete randomized blocks. Each group of four plants from the same genotype received, together with irrigation, one of four nutritional solutions, initially at 100 mL/plant, increasing over the time to 300 mL/plant. The nutritional solution were 0 mg/L (distilled water, as a control), 75 mg/L, 150 mg/L, and 300 mg/L of 15N: 5P: 15K Cal-Mag, Water Soluble Fertilizer Miracle-Gro® - Excel Fertilizer.

All plants were evaluated concerning with the following parameters presented:

a. Number of days to first anthesis from seed sowing: All plants were tagged when the first flower reached anthesis, and the number of days between first flower anthesis and seed sowing date were calculated. This evaluation ended eighty-three days after sowing.

b. Number of flowers per plant: Eighty-three days after sowing all flowers per plant were counted.

c. Number of branches per plant: Eighty-four days after sowing all stems that arose from the substrate surface and reached 8 cm up from the substrate surface, were counted on all plants.

d. Plant height: Eighty-four days after sowing all plants were measured from the substrate surface to the highest apical meristem.

e. Relative amount of chlorophyll in the leaves: Eighty-five days after sowing six representative leaves per plant had the relative chlorophyll amount per plant indirectly estimated using the SPAD-502 device (Specialty Product Agricultural Division - Minolta Camera Company, Ramsey, NJ, USA). Each individual measurement evaluated one area of 6 mm^2 (2 mm x 3mm). The SPAD-502 value per plant was obtained through the average of six sample measurements taken on six different leaves per plant, which were located in the first 8 cm above the substrate surface.

f. Average length of the longest root, average shoot biomass, average root biomass: Eighty-six days after sowing all shoots were cut at the level of the substrate surface and the root system was washed with water to remove the growing media. The length of the longest root was measured, and shoots and roots were placed apart in paper bags and dried in a Blue-M Drying Oven (Blue-M Electric Co., Blue Island, IL, USA) at 70°C for five days. The mass of the dried shoots and dried roots was determined using a Mettler PM 2000 balance (Mettler-Toledo Inc., Hightstown, NJ, USA). After shoots and roots biomass data were obtained, the ratio of the shoot biomass to the root biomass was calculated.

All data were statistically analyzed by calculating means, standard errors and factorial analysis using the SAS means procedure (SAS 6.0, Cary, NC, USA). The factorial analysis was performed with the Tukey's Honest Significant Difference Test. This research was conducted at University of Florida (Gainesville, FL, USA).

3. RESULTS AND DISCUSSION

The parameters analyzed demonstrated that wild-type plants showed the best vegetative and reproductive growth when fertilized with 150 mg/L of 15N: 5P: 15K, such as will be following demonstrated. At 0 mg/L and 75 mg/L of 15N: 5P: 15K nutritional deficiency symptoms were observed.

Roots are the primary sites of cytokinin biosynthesis, although there is some cytokinin biosynthesis in the shoots (HARE et al., 1997). Local cytokinin accumulation is known to increase the sink strength stimulating the import of photosynthetic products, and biomass accumulation to specific locations (RONZHINA & MOKRONOSOV, 1994). Indeed P_{SAG12}–*ipt* lines under absence of nutrient produced more root biomass than wild-type plants (Table 1). It is possible to suggest that nutritional deficiency activated the SAG12 promoter, and contrarily to the regular metabolism, these plants increased the cytokinin biosynthesis alleviating the restricted mineral supply symptoms. This supposition is consistent with the results verified in P_{SAG12}–*ipt* tobacco plants grown under limiting nitrogen supply, which showed higher cytokinin level than wild-type

plants (JORDI et al., 2000). At 75, 150, and 300 mg/L of fertilizer, all genotypes showed similar root biomass (Table 1). Under absence of nutrients, the data obtained for the length of the longest root agrees with the root biomass results, P_{SAG12}–*ipt* lines showed longest root system in comparison to the wild-type plants (Table 1). At 75 and 150 mg/L of fertilizer, line 1 plants showed longest root system in comparison to the wild-type plants (Table 1).

