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Transgenic *Brassica napus* plants overexpressing aluminium-induced mitochondrial manganese superoxide dismutase cDNA are resistant to aluminium

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ABSTRACT

We investigated the role that manganese superoxide dismutase (MnSOD, EC 1·15·1·1), an important enzyme of the antioxidant pathway, may play in aluminium (Al) toxicity/resistance. A wheat (*Triticum aestivum*) cDNA (*WMnSOD*) was cloned and up-regulation of the transcript was observed in root tips after 24 h exposure to 100 μM Al. The *WMnSOD1* under the control of the CaMV*35S* promoter was expressed in canola (*Brassica napus*). Transgenic plants were phenotypically normal. Northern analyses showed enhanced levels of the *WMnSOD1* and *WMnSOD1-GUS* transcripts and total SOD activity was 1·5- to 2·5-fold greater in transgenic plants than in wild type (WT) plants. Transgenic (T_1) leaf discs showed increased retention of chlorophyll and reduced electrolyte leakage when exposed to methyl viologen (MV) as compared with WT leaf discs, suggesting that transgenic plants were more resistant to oxidative stress. When WT canola plants were exposed to aluminium (0–200 μM), inhibition of root growth, higher SOD activity and increased levels of malondialdehyde (MDA, an indicator of lipid peroxidation) were observed in roots. Aluminium-induced inhibition of root growth and accumulation of MDA was lower in homozygous transgenic plants (T_2) compared with WT plants. Transgenic lines also showed lower synthesis of 1,3-β-glucans (callose, a sensitive marker for Al injury) as compared with WT. These data suggest that resistance to Al toxicity can be improved by overexpressing *WMnSOD1*.

Key-words: Aluminium; *Brassica napus*; callose; gene expression; lipid peroxidation; oxidative stress; resistance; superoxide dismutase; transgenic plants.

INTRODUCTION

Exposure of plants to various environmental perturbations, including intense light, drought, temperature stress, the presence of herbicides and metal toxicity, can lead to the generation of activated oxygen species (AOS). These AOS in turn generate hydroxyl radicals, which can affect a variety of biological macromolecules (Halliwell & Gutteridge 1984; Kampfenekel, Van Montagu & Inzé 1995). For example, AOS can react with unsaturated fatty acids in the plasma membrane to cause peroxidation of essential membrane lipids, which in turn may lead to severe cellular damage. Protection against metal toxicity in plants is thought to occur via a number of mechanisms, including control of the uptake, detoxification by phytochelatins (Briat & Lebrun 1999), or chelation by organic acids or amino acids (Basu, Basu & Taylor 1994a; Basu, Godbold & Taylor 1994b; Basu *et al*. 1999). When these systems become overloaded, metal-dependent detoxification mechanisms are activated. Under these conditions, metal-induced tissue damage, enhanced levels of lipid peroxidation, alteration of antioxidant enzyme activities and higher antioxidant levels have been observed (reviewed in Briat & Lebrun 1999).

Aluminium (Al) is one of the most important factors limiting plant productivity on acid soils. The primary site of Al accumulation and toxicity is the root meristem, where inhibition of root elongation has been recognized as a primary symptom of Al toxicity (Foy, Chaney & White 1978; Taylor 1988; Matsumoto 2000). The primary lesions leading to the inhibition of root growth have not been elucidated fully (Kochian 1995). Aluminium ions have been shown to cause increased peroxidation of membrane lipids. For example, Yamamoto *et al*. (1996, 1997) observed increased levels of lipid peroxidation in *Nicotiana tabacum* cells in the presence of Al and iron (Fe). They suggested that lipid peroxidation might be the primary lesion involved in Al-induced inhibition of growth. Furthermore, recent studies with *Pisum sativum* roots suggested that lipid peroxidation is a relatively early symptom induced by the accumulation of Al (Yamamoto, Kobayashi & Matsumoto 2001). Perhaps not surprisingly, Al toxicity increases the activity of superoxide dismutase and peroxidase (Cakmak & Horst 1991). Several Al-induced genes, including peroxidase, glutathione-S-transferase and SOD, have also been found to be induced by oxidative stress (Richards *et al*. 1998). Ezaki *et al*. (2000) recently demonstrated that overexpression of four Al-induced genes [*Arabidopsis* blue-copper-binding protein, *N. tabacum* glutathione S-transferase (*parB*), *N. tabacum* GDP-dissociation inhibitor gene (*NtGDI1*) and *N. tabacum* peroxidase gene (*NtPox*)] could ameliorate Al toxicity in *Arabidopsis*. Three of these genes also provided protection against oxidative stress, providing further evi-

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dence of a link between Al stress and oxidative stress in plants.

Defence against oxidative stress in plants involves complex metabolic adjustments, but the primary control occurs through SODs (Scandalios 1993; Bowler *et al*. 1994). Superoxide dismutases directly determine the cellular concentrations of O_2 and H_2O_2 by dismutating superoxide radicals. Superoxide dismutases are classified according to their metal cofactor and their subcellular localization. The predominant forms of SOD are the mitochondrial MnSOD, the cytosolic Cu/ZnSOD and the chloroplastic Cu/Zn and FeSODs (Bowler *et al*. 1994). Since SOD provides protection from AOS, different SOD genes have been expressed in transgenic plants (Allen 1995). Bowler *et al*. (1991) developed transgenic *N. tabacum* by expressing a chimeric gene derived from *Nicotiana plumbaginifolia* in which the mitochondrial transit peptide from a MnSOD cDNA was replaced by a chloroplastic transit peptide from *Arabidopsis* Rubisco (ribulose 1·5-bisphosphate carboxylase/oxygenase). Plants carrying the novel chloroplastic MnSOD showed reduced cellular damage when treated with the oxidative stress agent methyl viologen (MV). Transgenic *Medicago sativa* L. plants overexpressing the same chloroplastic MnSOD had increased vigour after freezing stress and increased winter survival under field conditions (McKersie, Bowley & Jones 1999). The targeting of a mitochondrial MnSOD to the chloroplasts provided protection to the plasmalemma but not to photosystem II in transgenic *Nicotiana plumbaginifolia* (Slooten *et al*. 1995). Recent studies by Kingston-Smith & Foyer (2000) reported that overexpression of MnSOD in maize leaves could lead to increased activity of the enzymes of the ascorbate–glutathione cycle (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase).

