STABY

1994 BACHEM

Antisense Expression of Polyphenol Oxidase Genes Inhibits Enzymatic Browning in Potato Tubers

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Spoilage caused by post-harvest enzymatic browning is a problem of considerable importance to food growers, processors and retailers. Here we show that antisense inhibition of polyphenol oxidase (PPO) gene expression abolishes discoloration after bruising of potato tubers in individual transgenic lines grown under field conditions. Using appropriate promoters to express antisense PPO RNA, melanin formation can be specifically inhibited in the potato tuber. This lack of bruising sensitivity in transgenic potatoes, and the absence of any apparent detrimental side effects open the possibility of preventing enzymatic browning in a wide variety of food crops without resorting to treatments such as heating or the application of antioxidants.

Received 31 May 1994; accepted 1 August 1994.

The development of brown discoloration in a wide range of fruit and vegetables reduces consumer acceptability and is thus of significant economic importance to the primary producer and the food processing industry[1]. As well as affecting the harvested produce, brown staining of processed products such as in juices, pulp, and homogenates currently necessitates the use of various food additives. Traditionally, browning in foods has been controlled by the use of sulfiting agents. Such food additives have been used in a wide range of fresh, frozen, and processed products, including potatoes, lettuce, mushrooms, avocados, grapes, many baking products, wine, beer, and seafood, in which the process of enzymatic browning is a significant problem. Recently, however, doubt has been cast on the safety of sulfites for human consumption. The U. S. Food & Drug Administration, for example, has rescinded the GRAS (Generally Recognized As Safe) listing for several sulfiting agents for use on fruit and vegetables and more are being reviewed[2].

In potato tubers, injury during mechanical harvesting and subsequent handling causes areas of the tuber to develop discolored patches (blackspot) which extend from the site of impact. Although potato blackspot is frequently not associated with visible tissue damage, it can be the cause of severe crop losses during grading for both tablestock and frozen products[3,4]. The discoloration of the damaged tissue results from the enzymatic production of complex polyphenolics[5], also referred to as melanins. In bacterial and mammalian systems, melanins are regarded as the oxidation products of tyrosine derived from monophenol mono-oxygenase activity. In plants, this activity is often not detectable. Furthermore, the colored oxidation products of PPO activity can result from polymerization of a wide variety of different phenolic compounds. In this paper we use the term melanin in the broad sense to denote polyphenolic pigments formed by auto-oxidation of PPO-derived quinones.

A large number of interacting genetic and environmental factors influence enzymatic browning in potatoes including tuber dry matter as well as availability and levels of substrate[5,6]. The first steps in the pathway leading to the formation of melanins involve the oxidation of monophenols and *o*-diphenols to *o*-quinones. Further oxidative reactions, thought to be largely non-enzymatic, then give rise to polyphenolic melanin-like compounds[7,8]. The enzyme thought to be responsible for initial steps in this pathway is polyphenol oxidase[1]. Plant PPOs are nuclear-encoded copper metalloproteins, with a molecular mass of circa 59,000 and are localized in membranes of plastids[9]. Plant genes encoding this enzyme have been recently cloned and characterized[10-13]. Although the sequences of plant PPO genes are very similar, only the putative copper binding sites are conserved when the plant genes are compared to mammalian, bacterial, or fungal tyrosinases[12]. While PPO enzyme activity has been implicated in the browning of plant tissues after damage, no biological function has been unequivocally assigned to PPO in intact plant tissue. Interest in the biological function of PPO as well as the need to ameliorate the severe losses caused by PPO-mediated browning in potato and other agricultural commodities led us to evaluate the possibility of engineering blackspot resistance in potatoes using molecular techniques. One of the most successful methods developed in recent years to inhibit gene expression in plants has been concomitant expression of an introduced antisense gene[14]. In this paper we describe the isolation of tuber-specific PPO cDNAs and the inhibition of PPO expression in transgenic potato plants by expressing a series of antisense PPO gene constructs driven by constitutive and tissue specific promoters.

