Enhanced drought tolerance in transgenic potato expressing the *Arabidopsis thaliana* Cu/Zn superoxide dismutase gene

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All aerobic organisms must possess the means to protect themselves from the toxic effects of reduced oxygen species generated during the normal metabolic activity of cells or as a result of environmental stresses such as drought. Cells are protected from the deleterious effects of free oxygen radicals by Cu/Zn superoxide dismutase (SOD), which catalyses the initial step in neutralizing activated oxygen species. In the study reported here, the potato cultivar Aviva was transformed with a cytosolic Cu/Zn superoxide dismutase gene from *Arabidopsis thaliana* using *Agrobacterium*-mediated gene transformation. Four transgenic potato lines were identified and evaluated for drought tolerance in the greenhouse. Two transformed lines could withstand drought in the greenhouse for two weeks longer than the untransformed plants and one week longer than two other transformed lines. These findings were confirmed by data from enzyme activity as well as by 2,3,5-triphenyltetrazolium chloride reduction.

Introduction

Cells are protected from the deleterious effects of oxygen free radicals by superoxide dismutase (SOD), which catalyses the initial step in neutralizing activated oxygen species. The superoxide anion radicals are reduced to hydrogen peroxide and molecular oxygen.[1–4] A positive correlation between enzymes from the antioxidative system and drought tolerance was reported for maize,[5] tobacco[6] and alfalfa.[7] As a result, SOD has become the object of intensive research on the physiology, biochemistry, and the molecular and cell biology of plants. The superoxide dismutases are a divergent class of metalloenzymes, which form as distinct isozymes in different subcellular compartments. The manganese-SOD (Mn-SOD) is usually found within the mitochondrial matrix, whereas the iron-SOD (Fe-SOD) occurs in plastids. Copper/zinc-SOD (Cu/Zn-SOD) is localized in both the cytosol and plastids.[8]

Correlations between elevated SOD activity and stress tolerance suggest that the regulation of SOD levels provides plants with a mechanism against oxygen toxicity. However, direct proof of this effect is lacking.[9,10] A true evaluation of the effects of changing SOD activity in plants may be obtained by genetic engineering.[2] The effect of overproduction of the SOD enzyme (increased copy number), or lack of this activity (antisense technology), during drought stress may enhance our understanding of the role of Cu/Zn-SOD activity.

The genetic manipulation of SOD in plants was first described for tobacco and tomato. The regenerants overproduced a chloroplastic Cu/Zn-SOD derived from petunia. There was no significant difference between either tobacco or tomato plants that produced elevated Cu/Zn-SOD levels and the control plants. These researchers concluded that enhanced activity of SOD alone in the chloroplasts was not adequate to protect the cells against oxygen toxicity caused by ozone, fumigation or the herbicide, methyl viologen.[11] Different results were obtained when a chloroplastic Cu/Zn-SOD from pea was introduced into tobacco and potato. The transgenic plants were more tolerant when exposed to methyl viologen; membrane damage was measured by electrolyte leakage.[12] Additionally, tobacco plants that express a chimeric gene which encodes chloroplast-localized Cu/Zn-SOD from pea were more tolerant to chilling and high light intensity.[1] Transgenic tobacco plants that over-expressed mitochondrial Mn-SOD as well as a chloroplast-targeted Mn-SOD were more resistant to methyl viologen.[13] Transgenic potato plants that expressed tomato Cu/Zn-SODs had enhanced protection against methyl paraquat toxicity.[9] Transgenic alfalfa (*Medicago sativa*) that expressed Mn-SOD was more drought tolerant than control plants. A three-year field trial showed that the yield and survival of transgenic plants improved significantly, demonstrating for the first time that greater tolerance to oxidative stress is also successfully adapted in the field.[7]

Van der Mescht *et al.*[14] showed that the potato cultivar Aviva had only 50% Cu/Zn-SOD activity compared with eleven other cultivars, which differed in growth period and drought tolerance. Aviva is a drought-tolerant cultivar with a short growth period intended for the crisp market. The cultivar was bred at the Agricultural Research Council (ARC)-Vegetable and Ornamental Plant Institute (VOPI), Roodeplaat, Pretoria. The aim of the study reported here was to transform Aviva plants with an *A. thaliana* Cu/Zn-SOD gene in an attempt to enhance its drought tolerance.

