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Biochemical side effects of the herbicide FINALE® on *bar* gene-containing transgenic pineapple plantlets

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Abstract Pineapple is one of the most important tropical fruits and therefore intensive genetic improvement programs are being carried out in many countries, including Cuba. Our research team has previously introduced the *bar* gene, along with *chitinase* and *AP24* genes, into the pineapple genome. Herein, we report on the biochemical side effects of the herbicide FINALE® on these transgenic plantlets during hardening. Levels of aldehydes and chlorophylls, and peroxidase activity were recorded. The transformed clone studied here, not sprayed with FINALE®, showed the following side effects because of transgenesis only. Levels of malondialdehyde, other aldehydes, chlorophyll b, and total chlorophyll pigments decreased. The most remarkable biochemical differences between transgenic and non-transgenic plantlets after application of FINALE® follow. Levels of malondialdehyde and other aldehydes in transgenic material were not decreased by FINALE®, perhaps because these levels were already low as a result of transformation. FINALE® increased peroxidase activity in transgenic plantlets but such increase was higher in non-transgenic material. The herbicide increased contents of chlorophyll pigments (a, b, total) in transformed plantlets. However, as expected, non-transgenic plantlets decreased levels of chlorophylls (a, b, total) after application of FINALE®. The genetic transformation of pineapple with the *bar* gene not only conferred resistance to the herbicide FINALE®, but also promoted other biochemical changes.

Keywords *Ananas comosus* (L.) Merr. · Aldehydes · Chlorophyll · Peroxidases

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Introduction

Pineapple world production reached 18.2 million tons in 2006 (FAOSTAT 2008). Therefore, several research groups are developing basic and applied studies to create new varieties with better agronomic performance. We previously developed a protocol for pineapple genetic transformation introducing the *chitinase*, *AP24* and *bar* genes into the pineapple genome under the control of the following promoters: OCS-35S CaMV-rice actin I, 35S CaMV and maize Ubi1, respectively (Espinosa et al. 2002). These promoters have been described as constitutive transcription promoters (Franck et al. 1980; Christensen et al. 1992).

Chitinase and *AP24* have been described as antifungal genes. The *chitinase* gene (from *Phaseolus vulgaris*) product degrades chitin: an essential compound of most of the fungal cell walls (Broglie et al. 1986; Schlumbaum et al. 1986). The *AP24* gene

(from *Nicotiana tabacum*) codes for a protein that destabilizes the fungal membrane (Singh et al. 1989; Woloshuk et al. 1991). We introduced these two antifungal genes into the pineapple genome as an attempt to reduce the great losses caused by *Phytophthora nicotianae* var. *parasitica* (Kamoun 2001). These phytopathological studies are in progress in our field experimental station. Regarding the safety profile studies of these two genes, we have not found reports about *AP24*. However, class-I chitinases have been identified as the major panallergens in fruits associated with the latex-fruit syndrome, such as the uncooked-consumed avocado, banana and chestnut (Blanco et al. 1994; Brehler et al. 1997; Sanchez-Monge et al. 2000).

We used the *bar* gene as a selectable marker. It was cloned from *Streptomyces hygroscopicus* and encodes for phosphinotricin acetyltransferase (Thompson et al. 1987). This enzyme is capable of inactivating phosphinotricin that is the active compound of the non-selective herbicide FINALE® (Torres et al. 1999). After application of FINALE® onto non-transformed plants, phosphinotricin inhibits the enzyme glutamine synthetase (Mohapatra et al. 1999). Such an inhibition causes plant toxicity to ammonium provoking death (D'Halluin et al. 1992). Phosphinotricin acetyltransferase proteins have been deeply studied (Wehrmann et al. 1996) and have an excellent safety profile (Herouet et al. 2005).

The above mentioned references indicate that genes and promoters used for pineapple genetic transformation are well known, at least in their primary effect. However, biochemical side effects of the herbicide FINALE® on *bar* gene-containing transgenic pineapple plantlets have not been explored to date. Although several metabolic studies on transgenic plants have shown effects of transformation (Momma et al. 1999; Wilson and Latham 2006), investigation of the biochemical unexpected side effects could help to positively impact the public perception on genetically modified plant food (Kuiper et al. 2001).