Results

Isolation and characterization of tuber-specific potato PPO cDNAs. Two PPO cDNAs have been isolated from potato[11]. In order to analyze genes that are expressed in the

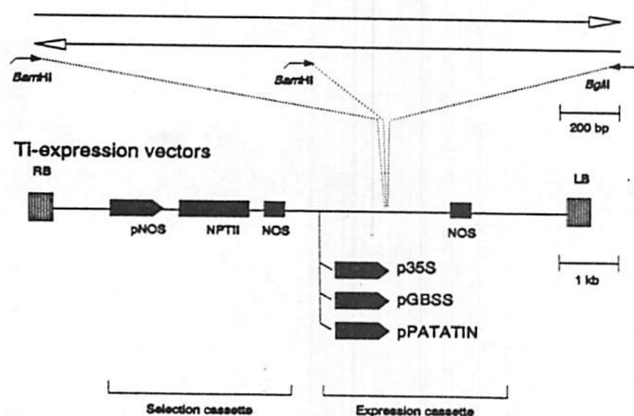

FIGURE 1. Construction of the sense and antisense PPO plasmids. All PPO gene fragments were derived from the original cDNA clones using PCR assisted cloning. The approximate positions of primers for PCRs are indicated by small arrows and the restriction sites incorporated into the primers are shown. The large arrows show the direction of translation of the PPO gene. Right and left borders of the T-DNA vector are indicated by RB, and LB respectively. pNOS, NPTII and NOS are abbreviations for the nopaline synthase promoter, the neomycin phosphotransferase II gene and the nopaline synthase 3′ transcription terminator region.

TABLE 1. Classification of cDNA clones isolated from a potato tuber library. The sizes of the clones were estimated by gel electrophoresis or by sequence determination in the case of pKG45-8 and pKG59-4. 5′ terminal sequencing (circa 500 bp) was carried out on all listed clones to determine the identity of the gene giving rise to the cDNA clone. When sequence identity was found between individual clones a putative PPO gene (A–D) was assigned to the sequence. Classes (I or II) were assigned to a cDNA clone when it revealed more than 75% sequence identity to either pKG45-8 (Class I) or pKG59-4 (Class II).

Class	Gene	CDNA clone	size in bp
I	A	pKG45-5	1850
		pKG45-8	1875
II	B	pKG59-4	1931
		pKG45-6	1800
		pKG45-4	1600
		pKG45-7	1600
		pKG45-9	1400
		pKG59-1	500
II	C	pKG45-11	1800
		pKG45-3	1500
II	D	pKG45-10	1300
II	E	pKG59-2	800

tuber, a cDNA library from developing tubers[15] was screened with leaf PPO cDNAs[11]. Partial sequence analysis was carried out on the 12 largest clones isolated. The analysis of sequence data showed that all cDNA clones fell into two distinct classes. The complete sequences were determined from one clone of each class (pKG45-8 [Class I], pKG59-4 [Class II]). The Class I clone from tuber (pKG45-8) is highly homologous to the potato leaf clones (pPPO-PI[11]; 98.8% sequence identity), while the Class II clone (pKG59-4) shows more similarity to the tomato PPO clone; (PPO F[12,13]; 80.1% sequence identity) than to any potato cDNA clones isolated to date. Class I and Class II share 72.4% homology. At least five different PPO genes or allelic variants of these genes are expressed in the potato tuber (Table 1; A–E). The most abundantly represented transcript in this tuber cDNA library belongs to the Class II gene family (B–E). In this group, transcripts from the B gene occur at the highest frequency.

Construction of T-DNA vectors carrying antisense PPO cDNAs. In order to maximize the chances of achieving a high level of antisense inhibition of PPO gene expression, we designed a series of antisense constructs which contain either the full-length PPO gene or a 5′-800 bp section of both classes of PPO genes. We used the CaMV 35S promoter which gives high expression levels throughout the plant[16], as well as two promoters which direct expression more specifically to the potato tuber: the granule-bound starch synthase G28[17] (GBSS) and patatin type I[15] promoters. As a control, a construct was used in which the Class I PPO gene was inserted in a sense orientation under the transcriptional control of the CaMV 35S promoter. Constructs were also included that lacked a PPO gene and carried the GUS marker gene (pBI121)[18]. The construction of plasmids used for transformation is represented in Figure 1.

Transformation and analysis of transgenic material. Two commercial tetraploid potato varieties were chosen for the transformation experiments: Diamant and Van Gogh. Both varieties have been selected for a reasonably good level of blackspot resistance when compared to other varieties. Thus, the challenge was to assess whether molecular approaches could increase blackspot resistance over and above what traditional breeding techniques have achieved.