Materials and methods

See Appendix.

Results

Kanamycin tolerance experiments

Callus regeneration was observed after seven weeks (Table 1). The addition of cefotaxime to the regeneration medium did not appear to suppress regeneration as indicated by the formation of callus. In the case of Aviva, cefotaxime stimulated shoot production, because four explants produced callus and shoots after seven weeks, whereas explants incubated on regeneration medium only produced callus only. The addition of kanamycin to the medium killed off the leaf explants, even at the lowest concentration of 25 mg l^{-1}. Thus, we decided to use this concentration to select transformed cells following plant transformation.

Table 1. Regeneration* from Aviva leaf explants incubated with various antibiotic concentrations.

Treatment	Explants	Dead	Callus only	Callus + shoots
RIAT + 0 mg l^{1} Km	20	0	20	0
RIAT + 25 mg l^{1} Km	20	20	0	0
RIAT + 50 mg l^{1} Km	20	20	0	0
RIAT + 75 mg l^{1} Km	20	20	0	0
RIAT +100 mg l^{1} Km	20	20	0	0
RIAT + 250 mg l^{1} Cx	20	0	16	4

*Table entries represent numbers.
Km, kanamycin; Cx, cefotaxime.
RIAT, two-step *in vitro* regeneration medium procedure for potato plantlets.

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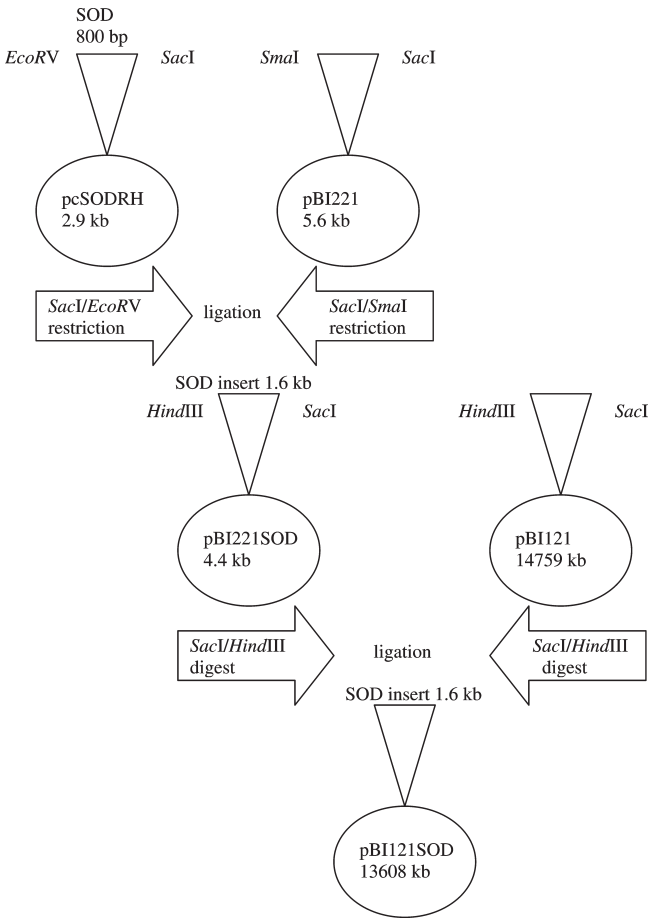

Fig. 1. Schematic representation of the steps in the cloning of the *Arabidopsis thaliana* superoxide dismutase (SOD) gene into the plant transformation vector pBI121 to produce pBI121SOD.

Cloning of the *A. thaliana* Cu/Zn-SOD cDNA into transformation vectors

Following transformation, 16 putative transformed DH5α colonies were observed. Fourteen of the sixteen DH5α clones exhibited an identical electrophoretic pattern. Two bands (one corresponding to 1.6 kb and one to 2.8 kb) were expected because the pBI 221 SOD construct contains two *Pst* 1 sites at 1.6 kb and 2.8 kb. These results showed that 14 of the 16 DH5α clones were successfully transformed with the SOD gene (0.8 kb). This represents an 88% transformation rate. These transformed DH5α cells were termed pBI 221 SOD (4.6 kb) (Fig. 2).

