

Safety assessment of nonbrowning potatoes: opening the discussion about the relevance of substantial equivalence on next generation biotech crops

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Summary

It is expected that the next generation of biotech crops displaying enhanced quality traits with benefits to both farmers and consumers will have a better acceptance than first generation biotech crops and will improve public perception of genetic engineering. This will only be true if they are proven to be as safe as traditionally bred crops. In contrast with the first generation of biotech crops where only a single trait is modified, the next generation of biotech crops will add a new level of complexity inherent to the mechanisms underlying their output traits. In this study, a comprehensive evaluation of the comparative safety approach on a quality-improved biotech crop with metabolic modifications is presented. Three genetically engineered potato lines with silenced polyphenol oxidase (*Ppo*) transcripts and reduced tuber browning were characterized at both physiological and molecular levels and showed to be equivalent to wild-type (WT) plants when yield-associated traits and photosynthesis were evaluated. Analysis of the primary metabolism revealed several unintended metabolic modifications in the engineered tubers, providing evidence for potential compositional inequivalence between transgenic lines and WT controls. The silencing construct sequence was *in silico* analysed for potential allergenic cross-reactivity, and no similarities to known allergenic proteins were identified. Moreover, *in vivo* intake safety evaluation showed no adverse effects in physiological parameters. Taken together, these results provide the first evidence supporting that the safety of next generation biotech crops can be properly assessed following the current evaluation criterion, even if the transgenic and WT crops are not substantially equivalent.

Keywords: biotech crop, potato quality improvement, GMO safety assessment, *Solanum tuberosum*, tuber browning, polyphenol oxidase.

Introduction

Polyphenol oxidases (PPO; EC 1.14.18.1 or EC 1.10.3.1) are very intriguing enzymes. Although they have been extensively studied for many years and the reactions they catalyse are well understood, their precise localization in defined metabolic pathways is still ambiguous. In plants, PPO are plastid-localized enzymes ubiquitous among angiosperms that catalyse the oxygen-dependent

conversion of phenolics to quinones (Thygesen *et al.*, 1995). In potato (*Solanum tuberosum*), PPO are coded by a multigene family consisting of at least six members with a complex pattern of spatial and temporal gene expression. Three of them, *Pot32* (U22921), *Pot33* (U22922) and *Nor333* (M95196), are expressed in tubers (Thygesen *et al.*, 1995). Additionally, *Nor333* is also expressed in flowers and leaves (Thygesen *et al.*, 1995). The phenolic substrates of PPO are localized in the vacuole and come

into contact with the enzyme when tissue damage occurs, being oxidized by PPO and precipitating as dark-coloured melanin-like polymers (Thygesen *et al.*, 1995), a phenomenon called enzymatic browning.

The brown discoloration caused by the enzymatic browning process is an important problem for primary potato producers, because as much as 20% of the production can be compromised during harvest and post-harvest procedures (shipping, storage, distribution and manipulation) (Whitaker and Lee, 1995). Furthermore, browning reduces consumer acceptance as it negatively affects appearance and nutritional quality (Tomás-Barberán and Espín, 2001) and also alters organoleptic properties of food (Friedman, 1996; Tomás-Barberán and Espín, 2001). In addition, browning is especially problematic for the fresh and processing potato industry, and its avoidance causes increased production costs. Browning inhibition can be achieved adding either natural or chemical antioxidants, being sulphite-containing additives the most extensively used in many countries, although they have some adverse effects on human health (Peroni and Boner, 1995). Recently, it has been suggested that direct selection for different *Pot32* allelic combinations may help in achieving nondiscolouring cultivars (Werij *et al.*, 2007). However, in potato, the development of a new variety by conventional breeding may take up to 15 years (Mullins *et al.*, 2006).

Considering all these reasons together and taking into account that potatoes are currently the fourth most important food crop consumed worldwide and a critical alternative to the main cereal crops for feeding the world's population (Haas *et al.*, 2009), genetic engineering stands as an attractive alternative to avoid enzymatic browning without implementation of additional antibrowning procedures. Since the pioneer report showing that antisense expression of *Ppo* genes prevents tuber enzymatic browning (Bachem *et al.*, 1994), other reports have shown similar strategies for silencing this enzyme to enhance food quality (Graham *et al.*, 2000; Coetzer *et al.*, 2001).

Although first generation genetically engineered (GE) crops are being increasingly adopted worldwide since 1996 (James, 2008), concerns among scientists, consumers and regulatory authorities about the safety of GE crops have led to the development of the 'comparative safety' and 'substantial equivalence' concepts (Kok and Kuiper, 2003). In this regard, the concept of comparative safety comprises the analytical nature of the first step of the GE food safety assessment in combination with consecutive

toxicological and nutritional evaluations (Kok and Kuiper, 2003). The concept of substantial equivalence, considered part of the comparative safety assessment (Kok and Kuiper, 2003), embodies the idea that parental organisms can serve as a basis for comparison when assessing the safety of a modified organism (Fernie *et al.*, 2004). The rationale for this comparison is based on the assumption that conventional counterparts are generally regarded as safe because of their history of use. The criterion is therefore to establish the same level of safety as that accepted for traditional foods. To apply these concepts, it is mandatory to make a comparative analysis of their metabolism, including photosynthesis, growth and yield (Kuiper *et al.*, 2003). If the engineered plant has been comparatively modified or uncertainties remain with respect to the occurrence of unintended toxicological or nutritional effects, animal feeding studies in a model organism should be considered (EFSA, 2008). To evaluate possible unintentional alterations, transcriptomics, proteomics and metabolomics analyses are currently used (Cellini *et al.*, 2004), being metabolomics the most useful approach (Colquhoun *et al.*, 2006), because it allows a general overview of the primary metabolism and its modifications (Roessner *et al.*, 2000; Baker *et al.*, 2006). Additional major issues that need to be addressed are potential toxicity or allergenicity risks that may arise from the modified crop (Lehrer and Bannon, 2005; EFSA, 2008). In this regard, bioinformatics and animal feeding safety studies fulfil a sentinel role to identify possible unintended effects of allergenic, toxicological and nutritional relevance (EFSA, 2008).

The next generation of biotech crops displaying enhanced quality traits will provide perceivable benefits to end-users and is expected to improve public perception of genetic engineering technology (Graff *et al.*, 2009). Nevertheless, while this next generation of biotech crops is being developed, it is important to establish whether the current safety assessment criterion is appropriate to evaluate them. In contrast with the first generation of biotech crops, where only a single trait is modified, and the traits introduced so far are not considered to have significant effects on the metabolism of the plant, the next generation of biotech crops will add a new level of complexity inherent to the mechanisms underlying their output traits. Scientific studies tackling these concerns are mandatory and may help in the aim of increasing public consciousness about the benefits and safety of genetically engineering technology. In this study, a detailed physiological and metabolic characterization, as well as the intake safety evaluation, of GE potato plants silenced for PPO-encoding

genes is presented. Our primary focus was to appraise whether the current evaluation model for the risk assessment of transgenic crops is suitable for a new biotech crop where substantial equivalence with respect to the wild-type (WT) progenitor is not likely to be the case. This study suggests that the current procedures can provide sufficient information to assess the safety of next generation biotech crops and opens the discussion about the relevance of the substantial equivalence principle on complex GE crops.

Results

Construction of *Ppo*-silencing plasmid, potato transformation and selection of transgenic lines

To silence several members of the *Ppo* gene family, a hairpin construct with 100%, 84% and 71% identity to *Pot32*, *Pot33* and *Nor333*, respectively, was designed (Wesley *et al.*, 2001). To obtain the PPO interference cassette, a 376-bp fragment from the 5' region of the *Ppo* gene *Pot32* was amplified from potato genomic DNA and

fused in sense and antisense orientation flanking both sides of the 838-bp intron I of the phosphoenolpyruvate carboxylase I of *S. tuberosum* (X90982). This construct was inserted between the cauliflower mosaic virus 2x35S promoter and the nopaline synthase terminator. This cassette was then cloned into the pPZP-Hyg binary vector (Romano *et al.*, 2001) harbouring the hygromycin phosphotransferase gene (*Hpt*), and the resulting plasmid was named pPPOi (Figure 1c). The pPPOi construction was confirmed by restriction mapping and DNA sequence analysis and used to transform potato plants by *Agrobacterium* coculture (Sheerman and Bevan, 1988). After hygromycin selection, 39 independent lines were obtained. The analysis of PPO enzymatic activity of *in vitro*-growing plants identified 26 lines displaying leaf PPO activities 30% lower than that observed in WT plants (Figure 1a). Among the transgenic plants with lowest PPO activity, three lines (named j8, j14 and j20), containing a single copy of the silencing cassette (Figure 1b) adequately integrated, were selected for further experiments. The appropriate recombination of the PPO interference cassette was confirmed by PCR in the selected transformants (Figure 1d).