Increases in fertilizer concentration in petunia plants are directly related with increase in plant biomass accumulation (IERSEL-VAN et al., 1998), such as was verified for all genotypes (Table 1). At 0 and 75 mg/L of fertilizer, no significant difference was verified for shoot biomass of genotypes. At 300 mg/L of fertilizer, wild-type plants produced more shoot biomass than P_{SAG12}–*ipt* lines (Table 1). Less shoot biomass accumulated in P_{SAG12}–*ipt* lines comparatively with wild-type plants grown under 300 mg/L is hypothesized by JORDI et al. (2000). These authors hypothesized that under field conditions P_{SAG12}–*ipt* plants might have lesser utilization of nutrients than wild-type plants. This hypothesis is supported because, to optimize light and nutrients absorption, nitrogen distribution should be direct to the youngest leaves, where there is higher light intensity instead of to the oldest leaves, however a reversal of nutrient accumulation was observed in P_{SAG12}–*ipt* tobacco plants (JORDI et al., 2000).

Genotypes affected shoot to root ratio response to fertilization (Table 1). Smaller shoot to root ratios were observed for the lower nutrient concentrations tested (Table 1). These results were expected, considering the plant adaptation to sub-optimal growing conditions, with relatively more biomass accumulation to the root system instead of the shoots (MARDANOV et al., 1998). Comparing the shoot to root ratio, transgenic P_{SAG12}–*ipt* lines showed better adaptation to sub-optimal conditions than wild-type plants (Table 1).

Table 1. Means for shoots biomass, root biomass, length of the longest root, and ratio biomass of shoot to root, for wild-type and P_{SAG12}–*ipt* transgenic lines of petunia grown under various concentrations of fertilizer

	Genotypes	Fertilizer (mg/L)			
		0	75	150	300
Shoot biomass (g)	Wild-type	1.38 Ad	6.82 Ac	12.76 Abb	18.86 Aa
	Line 1	1.60 Ad	7.24 Ac	13.39 Ab	17.99 Ba
	Line 2	1.96 Ad	7.01 Ac	12.43 Bb	17.26 Ca
Root biomass (g)	Wild-type	0.25 Bd	0.42 Ac	0.61 Ab	1.12 Aa
	Line 1	0.51 Ac	0.54 Ac	0.72 Ab	0.99 Aa
	Line 2	0.42 Ac	0.47 Ac	0.74 Ab	1.00 Aa
Length of the longest root (cm) ...	Wild-type	20.48 Cc	22.22 Bb	26.08 Ba	25.78 Aa
	Line 1	22.83 Ac	23.03 Ac	27.46 Aa	26.08 Ab
	Line 2	21.85 Bc	22.31 Bc	26.27 Ba	25.10 Bb
Shoot to root ratio	Wild-type	5.52 Ac	16.23 Ab	20.92 Aa	16.84 Bb
	Line 1	3.14 Cc	13.41 Cb	18.60 Ba	18.17 Aa
	Line 2	4.67 Bc	14.90 Bb	16.80 Ca	17.26 Ba

Means within a column or a within a row followed by the same capital or small letter, respectively, are not significant different at p = 0.05, according to Tukey test.

Genotypes affected plant response to fertilization for plant height (Table 2). Under absence of nutrient, line 1 plants were shorter than wild-type plants (Table 2), as was observed in plants overproducing cytokinin (GAN & AMASINO, 1996). Shorter stature is a desirable commercial feature for petunia plants, which need applications of growth regulator to reduce stem growth. In the other concentrations, no significant difference was observed for height of wild-type plants and P_{SAG12}–*ipt* lines (Table 2).