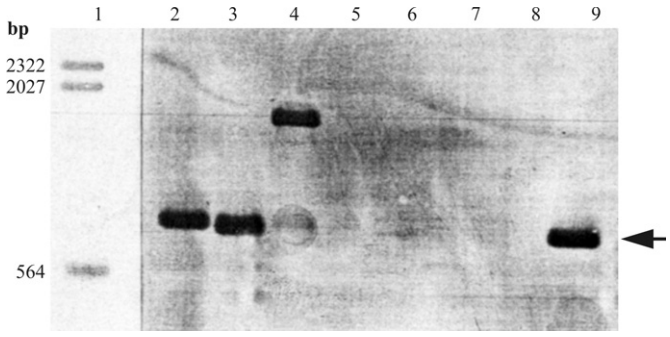

Fig. 2. Southern blot, using the DIG labelling and detection kit as method to confirm transformation of pB1 121 with the *Arabidopsis thaliana*-SOD gene (indicated by arrow). Lane 1: Molecular weight marker II (Roche); lanes 2, 3, 4: *BamH*I, *EcoR*I, and *Pst*I restriction of pBI 121 SOD, respectively; lanes 5, 6, 7: *BamH*I, *EcoR*I, and *Pst*I restriction of pBI 121, respectively; lane 9: *EcoR*I restriction of pcSODRH.

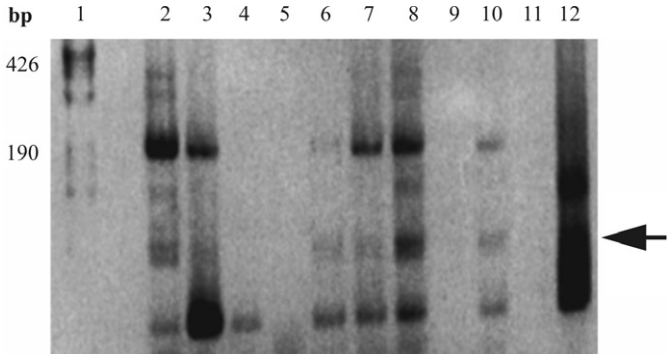

Fig. 3. PCR analysis of putative *Arabidopsis thaliana*-SOD-transformed potato plants using SOD-specific primers. Lane 1: Molecular weight marker III (Roche); lanes 2–8: putative transformed plants; lane 9: untransformed control plant; lane 10: negative control plasmid; lane 11: water; lane 12: pB1121 SOD positive control plasmid. Arrow indicates confirmation of transformed potato clones.

Molecular confirmation of transformed potatoes

The expression of the SOD gene in the plants was studied by means of PCR analysis as well as Southern blot analysis. The PCR results with the SOD-specific primers indicated that these primers were not specific enough, because the *Arabidopsis* SOD gene has high homology with the native potato SOD. The PCR fragment representing the SOD gene was present in all of the putative transformants, except transformant SOD3, but also to a lesser extent in the untransformed control plant. All other negative controls yielded no bands, as expected. Because of these results, a PCR with the NptII primers was performed, because the transformation cassette also contained a kanamycin marker gene. The PCR with the NtpII primer gave positive results in all of the putative transformants tested, namely, SOD1, SOD2, SOD6 and SOD7 (Fig. 3). No amplified fragments were observed in any of the negative controls, as expected.

Southern blot analysis was performed with digested plant DNA, using the SOD DIG-labelled probe. Bands were observed in all of the putative transformants, however, as well as in the control plant (results not shown). This was ascribed to the fact that the *Arabidopsis* SOD gene has a high homology with the native potato SOD. These results confirmed what was observed in the PCR screening for the SOD gene. The Southern blot gave the expected banding pattern for the putative transformants, namely, SOD1, SOD2, SOD6 and SOD7 (Fig. 4). All the negative controls yielded no bands, as expected. These results indicated that the SOD gene had been integrated into the genome of SOD1, SOD2, SOD6 and SOD7.

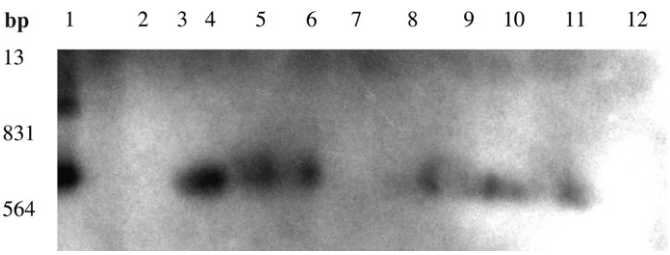

Fig. 4. Autoradiograph of Southern blot of putative *Arabidopsis thaliana*-SOD-transformed potato plants using a DIG labelled nptII probe and DNA extracted from the putative transformed plants. Lane 1: Molecular weight marker III (Roche); lane 2: pBI 121 SOD; lanes 3, 4: untransformed control plants; lanes 5–12: putative transformed plants; lane 13: negative plasmid control.