Based on these prospects, we studied some of the biochemical side effects of pineapple genetic transformation in interaction with the herbicide FINALE®. The present report is focused on the evaluation of the early stage of greenhouse hardening of transformed and non-transformed pineapple plantlets. Levels of aldehydes and chlorophyll, and the activity of peroxidases were recorded. We selected these compounds because they are related to a wide range of important biochemical and physiological pathways such as plant response to stress and photosynthesis (Porras 1991; Gross et al. 2000; Moller 2001; Yaginuma et al. 2002). We explored other biomarkers different from those recommended by the OECD (1993) because we evaluated very young pineapple plantlets far for fruiting and marketing. Plant survival was also evaluated.

Materials and methods

Pineapple c.v. Serrana Smooth Cayenne was used. Transgenic plantlets were obtained according to Espinosa et al. (2002). Embryogenic calli were gently passed through polypropylene meshes (2000 μm pore diameter, Spectrum) and dried for 15 min in the laminar flow cabinet. *Agrobacterium tumefaciens* suspension was then added (strain AT2260, pHCA58, containing a *bar* gene controlled by maize Ubi 1 promoter, a class-I bean *chitinase* gene controlled by a hybrid OCS-35S CaMV-rice actin I promoter, and a tobacco *AP24* gene controlled by 35S CaMV promoter). After 10 min, infected calli were washed with distilled water and dried with filter paper. Co-culture was allowed for 24 h (darkness, 25°C). Calli were then transferred to the callus proliferation medium supplemented with 0.2 g l^{-1} cefotaxime (4 weeks, 25°C, 16 h light photoperiod, 2000 lux). Calli were transferred to temporary immersion bioreactors for plant regeneration in a selective medium (2.5 mg l^{-1} phosphinotricin). After 45 days, phosphinotricin-resistant plantlets were recovered. Non-transformed plantlets (control treatment) were obtained following the protocol described above but avoiding contact with *Agrobacterium tumefaciens* and phosphinotricin.

About 120 non-transformed and 120 transformed (one clone) plantlets were transferred to a greenhouse for hardening according to Yanes et al. (2000). Plantlets were placed in plastic trays containing 82 cm^3 of a mixture zeolite ± filter cake (1:1). Microject automated irrigations for 25 s every 30 min were applied. Plantlets were kept under a photosynthetic photon flux density of 458 μmol m^{-2} s^{-1}. Standard phytosanitary controls were applied. About 60 non-transformed and 60 transformed plantlets were sprayed with the herbicide FINALE® at 12 l ha^{-1}

as recommended by Bayer CropScience (2005). Evaluations started immediately after application of the herbicide (day 0: less than 1 min after herbicide application) and after 15 or 30 days. The experimental design was completely randomized. In a previous experiment (data not shown) we compared resistance to herbicide FINALE® of 100 transformed clones. In the present study we used the transgenic clone that previously showed the lowest foliar damage after application of FINALE®.

Plant survival was recorded. Leaf samples were stored in liquid nitrogen at 15 days after application (or not) of the herbicide. Each biochemical determination started from three independent pooled samples (100 mg each). They were finely grounded in liquid nitrogen. Contents of malondialdehyde and other aldehydes (Heath and Packer 1968), chlorophyll (a, b, total; (Porras 1991)), and peroxidase activity (Hammerschmidt et al. 1982) were measured. The experiment was repeated 3 times.

Results and discussion

Genetic transformation with the *bar* gene protected the plantlets from the effect of herbicide FINALE®. Most of non-transformed plantlets had died at 15 days after the herbicide application (Fig. 1a).

Biochemical analyses of surviving plant leaves (15 days), showed a detrimental effect of FINALE® on malondialdehyde level in non-transformed plantlets. However, this effect of the herbicide was not observed in transgenic plantlets. Level of malondialdehyde in non-transformed plantlets, not spayed with FINALE®, was relatively high (Fig. 1b). Regarding content of other aldehydes, results were similar to malondialdehyde evaluations (Fig. 1c). FINALE® increased peroxidase activity in both non-transformed and transformed plantlets but its effect on non-transgenic plantlets was more remarkable (Fig. 1d).