We previously purified a 23 kDa protein that is exuded by the Al-resistant *Triticum aestivum* cv. Maringa, grown under conditions of Al stress (Basu *et al*. 1994a). This protein cosegregated with the Al resistance phenotype in segregating populations arising from a cross between doubled-haploid Katepwa (Al-sensitive) and doubled-haploid Alikat (Al-resistant) (Basu *et al*. 1999). Analysis of peptide microsequences revealed that the 23 kDa protein was similar to a variety of MnSODs from bacterial and plant sources. We have not been able to isolate the gene encoding our putative extracytosolic MnSOD, perhaps because of the low abundance of the transcript. However, we have observed that a gene for the mitochondrial MnSOD (*WMnSOD*) from *T. aestivum* cv. Maringa also showed higher expression under conditions of Al stress. Given the putative role of oxidative stress in Al injury, we decided to overexpress the *WMnSOD* gene in *Brassica napus* cv. Westar. Unpublished data from our laboratory has demonstrated the sensitivity of *B. napus* to acid soil and Al toxicity. Overexpression of *WMnSOD* in *B. napus* resulted in increased resistance to Al toxicity.

MATERIALS AND METHODS

Cloning of *WMnSOD* cDNA

To isolate the *MnSOD* gene, degenerate oligonucleotides [sense primer SOD1; 5′-CA(TC)CA(TC)CA(GA)AA (GA)CA(TC)CA(TC)(GC)-3′ and antisense primer SOD2; 5′-AG(AT)TA(AG)TA(AGCT)GC(AG)TG(TC) TCCCA(AGCT)AC-3′] were designed on the basis of conserved sequences from plant MnSODs. These synthetic oligonucleotides were used as primers to amplify cDNA fragments (RT-PCR) from poly $(A)^+$ RNA isolated from roots of *T. aestivum* cv. Maringa treated with Al (solutions and growth conditions as described in Basu *et al*. 1999). A fragment of the expected length (434 bp) was obtained, cloned into T-vector (pBluescript; Stratagene, La Jolla, USA), sequenced and used as a probe to screen a cDNA library.

The cDNA library was constructed in Lambda Zap II (Stratagene) using poly $(A)^+$ RNA isolated from roots of cv. Maringa grown under conditions of Al stress. Stable cDNA was obtained from mRNA using Superscript II RNase H reverse transcriptase (GIBCOBRL). *Eco*RI and *Xho*I linkers were attached to the 5′ and 3′ ends of the ds-cDNA, which allowed for directional ligation of cDNA into the Lambda Zap II vector. Recombinants were packaged using Gigapack II Gold packaging extract (Stratagene). Following amplification in XL-1 Blue cells, recombinant phages were screened by nucleic acid hybridization using a gel-purified insert of the MnSOD probe. DNA in plaque lifts (Hybond N membranes from Amersham Pharmacia Biotech Inc., Quebec, Canada) were denatured and fixed according to Sambrook, Fritsch & Maniatis (1989) before prehybridizing and hybridizing at 65 °C. Using the *in vivo* excision properties of Lambda Zap II, positive clones were excised with ExAssist helper phage in SOLR (SOLR™ strain, Stratagene) cells and recircularized to generate subclones (phagemids) in the pBluescript SK (–) vector. Several clones were sequenced and the deduced amino acid sequence from the full-length cDNA clone (*WMnSOD*, Genbank accession number AF092524) was aligned with other known mitochondrial MnSOD sequences using the basic local alignment search tool (BLAST) algorithm (Altschul *et al*. 1990).

Construction of plant transformation vectors

The full-length clone (*WMnSOD*) was amplified using PCR with primers [sense primer WSOD1; 5′-GCTCTAGA CATGGCGCTCCGCACGT-3′ and antisense primer WSOD2; 5′-CGGGATCCAACAGCACTAGCGAAT-3′] designed to create a *Xba*I site at the 5′ end and *Bam*HI site at the 3′ end (underlined above). The amplified fragment was gel-purified and cloned into T vector, resulting in *WMnSOD1*. The *Xba*I/*Bam*H1 fragment from *WMnSOD1* was ligated into *Xba*I/*Bam*H1 digested pBI121 vector (Clontech Laboratories Inc., USA) to create the pBI121-*WMnSOD1* vector. This vector will express *WMnSOD1* under the control of the cauliflower mosaic virus promoter

(CaMV) with GUS (*Uid A*) as a reporter gene and *NPT II* as a selectable marker. The plasmid was sequenced through the *CaMV35S* promoter–*WMnSOD1* junction to verify construct assembly. The recombinant plasmid was transferred into *Agrobacterium tumefaciens* strain c58pmp90.

Plant transformation

Seeds of *B. napus* cv. Westar were surface-sterilized for 15 min in a 15% (v/v) bleach solution, followed by 2 min in 70% (v/v) ethanol and three rinses in sterile distilled water. Seeds were then germinated under aseptic conditions on germination medium in 60 × 15 mm Petri dishes for 5 d. The germination medium contained half-strength MS media (Murashige & Skoog 1962), 10 g L^{-1} sucrose and 7 g L^{-1} agar (pH 5·8). The *Agrobacterium* strain c58pmp90 containing pBI121-*WMnSOD1* was grown overnight at 28 °C in yeast extract peptone (YEP) medium containing 25 mg L^{-1} kanamycin and 20 mg L^{-1} gentamycin. Cotyledons with cotyledonary petioles from 5-d-old seedlings were used as explants for transformation following the protocol of Moloney, Walker & Sharma (1989). After 2 d of cocultivation in the dark at 22 °C, explants were transferred to regeneration medium consisting of MS macro and micro nutrients, 30 g L^{-1} sucrose and 0·5 mg L^{-1} benzyladenine (BA).