Table 2. Cu/Zn-SOD levels† during drought stress and control conditions in a comparison between untransformed Aviva plants and four *Arabidopsis thaliana*-SOD-transformed Aviva lines (SOD1, SOD2, SOD6 and SOD7).

Cultivar	Treatment	Week 1*		Week 2		Week 3		Week 4		Week 5	
Aviva	control	1.98	n.s.	1.62	n.s.	2.07	*↓	lethal stress			
	stress	1.71		1.50		1.67					
SOD1	control	2.18	n.s.	2.07	n.s.	2.00	n.s.	2.18	*↓	2.02	*↓
	stress	2.17		2.12		1.16		1.78		1.27	
SOD2	control	2.14	n.s.	2.37	*↓	1.96	n.s.	2.53	*↓	2.18	*↓
	stress	2.15		1.91		1.73		1.77		1.92	
SOD6	control	2.35	n.s.	2.20	n.s.	2.22	n.s.	2.28	n.s.	lethal stress	
	stress	2.12		2.03		2.32		2.44			
SOD7	control	2.95	n.s.	2.88	*↓	2.53	*↓	2.61	n.s.	lethal stress	
	stress	2.90		2.54		2.23		2.54			

*Weeks without water; * ↓ significant decrease; n.s., not significant.
†Enzyme activity was measured in units/gram dry weight.

Cu/Zn-superoxide dismutase activity

The Cu/Zn-SOD activity was measured in the leaves that were harvested at weekly intervals during the six weeks that water was withheld. After two weeks without water, a significant decrease in Cu/Zn-SOD activity was observed in the transformed lines SOD2 and SOD7; after three weeks without water in the Aviva plants and in the transformed line SOD7; after four and five weeks without water in the SOD1 and SOD2; whereas SOD6 and SOD7 showed no significant differences after four weeks without water (Table 2).

2,3,5-Triphenyltetrazolium chloride reduction

Viability, as estimated by 2,3,5-triphenyltetrazolium chloride (TTC) reduction to the formazan salt, was measured every 30 min over a period of 3 h following the induction of the lethal stress. The formazan concentrations were higher as a result of the stress treatment than in the controls in three of the transformed plants, namely, SOD2, SOD6 and SOD7, indicating a tolerant reaction. In the sensitive reaction, the formazan concentrations over time were lower in the stress treatment than in the controls, that is, for the untransformed Aviva cultivar and the transformed SOD1 plants. The area between the corresponding graphs was estimated as the difference between the mean after the stress treatment, and the corresponding values for the controls, over time.[24] These results are presented in Fig. 5. The more negative values are an indication of a more sensitive cultivar, such as untransformed Aviva. We therefore concluded that all of the transformed potato plants (SOD1, SOD2, SOD6 and SOD7) were more drought-tolerant than the untransformed ones.

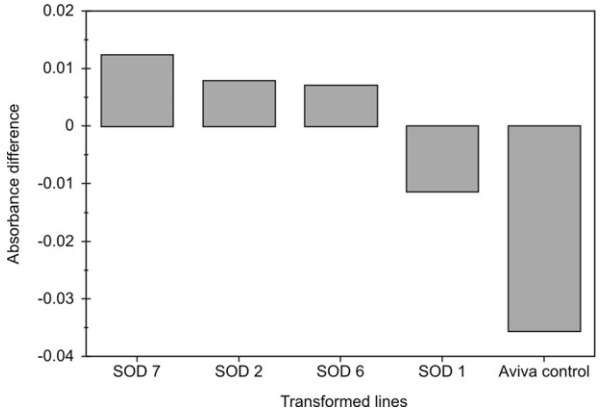

Fig. 5. The mean difference between the stress and control treatments of leaf discs obtained from *Arabidopsis thaliana*-SOD-transformed plants, and the untransformed Aviva line. The positive values indicate a drought-tolerant reaction whereas the negative values represent a sensitive reaction to drought when measured by triphenyltetrazolium chloride (TTC) reduction.