Differences between two transgenic lines were expected because each transgenic plant produced came from a single transformation event. It is known that for each transformation event the number of transgene copies inserted can vary, the transgene can be inserted in different sites, and also gene silencing can occur (MAESSEN, 1997). These factors affect so the amount of cytokinin produced, and in consequence the plant performance under nutritional deficiency. This is the reason why it is necessary to evaluate several transgenic plants obtained, and its progeny, to verify which event will show the agronomic features desirable, and whether this feature will be stable over the generations.

All plants showed similar number of branches per plant in absence of fertilizer (Table 2). At 75 and 150 mg/L of fertilizer, transgenic P_{SAG12}–*ipt* lines produced a higher number of branches than wild-type plants (Table 2). Effects of cytokinin overproduction leading to higher number of branches are known (DERVINIS, 1999). Increases in branch numbers are commercially interesting to petunia because it leads to a compact growth habit.

Genotypes affected the plant response to fertilization for the time to first flower anthesis (Table 2). In the absence of fertilizer, P_{SAG12}–*ipt* lines required more time to reach the first flower anthesis than wild-type plants (Table 2). At 75 and 150 mg/L of fertilizer, line 2 plants required more time to reach first flower anthesis than wild-type plants (Table 2). Delays in the onset of flowering in ornamental plants increase the production time, affecting profitability. Effects of cytokinin on the onset of flowering have often been conflicting (MOK & MOK, 1994).

Reports have shown that increases in cytokinin delay flowering onset or accelerate it (CHAURDHURY et al., 1993). Petunia plants containing the P_{SAG12}–*ipt* gene had several morphological characteristics affected, such as plant height, number of branches per plant, length of the longest root, as well biomass allocation. Plant size is a characteristic closely related with flowering onset (POETHIG, 1990), and flowering and root initiation and/or elongation are usually antagonistic processes (POETHIG, 1990). All plants had flowering anthesis delayed under plentiful nutrient supply, 300 mg/L of fertilizer, comparatively with 150 mg/L of fertilizer, probably due to increased vegetative growth under high fertilizer concentrations in detriment of flowering, due to excess of nitrogen level (SALISBURY & ROSS, 1992).

Genotypes affected plant response to fertilization for the number of flowers per plant (Table 2). Wild-type plants and transgenic P_{SAG12}–*ipt* lines did not show significant difference in the number of flowers per plant in the absence of fertilizer (Table 2), because of inhibitory effects of essential mineral element deficiencies (MARSCHNER, 1986). At 75 and 300 mg/L of fertilizer, line 1 plants produced fewer flowers than wild-type plants (Table 2). At 75, 150, and 300 mg/L of fertilizer, line 2 plants produced fewer flowers than wild-type plants (Table 2). It is clear that the cytokinin overproduction reduced the number of flowers per plant, even under a good nutrient supply. At 150 mg/L of fertilizer, all plants produced more flowers than at 300 mg/L of fertilizer (Table 2). Nutritional deficiency or excess were evidently harmful to the number of flowers per plant (FRETT, 1985).

In the absence of fertilization, P_{SAG12}–*ipt* lines retained more chlorophyll in the lower leaves than wild-type plants (Table 2). Exogenous applications of cytokinin (SMICIKLAS & BELOW, 1992), post-harvest treatments (IRVIN & JOYCE, 1995), and *ipt* gene inserted into plants (DERVINIS, 1999; JORDI et al., 2000) have all been shown to reduce relative chlorophyll degradation. Cytokinin acts stimulating chloroplast development and maintenance (SALISBURY & ROSS, 1992). Similar results have been observed in plants grown under nutritional deficient solution containing cytokinin, where the nutritional deficiency symptoms were alleviated (KUIPER et al., 1989). At the other fertilizer concentrations, P_{SAG12}–*ipt* lines had a similar amount of chlorophyll in leaves as wild-type plants (Table 2).