After 1 week on regeneration media, explants were transferred to selection media containing the same basal components as the regeneration media, with 50 mg L^{-1} kanamycin and 500 mg L^{-1} carbenicillin. After 4–6 weeks on selection medium, regenerated green shoots (putative transformants) were transferred to a rooting medium containing 0·5 mg L^{-1} IBA. When rooting was well established, putative transformants (designated T_0) were transferred to soil and grown in a controlled-environment chamber (16 h light 19 °C/8 h dark 14 °C). Sixteen independent lines were produced. The presence of the cotransformed gene was tested by polymerase chain reaction (PCR) analysis. The expression of the transcript for the transgene was confirmed in eight plants. Four of these plants (T_0) were fertile. At least 15 T1 seedlings were grown from each primary transgenic line and checked for the presence of the transgene by PCR. The segregation ratio (3 : 1) suggested the integration of the transgene at a single locus. To select homozygous lines, up to four different T1 plants from each primary transgenic line were screened. In the T2 generation, a minimum of 20 seedlings from different T1 plants were screened by PCR for the presence of the transgene to ensure their homozygosity.

Genomic DNA PCR and Southern analysis

Genomic DNA was isolated by grinding 100 mg of cotyledonary tissue using a DNeasy kit (Qiagen Inc., Mississauga, Ontario, Canada). PCR was performed using the following oligonucleotides: [sense primer CaMV; 5′-CCACTGACG TAAGTGGACGCACA-3′ and antisense primer WSOD2; 5′-CGGGATCCAACAGCAC TAGCGAAT-3′] or [sense primer WSOD1; 5′-GCTCTA GACATGGCGCTCCGC ACG-3′ and antisense primer GUS; 5′-TCATTGTTTGCC TCCCTGCTGC-3′]. An *Agrobacterium* strain containing the pBI121-*WMnSOD1* was used as a positive control template and wild type (WT) canola genomic DNA was used as a negative template. Products of PCR were analysed on a 1% agarose gel containing ethidium bromide. For Southern analysis, 10 μg of genomic DNA isolated from WT plants and transformants was digested with *Bam*H1 and probed with a gel-purified fragment of *WMnSOD1*.

Northern analysis and measurement of SOD activity of transformants

Transformed plants were analysed for the presence of *WMnSOD1* and *Uid A* transcripts. Total RNA was prepared from leaves and roots of both WT and transgenic plants using an RNeasy Plant mini kit (Qiagen). Total RNA samples (10 μg) were then resolved on a formaldehyde/agarose (1%) gel and transferred onto Genescreen Plus membranes (NEN Life Sciences Products, Boston, MA, USA). Membranes were probed with a gel-purified fragment of *WMnSOD1 and* exposed to Kodak X-OMAT film at 70 °C using an intensifying screen. All autoradiographs and EtBr-stained gels (for loading analysis and corrections) were scanned and converted to digital images using an Alpha Innotech Imaging System (Alpha Innotech Corporation, San Leandro, CA, USA). The same membranes were then reprobed with *Uid A* and *16S rRNA* probes.

For the determination of total SOD activity, 0·5 g of leaves were homogenized with 2 mL of 100 mM potassium phosphate buffer (pH 7·8) containing 0·1 mM ethylenediaminetetraacetic acid (EDTA), 0·1% Triton X-100 and 1% polyuinglpolypyrrolidone (PVP) as described by Sen Gupta *et al.* (1993). After centrifugation at 15 000 × g for 20 min, extracts were dialysed against 5 mM potassium phosphate buffer (pH 7·8) overnight. The SOD activity was measured spectrophotometrically at 340 nm as described by Paoletti & Mocali (1990). The cyanide-insensitive SOD activity was determined by preincubation of the extract with 1 mM potassium cyanide (KCN) for 30 min at room temperature. The activity inhibited in the presence of KCN is considered to be copper zinc SOD activity. Protein concentration was determined by the Bradford (1976) method, using bovine serum albumin (BSA) as a standard.

Oxidative stress resistance in transgenic lines

Healthy and fully expanded leaves from WT and selected T_1 plants were detached and washed briefly in deionized water. Leaf discs of 2·3 cm diameter (weighing ~100 mg) were excised and incubated overnight in the dark at room temperature with 40 mL of water or an aqueous solution (0, 0·25, 0·5, 0·75, 1·0 and 2·0 μM) of the oxidative stress agent, methyl viologen (paraquat-1,1′-dimethyl-4-4′-bipyridinium chloride). The discs were then illuminated for 2 h under

cool white fluorescent tubes (30 μmol m^{-2} s^{-1}) and subsequently incubated in the dark for another 20 h at 28 °C. The MV-dependent ion leakage was measured as an increase in the conductance (μS cm^{-1} 100 mg^{-1}) of the incubation media.

Damage caused by treatment with MV was also measured by the degree of bleaching occurring in the leaf tissues. Leaf discs of 2·3 cm diameter were cut and floated in 20 mL of MV (0, 2·5, 5·0 and 10·0 μM) under continuous white light (50 μmol m^{-2} s^{-1}) at 22 °C for 48 h. The amount of chlorophyll *a* and *b* in the leaf tissue was determined spectrophotometrically following extraction in 80% acetone (Lichtenthaler 1987).

Root growth assay

Seedlings from homozygous lines were tested for their sensitivity of root growth to Al. After germinating seeds on moist sand for 3 d, individual seeds were planted on cotton plugs stuffed into 1 mL syringes (with caps removed). Plants were grown in complete nutrient solution (Basu *et al*. 1994b, 1999), but Fe was omitted from the media. Individual seedlings were selected for uniformity after 24 h, initial root lengths were recorded and seedlings were placed in 50 mL of the treatment solution in disposable centrifuge tubes. Growing plants in syringes meant they could be transferred easily from control to treatment solutions without damaging roots. Furthermore, roots were forced to grow in a straight line, allowing root length to be measured more accurately. Plants were incubated in the treatment solution for 48 h with constant shaking.