Discussion

Injury to plants, as caused by environmental stress, is associated with oxidative stress at the cellular level.[12,25] Perl *et al.*[9] postulated that transgenic plants with constitutively elevated levels of SODs should be more tolerant to photo-oxidative damage. However, the beneficial effects of the overproduction of SOD was achieved mainly by using methyl viologen as a stress factor. This result is only an indication of the biological importance of SOD, because paraquat is a superoxide-generating redox cycler, compared to environmental stress conditions that have a pleiotropic effect on plants.[26,27]

Van der Mescht *et al.*[14] demonstrated a correlation between increased Cu/Zn-SOD activity during drought stress and drought sensitivity. The results presented here confirm this observation. Although there was a slight increase in enzyme activity in the four transformed lines when they were watered, Cu/Zn-SOD activity under drought stress conditions showed either a non-significant response or a significant decrease compared with non-stressed plants. Additionally, some transformed lines (SOD 1 and SOD 2) could withstand drought in the greenhouse two weeks longer than the untransformed plants and one week longer than the transformed lines SOD 6 and SOD7. High levels of Cu/Zn-SOD activity (50-fold) in transgenic tobacco, according to Tepperman *et al.*,[11] did not confer tolerance to oxidative stress, while a small increase in Cu/Zn-SOD activity provided resistance to methyl viologen in human and mouse cells. It is possible that glutathione reductase and ascorbate peroxidase activity were limiting factors when SOD activity reached very high levels. In the results presented here, the copy number of the Cu/Zn-SOD gene in the transgenics could not be determined. This is ascribed to the high homology between the *A. thaliana* and the potato SOD genes, as well as the tetraploid nature of potato. Under control conditions, however, SOD6 and SOD7 displayed significantly higher Cu/Zn-SOD activity than Aviva, SOD1 and SOD2. Interestingly, it was the transformed lines that did not differ significantly from the control plants that survived the longest.

Triphenyltetrazolium chloride reduction gives an indication of cell viability. Additionally, the inhibition of TTC reduction is a measure of dehydrogenase inactivation that results in reduced formazan production.[28] It has been shown that during acclimation experiments, a higher formazan concentration in stressed plants compared to control plants over time, represents a tolerant reaction in cotton, whereas less formazan production in the stress treat-

ment compared to controls is an indication of sensitivity.[24] Leaves subjected to an osmotic stress of 0.5 M mannitol (–1.24 MPa) exhibited a tolerant response, with the exception of the transformed plant SOD1 and the untransformed Aviva plants. Although line SOD1 was drought sensitive, it was still more stress tolerant than the untransformed control plants. A statistical analysis was not performed on our results, because there was a high associated standard deviation as a result of the measurements being taken over time. In this case, the tendencies (tolerant versus sensitive) were important.[24] We concluded that the plants transformed with the Cu/Zn-SOD gene were more tolerant to drought than untransformed plants.

The TTC results were compared with the greenhouse findings, but a direct correlation was not obvious. According to these results, SOD2, SOD6 and SOD7 were drought tolerant, whereas SOD1 (although less sensitive) and Aviva were drought sensitive. On the other hand, the greenhouse results indicated that SOD1 and SOD2 were more drought tolerant than SOD6 and SOD7, which in turn were more tolerant than Aviva controls. Finding a correlation between the two experiments may have been complicated by the intensity of the drought stress. In the greenhouse, water was withheld from the plants, resulting in the progressive increase in drought stress, whereas TTC reduction was measured only under one water regime that represented a mild drought stress. We conclude, however, that the transformed lines were able to withstand drought longer than the untransformed cultivar before a lethal stress was induced.

It seems reasonable to conclude from these data that SOD plays a significant role in protecting living cells against the toxicity and mutagenicity of active oxygen species by virtue of their capacity to savage the superoxide radical. Van der Mescht *et al.*[14] showed previously that the Aviva cultivar displayed only half the Cu/Zn-SOD activity of 11 other cultivars differing in growth period and drought tolerance. Thus, we have shown for the first time that drought tolerance can be enhanced in a potato cultivar with sub-optimal Cu/Zn-SOD activity.

We are grateful to the Water Research Commission for financial support and to A. Slusarenko (Aachen) for the supply of the SOD *Arabidopsis* gene.