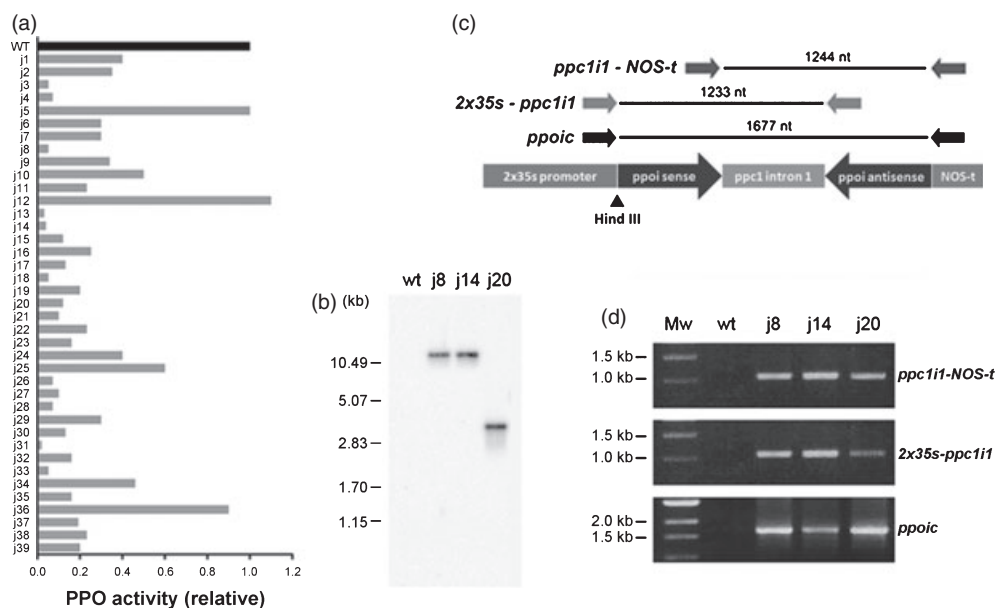


Figure 1 Obtaining of -PPO transgenic plants. (a) PPO activity of putative -PPO transgenic lines grown in solid hygromycin-enriched medium. The data are the average of three independent measurements and are presented as the relative activities compared to wild type (WT). (b) Southern blot analysis of selected j8, j14 and j20 lines harbouring a single copy of the PPO-silencing cassette. (c) Schematic representation of the hairpin PPO interference cassette. The arrows represent the primers used to analyse the integrity of the recombined construct in the selected transgenic lines. 2x35S promoter is the cauliflower mosaic virus 2x35S promoter. *ppoi* sense and *ppoi* antisense are the sense-oriented and antisense-oriented sequences directed to silence the *Ppo* genes. *ppc1* intron 1 is the spliceable intron 1 of phosphoenolpyruvate carboxylase I of *Solanum tuberosum*. NOS-t is the nopaline synthase terminator. The bottom arrowhead indicates the Hind III restriction site. (d) PCR analysis of gDNA from WT and transgenic plants. The selected transgenic lines were examined for the integrity of the recombined PPO interference cassette using the three primer combinations shown in 1C. *ppc1i1*-NOS-t is an amplicon of 1244-nt expected length. *2x35s-ppc1i1* is an amplicon of 1233-nt expected length. *ppoi* is an amplicon of 1677-nt expected length.

Bioinformatics analyses to assess potential allergenic cross-reactivity

Gene-silencing constructs are not designed to translate into protein, and there are no previous reports demonstrating that hairpin constructs are capable of producing proteins. Nevertheless, as the allergenicity assessment is a very important part of the safety assessment process for GE crops (Goodman, 2006; Goodman *et al.*, 2008), the hairpin sequence introduced in the -PPO potato plants was evaluated in all the six frames using bioinformatics approaches to identify any potential sequence matches to known allergenic proteins (Goodman, 2006; Singh *et al.*, 2009). The sequence homology searches against Structural Database of Allergenic Protein (SDAP), Food Allergy Research and Resource Program (FARRP) and NCBI-Entrez protein databases did not identify any significant alignment with known allergenic sequences. The maximum scoring aligned sequence (32.5%) in the SDAP database, with allergen Asp f 5 (CAA83015) of *Aspergillus fumigatus*, is below the 50% identity level that is likely to indicate cross-reactivity (Aalberse, 2000) and below the 35%

identity level over 80 or more amino acids suggested by the FAO/WHO Codex Alimentarius guidelines (Codex Alimentarius Commission, 2003). In the FARRP allergen database, the best e-value obtained, (1.1), was with the alpha-type gliadin precursor protein (170710) of *Triticum aestivum* (bread wheat), which is 55 times higher than the significant value of 0.02 for two homologous proteins (Pearson, 1996; Goodman, 2006; Singh *et al.*, 2009). Furthermore, no significant alignments with allergenic proteins were found using the NCBI-Entrez protein database (Goodman *et al.*, 2008).

Silencing of *Ppo* genes results in a decrease in PPO protein, enzyme activity and tuber browning but has no effects on growth, development and photosynthesis

All GE plants presented normal phenotypes (Figure 2a) and were indistinguishable from WT at all developmental stages under our greenhouse conditions. Furthermore, no differences were observed in morphology or size between -PPO and WT tubers (Figure 2b). However, PPO activity in

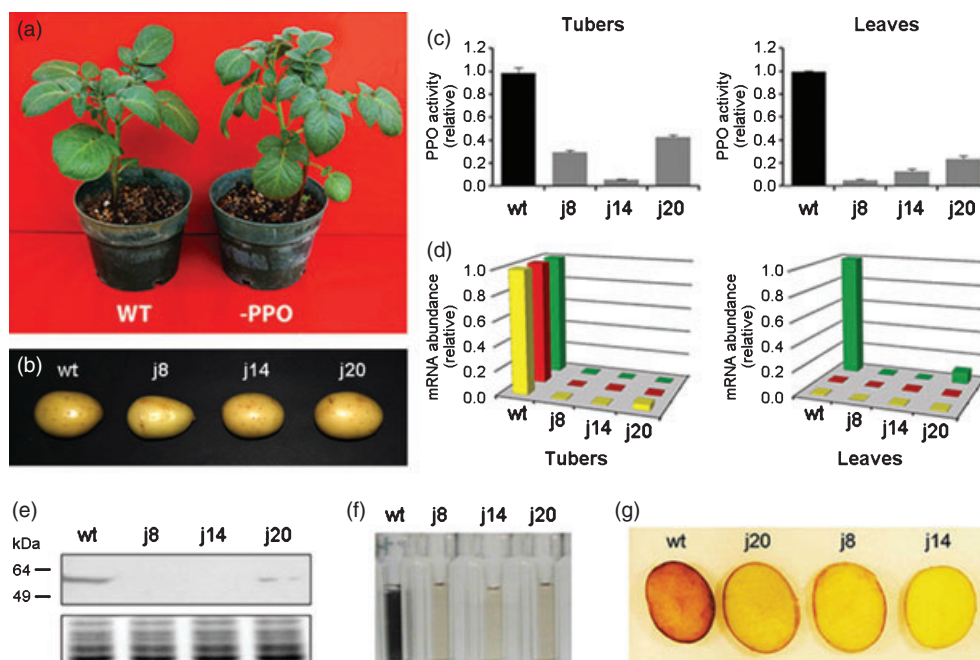


Figure 2 Analysis of -PPO lines. (a) Representative appearance of 1-month-old wild-type (WT) and -PPO plants. (b) Representative appearance of WT and -PPO tubers. (c) PPO activity of tubers and leaves of greenhouse-grown plants. Data are presented as the relative activities compared to WT. Error bars represent the SEM from six individual plants per line. All -PPO lines were significantly different ($P \leq 0.005$) from the WT according to the *t*-test. (d) qRT-PCR expression analysis of individual members of the *Ppo* gene family. Green, red and yellow bars depict *Nor333*, *Pot33* and *Pot32* genes, respectively. The results shown are the average of six independent PCRs. Values are normalized to those obtained for *Ef-1 α* and are expressed as relative mRNA abundance compared to WT. All standard deviations are below 5%. (e) Immunoblot analysis of PPO protein from greenhouse-grown tubers. The bottom panel shows Coomassie blue gel staining of total protein. (f) Appearance of potato protein extracts after 30 min at room temperature. (g) Browning phenotype of WT and -PPO sliced tubers.

tubers and leaves of the selected lines was significantly decreased in both tissues when compared to the WT controls (Figure 2c). To examine the effectiveness of RNA silencing on various individual members of the *Ppo* gene family, quantitative reverse transcription PCR (qRT-PCR) was performed using primers designed to amplify specific regions of the *Ppo* genes *Pot32*, *Pot33* and *Nor333*. This experiment revealed that, as expected, all three genes were expressed in tubers but only *Nor333* was detected in leaves and that all the *Ppo* transcripts evaluated were highly reduced in both tissues of the selected -PPO lines (Figure 2d). The presence of PPO protein was determined by Western blot analysis on total soluble protein extracted from tubers. Immunoblot analysis showed a band corresponding to PPO (~60 kDa) in WT samples. PPO was barely detectable in some samples of the j20 line, and no PPO protein was detected in the j8 and j14 lines (Figure 2e). In parallel, a reduction in the oxidation was visually evident in the transgenic protein extracts when compared to WT (Figure 2f). To test the browning propensity of mature -PPO tubers, 4-month-old greenhouse-grown tubers were cut into slices and incubated for 48 h at room temperature in a humid chamber. As observed in Figure 2g, blackening was greatly inhibited in all -PPO tubers when compared to WT.