Lipid peroxidation

The amount of lipid peroxidation was assessed using thiobarbituric acid (TBA) assay, in which malondialdehyde (MDA) is quantified as an end product (Heath & Packer 1968). Root tissue (~30–50 mg) was homogenized in 1 mL 0·1% trichloroacetic acid (TCA); 1 mL 0·6% aqueous solution of TBA was then added to each sample. The mixture was heated at 95 °C for 20 min and the reaction was stopped by placing the tubes in an ice water bath. Samples were centrifuged at 10 000 × *g* for 5 min and the absorbance of the supernatant was determined at 532 and 600 nm. The value for non-specific absorption at 600 nm was subtracted from the value at 532 nm. The amount of MDA–TBA complex was calculated using the extinction cofficient 155 $mM^{-1}cm^{-1}$.

Spectrofluorometric estimation of callose

Synthesis of 1,3-β-glucans (callose) in 5 mm root tips was employed as a marker for Al-induced injury using the spectrofluorometric procedure described by Zhang, Hoddinott & Taylor (1994). Pachyman (calbiochem, La Jolla, CA, USA) was used as an external standard and callose content was expressed as μg pachyman equivalents (PE) g^{-1} fresh weight (FW).

RESULTS

Up-regulation of WMnSOD in response to Al in wheat

We isolated a full-length cDNA (*WMnSOD*) encoding a mitochondrial MnSOD from *T. aestivum*. The predicted protein sequence contained 231 amino acids with a putative 21-amino-acid mitochondrial transit peptide present at the N-terminus and was 99% identical at the protein level to two other wheat mitochondrial MnSODs (*U73172, U72212*; Wu *et al*. 1999). To determine if *WMnSOD* responded to Al stress, total RNA was prepared from roots of cv. Maringa grown in the presence of 100 μM Al for different lengths of time. This concentration of Al does not seem to inhibit root growth in Maringa after 24 h of exposure (data not shown). A MnSOD transcript of ~0·9 kb was detected and up-regulation of the transcript was observed in root tips after 24 h of Al treatment (Fig. 1). This analysis confirmed that *WMnSOD* is up-regulated in roots in the presence of Al.

Overexpression of *WMnSOD1* in transgenic canola

Sixteen independent lines were produced by transforming *B. napus* cv. Westar with pBI121-*WMnSOD1* (details of the construct shown in Fig. 2a) using *Agrobacterium*-mediated transformation of cotyledonary petioles. Four of these lines were fertile and used for further studies. We used genomic PCR to confirm the presence of the *WMnSOD1* gene into *Brassica napus*. With WSOD1 and GUS as primers, an expected ~2·8 kb band was found in transgenic plants and in the positive control, but not in WT plants (Fig. 2b). A ~0·9 kb band was amplified using CaMV and WSOD2 as primers in transgenic plants and in the positive control, but not in the WT plants (Fig. 2b). In Southern analysis, a band of expected size (~0·9 kb, corresponding to *WMnSOD1* cDNA fragment) was detected in all transgenic lines, but not in the WT plants, after the digestion of genomic DNA with *Bam*H1

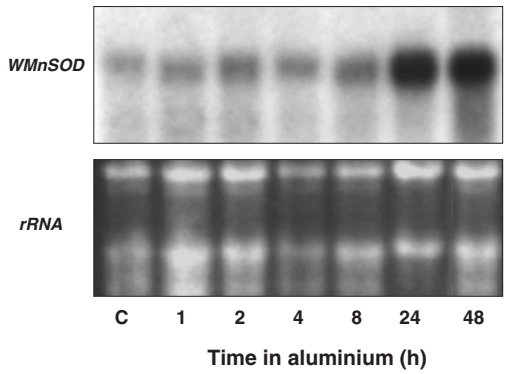

Figure 1. Expression of *WMnSOD* transcripts in root tips of *T. aestivum* cv. Maringa in response to 100 μM Al over time (0–48 h). The upper panel shows the results of the probe with wheat *WMnSOD*; the lower panel shows ethidium bromide staining of the same gel. The experiment was repeated at least two times.