Received 30 March 1999. Accepted 8 April 2007.

1. Sen Gupta A., Heinen J.L., Holaday A.S., Burke J.J. and Allen R.D. (1993). Increased resistance to oxidase stress in transgenic plants that overexpress chloroplastic Cu/Zn superoxide dismutase. *Proc. Natl Acad. Sci. USA* **90**, 1692–1633.
2. Bowler C., Van Montagu M. and Inzé D. (1992). Superoxide dismutase and stress tolerance. *Annu. Rev. Pl. Physiol. Pl. Mol. Biol.* **43**, 83–116.
3. Scandalios J.G. (1993). Oxygen stress and superoxide dismutase. *Plant. Physiol.* **101**, 7–12.
4. Zhu D. and Scandalios J.G. (1994). Differential accumulation of manganese – superoxide dismutase transcripts in maize in response to abscisic acid and high osmoticum. *Plant Physiol.* **106**, 173–178.
5. Malan C., Greyling M.M. and Gressel J. (1990). Correlation between Cu/Zn superoxide dismutase and glutathione reductase, and environmental and xenobiotic stress tolerance in maize inbreds. *Plant Sci.* **69**, 157–166.
6. Van Rensburg L. and Krüger G.J.H. (1994). Evaluation of components of oxidative stress metabolism for use in selection of drought tolerant cultivars of *Nicotiana tabacum* (L). *J. Pl. Physiol.* **143**, 730–737.
7. McKersie B.D., Bowley S.R., Harjanto E. and Leprince O. (1996). Water deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol.* **111**, 1177–1181.
8. Sakamuto A., Ohsuga H., Wakaura M., Mitsukawa N., Hibino T., Masumura T., Sasaki Y. and Tanaka K. (1993). cDNA Cloning and expression of the plastidic copper/zink – superoxide dismutase from spinach (*Spinacea vleracea* L.) leaves. *Plant Cell Physiol.* **34**(6), 965–968.
9. Perl A., Perl-Treves R., Galili S., Aviv D., Shalgi E., Malkin S. and Galum E. (1993). Enhanced oxidative-stress defence in transgenic potato expressing tomato Cu/Zn superoxide dismutases. *Theor. Appl. Genet.* **85**, 568–576.
10. Liu H-T., Liu T-T., Pan Q-H., Yang H-R., Zhan J-C. and Huang W-D. (2006). Novel interrelations between salicylic acid, abscisic acid, and PIP_2-specific phospholipase C in heat acclimation-induced thermotolerance in pea leaves. *J. Exp. Bot* **57**(12), 3337–3347.
11. Tepperman J.M. and Dunsmur P. (1990). Transformed plants with elevated levels of chloroplastic SOD are not more resistant to superoxide toxicity. *Plant Mol. Biol.* **14**, 501–511.
12. Allen R.D. (1995). Direction of oxidative stress tolerance using transgenic plants. *Plant Physiol.* **107**, 1049–1054.
13. Bowler C., Slooten L., Vandenbranden S., De Rycke R., Booterman J., Sysbesma C., Van Montagu M. and Inzé D. (1991). Manganese superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J.* **10**, 1723–1732.
14. Van der Mescht A., De Ronde J.A. and Rossouw F.T. (1998). Cu/Zn superoxide dismutase, glutathione reductase and ascorbate peroxidase levels during drought stress in potato. *S. Afr. J. Sci.* **94**, 496–499.
15 Murashige T. and Skoog F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
16. Hinges R. and Slusarenko A. (1992). cDNA and derived amino acid sequence of a cytosolic Cu/Zn superoxide dismutase from *Arabidopsis thaliana* (L.) Heyhn. *Plant Mol. Biol.* **18**, 123–125.
17. Sambrook J., Fritsch E.F. and Maniatis T. (1989). *Molecular Cloning, a Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.
18. Chung C.T. and Miller R.H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Res.* **16**, 3580.
19. Armitage P. (1988). Transformation of dicotyledonous plant cells using the Ti plasmid of *Agrobacterium tumefaciens* and Ri plasmid of *A. rhizogenes.* In *Plant Genetic Engineering and Gene Expression: A laboratory manual,* eds J. Draper, R. Scott, P. Armitage and R. Welden, pp. 69–160. Blackwell Scientific Publications, Oxford.
20. Murray S.L., Burger J.T., Oelofse D., Cress W.A., Van Staden J. and Berger D.K. (1998). Transformation of potatoes (cv Late Harvest) with the potato leafroll virus coat protein gene, and molecular analysis of transgenic lines. *S. Afr. J. Sci.* **94**, 263–268.
21. McGregor C.E., Lambert C.A., Greyling M.G., Louw H. and Arnich L. (2000). A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP, SSR) in tetraploid potato germplasm. *Euphytica* **113**(1), 135–144.
22. Ish-Horowicz D. and Burke J.F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acids Res.* **9**, 2989–2998.
23. Van der Mescht A., Visser A.F., De Ronde J.A. and Vorster H.J. (1992). Protein profiles during drought stress in potato. *J. S. Afr. Soc. Hort. Sci.* **2**(1), 55–57.
24. De Ronde J.A., Van der Mescht A. and Cress W.A. (1994). The biochemical responses of six cotton cultivars to heat stress. *S. Afr. J. Sci.* **91**, 363–366.
25. Kasukabe Y., He I., Watakabe Y., Otani M., Shimada T. and Tachibana S. (2006). Improvement of environmental stress tolerance of sweet potato by introduction of genes for spermidine synthase. *Plant Biotechnol.* **23**, 75–83.
26. Van Camp W., Willekens H., Bowler C., Van Montagu M., Inzé D., Reupold-Popp P., Sandermann H Jr. and Langebartels C. (1994). Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. *Bio/Technology* **12**, 165–168.
27. Cassels A.C. and Doyle B.M. (2003). Genetic engineering and mutation breeding for tolerance to abiotic and biotic stresses: science, technology and safety. *Bulg. J. Plant Physiol.* (Special Issue), 52–82.
28. Chen H.H., Shen Z.Y. and Li P.H. (1982). Adaptability of crop plants to high temperature stress. *Crop Sci.* **22**, 719–725.