Using potato tissue explants (internodes) in co-cultivation experiments we produced 50 independent transformants per construct and variety, yielding 1400 transgenic lines[19]. In order to verify the transformation protocol and to obtain data on the average copy number of transgenes in the transformed lines, Southern blot analysis[20] was carried out at random on a sample of 50 lines, including representatives from each construct type for both varieties. The average copy number was 3 and the predicted restriction patterns were obtained for all lines tested, confirming transformation and the integrity of the insert (data not shown).

For an initial elimination screening of all 1400 transgenotes, the lines were cultured for microtuber production, and these tissues were then used in a PPO enzyme assay. We found no statistically significant differences between the ability of different antisense PPO genes (Class I and II) to suppress PPO activity, nor were there significant differences with respect to the size of the PPO gene-sections used in the constructs. Thus, in the analysis of results presented below, these variants are grouped together, and the means compared to GUS-transformed controls. In the cultivars Diamant and Van Gogh, 74% and 72% of antisense transformants, respectively, gave lower PPO enzyme activity than the GUS-transformed controls. In total, thirty-two lines harboring antisense PPO constructs had no detectable PPO activity. Notably, only one of these lines was transformed with the patatin-promoter construct. Conversely, very high enzyme activity was found in individual lines expressing the PPO gene in a sense orientation. PPO enzyme activity in these transgenic plants reached levels up to 7-fold higher than GUS-transformed controls in Diamant and up to 10-fold in Van Gogh lines. In Figure 2 the mean PPO enzyme activities in microtubers are shown for the three promoters used and for the two potato varieties separately. Each bar represents the mean value of 200 transgenic lines. Both potato varieties show reduced mean enzyme activities when either the CaMV 35S or GBSS promoters are used. In contrast, transgenic plants expressing antisense PPO genes from the patatin-promoter constructs do not show statistically significant reductions.

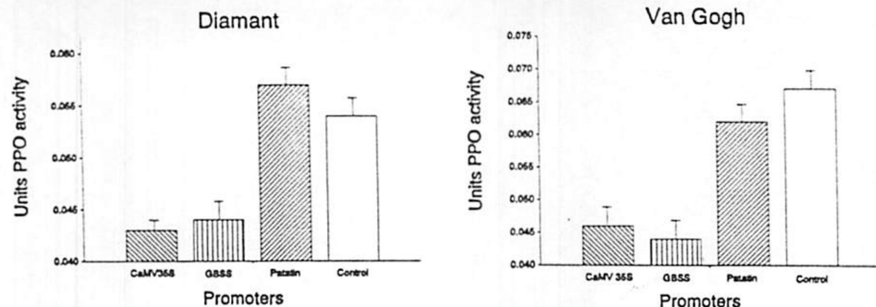

FIGURE 2. Mean units of microtuber PPO activity in the transgenic lines expressing antisense PPO genes are significantly reduced when the 35S CaMV and GBSS-G28 promoters are used to drive the antisense PPO gene, when compared to both the patatin promoter and the control. The data represents the means of 50 replicate lines, 4 different PPO genes per promoter per variety. Data was statistically examined by analysis of variance (ANOVAII) and subsequently tested with the Student-*t* test. Standard errors of the means are indicated.

Transcript analysis in transgenic potato lines. To verify the data obtained from the enzyme assays and to obtain some understanding of the kinetics of PPO expression in the transgenic potatoes, we analyzed mRNA isolated from young leaves, stolon tips initiating tuber formation, and young potato tubers (Fig. 3A, B, and C, respectively). The transgenics chosen for this experiment were nine Van Gogh lines (three lines from every promoter combination) containing full length antisense PPO genes and showing the lowest PPO enzyme activity in microtubers. Constructs expressing a CaMV 35S driven PPO gene in sense orientation and a CaMV 35S-GUS transformed control were also included. When poly-A$^+$ RNA isolated from either leaf, stolon or tuber of plants harboring the CaMV 35S promoter antisense PPO constructs was probed with an internal double stranded DNA fragment of pKG59-4, virtually no signal could be detected in any of the lines tested (Fig. 3ABC; lanes 3–5). However, PPO-gene transcript was detected in leaves of plants transformed with antisense constructs driven by both GBSS-G28 and patatin promoters (Fig. 3A, lanes 6–11). The weakly reduced transcript levels in leaves of the pGBSS/antisense PPO plants may well reflect the low level of GBSS promoter activity in these tissues. Interestingly, in stolon tips initiating tuber formation, PPO transcript was detected in the poly-A$^+$ RNA from all lines containing patatin promoter constructs (Fig. 3B; lanes 9–11) which disappears during further tuber development (Fig. 3C; lanes 9–11).