To evaluate tuber yield, WT and -PPO size-normalized seed tubers were used in two experiments. These were performed in different seasons (winter and summer) to

obtain a reliable data set. As shown in Figure 3a–c, there were no differences in plant yield (g/plant), individual tuber weight or tuber number per plant between -PPO and WT plants. Additionally, to investigate any possible effect of the decrease in PPO activity on the photosynthetic capacity of potato, 5-week-old plants were tested for CO₂ assimilation, stomatal conductance and transpiration. As shown in Figure 3d–f, the data obtained suggest that none of these parameters differed significantly between WT and -PPO plants.

Tuber metabolite levels are dissimilar in WT and -PPO lines

Having demonstrated that silencing *Ppo* genes in potato has no effects on plant growth and development or yield traits, we next turned our attention to the analysis of the metabolite composition of the tubers of these transgenic plants. A targeted metabolite profile analysis was performed by applying a combination of spectrophotometric and gas chromatography coupled with mass spectrometry (GC–MS) techniques (Roessner *et al.*, 2000). Samples for these analyses were prepared and analysed in one group including the selected -PPO lines and the WT control from the same greenhouse experiment for which the molecular, photosynthetic, growth and development analyses were carried out. A high level of variation between the -PPO lines and the WT control tubers was observed, and a num-

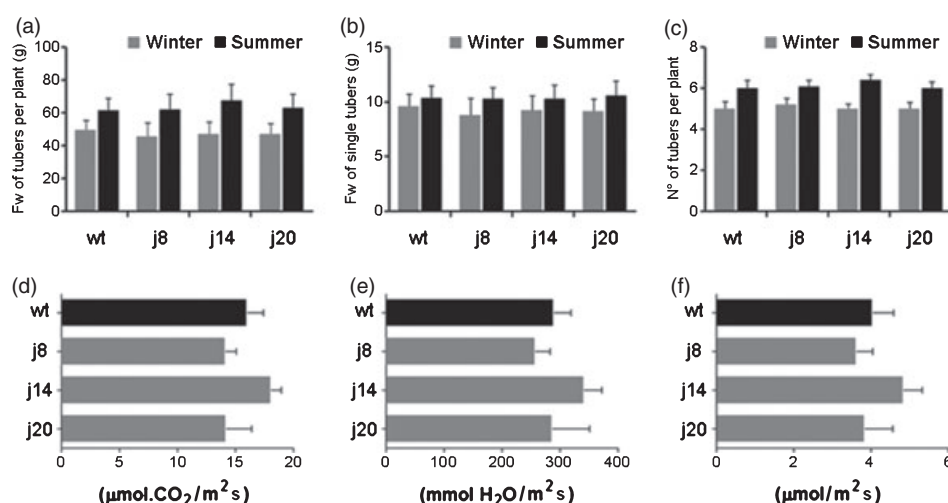


Figure 3 Evaluation of yield and photosynthetic parameters. Potato plants were greenhouse-grown on 4-L pots for 4 months. Total tuber fresh weight per plant (a), single tuber fresh weight (b) and tuber number per plant (c) were evaluated in a winter and a summer trial. Error bars represent the SEM of 25 plants per line. No statistically significant differences ($P \leq 0.05$) were found between the wild-type (WT) and the transgenic lines according to the *t*-test. (d) Net CO₂ uptake per unit area. (e) Stomatal conductance. (f) Transpiration. Error bars represent the SEM of six independent plants per line. No statistically significant differences ($P \leq 0.05$) were found between the WT and the transgenic lines according to the *t*-test.

ber of metabolites showed significant differences in the transgenic lines, all of them showing the same trend regarding increases or decreases (Figure 4a,b). Particularly, amino acid contents diminished, while a group of soluble sugars composed of glucose, fructose, maltose and trehalose increased in all the -PPO lines. Only a few organic acids (2-amino-adipate, dehydroascorbate, chlorogenate and threonate) and fatty acids (palmitoleate, stearate and lignocerate) showed significant changes in the -PPO tubers with respect to WT controls (Figure 4a,b/Table S1). To

better interpret the differences in metabolism between lines, a nonparametric analysis was applied. A model of principal component analysis (PCA) explaining about 60% of the data variance is shown in Figure 4c. However, only the first component (PC1; accounting for 32.6% of the variance) allowed discrimination of -PPO and WT tuber samples. Investigation into the relative contribution (loadings) of individual variables in the PC1 dimension highlighted eight metabolites with a significant impact on genotype separation (≥ 0.19 and ≤ -0.19). All of these