Appendix

Materials and methods

Maintenance of in vitro *plantlets*

Aviva plantlets were obtained from the Potato gene bank (ARC-Roodeplaat) and were multiplied on the growth medium of Murashige and Skoog (MS)[15] in glass bottles under sterile conditions. Explants were cultured at 21–24°C with a photoperiod of 16 h in a photosynthetic photon flux (PPF) of 40 μmol m^{-2} s^{-1} (GEC Alsthom cool white fluorescent light).

Leaf disc regeneration

Leaves were excised from 4–5-week-old *in vitro* plantlets; the apical and basal 3 mm was excised and placed abaxial side down on the medium, which had been poured into Petri dishes under sterile conditions. Twenty-five leaves were used, the leaf explants were sub-cultured onto fresh medium once a week and incubated in a growth room under the same conditions as for the explants (see above).

The two-step *in vitro* regeneration medium procedure for potato plantlets (RIAT) was used. Leaf explants were first incubated on RIAT medium (MS stocks 1–6, 20 mg ml^{-1} sucrose, 2 μg ml^{-1} zeatin, 0.02 mg l^{-1} NAA, 0.02 μg ml^{-1} GA_3, 7.5 mg ml^{-1} agar, pH 5.8) until visible callus was produced on the cut edges of the leaf. Explants were subsequently transferred to RIAT medium with the auxin component (NAA) removed.

Kanamycin tolerance experiments

To determine the optimal kanamycin concentration for selection of transformed cells, Aviva leaf explants were incubated on regeneration media containing various levels of kanamycin (0, 25, 50 , 75 and 100 μg ml^{-1}). Regeneration on medium containing 250 μl ml^{-1} cefotaxime (the antibiotic was added to control growth of *Agrobacterium tumefaciens* after transformation) was also evaluated. Results were recorded after seven weeks.

Cloning of an A. thaliana *Cu/Zn-SOD cDNA into plant transformation vectors*

The plasmid pcSODRH consists of a 788-bp cDNA clone of a cytosolic Cu/Zn-SOD from *A. thaliana* in the *Eco*R1 site of p Bluescript (SK$^+$). The insert consists of a full coding sequence and 112-bp 5′ and 206-bp 3′ untranslated region + 14 bases of poly A$^+$ tail as previously described by Hinges and Slusarenko.[16] All restriction enzyme digests were performed according to standard procedures.[17] The pcSODRH was first restricted with *Sac*1 and, after precipitation, with *Eco*RV to yield the SOD insert (0.8 kb). The pBI 221 plasmid (5.7 kb) was first restricted with the *Sac*1 restriction enzyme and then with *Sma*I to yield the pBI 221 vector (3.8 kb) (Fig. 1).