The herbicide dramatically decreased content of chlorophyll a in non-transformed plantlets. This may be the result of the deleterious effect of FINALE®. Contrastingly, the herbicide increased levels of chlorophyll a in transgenic plantlets. This experimental treatment reached the highest chlorophyll a level observed (Fig. 1e).

Levels of chlorophyll b and total chlorophyll contents were modified by FINALE® in the same way (Fig. 1f, g). Transgenic plantlets sprayed with the herbicide showed the highest total chlorophyll content recorded (Fig. 1g).

The transformed clone studied here, not sprayed with FINALE®, showed the following side effects because of transgenesis with *chitinase*, *AP24* and *bar* genes. Levels of malondialdehyde, other aldehydes, chlorophyll b, and total chlorophyll pigments decreased. For plants generated by recombinant technology, side effects (such as those observed in our experiment) may arise from the process of introducing foreign genes or as a result of the interaction among the transgene, the genetic background of the plant and the environment (Meyer 1999). Moreover, random insertion of DNA sequences can cause modification, interruption or silencing of existing genes as well as activation of silent genes (Codex 2003).

The most remarkable biochemical differences between transgenic and non-transgenic plantlets after application of FINALE® follow. Levels of malondialdehyde and other aldehydes in transgenic material were not decreased by FINALE®, perhaps because these levels were already low as a result of transformation. FINALE® increased peroxidase activity in transgenic plantlets but such increase was higher in non-transgenic material. The herbicide increased contents of chlorophyll pigments (a, b, total) in transformed plantlets. However, as expected, non-transgenic plantlets decreased levels of chlorophylls (a, b, total) after application of FINALE®.

Levels of malondialdehyde and other aldehydes, and peroxidase activity, have been described to be closely connected. Malondialdehyde is one of the primary metabolite of plant response to stress (e.g. herbicides, (Dumet and Benson 2000). It results from peroxidation of cell membrane lipids, and promotes formation of other aldehydes (Moller 2001). It is also well documented that as a result of reactive oxygen species action, besides aldehydes, hydrogen peroxide is formed. Then peroxidase activity is increased (Gross et al. 2000; Breusegem et al. 2001; Moller 2001; Arora et al. 2002; De Jong et al. 2002; Kuo and Kao 2004). According to these references, levels of malondialdehyde and other aldehydes, and peroxidase activity were expected to increase after FINALE® application. However, results shown in Fig. 1 (b, c) do not support these statements. Levels of malondialdehyde and other aldehydes were not