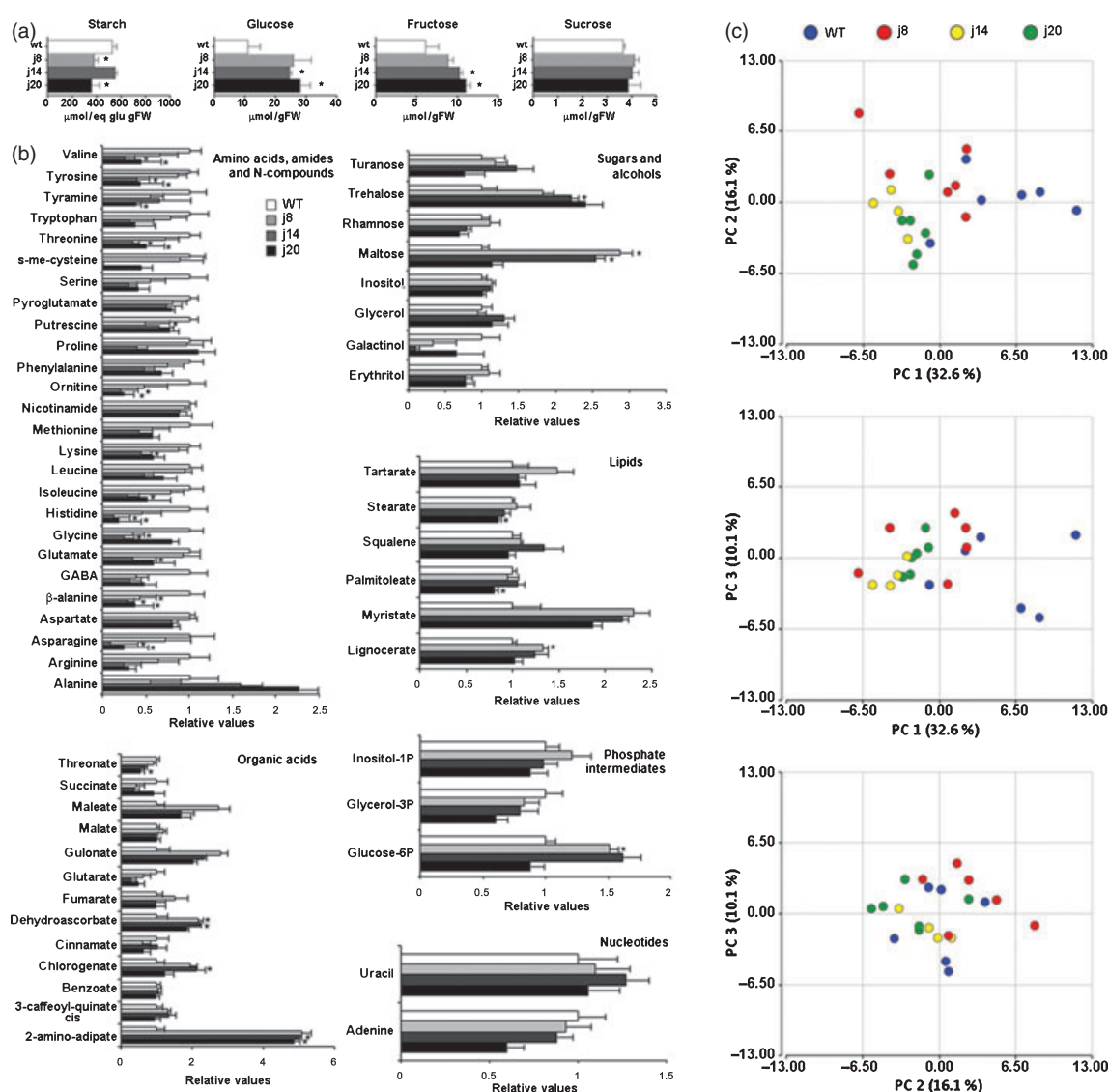


Figure 4 Metabolic analyses of -PPO and wild-type (WT) tubers. (a) Total values of starch, glucose, fructose and sucrose measured spectrophotometrically. (b) Relative values of metabolites measured by GC-MS. Values are normalized with respect to the mean response calculated for the WT. Error bars represent the SEM. (c) Principal component analysis (PCA) of the metabolic profiles of WT and -PPO lines. Distances between samples were determined using the log-transformed, normalized data of the single measurements from which the mean values in Table S1 were derived. PCA vectors span an 11-dimensional space to afford best sample separation. Vectors 1, 2 and 3 including 58.8% of the metabolic variance are presented.

variables correspond to six amino acids (methionine, phenylalanine, histidine, ornithine, arginine and serine) and to two organic acids (glutarate and dehydroascorbate).

Mice fed -PPO tubers consume more potato than mice fed WT tubers and present normal physiological parameters

To investigate whether the reduced enzymatic browning levels in the -PPO potato tubers produce a particular effect on palatability and diet, and considering that an influence of (and on) food intake could happen as a result of the differences originated by the genetic modification (browning inhibition and metabolite levels), feeding studies were conducted in mice. Because a hairpin silencing strategy was used and no novel protein, other than the already proven safe hygromycin phosphotransferase (HPT) (Petersen *et al.*, 2005; Lu *et al.*, 2007; Zhuo *et al.*, 2009), was introduced in the -PPO potatoes, a whole food feeding approach was decided. Animals were fed diets consisting of pellet food supplemented with WT or -PPO potato tubers (both pellet food and potato lines were available *ad libitum*). Mice were controlled every day, and no differences on their general aspect or behaviour were observed between groups. Interestingly, mice fed diets supplemented with -PPO tubers showed a significantly greater daily tuber intake than those fed diets supplemented with WT tubers (Figure 5a,c). However, no significant differences were observed in pellet food consumption between groups (Figure 5b,d). Despite the differential potato consumption, mice fed -PPO tubers showed no additional weight gain compared to those fed WT tubers (Figure 5e). Once feeding experiments were finished, mice were examined for organ weights and macroscopic findings, and no differences were found between groups (Figure 5f/Table 1). Additionally, no statistically significant differences ($P \leq 0.05$) were found in relative organ weights between control and experimental groups (Table 1), and data were comparable to reference values for healthy mice (<http://jaxmice.jax.org/strain/000651.htm>), suggesting no signs of toxicity. Liver and kidney organs were analysed for histopathological findings. The morphological study did not evidence any architectural or cytological alterations. Normal structures were preserved in liver and renal tissue of WT-fed and -PPO potato-fed mice (Figure 5g). Haematological and blood biochemistry values were found to be similar between all groups (Table 2). Additionally, analysis of faecal pellets showed that the microbiota (colony-forming units/mg of faecal sample) of control and experimen-

tal samples were comparable (data not shown), indicating no adverse effects on gut microbiota (Osusky *et al.*, 2000).

Discussion

Crop improvement via genetic engineering is a subject of public concern that has led to the development of evaluation steps before a GE crop can be regarded as safe. The current evaluation model has been extensively applied and has proven to be satisfactory for first generation biotech crops. However, as the more complex next generation biotech crops are being developed, the question of whether the current evaluation model is also suitable to evaluate them has not been tackled in a practical case. To undertake this concern with a proof of concept, we engineered potato plants to down-regulate the expression of polyphenol oxidase (*Ppo*) transcripts and subjected three lines with reduced PPO activity and diminished tuber browning to the present safety evaluation criterion. This is a suitable model to evaluate our working hypothesis because on one hand, an enhanced quality trait is obtained and on the other hand, the emphasis is laid on down-regulating several members of a gene family coding for an enzyme with unknown biological function, thus potentiating the occurrence of unpredictable unintended metabolic modifications. Only a few reports have studied the substantial equivalence concept in quality-improved biotech crops (Catchpole *et al.*, 2005; Baker *et al.*, 2006) and this is, to our knowledge, the first report exploring the comparative safety assessment in a transgenic crop modified for a quality trait.

Half of the world's vegetable crops are lost as a result of post-harvest deteriorative reactions (Martinez and Whitaker, 1995). In the case of potatoes, tuber browning inhibition is currently achieved by application of antioxidants, such as sulphite-containing additives that are still widely used despite their adverse health effects (Peroni and Boner, 1995). The implementation of nonbrowning potato cultivars could alleviate the industrial use of antibrowning additives, reducing production costs and improving public health. Furthermore, their adoption could positively impact plant productivity while decreasing harvest and post-harvest losses and extending product shelf life. Although -PPO plants are transformed with a hairpin construct and no gene product is expected to originate from it, to exclude any possible allergenic cross-reactivity from spurious translation, bioinformatics analyses of the hairpin sequence translated in all the six reading frames

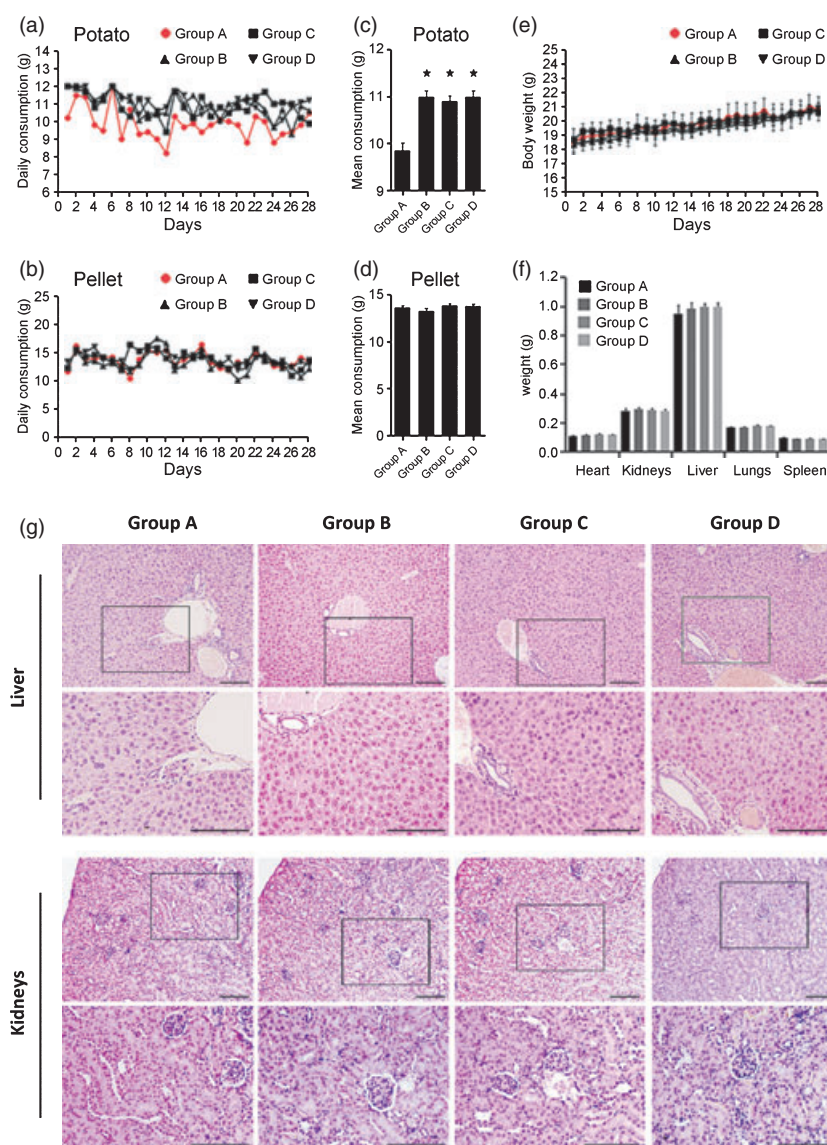


Figure 5 Mice feeding studies. Groups A, B, C and D were fed with wild-type (WT), j8, j14 and j20 lines, respectively. (a) Daily potato consumption. (b) Daily pellet consumption. (c) Mean potato consumption. (d) Mean pellet consumption. All groups fed -PPO lines consumed significantly more potato than groups fed WT potatoes, but there were no significant differences in pellet consumption between groups according to the *t*-test ($P \leq 0.05$). Error bars represent the SEM. (e) Mouse body weights. No significant differences were found between groups according to the repeated measures ANOVA test. (f) Mouse organ weights. No statistically significant differences ($P \leq 0.05$) were found between groups according to the *t*-test. Error bars represent the $\pm$ SEM. (g) Representative histological findings of liver and kidney tissues. Bar, 100 μ m. Two experiments were performed, and one representative experiment is shown.

were performed in several databases. Results from the recommended search methods did not identify any significant homology to known allergens, suggesting that there is no expected risk for allergenic cross-reactivity derived from the hairpin sequence used in this study. Furthermore, neither toxicity nor allergenicity is expected to occur from the *Hpt* selector gene product, because its safety has been previously established (Petersen *et al.*, 2005; Lu *et al.*, 2007; Zhuo *et al.*, 2009). Future analysis of the hairpin

flanking sequences may shed light on the potential occurrence of plant gene disruptions and unintended generation of fusion proteins.