After ligation (1 insert : 1 vector),[17] a transformation experiment was performed according to Chung and Miller.[18] The successful transformation of *E. coli* DH5α yielded ampicillin-resistant colonies. Plasmid DNA was extracted using the JAT preparation protocol. The pBI 121 plasmid was first restricted with the *Sac*I restriction enzyme and then with *Hind*III to yield the pBI 121 vector (10.3 kb). Ligation and transformation yielded the pBI 121 SOD vector (Fig. 1). The method of Armitage[19] was used for the triparental mating procedure.

Growth of Agrobacterium tumefaciens

An aliquot of frozen *A. tumefaciens* LBA 4404 cells, into which the pSOD plasmid had been inserted by triparental mating, was inoculated and incubated in YM medium (Gibco BRL) supplemented with 50 μg ml^{-1} kanamycin and 100 μg ml^{-1} rifampicin on a shaker at 28°C for 36 h. Before plant transformation, the bacterial cells were pelleted by centrifugation (3300 rpm, 25 min, 4°C) and resuspended in YM medium only.

Leaf disc transformation and regeneration

Potato leaf discs were pre-incubated on MS plates for 48 h. Following this, leaves were immersed in *A. tumefaciens* cell suspensions for various periods, blotted on sterile filter paper and re-plated onto the MS plates for two days of co-cultivation, as had previously been determined for potatoes.[20] Following co-cultivation, explants were transferred to the RIAT two-step indirect regeneration medium. Leaf explants were first incubated on RIAT medium (MS stocks 1–6, 20 mg ml^{-1} sucrose, 2 μg ml^{-1} zeatin, 0.02 μg ml^{-1} NAA, 0.02 μg ml^{-1} GA_3, 7.5 mg ml^{-1} agar, pH 5.8) until callus production was visible on the cut edges of the leaf. Explants were subsequently transferred to RIAT medium with the auxin component (NAA) removed (designated RIAT-).

Molecular confirmation of transformed potatoes

DNA was extracted[21] from seven putative transgenic tissue culture Aviva plantlets containing the SOD gene as well as from a control Aviva plant. The method of Ish-Horowicz and Burke[22] was used to isolate the SOD plasmid DNA, which was quantified with the use of a fluorometer. Electrophoresis of isolated DNA was performed on a 1% agarose gel.

To verify that the transgene had been integrated into the potato genome, PCR analysis was performed using SOD-specific primers. NptII-specific primers were also used. The PCR reactions were carried out in 10 μl volumes containing the following final concentrations: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $mgCl_2$, 0.025 mM each of dATP, dCTP, dGTP, and dTTP, 0.5 μM of each primer, and 0.5 U *Taq* polymerase. Standard amounts of DNA added were 40 ng plant DNA and 10 ng pBI 121 plasmid DNA per reaction.

The PCR cycling conditions included an initial denaturation step of 94°C for 2 min. This was followed by 35 cycles with denaturation at 94°C for 30 s, annealing at 56°C and 64°C for 30 s, for the SOD and NptII genes, respectively, and elongation at 72°C for 45 s. A negative control containing all PCR reagents, except template DNA, was included in all experiments. The PCR products were electrophoresed on a 1% agarose gel, stained with ethidium bromide and the DNA visualized under ultraviolet light.

Southern blot analysis was performed with SOD as well as NptII probes, which were DIG labelled according to the manufacturer's instructions using Boehringer Mannheim's DIG labelling and detection kit. Plant DNA was digested with *Hind*III and *Eco*RI in preparation for the Southern blot.[17]

Hardening off and drought stress of transformed plants

Four transgenic and one control (untransformed) tissue culture Aviva plantlets containing the SOD gene were hardened off.[20] The potatoes were grown in a greenhouse under conditions as described previously by Van der Mescht *et al.*[23]

Enzyme analysis

Cu/Zn-SOD extractions were performed as described by Van der Mescht *et al.*[14]

2,3,5-Triphenyltetrazolium chloride reduction

The accumulation of formazan was measured as described by De Ronde *et al.*[24]