The results presented provide direct evidence that P_{SAG12}–*ipt* lines show better adaptations to grow under poor nutrient supply than wild-type plants. This is consistent with the hypothesis that throughout evolution, increases in cytokinin level have helped plants to become adapted to different habitats

(AUER, 1997). The P_{SAG12}–*ipt* system may be also advantageous in short periods of nutritional deficiency that plants are subjected during the crop production. In nature nitrogen is slowly released, and is gradually made available to plant roots by various soil processes, most often in amounts low enough to limit the growth (THORSTEINSSON & ELIASSON, 1990). Nutritional deficiencies are usually observed during the crop production and in retail environments. Decreases in cytokinin level are not merely a transient phenomenon found only in starvation experiments, but can occur in adapted cultures growing at sub-optimal rates (THORSTEINSSON & ELIASSON, 1990). Cytokinin affects developmental and physiological processes that also may be favored with the P_{SAG12}–*ipt* technology. These may include increased tolerance to early-season water stress (AMBLER et al., 1992), and leaf diseases (AUER, 1997).

One conflicting question has been the occurrence of developmental abnormalities associated with *ipt* gene expression. Transgenic constructs containing the SAG12 promoter have been fine-tuned, reducing senescence symptoms in particular tissues or in stressing periods. Tobacco plants transformed with the P_{SAG12}-*ipt* gene did not show any abnormalities (GAN & AMASINO, 1995; JORDI et al., 2000). Nevertheless, in these petunia plants, containing the same system, the SAG12 promoter did not present complete regulation, and

Table 2. Means for plant height, number of branches per plant, number of days to anthesis, number of flowers per plant, and relative chlorophyll amount for wild-type and P_{SAG12}–*ipt* transgenic lines of petunia grown under various concentrations of fertilizer

	Genotypes	Fertilizer (mg/L)			
		0	75	150	300
Plant height (cm)	Wild-type	22.36 Ab	27.54 Aa	28.83 Aa	27.75 Aa
	Line 1	14.79 Bb	23.83 Aa	25.50 Aa	25.00 Aa
	Line 2	21.21 Ab	24.50 Aab	26.04 Aa	24.25 Aab
Number of branches per plant ...	Wild-type	1.00 Ad	1.67 Cd	5.17 Cb	7.17 Ba
	Line 1	1.00 Ad	4.33 Ad	6.55 Ab	8.33 Aa
	Line 2	1.00 Ad	2.83 Bd	5.83 Bb	6.83 Ba
Number of days to anthesis	Wild-type	72.73 Cb	72.25 Bb	73.58 Bb	77.58 Aa
	Line 1	80.08 Aa	73.75 ABb	74.09 Abb	79.33 Aa
	Line 2	76.75 Bb	76.58 Ab	77.33 Ab	80.83 Aa
Number of flowers per plant	Wild-type	4.82 Ac	13.67 Ab	26.17 Aa	9.33 Ac
	Line 1	2.50 Ab	6.08 Bb	21.91 Aa	1.75 Bb
	Line 2	3.08 Ab	5.83 Bb	12.75 Ba	3.25 Bb
Chlorophyll amount	Wild-type	4.36 Bc	32.95 Ab	43.49 Aa	47.21 Aa
(SPAD value)	Line 1	15.73 Ad	33.93 Ac	43.36 Ab	48.69 Aa
	Line 2	11.98 Ad	32.07 Ac	41.92 Ab	50.43 Aa

Means within a column or a within a row followed by the same capital or small letter, respectively, are not significant different at p = 0.05, according to Tukey test.

some *ipt* gene expression occurred without nutritional deficiency. This consequence was especially detrimental because fewer flowers were produced in P_{SAG12}–*ipt* plants, a very important feature to this specie. Apparently, this system did not work in the same way in tobacco and in petunia plants produced in earlier experiments. The remained question to be investigated is why the SAG12 promoter was differently regulated in this trial. Future researches involving transgenic P_{SAG12}-*ipt* plants including observations directed at investigating different stress mechanisms, and their effects on these plants, and the molecular analysis of cytokinin action might elucidate the remained questions about the involvement of this hormone coordinating plant response to stresses.

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