Transgenic plants showed significant reduced levels of PPO activity and several transcript members of the *Ppo* gene family resulted down-regulated in both tuber and leaf tissues. In agreement, PPO protein levels and tuber browning were markedly reduced in the -PPO lines. It is worth mentioning that the morphology and growth

Table 1 Organ and relative organ weight of mice fed diets supplemented with wild-type (WT) or -PPO potato tubers

	Group A	Group B	Group C	Group D
Heart	0.1101 ± 0.015	0.1166 ± 0.008	0.1196 ± 0.012	0.1189 ± 0.009
% Heart	0.52 ± 0.023	0.55 ± 0.050	0.57 ± 0.073	0.56 ± 0.035
Kidneys	0.2834 ± 0.036	0.2963 ± 0.026	0.2912 ± 0.017	0.2833 ± 0.023
% Kidneys	1.35 ± 0.062	1.41 ± 0.126	1.40 ± 0.058	1.35 ± 0.084
Liver	0.9471 ± 0.141	0.9833 ± 0.101	0.9996 ± 0.046	0.9945 ± 0.070
% Liver	4.53 ± 0.262	4.70 ± 0.557	4.83 ± 0.205	4.74 ± 0.321
Lungs	0.1694 ± 0.010	0.1707 ± 0.016	0.1785 ± 0.015	0.1786 ± 0.007
% Lungs	0.81 ± 0.091	0.81 ± 0.071	0.86 ± 0.086	0.85 ± 0.047
Spleen	0.0999 ± 0.011	0.0884 ± 0.010	0.0932 ± 0.005	0.0905 ± 0.006
% Spleen	0.47 ± 0.048	0.42 ± 0.039	0.45 ± 0.019	0.43 ± 0.037

Organ-named rows are organ weights (g). % Organ-named rows are organ percentages of body weight. Groups A, B, C and D were fed with WT, j8, j14 and j20 fresh potato tubers, respectively. No statistically significant differences ($P \leq 0.05$) were found between WT-fed and -PPO-fed groups according to the *t*-test. Values are means ± SD. This experiment was performed twice, and one representative experiment is shown.

Table 2 Blood biochemistry and haematological values of mice fed diets supplemented with wild-type (WT) or -PPO potato tubers

	Group A	Group B	Group C	Group D
Blood biochemistry				
ALT	42.80 ± 12.01	41.80 ± 15.34	41.20 ± 7.39	39.60 ± 2.61
AST	112.2 ± 7.12	113.6 ± 18.31	115.4 ± 40.90	96.8 ± 24.04
ALP	253.3 ± 21.75	274.6 ± 40.87	270.6 ± 26.62	267.6 ± 42.10
γ-GTP	1.50 ± 1.29	1.60 ± 1.51	1.80 ± 0.83	2.0 ± 1.00
T-CHO	105.5 ± 6.80	110.2 ± 5.49	104.6 ± 7.98	113.2 ± 11.39
SA	3.12 ± 0.43	3.4 ± 0.18	3.38 ± 0.08	3.47 ± 0.09
TP	4.47 ± 0.73	4.92 ± 0.30	4.88 ± 0.14	5.00 ± 0.08
Haematology				
WBC	3.28 ± 0.25	4.16 ± 1.88	3.70 ± 0.77	4.14 ± 1.38
RBC	7.69 ± 0.69	7.87 ± 0.60	8.09 ± 0.30	8.08 ± 0.18
HGB	13.28 ± 0.42	12.92 ± 0.39	13.56 ± 0.34	13.72 ± 0.39
HCT	37.40 ± 3.46	38.42 ± 3.16	39.10 ± 1.53	39.46 ± 0.79
MCV	48.60 ± 0.37	48.76 ± 0.40	48.32 ± 0.31	48.82 ± 0.40
MCH	15.46 ± 1.46	15.70 ± 1.86	16.76 ± 0.64	17.00 ± 0.44
MCHC	31.82 ± 2.87	32.28 ± 4.05	34.70 ± 1.34	34.04 ± 1.91
RDW	17.06 ± 0.35	16.80 ± 0.33	16.88 ± 0.27	16.68 ± 0.25
PLT	682.2 ± 112.4	635.8 ± 149.4	615.0 ± 294.7	705.2 ± 98.00
MPV	5.68 ± 0.31	5.66 ± 0.58	5.36 ± 0.11	5.60 ± 0.18
PCT	0.25 ± 0.15	0.24 ± 0.09	0.33 ± 0.16	0.42 ± 0.08
PDW	16.46 ± 1.20	16.88 ± 0.98	16.28 ± 0.63	16.54 ± 0.71
NE%	16.95 ± 0.46	16.75 ± 1.25	16.08 ± 1.49	16.82 ± 0.81
LY%	85.73 ± 11.00	86.95 ± 10.45	82.88 ± 10.22	86.48 ± 10.25
MO%	1.67 ± 1.76	1.74 ± 0.94	1.67 ± 1.12	1.62 ± 1.07
EO%	0.75 ± 0.44	0.76 ± 0.16	0.77 ± 0.42	0.68 ± 0.16
BA%	0.52 ± 0.24	0.47 ± 0.26	0.55 ± 0.80	0.42 ± 0.45

Groups A, B, C and D were fed with WT, j8, j14 and j20 fresh potato tubers, respectively. No statistically significant differences ($P \leq 0.05$) were found between WT-fed and -PPO-fed groups according to the *t*-test. Values are means ± SD.

ALT, alanine aminotransferase (IU/L); AST, aspartate aminotransferase (IU/L); ALP, alkaline phosphatase (IU/L); γ-GTP, γ-glutamyl-transpeptidase (IU/L); T-CHO, total cholesterol (mg/dL); SA, serum albumin (g/dL); TP, total protein (g/dL); WBC, white blood cell count (10^3 cells/μL); RBC, red blood cell count (10^6 cells/μL); HGB, haemoglobin (g/dL); HCT, haematocrit (%); MCV, mean cell volume (fL); MCH, mean cell haemoglobin (pg); MCHC, mean cell haemoglobin concentration (g/dL); RDW, red cell distribution width (%); PLT, platelet (10^3 cells/μL); MPV, mean platelet volume (fL); PCT, total platelet mass (%); PDW, platelet distribution width (%); NE%, percentage of neutrophils (%); LY%, percentage of lymphocytes (%); MO%, percentage of monocytes (%); EO%, percentage of eosinophils (%); BA%, percentage of basophils (%).

characteristics, as well as the yield-associated traits of the -PPO lines, were indistinguishable from those of WT controls, indicating equivalence between both groups at this level. In addition, photosynthesis performance was comparable between -PPO and WT lines, revealing a substantial conservation of this anabolic process in the transgenic lines. Thus, our results are in accordance with a previous report, where reducing PPO activity does not appear to interfere with normal plant growth and development (Coetzer *et al.*, 2001).

A large proportion of the measured metabolites showed significant changes in the -PPO tubers with respect to their WT controls, and both the number of metabolites showing variations and the magnitude of these changes correlate well with the levels of PPO enzymatic activity. As the compositional comparison presented in this work was assessed under controlled environmental conditions, this observation suggests that the trait modification is responsible, at least in part, for the metabolite variation observed. Nevertheless, individual variability must be taken into account, because high compositional heterogeneity has been highlighted by recent reports (Catchpole *et al.*, 2005; Baker *et al.*, 2006). Even when the precise localization in defined metabolic pathways of PPO-catalysed reactions is still ambiguous, our results from the targeted profiling approach provide compelling evidences that the observed metabolic differences are the consequences of the reduction in the activity of this enzyme: (i) the principal substrate for enzymatic browning, chlorogenate (Friedman, 1997), was accumulated in the transgenic tubers; (ii) the levels of the oxidative stress sensor metabolite dehydroascorbate (Pignocchi and Foyer, 2003) were altered in the -PPO tubers; and (iii) a high correlation between the free amino acid contents and the level of tuber discoloration was observed. This is in agreement with a previous report where potato genotypes with different internal blackspot resistance were evaluated (Corsini *et al.*, 1992).