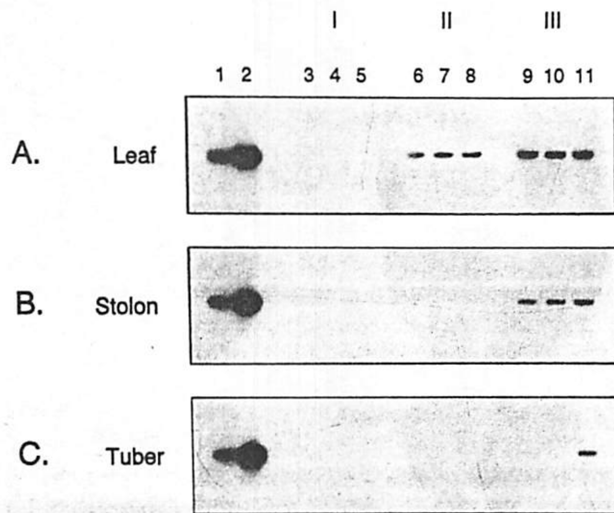

FIGURE 3. Northern analysis of transgenic potato plants. Expression of PPO mRNA in leaves (A), stolon tips initiating tubers (B) and young potato tubers (C). The two controls are poly-A$^+$ RNA from a GUS-transformed line (lane 1) and from a sense construct (lane 2). The first block of three lanes (I; lanes 3–5) is poly-A$^+$ RNA from plant tissues expressing PPO under control of the 35S CaMV promoter, the second block (II; lanes 6–8) from the GBSS promoter and block III (lanes 9–11) from the patatin promoter. The filter was probed with an 800 bp internal fragment of the Class II PPO gene labeled with ^{32}P.

Immunoblot analysis of PPO proteins in tubers of transgenic potatoes. Protein was extracted from microtubers of the same lines as those used in the transcript analysis, and immunoblot analysis was carried out using a polyclonal antibody raised against purified *Solanum berthaultii* PPO[21]. The results (Fig. 4) show abundant PPO protein in the sense construct (lane 2) when compared to the GUS-transformed control. Virtually no PPO protein could be detected in any lines carrying the CaMV 35S and GBSS constructs. However, control levels of PPO protein were revealed in the patatin-driven antisense PPO lines.

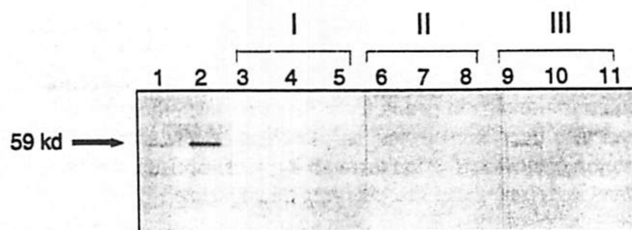

FIGURE 4. Immunoblot analysis of PPO protein from microtubers in the same lines as those used in the northerns. The two controls are protein from a GUS-transformed line (lane 1) and from a sense PPO construct (lane 2). The first block of three lanes (I; lanes 3–5) is protein from plant tissues expressing PPO from the 35S CaMV promoter, the second block (II; lanes 6–8) from the GBSS promoter and block III (lanes 9–11) from the patatin promoter. Ten micrograms of total protein was loaded per lane and the filter was probed with polyclonal antibody raised against purified PPO from *Solanum berthaultii* as described[11].