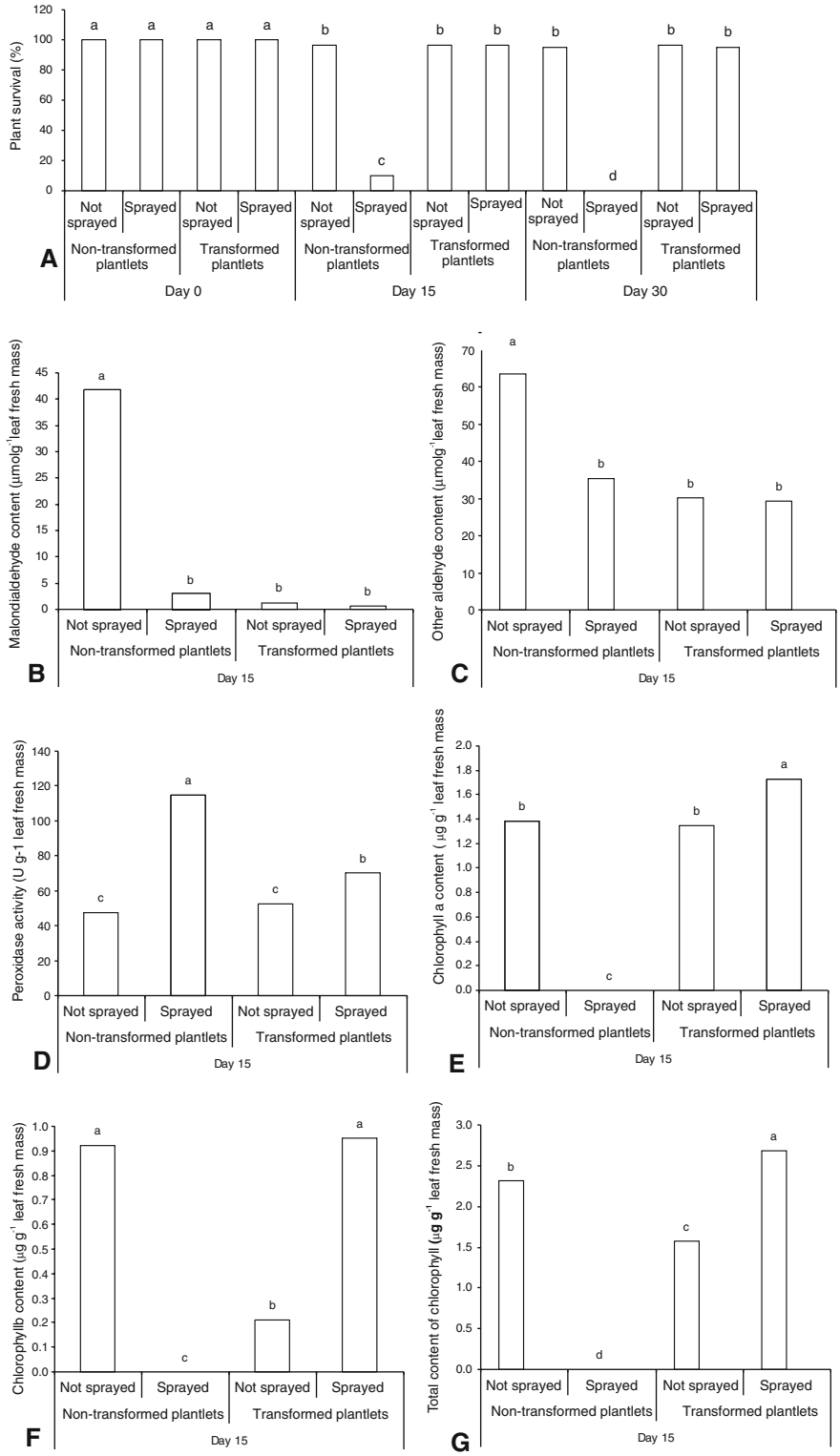

Fig. 1 Survival, levels of aldehydes and chlorophylls, and peroxidase activity during hardening of transformed or non-transformed pineapple plantlets, sprayed or not with herbicide FINALE®. For each indicator, results with the same letter are not statistically different (ANOVA, Tukey, $P > 0.05$, Statistical Package for Social Sciences, version 8.0 for Windows, SPSS Inc.). Plant survival percentages were transformed, for statistical analysis only, according to $y' = 2 \times (\text{arcsine } (y/100)^{0.5})$

increased by the herbicide. Perhaps, biochemical evaluations were performed too late (15 days) after application of the herbicide and therefore, the initial changes were not recorded. Only measurements of peroxidase activity agreed these previous reports (Fig. 1d).

Regarding measurements of chlorophyll pigments, it is convenient to take into consideration that reaction of glutamine synthetase 2 takes place in chloroplasts (Maldonado 1993) and this is the target enzyme inhibited by phosphinotricin (Metz et al. 1998). Therefore, it was expected that chlorophyll structures were affected in non-transformed pineapple plantlets after herbicide application (Fig. 1e, f, g). Contrastingly, the positive effect of FINALE® on formation of chlorophyll pigments (a, b, total) in transgenic plantlets was not previewed. It might be that stress caused by the herbicide on transgenic material, somehow destabilized temporarily the chloroplast structure. Then, transformed plantlets synthesized more chlorophyll to keep photosynthesis efficiency at the same level as under non-stress conditions. This kind of general physiological response to compensate for damages has been previously described (Scarpari et al. 2005). At present, pineapple transgenic plants are being studied at the Bioplant Center's Field Experimental Station.

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