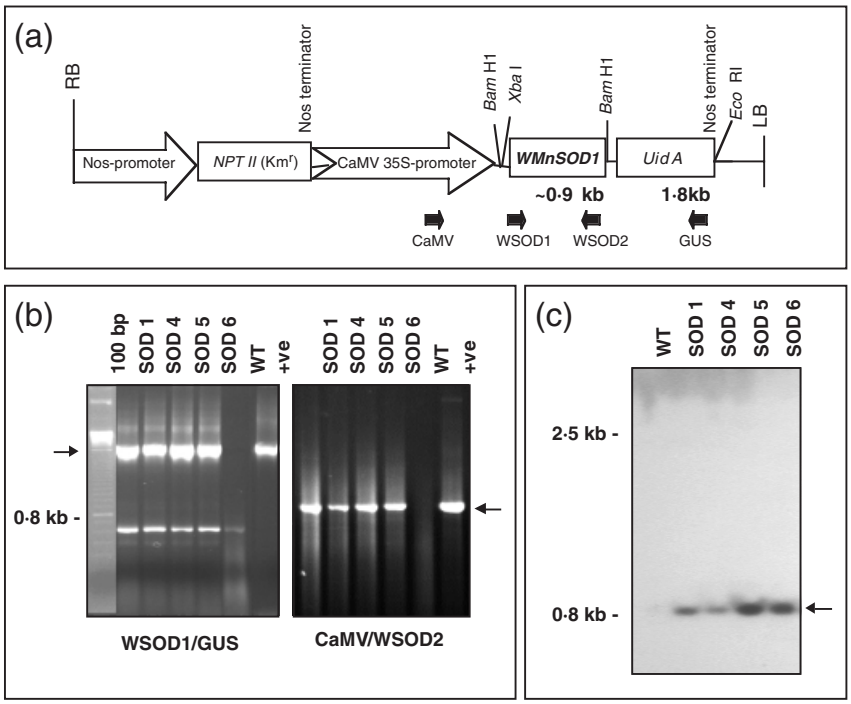

Figure 2. (a) Schematic representation of the construct, pBI121-*WMnSOD1* carrying a gene for *T. aestivum* mitochondrial *WMnSOD1* under the control of a *CaMV* promoter and with *nptII and UidA* as selectable markers (see Materials and methods). Two pairs of primers (WSOD1/GUS and CaMV/WSOD2) are indicated below the construct. (b) Genomic PCR of primary transformants (SOD 1, SOD 4, SOD 5, SOD 6). Genomic DNA was used as a template for PCR using either WSOD1/GUS (expected size of the product ~2·8 kb) or CaMV/WSOD2 (expected size of the product ~0·9 kb) as the primers. The *Agrobacterium* strain containing the construct pBI121-*WMnSOD1* was used as a positive control template and wild type DNA was used as a negative template. (c) Southern analysis of primary transformants (SOD 1, SOD 4, SOD 5, SOD 6). Genomic DNA (10 μg) isolated from the leaves of kanamycin-resistant *B. napus* plants transformed with pBI121-*WMnSOD1* were digested with *Bam*H1 and probed with radio-labelled *WMnSOD1*.

(Fig. 2c). In addition, restriction digestion of genomic DNA with *Eco*RI resulted in the appearance of bands at different positions in the different transgenic lines, suggesting that these lines were produced from different transformation events. A weak hybridization of wheat probe was also observed with endogenous SOD (data not shown).

Plants transformed with pBI121-*WMnSOD1* were analysed for the presence of *WMnSOD1* and *UidA* transcripts. Figure 3a shows two transcripts of ~0·9 and ~2·8 kb when total RNA from transgenic plants was probed with *WMnSOD1*. The presence of a ~0·9 kb transcript indicates that transcription initiation and termination of *WMnSOD1* occurred on its own, which was expected because of the presence of initiation (ATG), termination (TGA) and poly $(A)^+$ signals in the cloned *WMnSOD1* fragment. However, the *WMnSOD1* probe also showed weak hybridization with endogenous SOD and this was apparent from a weak signal of the ~0·9 kb transcript in the WT despite a greater loading. The ~2·8 kb transcript (upper band) represents the fusion transcript (MnSOD-GUS) between *WMnSOD1* and *UidA* (Fig. 3a). The same membrane was reprobed with probes for *UidA* and *rRNA*, which demonstrated the lack of GUS in WT plants and near-equal loading of lanes in transgenic lines. The SOD-GUS and *UidA* transcripts were not observed in RNA from WT plants. The expression of the transgene was also confirmed in roots and shoots from several T_1 plants and homozygous T_2 plants by probing with *WMnSOD1* and *Uid A* (data not shown).

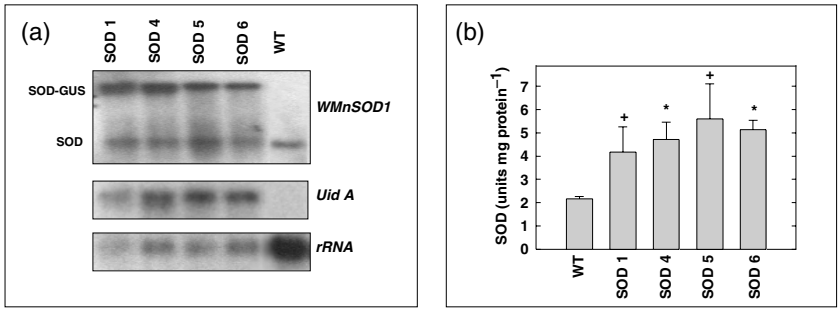

Figure 3. Northern analysis of *B. napus* primary transformants (SOD 1, SOD 4, SOD 5, SOD 6) (a) and the levels of SOD activity in T_2 and the WT plants (b). Northern blots of total RNA (10 μg) isolated from transgenic and WT plants were hybridized with radio-labelled *WMnSOD1* (expected size ~2·8 kb for SOD-GUS transcript and ~0·9 kb for WMnSOD and endogenous SOD), *UidA* (expected size ~2·8 kb for SOD-GUS transcript) and *rRNA* (~0·7 kb) gene probes. The same blot was hybridized with each probe after stripping the earlier probes. Enzyme activity was measured in young leaves from T_2 plants (see Materials and methods). Vertical bars represent standard errors ($n = 3$ per experiment). Each experiment was repeated at least three times. Values significantly different from the WT according to *T*-test are indicated by asterisks (+, $P \leq 0{\cdot}1$, *, $P \leq 0{\cdot}05$).

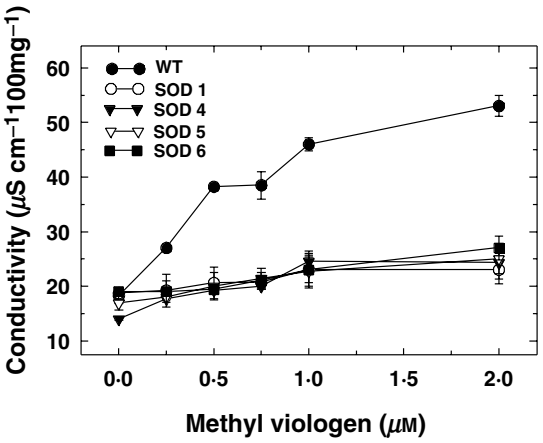

Figure 4. Effect of methyl viologen (MV) on ion leakage as assessed by the conductivity of assay solutions supporting WT and transgenic plants. Leaf discs from selected T_1 and WT plants were incubated in solutions with increasing concentrations of MV (0–2 μM) overnight in the dark at room temperature. Leaf discs were then illuminated for 2 h at 30 μmol m^{-2} s^{-1} and subsequently incubated in the dark for another 20 h at 28 °C before conductivity was measured (see Materials and methods). Vertical bars represent standard errors (n = 3 per experiment). The experiment was repeated at least three times.