Field evaluation. In conventional potato breeding practice, standardized tests are carried out to determine the extent of discoloration after bruising in tubers from breeding lines[22]. An index (BI) is calculated for blackspot sensitivity which takes into account the level of tuber discoloration after subjecting them to standardized mechanical damage and subsequent storage at low temperature. The resulting index ranges from 0 to 50. Indices from tubers of 50 lines were determined after *in vitro* propagation and planting in field trials in Metslawier, northern Holland, in 1992 (Fig. 5). Lines were selected on the basis of enzyme assays described above. A significantly lower level of discoloration was noted on visual scoring, after tubers had been peeled, in lines carrying either CaMV 35S—or GBSS promoter driven

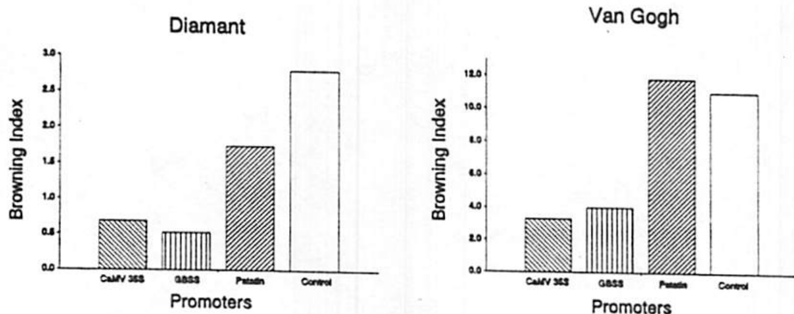

FIGURE 5. Discoloration indices of (BI) the field grown transgenic potato lines show a significant decrease in values when either the 35S CaMV or GBSS-G28 promoters were used in the constructs. Although the patatin containing lines had been selected from the total group of transgenics on the basis of low enzyme activity, no significant differences could be established from the controls in either variety.

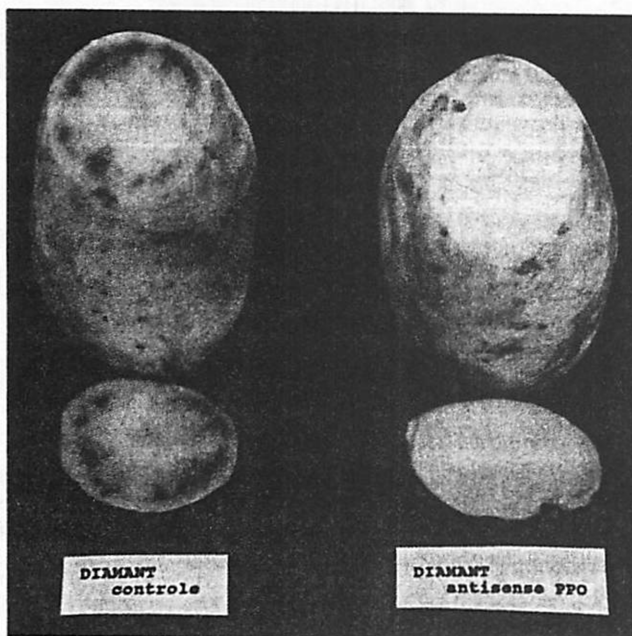

FIGURE 6. Bruising phenotype of an untransformed control and a transgenic line of the variety Diamant showing the medullary browning in the control and the pale color in the transgenic. Both potato tubers had been treated identically prior to photography.

antisense PPO genes. These results were further substantiated in the discoloration indices, where significantly lower indices were calculated in these transformants for both varieties when compared to the patatin promoter constructs, even though the latter had also been preselected on the basis of low enzyme levels. Figure 6 shows a section through a typical bruised tuber from a Van Gogh transgenic line carrying an antisense PPO gene under the control of the GBSS promoter with a non-transformed control depicted next to it.

Discussion

Modulating gene expression using antisense technology is rapidly becoming an important approach for achieving targeted alterations in plant biochemical pathways. Commercial applications now include alterations of flower color[14], virus resistance (reviewed in ref. 23) and fruit ripening[24]. Our results extend the possible uses of antisense technology to an area of food quality not previously investigated.

The two varieties used in the transformation experiments show an initial difference in their bruising phenotype with the variety Diamant having a lower browning susceptibility than Van Gogh. This was reflected in the antisense transgenics, where a significantly larger reduction in both enzyme activity and bruising phenotype was achieved in the latter variety. The conclusion that can be drawn from these tests is that a high percentage of blackspot resistant lines can be selected from transgenic potatoes expressing an antisense PPO gene under the control of CaMV 35S or GBSS promoters. These results are in contrast with previous attempts to select blackspot resistance from tissue culture-derived somaclonal variant potato lines, which proved unsuccessful (F.T.M. Verheggen; unpublished data).