Given the differences found in the tuber metabolites of -PPO lines with respect to the WT controls, it was critical to investigate the effect of -PPO potato consumption in a dietary context to identify potential unintended effects of toxicological or nutritional relevance. In addition, it was tempting to speculate whether -PPO potatoes might taste different from WT potatoes and hence have an influence on ingestion. Interestingly, when mice feeding experiments were performed, mice fed diets supplemented with -PPO potatoes consumed an average of ~11% more potato

than mice fed diets supplemented with WT potatoes. This may be originated from differential organoleptic properties between -PPO and WT tubers, arising from dissimilarities such as those observed in sugars and acid metabolite levels. Alternatively, the differences in potato ingestion may be also attributable, in part, to the reduced browning in -PPO tubers, allowing a slower deterioration and making them palatable for longer periods than WT tubers. Noticeably, no significant differences were observed in pellet food consumption between groups, and no additional weight gain occurred in the -PPO-fed mice. As potato tubers are about 80% of water, we can speculate that the observed increased -PPO potato consumption might not represent a significant caloric intake.

When organ and blood physiological parameters were analysed, neither signs of toxicity nor alterations on metabolism were found. Furthermore, no adverse effects on gut microbiota were observed in the -PPO-fed groups. Considering an average human body weight of 70 kg, the worldwide average annual per capita consumption of potatoes (estimated in 31.3 kg; <http://www.potato2008.org/en/world/index.htm>) and that in our experiments mice presented an average weight of 19.6 g and denoted an average potato daily consumption of 2.2 g, it can be expected that a 91.4-fold safety margin of exposure was achieved in the feeding experiments, equivalent to a human ingestion of 7.8 kg potato/person/day. Together, these results suggest that consumption of nonbrowning potatoes presents no risk for adverse health effects. Further 90-day feeding studies (EFSA, 2008) will provide an additional level of safety to our preliminary results.

Acceptability of next generation biotech crops will require them to be safe. Here, we tested three nonbrowning potato lines grown under controlled conditions with the current safety criterion. We conclude that the current procedures can provide sufficient information to assess the safety of new biotech crops, even if the transgenic and WT crops are not substantially equivalent.

Experimental procedures

Vector and plant transformation

The hairpin construct (Wesley *et al.*, 2001) contains a CaMV 2x35S promoter, a sense 5' polyphenol oxidase gene (GenBank accession number: U22921) fragment of 376 nucleotide (ppoi), an 840 nucleotide of the intron 1 of the phosphoenolpyruvate carboxylase 1 of *S. tuberosum*, (ppc1 intron 1; GenBank accession number: X90982), the antisense 5' *Ppo* gene fragment and a NOS terminator. The sense and antisense ppoi and the ppc1 intron 1

fragments were obtained by PCR amplification of potato genomic DNA. The sense ppoi fragment was amplified using the primers Pot32senseFwd (5'-CCACTCGAGTGAGCAATAATGGCAAGCT-TGTG-3') and Pot32senseRev (5'-CCGGATATCATCATCAGGCT-TAGGCGCGC-3'). The ppc1 intron 1 fragment was amplified using the primers Ppc1i1Fwd (5'-GTATGCATTTTCCCAATTTATG-3') and Ppc1i1Rev (5'-ACCTAATGTGAGATTGAAAATATC-3'). The antisense ppoi fragment was amplified using the primers Pot32antisenseFwd (5'-AAAAGTGCAGCTTCTCCATATCATCAGGCTTAG-3') and Pot32antisenseRev (5'-TCCTCTAGATGAGCAATAATGG-CAAGCTTGTG-3'). All PCRs were carried out in 20 µL volume, and cycling conditions were 95 °C for 10 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 or 2 min and a final 10-min extension at 72 °C. The binary vector pPZP-Hyg (Romano *et al.*, 2001) harbouring the desired construct was transferred into the *Agrobacterium tumefaciens* strain GV3101 by electroporation. Transgenic potato plants were obtained by *Agrobacterium*-mediated tuber disc transformation (Sheerman and Bevan, 1988), with the addition of 10 mg/L acetosyringone to the co-cultivation medium. Putative transgenic shoot-buds were selected on solid medium containing 4 mg/L hygromycin.

Protein extraction, PPO activity assay and immunoblot analysis

Protein extracts were obtained from leaf or tuber tissue homogenized at a ratio of 200 mg fresh weight to 1 mL homogenization buffer [50 mM Tris-HCl, pH 7.5, 2 mM β-Me, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/mL leupeptin, 3% (w/v) polyvinylpyrrolidone (PVP), and 1 mM Na₂ EDTA, 20% glycerol]. The homogenate was centrifuged at 10000 *g* at 4 °C for 20 min, and the supernatant was used for protein quantification and assessment of PPO activity. Total protein was quantified by the Bradford method (Bradford, 1976), using bovine serum albumin as standard. PPO activity assays were performed with 2-µg protein extracts in 1000 µL of 20 mM phosphate buffer (pH 6) containing 4 mM MgCl₂ and 10 mM L-DOPA. PPO activity was followed by the change in absorbance at 475 nm as a result of the oxidation of L-DOPA. Catalase (84 U/µL) was added to samples before analysis to avoid oxidation from peroxidases. Protein extracts were denatured by boiling for 5 min in sample loading buffer (Laemmli, 1970), and 10 µg of protein was loaded in each lane. Proteins were resolved in SDS/PAGE and immobilized onto polyvinylidene fluoride (PVDF) membranes (Perkin Elmer, Waltham, MA, USA). PPO was detected with a rabbit polyclonal PPO antibody (Marqués *et al.*, 1994) in combination with alkaline phosphatase-conjugated goat anti-rabbit IgG. Coomassie blue-stained gels were run in parallel to verify equal loading.

DNA and RNA analysis

DNA was obtained as described by Edwards (Edwards *et al.*, 1991) for PCR use or isolated as described by Murray (Murray and Thompson, 1980) for Southern blot analysis. Transgenic and the approximate copy number of the silencing construct were determined by Southern blot analysis (Southern, 1975) using

10 µg of genomic DNA digested with 20 U of Hind III restriction enzyme (New England Biolabs, Ipswich, MA, USA). A PCR-amplified 543-bp fragment of the 2x35S promoter was used as a probe. The integrity of the silencing cassette in transgenic plants was determined by PCR using primers 2x35Sfwd3' (5'-ATCTCCACTGACGTAAGGA-3'); NOS-trev5' (5'-TGATAATCATCG-CAAGACCG-3'); PPC1i1Fwd (5'-GAGAGGATATCAAAGAAACAG-3'); and PPC1i1Rev (5'-GCTCATAACACTCTTGGACC-3'). Products were analysed by electrophoresis on 1% agarose gels and stained with ethidium bromide. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Purified RNAs were quantified by spectrophotometry, and RNA integrity was evaluated by agarose gel electrophoresis. RNA samples were treated with RNase-free DNase I (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions, and cDNA was generated with the Superscript III Reverse Transcriptase Kit (Invitrogen) according to the manufacturer's instructions, using random primers plus 2 µg of total RNA. Expression of *Ppo* gene family members was monitored by qRT-PCR using the primers POT32RTfwd (5'-CATGCAAGGTTACCAATAATAACG-3'); POT32RTrev (5'-GCAACACCATAAAGACCACT-3'); POT33RTfwd (5'-TTTCTAATAGTGGTGACCAAAACC-3'); POT33RTrev (5'-GCATTAGCAACACCATAAAGACC-3'); NOR333RTfwd (5'-AAGAAGGTGTGATGTGTCATAC-3'); and NOR333RTrev (5'-CGGATGCGGAGTTAGTCAT-3'). The elongation factor 1-α gene (*Ef-1α*; GenBank accession number: AB061263.1) of *S. tuberosum* was used as an endogenous control as it has been demonstrated to be the most suitable gene for qRT-PCR gene normalization expression in potato (Nicot *et al.*, 2005). *Ef-1α* was measured using the primers EF-1αFwd (5'-TGAGGCAAAGTGTGCTGTC-3') and EF-1αRev (5'-TGGAAACACCA GCATCACAC-3'). qRT-PCR was performed in a total volume of 20 µL using SYBR Green I (Invitrogen) technology on a Rotor-Gene 6000 instrument (Corbett Life Science, Mortlake, Australia) with 10 ng of cDNA and 500 nM of each specific sense and antisense primers. Cycling conditions were 95 °C for 10 min followed by 40 cycles of 30 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C. All gene fragments were amplified in triplicate from the same RNA preparation, and the mean value was considered. PCR for each gene fragment was performed alongside standard dilution curves of total cDNA (Gomes *et al.*, 2006). All data were normalized to the level of the endogenous reference gene *Ef-1α* and to WT expression values.