SOD was measured in homozygous T_2 plants arising from each primary transgenic. Transgenic plants consistently had 1·5- to 2·5-fold higher levels of total SOD activity (+, $P \leq 0{\cdot}1$; *, $P \leq 0{\cdot}05$) than WT plants (Fig. 3b). The SOD activities were also found to be higher in T_0 and T_1 plants compared with WT plants (data not shown). We also observed the GUS staining in leaf tissues from transgenic plants but not those from WT plants (data not shown).

Resistance to oxidative stress in transgenic lines

Cellular resistance to oxidative stress was tested in selected individuals from the T_1 generation by exposing leaf discs to MV and measuring MV-dependent ion leakage as an increase in the conductance of assay solutions. The conductivity of assay solutions increased with increasing concentrations of MV in WT plants, with a 180% increase observed at 2·0 μM MV. In contrast, an increase of only 22–38% was observed in the four transgenic lines at 2·0 μM MV, suggesting enhanced tolerance to MV (Fig. 4). Oxidative damage caused by MV was also reflected in the degree of bleaching observed in the leaf tissue (Fig. 5a). Leaf discs from WT plants showed bleaching at concentrations of ≥2·5 μM MV. In contrast, transgenic plants showed little sign of bleaching, even at 10 μM MV (Fig. 5a). Measurement of chlorophyll content after 48 h of treatment with MV provided further support for these phenotypic differences (Fig. 5b). Methyl viologen-induced loss of chlorophyll was lower in transgenic plants than in WT plants (Fig. 5b).

Effect of Al on root growth, lipid peroxidation and SOD activity in WT canola

When WT seedlings were exposed to different concentrations of Al (0–200 μM), a progressive inhibition of root growth was observed (Table 1). Root growth was inhibited by 66% at 100 μM Al and by 92% at 200 μM Al. MDA content, an indicator of lipid peroxidation, increased significantly: by two- and three-fold in roots in response to 100 and 200 μM Al, respectively (Table 1). Total SOD activity showed significant increases: two-fold in roots when exposed to 100 and 200 μM Al (Table 1). Similarly, KCN-insensitive SOD activity also showed significant (two-fold) increases with exposure to 100 and 200 μM Al (data not shown). In shoots, both MDA levels and SOD activity demonstrated an increasing trend with increasing concentrations of Al, but this increase was not statistically significant.

Resistance to Al in transgenic lines

We used root growth, lipid peroxidation and callose accumulation as indicators of Al stress to test the Al resistance

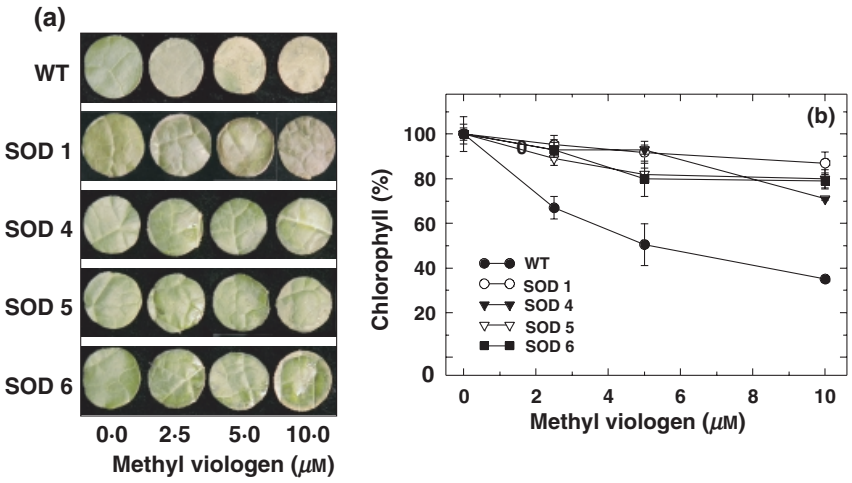

Figure 5. Effect of methyl viologen (MV) on symptoms of oxidative stress in leaf discs of transgenic and WT plants as shown by the degree of bleaching (a) and chlorophyll content (b). Leaf discs from selected T_1 and WT plants were incubated in solutions with increasing concentrations of MV (0–10 μM) overnight in the dark at room temperature. Leaf discs were then illuminated for 48 h at 50 μmol m^{-2} s^{-1}. Vertical bars represent standard errors (n = 3 per experiment). Each experiment was repeated at least three times.

Treatment (μM Al)	Root growth (mm)	MDA (μmol g FW^{-1})		Total SOD activity (Units mg protein^{-1})	
		Roots	Shoots	Roots	Shoots
0	45·0 ± 2·8^{a}	6·95 ± 1·4^{a}	6·91 ± 1·0^{a}	1·03 ± 0·1^{a}	3·20 ± 0·5^{a}
50	33·7 ± 4·4^{b}	8·40 ± 1·2^{a}	7·22 ± 0·6^{a}	1·48 ± 0·3^{a}	6·86 ± 2·3^{a}
100	20·0 ± 3·8^{c}	13·5 ± 0·8^{b}	9·70 ± 1·2^{a}	1·93 ± 0·1^{b}	9·55 ± 2·2^{a}
200	3·35 ± 0·5^{d}	18·3 ± 2·0^{b}	11·1 ± 2·3^{a}	2·10 ± 0·1^{b}	10·2 ± 3·2^{a}