Although the reason for the poor antisense inhibition of PPO expression in lines harboring the patatin promoter constructs remains unclear, it seems likely that the temporal expression pattern conferred on the introduced antisense PPO genes by the patatin promoter does not precisely coincide with the onset of endogenous PPO gene expression in the developing tuber. It was shown previously[11] that the expression of potato PPO gene is developmentally regulated; PPO mRNA can only be detected in early stages of organ development. The presence of endogenous PPO gene expression in stolons carrying the patatin antisense constructs indicates that the patatin promoter may not become fully active in this tissue in time to prevent accumulation of PPO mRNA. The early expression of endogenous PPO genes during organogenesis, taken together with the long half-life of the PPO protein, may well allow enough enzyme protein to be accumulated during tuber formation to give the high average activities in the patatin antisense lines described above. In young tubers (1–2 cm diameter), some transcript is detected in one of the patatin lines (Fig. 3C; lane 11). This is in agreement with the enzyme assays in which this line also showed higher PPO activities in microtubers. As expected, the sense PPO construct showed very high levels of PPO transcript in all tissues examined. These conclusions are also in agreement with studies of patatin and GBSS promoter activities[15,17]. Physical damage may be an additional factor reducing patatin promoter activity[25].

Of the large population of transgenic PPO lines generated, a small proportion of lines were not amenable to microtuber induction (<1%) and some of the lines chosen for field trials failed to grow. No correlation, however, could be established between the lack of viability, presumably due to somaclonal variation inherent to the transformation procedure, and decreased expression of PPO. Continuing field experiments in which more characters will be scored with regard to disease and pest resistance and biochemical characteristics may provide a better insight into the normal function of PPO activities in the biochemistry of intact tissues. Clearly, from an applied point of

view the lack of aberrant phenotypes associated with reduced PPO expression suggests that the approach described here may be broadly applicable to the reduction of enzymatic browning in a range of commercially important plants and their processed products.

Experimental Protocol

Plant materials. Potato plants (*Solanum tuberosum* cv. Van Gogh and Diamant) were grown *in vitro* on MS medium[26] supplemented with 30 g/l sucrose. Potato internode explants were transformed with *Agrobacterium tumefaciens* (strain GV3101)[27] containing the antisense-PPO Ti-plasmid constructs using the co-cultivation method essentially according to protocols described[19]. Plant material for molecular analysis was taken from plants grown in 17 cm pots under green house conditions. Poly-A+ RNA for Northern analysis was isolated from the first two internode leaves, from stolons initiating tuberization with 3–5 mm swollen tips and from tubers of 1–2 cm diameter harvested at the onset of flowering.

Molecular biology. Routine DNA manipulations were as described by Maniatis *et al.*[20]. Southern, Northern and Western analyses of potato DNA, PPO transcripts and proteins, respectively, was carried out as described previously[11]. Substrates for sequencing were produced using the *in vivo* excision protocol on lambda ZAPII clones (Stratagene, La Jolla, CA) isolated from a sink tuber cDNA library kindly supplied by L. Willmitzer. Poly-A+ RNA was extracted using poly-d[T]$_{15}$ oligonucleotides coupled to paramagnetic beads (Dynal A.S. Oslo, Norway). Five hundred ng poly-A+ RNA was loaded per lane and electophoretically separated RNA was capillary blotted onto Hybond N+ membrane (Amersham, UK) and probed with an internal DNA fragment of the PPO cDNA pKG59-4 labeled with ^{32}P. Protein was extracted from about 6 g of microtuber tissue of the transgenic lines used. 10 μg of protein was loaded per lane. Tandem Coomassie blue-stained gels were run to verify equal loading.