Plant material

WT and GE potato plants (*S. tuberosum* var. Spunta) regenerated *in vitro* were transferred to 4-L pots to be grown under greenhouse conditions (25 ± 3 °C). During the winter period, daylight was supplemented by sodium lamps providing 100–300 µmol/s/m² with a 16-h light/8-h dark photoperiod.

Bioinformatics analyses to assess potential allergenic cross-reactivity

Potential sequence homology to known allergens of the hairpin sequence introduced in the -PPO potato plants was evaluated in all

the six frames using bioinformatics approaches (Goodman, 2006; Singh *et al.*, 2009). The possible amino acid sequences were translated into protein sequence using the ExPASy tool (<http://www.expasy.ch/tools/dna.html>) and searched in the SDAP (Structural Database of Allergenic Protein; http://fermi.utmb.edu/SDAP/sdap_who.html) and FARRP (Food Allergy Research and Resource Program; <http://www.allergenonline.com/>) databases (Singh *et al.*, 2009). Full FASTA alignments and 80 amino acids sliding window FASTA alignments (Goodman *et al.*, 2008) were performed. Finally, BLASTP was used to identify any significant similarity to any newly reported allergen sequences not found in the SDAP or FARRP databases by searching the nonredundant sequences in the NCBI-Entrez Protein Database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Goodman and Wise, 2006; Goodman *et al.*, 2008).

Yield testing

Yield data were collected from two greenhouse experiments where -PPO lines were grown alongside WT controls using twenty-five 4-L pots per line. Tubers were harvested when the plants senesced after growth for approximately 4 months, in winter (September 2007) and summer (March 2008).

Photosynthetic measurements

Net CO₂ uptake, stomatal conductance and leaf transpiration were measured using the LI-6400 system (Li-Cor, Lincoln, NE, USA) under greenhouse conditions at a fixed irradiance (1500 µmol/m²/s). Parameters were calculated with the software supplied by the manufacturer. Measurements were taken on fully expanded leaves of the third node (counting from the top), and six plants were sampled for each line.

Metabolite levels

Tuber samples (four to six individual batches of samples pooled from 25 plants per genotype) were taken at harvest time, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. Extraction was performed by rapid grinding of tissue in liquid nitrogen and immediate addition of the appropriate extraction buffer. Levels of starch, sucrose, fructose and glucose were determined as described by Fernie *et al.* (2001). The levels of all other metabolites were quantified by GC-MS following the protocol described by Roessner *et al.* (2001). Potato tuber tissue (~100 mg) was extracted in 1400 µL of methanol, as described by Roessner *et al.* (2000) with the modifications proposed by Lisec *et al.* (2006); 60 µL of internal standard (0.2 mg/mL ribitol in water) was added for quantification. The mixture was extracted for 15 min at 70 °C, mixed vigorously with 1 volume of water, centrifuged at 2200 g and subsequently reduced to dryness in vacuo. The residue was redissolved and derivatised for 120 min at 37 °C (in 60 µL of 30 mg/mL methoxyamine hydrochloride in pyridine) followed by a 30-min treatment at 37 °C (with 120 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide). Sample volumes of 1 µL were then injected in a splitless mode, using a hot needle technique. The gas chromatography-time-of-flight mass spectrometry (GC-tof-MS) system was composed of an AS 2000 auto-

sampler, a GC 6890N gas chromatographer (Agilent Technologies, Santa Clara, CA, USA) and a Pegasus III time-of-flight mass spectrometer (LECO Instruments, St. Joseph, MI, USA). The mass spectrometer was tuned according to the manufacturer's recommendations, using tris-(perfluorobutyl)-amine (CF43). GC was performed on a MDN-35 capillary column, 30 m in length, 0.32 mm in inner diameter and 0.25 mm in film thickness (e.g. Macherey-Nagel or equivalent). The injection temperature was set at 230 °C, the interface at 250 °C and the ion source adjusted to 200 °C. Helium 5.0 was used as the carrier gas at a flow rate of 2 mL/min. The analysis was performed under the following temperature programme: 2 min of isothermal heating at 80 °C, followed by a 15 °C per min ramp to 330 °C and holding at this temperature for 6 min. Mass spectra were recorded at 20 scans per second with a scanning range of 70–600 m/z. Both chromatograms and mass spectra were evaluated using Chroma-TOF chromatography processing and mass spectral deconvolution software, version 3.00 (LECO Instruments). Identification and quantification of the compounds detected in the GC-tof-MS metabolite profiling experiment were performed with TagFinder 4.0 software (Luedemann *et al.*, 2008). Data sets measured at different times were not directly comparable because of variation of the tuning parameters of the GC-MS machine over time; we therefore normalized the data using the WT control of each measured batch as a reference.

Mice feeding studies

Two replicate studies were carried out using a total of forty 8-week-old female BALB/c mice (Animal Facility of the School of Veterinary Sciences, National University of La Plata, La Plata, Argentina). Animals were housed and kept under standard conditions (12-h light-dark cycle, air-conditioned room, 21 ± 1 °C, relative humidity 60 ± 10%) in accordance with the guide for the Care and Use of Laboratory Animals of the Public Health Service (USA). Group housing was used to improve animals' welfare. After acclimatization for 7 days, mice were randomly separated into groups of five animals per cage and fed for 28 days (Juberg *et al.*, 2009; Mathesius *et al.*, 2009) with diets differing in the potato tuber line supplied. Groups A, B, C and D were exposed simultaneously to fresh potato tubers (~1 × 4 cm sticks) WT, j8, j14 and j20, respectively, and pellet food (GEPSA Feeds, Pilar, Buenos Aires, Argentina) both *ad libitum*. Tap water was continuously available throughout the experiment. Body weight, pellet food and potato consumption were measured every day. The safety margin of exposure was calculated according to the following equation: Margin of exposure = [daily mice potato consumption (kg)/mice body weight (kg)] × [average human body weight (kg)/worldwide average daily human per capita potato consumption (kg)]. Haematological analyses were performed using an autoanalyzer Sysmex XT-1800i (Sysmex, Kobe, Japan) or determined microscopically, and serum biochemistry was determined using an autoanalyzer Hitachi 917 (Hitachi, Tokyo, Japan) after blood sample collection on day 28. Blood samples were collected via cardiac puncture under general anaesthesia by intraperitoneal administration of 0.01 mL/g of body weight of a cocktail of Ketamine (Ketalar, Parke Davis 0.23 mg/mL) and Xylazine (Rompum, Bayer, 0.14 mg/mL). Faecal pellets were collected on day 28 and cultured onto blood agar and Levine Eosin Methylene Blue (EMB)

agar for microbiota analysis. Vital organs such as heart, kidneys, liver, lungs and spleen of each mouse were weighed immediately after sacrifice, and kidneys and liver were subjected to histopathological examinations. Relative organ weights were calculated applying the following formula: Relative organ weight = [organ weight (g)/body weight (g)] × 100. Liver and kidney tissues were collected from sacrificed mice and fixed in 10% buffered formalin (v/v) for histological studies. Tissues were embedded in paraffin, sliced to 5-µm sections and stained with haematoxylin and eosin.

Photographic documentation

Macroscopic pictures were acquired with a Canon Ixus 70 camera (Canon, Tokyo, Japan). Tissue section pictures were captured using the in-line camera integrated in the Olympus BX41 microscope (Olympus, Tokyo, Japan).

Statistical analysis

Statistical significance was determined by *t*-test analysis, and repeated measures ANOVA test was used to determine significance in mouse body weight changes. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Metabolite content variations were assessed by *t*-tests analyses using EXCEL (Microsoft Corporation, Redmond, WA, USA) considering *P* < 0.01 as a significant value. PCA was carried out as detailed in Roessner *et al.* (2001) using the INFOStat software.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Table S1 Metabolite levels in transgenic PPO silenced potato tubers relative to wild type. Data presented are relative mean values $\pm$ standard error of measurements from four to six individual batch of samples pooled from 25 plants per genotype.