Table 1. The effect of different concentrations of Al on root growth, MDA content and total SOD activity in *B. napus* cv. Westar. Four-day-old seedlings were exposed to different concentrations of Al for 48 h. For the determination of SOD activity, 7-d-old seedlings were exposed to metals for 48 h. Data are averages ±SE (n = 10 for root growth and n = 3 for MDA and SOD activity). Each experiment was repeated at least three times. Values within a column followed by a different letter are significantly different according to Student Newman–Keuls multiple range ($P \leq 0{\cdot}05$)

of homozygous T_2 plants. In WT plants, root growth was reduced by 40% at 100 μM Al and by 78% at 150 μM Al. Root growth was less affected in the transgenic lines, declining by only 10–20% and 50–68% at 100 and 150 μM Al, respectively (Fig. 6a). The observed growth reductions in all transgenic lines were significantly less than the reductions in WT plants (+, $P \leq 0{\cdot}1$; *, $P \leq 0{\cdot}05$; **, $P \leq 0{\cdot}01$). Analysis of MDA contents provided additional support for the enhanced Al resistance observed in transgenic lines. At 150 μM Al, WT roots showed a three-fold increase in MDA content over the control. In transgenic lines, the increase was ~1·5-fold, significantly lower than that observed for WT (Fig. 6b).

Synthesis of 1,3-β-glucans (callose) provides an accurate and sensitive short-term marker for Al injury, which correlates well with the long-term effects of Al on root growth (Zhang *et al*. 1994). Callose production in root tips of WT plants increased by ~130% with exposure to 150 μM Al. In contrast, the accumulation of callose in the root tips of transgenic plants was significantly less than that in WT plants, increasing by only 15–60% (Fig. 6c).

DISCUSSION

Metals such as aluminium, cadmium, lead, copper, iron and nickel are directly toxic to plants and affect plant growth, development and yield. Among the many effects that metals have on plants, metal-induced production of AOS is known to disrupt a number of cellular processes (Foyer *et al*. 1997). As part of a suite of defences against oxidative stress, plant cells contain oxygen radical detoxifying enzymes such as SOD, peroxidase, glutathione-S-transferase and catalase. Aluminium has been shown to induce a number of oxidative stress genes in *Arabidopsis thaliana*, including peroxidase, glutathione-S-transferase and cytosolic Cu/ZnSOD (Richards *et al*. 1998). We previously identified an Al-induced, 23 kDa, root exudate protein in wheat (Basu *et al*. 1999). This protein cosegregated with the Al resistance phenotype in segregating populations arising from a cross between a doubled-haploid Katepwa (Al-sensitive) and a doubled-haploid Alikat (Al-resistant). Based on the sequence homology of three amino acid microsequences, our 23 kDa Al-induced protein appears to be a novel, extracytosolic MnSOD. Although we were unable to clone the gene for this extracytosolic SOD, we show here that the mitochondrial *WMnSOD* also responds to Al stress in a fashion similar to our putative extracytosolic MnSOD. In this study, we observed an up-regulation of a mitochondrial *WMnSOD* transcript in root tips of *T. aestivum* when plants were exposed to increasing concentrations of Al.

Elevated levels of SOD activity in plants has been found to correlate with plant resistance to oxidative stress (Allen 1995). In the current study, overexpression of the gene for mitochondrial *WMnSOD1* from *T. aestivum* in *B. napus* protected cellular membranes and significantly reduced damage induced by the oxidative agent, MV. Bowler *et al*. (1991) reported that overexpression of mitochondrial MnSOD in leaves of transgenic tobacco plants provided a high level of protection against oxidative stress on exposure to MV. In our studies, both the conductivity of assay solutions and the degree of bleaching of leaf discs were lower in the transgenic plants (compared with the WT) after incubation on different concentrations of MV solution.

The transgenic plants developed in this study showed a 1·5- to 2·5-fold increase in total SOD activity. We also used another vector, pCGN-*WMnSOD1*, to overexpress *WMnSOD* under the control of a double CaMV promoter without *UidA* (data not shown). Total SOD activities in transgenic plants developed using this vector were five-fold higher than in WT plants, but the transgenic plants failed to survive past the first generation. This may have been a result of the higher levels of SOD activity in the transgenic lines, which could have led to a higher production of H_2O_2 and, in turn, to more oxidative damage. The current literature does not provide a clear picture of the extent of overexpression required to protect plants from oxidative stress. Tepperman & Dunsmuir (1990) demonstrated a 30- to 50-fold increase in SOD activity in transgenic *N. tabacum* plants transformed with Cu/Zn SOD from petunia but failed to provide any detectable changes in oxidative stress resistance. Dramatically different results were obtained in transgenic *N. tabacum* plants transformed with Cu/Zn SOD from pea, where expression of the transgene resulted in a three-fold increase in total SOD activity (Sen Gupta *et al*. 1993) and led to a significant increase in resistance to light-mediated MV-induced membrane damage. In our study, a 1·5- to 2·5-fold increase in total SOD activity in transgenic