Plasmid constructions. To achieve tuber specific expression the Class I patatin[15] and GBSS-G28 (ref. 17) promoters were chosen. Fragments containing all sequences necessary to direct tissue specificity were isolated using PCR with standard protocols. Included in the PCR primers were restriction sites to facilitate cloning into the Ti-vectors. The GBSS promoter used was isolated from genomic DNA of the potato variety Bintje (from sequence data of the genomic clone G28)[17] and contained DNA from −1184 to −8. A HindIII site (5′) and a BamHI site (3′) were inserted at the termini by inclusion of the recognition sites in the PCR primers. This fragment was inserted into the gel purified Ti-vector (pKG1001; described below) after treatment of both fragment and vector with HindIII and BamHI. The Class I patatin promoter used, contained DNA from base −1514 to base −31[15] (cloned in a pUC8 plasmid kindly provided by L. Willmitzer). Restriction sites HindIII and BamHI were incorporated into the 5′ and 3′ ends using PCR to allow cloning into Ti-vector, pKG1001, after treatment with HindIII and BamHI. The CaMV 35S expression vector was constructed from the vector pBI121 (ref. 18). The modifications include replacement of the mutant NPTII gene in pBI121 and the deletion of the GUS coding region; the resulting vector (pKG1001) was also the basis for the other expression vectors described below. The two tuber-specific promoters were inserted into pKG1001 resulting in pKG1001/pat; containing the Class I patatin promoter and pKG1001/GBSS containing the GBSS promoter. Antisense constructs were made, using each of the full-length PPO genes. Another set of constructs were made using an 800 bp region around the translation initiation site. As a general strategy for cloning PPO genes into Ti-vectors, sequence specific PCR primers were designed against the required sites of the PPO cDNAs. Incorporated into these primers were recognition sites for restriction enzymes to be used in the cloning (BamHI and BglII, 5′ and 3′ termini, respectively). Tuber PPO sections from pKG59-4 and pKG45-8 were inserted into all three expression vectors described (pKG1001, pKG1001/pat and pKG1001/GBSS). In these experiments both the 5′ segment and the full length sections from the two cDNAs were used. All of the 14 potato PPO constructs were introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation and their integrity was rechecked by restriction enzyme analysis.

Enzyme assays. Five g fresh weight of microtubers from each line was homogenized in 5 ml buffer (10 mM Na acetate, pH 6.0). PPO enzyme assays were then performed on this extract. Fifty mM catechol was used as substrate for the assay in a total volume of 1 ml. Enzyme activity is expressed as the rate of change of OD at 520 nm/ml extract/min at 25°C. Two independent measurements were performed on each line and the means were used in the further analysis. Boiled extracts were tested and were shown to have no residual enzyme activity.

Browning assay and computation of discoloration indices. Potatoes harvested from each line, grown in separate plots, were subjected to bruising under standard conditions: 2–3 kg of potatoes are placed in a shaking device comprised of a wooden box with padded walls. The box is mechanically agitated for 30 seconds. After the bruising procedure, tubers are stored for 4 days at 8–10°C. Subsequently the potatoes are mechanically peeled until 80% of the skin is removed and the degree of browning is scored in terms of percent of the surface area affected by discoloration. The percentages are categorized into four classes and the number of tubers in each class are entered into the following formula from which the index is determined:

$$BI = \frac{L + 2 \times M + 3 \times Z}{6 \times (G + M + L + Z)} \times 100$$

Where G, L, M, and Z are the number of tubers categorised in a given class of surface browning (G; 0–0.2%, L; 0.2–0.5%, M; 0.5–2.0% and Z; >2%).

Acknowledgments

We thank M. Holwerda, H.T. Krijgsheld, S.H. van der Molen, M.A.F. Hornes and D. Pouwels for their technical help; M.T.J. de Both and L. Slootmaker for help and encouragement throughout the project; G. Simons, B. Horvath and T. Bisseling for critically reading the manuscript. The project was financed by Cebeco Handelsraad, RZ Research, De ZPC and the Ministry of Economic Affairs of the Netherlands. JCS acknowledges support from USDA-NRICGP. MDH is supported by a fellowship from the NSF/DOE/USDA Plant Science Centre.