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Table S1 Metabolite levels in transgenic PPO silenced potato tubers relative to wild type. Data presented are relative mean values $\pm$ standard error of measurements from four to six individual batch of samples pooled from 25 plants per genotype

Metabolite	WT	j8	j14	j20
<i>Amino Acids, Amides and N-compounds</i>				
alanine	1.00 $\pm$ 0.33	0.55 $\pm$ 0.34	1.59 $\pm$ 0.24	2.26 $\pm$ 0.22
arginine	1.00 $\pm$ 0.22	0.64 $\pm$ 0.23	0.25 $\pm$ 0.18	0.30 $\pm$ 0.08
asparagine	1.00 $\pm$ 0.28	0.73 $\pm$ 0.28	0.09* $\pm$ 0.31	0.24* $\pm$ 0.28
aspartate	1.00 $\pm$ 0.06	1.00 $\pm$ 0.08	0.80 $\pm$ 0.06	0.81 $\pm$ 0.07
GABA	1.00 $\pm$ 0.20	0.38 $\pm$ 0.14	0.32 $\pm$ 0.12	0.48 $\pm$ 0.13
glutamate	1.00 $\pm$ 0.12	0.92 $\pm$ 0.19	0.35* $\pm$ 0.26	0.58 $\pm$ 0.24
glycine	1.00 $\pm$ 0.15	0.35* $\pm$ 0.12	0.24* $\pm$ 0.11	0.79 $\pm$ 0.08
histidine	1.00 $\pm$ 0.20	0.46 $\pm$ 0.21	0.13* $\pm$ 0.18	0.19* $\pm$ 0.25
isoleucine	1.00 $\pm$ 0.15	0.78 $\pm$ 0.15	0.29* $\pm$ 0.12	0.52 $\pm$ 0.25
leucine	1.00 $\pm$ 0.14	0.95 $\pm$ 0.07	0.48 $\pm$ 0.10	0.70 $\pm$ 0.15
lysine	1.00 $\pm$ 0.11	0.88 $\pm$ 0.09	0.44* $\pm$ 0.11	0.59 $\pm$ 0.12
methionine	1.00 $\pm$ 0.26	0.57 $\pm$ 0.19	0.42 $\pm$ 0.14	0.57 $\pm$ 0.07
nicotinamide	1.00 $\pm$ 0.07	0.95 $\pm$ 0.05	0.89 $\pm$ 0.07	0.88 $\pm$ 0.14
ornithine	1.00 $\pm$ 0.17	0.48 $\pm$ 0.26	0.21* $\pm$ 0.19	0.24* $\pm$ 0.11

phenylalanine	1.00 ± 0.15	0.75 ± 0.18	0.48 ± 0.11	0.68 ± 0.12
proline	1.00 ± 0.25	0.97 ± 0.18	0.40 ± 0.10	1.10 ± 0.19
putrescine	1.00 ± 0.09	0.49* ± 0.27	0.66 ± 0.15	0.77 ± 0.10
pyroglutamate	1.00 ± 0.09	0.81 ± 0.15	0.74 ± 0.16	0.79 ± 0.03
serine	1.00 ± 0.20	0.55 ± 0.17	0.30 ± 0.09	0.41 ± 0.12
s-me-cysteine	1.00 ± 0.17	0.89 ± 0.26	nd	0.44 ± 0.12
β-alanine	1.00 ± 0.16	0.43* ± 0.18	0.29* ± 0.06	0.37* ± 0.21
threonine	1.00 ± 0.12	0.73 ± 0.14	0.35* ± 0.06	0.50* ± 0.20
tryptophan	1.00 ± 0.21	0.78 ± 0.18	0.32 ± 0.25	0.37 ± 0.23
tyramine	1.00 ± 0.19	0.55 ± 0.14	0.65 ± 0.36	0.39* ± 0.04
tyrosine	1.00 ± 0.09	0.86 ± 0.10	0.40* ± 0.13	0.43* ± 0.26
valine	1.00 ± 0.13	0.67 ± 0.19	0.26* ± 0.11	0.45* ± 0.22

Organic Acids

2-amino-adipate	1.00 ± 0.23	5.90 ± 0.24	<u>5.04*</u> ± 0.05	<u>4.87*</u> ± 0.16
benzoate	1.00 ± 0.10	1.02 ± 0.11	1.07 ± 0.08	0.98 ± 0.09
cinnamate	1.00 ± 0.35	0.61 ± 0.21	1.04 ± 0.23	0.64 ± 0.18
dehydroascorbate	1.00 ± 0.30	<u>2.17*</u> ± 0.13	<u>2.24*</u> ± 0.02	1.85 ± 0.06
fumarate	1.00 ± 0.16	1.52 ± 0.35	0.98 ± 0.27	0.97 ± 0.27

glutarate	1.00 ± 0.22	0.62 ± 0.20	0.29 ± 0.14	0.48 ± 0.18
gulonate	1.00 ± 0.36	2.80 ± 0.20	2.35 ± 0.05	2.03 ± 0.11
malate	1.00 ± 0.08	1.21 ± 0.06	0.96 ± 0.07	1.01 ± 0.09
maleate	1.00 ± 0.22	2.73 ± 0.37	1.69 ± 0.37	1.70 ± 0.27
3-caffeoyl-quinatate cis	1.00 ± 0.17	1.31 ± 0.09	1.35 ± 0.20	0.94 ± 0.16
3-caffeoyl-quinatate trans (chlorogenate)	1.00 ± 0.23	1.95 ± 0.20	<u>2.11*</u> ± 0.25	1.24 ± 0.24
succinate	1.00 ± 0.30	0.43 ± 0.24	0.38 ± 0.12	0.91 ± 0.31
threonate	1.00 ± 0.09	0.92 ± 0.08	0.70 ± 0.05	0.55* ± 0.09

Sugars and alcohols

erythritol	1.00 ± 0.07	1.09 ± 0.14	0.78 ± 0.08	0.77 ± 0.12
galactinol	1.00 ± 0.23	0.33 ± 0.31	0.09 ± 0.05	0.65 ± 0.36
glucose	1.00 ± 0.35	2.34 ± 0.53	<u>2.23*</u> ± 0.06	<u>2.54*</u> ± 0.29
glycerol	1.00 ± 0.13	0.94 ± 0.10	1.30 ± 0.13	1.13 ± 0.22
fructose	1.00 ± 0.25	1.49 ± 0.10	<u>1.67*</u> ± 0.06	<u>1.80*</u> ± 0.10
myo-inositol	1.00 ± 0.06	1.13 ± 0.04	1.10 ± 0.03	1.01 ± 0.03
maltose	1.00 ± 0.08	<u>2.88*</u> ± 0.16	<u>2.55*</u> ± 0.11	1.13 ± 0.14
rhamnose	1.00 ± 0.10	1.10 ± 0.13	0.79 ± 0.06	0.69 ± 0.11

starch	1.00 ± 0.06	0.71* ± 0.06	1.03 ± 0.03	0.67* ± 0.12
sucrose	1.00 ± 0.02	1.12 ± 0.05	1.10 ± 0.07	1.05 ± 0.14
trehalose	1.00 ± 0.19	1.82 ± 0.15	<u>2.22*</u> ± 0.08	2.41 ± 0.23
turanose	1.00 ± 0.31	1.18 ± 0.15	1.47 ± 0.22	0.76 ± 0.27

Phosphate Intermediates

glucose-6P	1.00 ± 0.08	<u>1.51*</u> ± 0.07	1.62 ± 0.14	0.88 ± 0.11
glycerol-3P	1.00 ± 0.13	0.83 ± 0.12	0.80 ± 0.14	0.60 ± 0.09
myo-inositol-1P	1.00 ± 0.11	1.21 ± 0.15	0.98 ± 0.11	0.88 ± 0.13

Lipids

lignocerate	1.00 ± 0.04	<u>1.33*</u> ± 0.05	1.24 ± 0.14	1.02 ± 0.08
myristate	1.00 ± 0.30	2.31 ± 0.17	2.18 ± 0.07	1.87 ± 0.09
palmitoleate	1.00 ± 0.04	0.94 ± 0.12	1.05 ± 0.08	0.80* ± 0.04
squalene	1.00 ± 0.08	1.09 ± 0.02	1.34 ± 0.20	0.96 ± 0.07
stearate	1.00 ± 0.02	1.04 ± 0.15	0.91 ± 0.06	0.85* ± 0.03
tartarate	1.00 ± 0.17	1.48 ± 0.17	1.07 ± 0.07	1.08 ± 0.17

Nucleotides

adenine	1.00 ± 0.15	0.93 ± 0.14	0.88 ± 0.09	0.60 ± 0.09
uracil	1.00 ± 0.22	1.10 ± 0.19	1.27 ± 0.13	1.06 ± 0.17

*significant value as determined by *t*-test ($p < 0.01$): underlined and bold numbers indicate increased and decreased metabolite levels relative to wild type controls respectively.