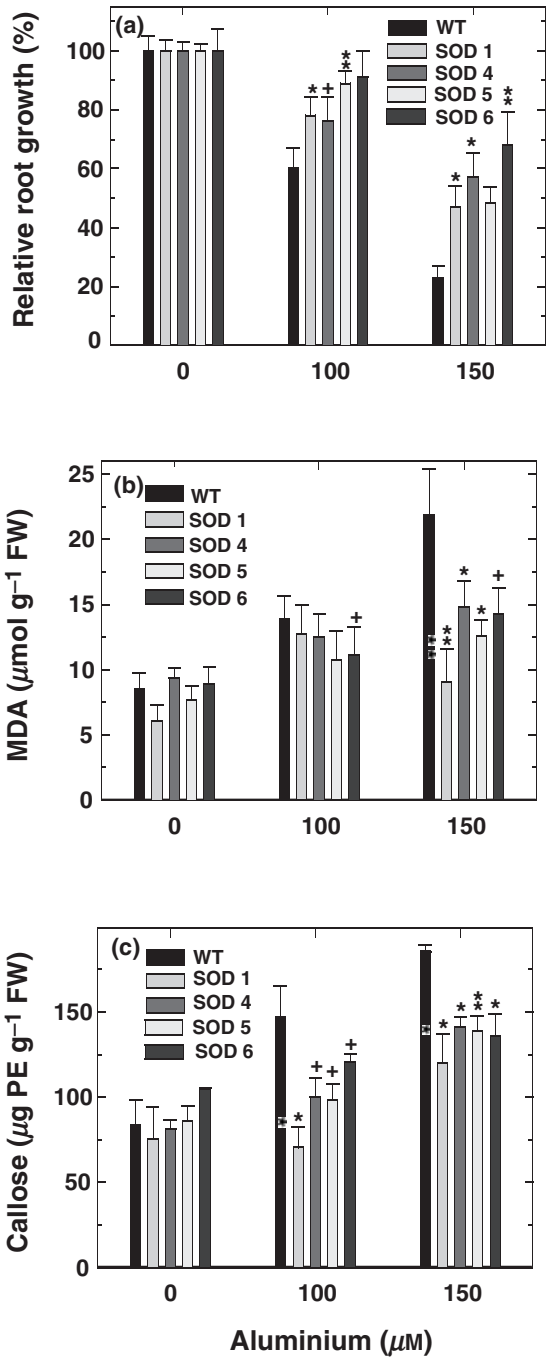

Figure 6. Comparison of the responses of homozygous transgenic lines (T_2) and WT plants to different concentrations of Al (0, 100, 150 μM). (a) Relative root growth; (b) MDA content; (c) Callose accumulation. Vertical bars represent standard errors for root growth (n = 6 per experiment) and for MDA and callose (n = 3 per experiment). Each experiment was repeated at least three times. Values significantly different from the WT according to the T-test are indicated by asterisks (+, $P \leq 0{\cdot}1$; *, $P \leq 0{\cdot}05$; **, $P \leq 0{\cdot}01$)

B. napus plants increased oxidative resistance compared with the WT plants.

Lipid peroxidation has been suggested as an early symptom of Al injury. Yamamoto *et al*. (1996) observed that *N. tabacum* cells treated with Al and Fe exhibit several responses indicative of increased lipid peroxidation and resultant cell death. When *N. tabacum* cells were treated with an antioxidant [DPPD (N, N′-diphenyl-p-phenylenediamine)], the degree of lipid peroxidation decreased and the resistance to Al increased (Yamamoto *et al*. 1997). Recent studies indicated spatial and temporal correlations between Al accumulation, lipid peroxidation, callose production and root elongation inhibition. Since oxidative stress has been implicated in Al injury, we would postulate that plants overexpressing the Al-induced mitochondrial MnSOD gene should also show enhanced resistance to Al stress. Ezaki *et al*. (2000) proposed that the enhanced Al resistance of transgenic lines of *Arabidopsis* overexpressing glutathione-S-transferase and peroxidase might be because of the restriction of lipid peroxidation, but levels of MDA were not determined. We observed increased lipid peroxidation and higher SOD activities in both roots and shoots of WT *B. napus* exposed to a range of Al concentrations. However, our transgenic *B. napus* plants that overexpressed mitochondrial *WMnSOD1* showed lower levels of MDA and callose and less inhibition of root growth with exposure to Al (compared with WT plants). As the primary site of Al accumulation and toxicity in plants is reported to be the root tip (Ryan, DiTomaso & Kochian 1993), the synthesis of callose in root tips can provide an accurate marker for Al injury (Zhang *et al*. 1994; Basu *et al*. 1999). Less Al-induced production of callose in root tips of transgenic *B. napus* plants compared with WT confirms the increased Al resistance of transgenic plants. The data clearly suggest a correlation between decreased lipid peroxidation and increased resistance to Al.

Superoxide dismutase produces O_2 and H_2O_2 from superoxide. Several recent studies have suggested a potential role for H_2O_2 as an active signalling molecule in plant cells (Foyer *et al*. 1997; Bowler & Fluhr 2000). Although the phytotoxicity of H_2O_2 at higher concentrations is well known (Dat *et al*. 2000), H_2O_2 has been shown to trigger increases in cytosolic calcium in tobacco (Price *et al*. 1994) and activate calcium-permeable channels in the plasmamembrane of *Arabidopsis* (Pei *et al*. 2000). Several studies have provided evidence linking rapid, Al-induced inhibition of apical calcium influx with Al inhibition of root growth (Kochian 1995). Nevertheless, Al injury is often accompanied by increases in cytosolic calcium (Bhuja 2000; Lindberg & Strid 1997; Nichol & Oliveira 1995). Furthermore, H_2O_2 has recently been found to be a potent activator of mitogen-activated protein kinases (MAPKs) in *Arabidopsis* leaf cells (Kovtun *et al*. 2000). Molecular complementation of an Al-sensitive yeast mutant with a MAP kinase gene *SLT2* has been shown to restore Al resistance (Schott & Gardner 1997). Therefore, the benefits of overexpressing *WMnSOD1* in our transgenic *B. napus* plants may be due not only to the removal of superoxide, but also to enhanced H_2O_2 production, which modifies signal transduction pathways.

Our results demonstrate that Al inhibits root growth, increases lipid peroxidation and increases callose accumu-

lation in WT *B. napus*. Transgenic Al-resistant *B. napus* plants overexpressing Al-induced mitochondrial *WMnSOD1* from wheat showed less inhibition of root growth and lower levels of both MDA and callose compared with the WT plants. Further work is required to explore the effects of the SOD transgene on the activity of other free-radical scavenging enzymes and antioxidants in *B. napus* transgenic lines and the potential for the modification of signal transduction pathways. It would also be useful to overexpress the SOD gene using tissue-specific and inducible promoters to improve the level of resistance. Further work to explore the role of other antioxidant enzymes in mediating Al resistance using yeast disruption mutants is now in progress.

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