References

1. Mayer, A. M. and Harel, E. 1991. Phenoloxidases and their significance in fruit and vegetables p. 373–398. *In:* Food Enzymology. Fox, P. F. (Ed.). Elsevier Science Publishers, New York.
2. The Federal Register. (year?) 51:25021–25026.
3. Gray, D. C. and Hughes, H. C. 1978. Tuber quality, p. 504–539. *In:* The Potato Crop: The Scientific Basis for Improvement. Harris, P. M. (Ed.). Chapman and Hall, London.
4. Vertregt, N. 1968. Relation between blackspot and the composition of the tuber. Eur. Potato J. 11:34–44.
5. Burton, W. G. 1969. Black spot, physiological aspects. Proc. of the 4th Triennial Conf. of the EAPR. (Brest), p. 79–92.
6. Stark, J. C., Corsini, D. L., Hurley, P. J. and Dwelle, R. B. 1985. Biochemical characteristics of potato clones differing in blackspot susceptibility Am. Pot. J. 62:657–666.
7. Katz, E., Thompson, C. J. and Hopwood, D. A. 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129:2703–2714.
8. Vanneste, W. H. and Zubrühler, A. 1974. Copper containing oxygenases, p. 371. *In:* Molecular Mechanisms of Oxygen Activation. Hayiashi (Ed.). Academic Press, N.Y.
9. Vaughn, K. C. and Duke, S. O. 1984. Function of polyphenol oxidase in higher plants. Physiol. Plant. 60:106–112.
10. Cary, J. W., Lax, A. R. and Flurkey, W. H. 1992. Cloning and characterisation of cDNAs for *Vicia faba* polyphenol oxidase. Plant Mol. Biol. 20:245–253.
11. Hunt, M. D., Eannetta, N. T., Yu, H., Newman, S. M. and Steffens, J. C. 1993. cDNA cloning and expression of potato polyphenol oxidase. Plant Mol. Biol. 21:59–68.
12. Newman, S. M., Eannetta, N. T., Yu, H., Prince, J. P., Carmen de Vince, M., Tanksley, S. D. and Steffens, J. C. 1993. Organisation of the tomato polyphenol oxidase gene family. Plant Mol. Biol. 21:1035–1051.
13. Shahar, T., Henning, N., Gutfinger, T., Hareven, D. and Lifschitz, E. 1992. The tomato 66.3 kD polyphenoloxidase gene: molecular identification and developmental expression. Plant Cell 4:135–147.
14. Mol, J. N. M., van der Krol, A. R., van Tunen, A. J., van Blokland, R., de Lange, R. and Stuitje, A. R. 1990. Regulation of plant gene expression by antisense RNA. FEBS 268:427–430.
15. Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Schell, J. and Willmitzer, L. 1989. Both developmental and metabolic signals activate the promoter of a class I patatin gene. EMBO J. 8:23–29.
16. Benfey, P.N. and Chua, N. 1990. The cauliflower mosaic virus 35S promoter: combinatoral regulation of transcription in plants. Science 250:959–966.
17. Rohde, W., Becker, D., Kull, B. and Salamini, F. 1990. Structural and functional analysis of two *waxy* gene promoters from potato. J. Genet. and Breed. 44:311–315.
18. Jefferson, R. A., Kavanagh, T. A. and Bevan M. W. 1987. GUS-fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6:3901–3907.
19. Ooms, G., Burrel, M.M., Bevan, M. and Hille, J. 1989. Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theor. Appl. Genet 73:744–750.
20. Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor NY.
21. Kowalski, S. P., Eannetta, N. T., Hirzel, A. T. and Steffens, J. C. 1992. Purification and characterisation of polyphenol oxidase from glandular trichomes of *Solanum berthaultii*. Plant Physiol. 100:677–684.
22. Meijers, C. P. 1986. Handleiding kwaliteitsbeoordeling veldgewasaardappelen (CKA-I), IBVL, Wageningen, The Netherlands.
23. Wilson, M. T. 1993. Strategies to protect crop plants against viruses: Pathogen derived resistance blossoms. Proc. Natl. Acad. Sci. USA 99:3134–3141
24. Bird, C. R. and Ray, J. A. 1991. Manipulation of plant gene expression by antisense RNA, p. 207–227. *In:* Biotechnology and Genetic Engineering Reviews. Vol. 9. Tombs, M. P. (Ed.). Intercept Ltd. Southampton, UK.
25. Belknap, W. R., Rickey, T. M. and Rockhold D. R. 1990. Blackspot bruise dependent changes in enzyme activity and gene expression in Lemhi Russet. Am. Pot. J. 67:253–265.
26. Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497.
27. van Larebeke, N., Engler, G., Holsters, M., van den Elsacker, S., Zaenen, J., Schilperoort, R.A. and Schell, J. 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature 252:169–170.