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Tobacco chloroplast transformants expressing genes encoding dehydroascorbate reductase, glutathione reductase, and glutathione-S-transferase, exhibit altered anti-oxidant metabolism and improved abiotic stress tolerance

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Summary

One approach to understanding the Reactive Oxygen Species (ROS)-scavenging systems in plant stress tolerance is to manipulate the levels of antioxidant enzyme activities. In this study, we expressed in the chloroplast three such enzymes: dehydroascorbate reductase (DHAR), glutathione-S-transferase (GST) and glutathione reductase (GR). Homoplasmic chloroplast transformants containing either DHAR or GST, or a combination of DHAR:GR and GST:GR were generated and confirmed by molecular analysis. They exhibited the predicted changes in enzyme activities, and levels or redox state of ascorbate and glutathione. Progeny of these plants were then subjected to environmental stresses including methyl viologen (MV)-induced oxidative stress, salt, cold and heavy metal stresses. Overexpression of these different enzymes enhanced salt and cold tolerance. The simultaneous expression of DHAR:GR and GST:GR conferred MV tolerance while expression of either transgene on its own didn't. This study provides evidence that increasing part of the antioxidant pathway within the chloroplast enhances the plant's ability to tolerate abiotic stress.

Introduction

Environmental stress is the major limiting factor in plant productivity. Much of the injury to plants caused by stress exposure is associated with oxidative damage at the cellular level resulting from increased production of reactive oxygen species (ROS). The term ROS embraces not only free radicals such as superoxide ($^{\cdot}O_2^-$) and hydroxyl ($^{\cdot}OH$) radicals, but also hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). While it is generally assumed that $^{\cdot}OH$ and $^1O_2$ are so reactive that their production must be minimized, H_2O_2 and $^{\cdot}O_2^-$ are synthesized at very high rates even under optimal conditions (Noctor and Foyer, 1998). In particular, organelles with an intense rate of electron flow such as chloroplasts and mitochondria are a major source of ROS production in plant cells (Mittler *et al.*, 2004). Therefore, a complex array of detoxification mechanisms has evolved to keep ROS production under control, including not only non-enzymatic components such as antioxidant ascorbate and glutathione, but also enzymatic scavengers. The major antioxidant enzymes are superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX), involved in the scavenging of ROS, while glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) are involved in the regeneration of ascorbate and glutathione.

Manipulation of the expression of enzymes involved in scavenging ROS by gene transfer technology has provided valuable information concerning the role of these enzymes by allowing direct investigation of their functions and interactions (Foyer *et al.*, 1994). It has also indicated that modifying ROS-scavenging systems of plants can lead to significant changes in oxidative stress tolerance, and improve plant performance under stress (Aono *et al.*, 1993; Sen Gupta *et al.*, 1993; Broadbent *et al.*, 1995; Foyer *et al.*, 1995). All these studies were performed using nuclear transformation with, in many cases, chloroplast-targeted recombinant protein. Elevated expression of antioxidant enzymes in chloroplasts, using this approach, has confirmed a key role for these enzymes in the protection of plants against these various oxidative stresses in this vulnerable cellular compartment (Badawi *et al.*, 2004). For instance, Yoshimura *et al.* (2004) reported that in transgenic tobacco plants expression of GPX protein in the chloroplast was more effective at providing stress tolerance than the expression of the same protein in the cytosol. More recently, some studies have aimed at developing transgenic plants expressing several chloroplast-targeted antioxidant enzymes simultaneously (Kwon *et al.*, 2002; Tang *et al.*, 2006; Lee *et al.*, 2007; Ahmad *et al.*, 2010). They demonstrated the effectiveness of manipulating more than one gene in improving plant resistance to stress.

The only attempt at expressing genes for ROS scavenging enzymes directly in the chloroplast via plastid transformation was carried out in our laboratory (Poage *et al.*, submitted). Two genes encoding MnSOD and GR were inserted into the plastome of tobacco plants. Transgenic plants overexpressing MnSOD showed an increased resistance to methyl viologen and UV-B stress, while GR-overexpressing plants were more tolerant

to cadmium and UV-B stress. These first results demonstrated that modifying levels of antioxidant enzymes by chloroplast genetic engineering is a promising strategy to study the ROS scavenging system in plants.

Compared with conventional transgenic technologies, plastid engineering offers several potential advantages such as high protein expression levels, absence of epigenetic effects (gene silencing, position effects) and gene containment through the lack of pollen transmission (Bock and Khan, 2004; Daniell, 2007). This method also provides additional advantages over nuclear transformation pertaining to oxidative stress. The antioxidant system is most commonly modified via nuclear transformation, requiring a transit peptide for translocation into the chloroplast, while plastid engineering provides the advantage of expression at the site of the majority of ROS production in the cell, the chloroplast, without the need for protein import. Chloroplast genetic engineering also offers the advantage of transgene stacking, i.e. simultaneous expression of multiple transgenes, creating the opportunity to express different ROS scavenging enzymes in a single transformation step (Bock, 2001; Daniell *et al.*, 2005).

In this work, three genes were selected for chloroplast transformation experiments: DHAR, glutathione-S-transferase (GST) and GR. They were chosen based on their roles within the ROS antioxidant pathway and indications from previous nuclear transformation studies that they showed potential for engineering multiple stress tolerance. Several studies have highlighted the importance of DHAR in the maintenance of the ascorbate pool and indicated that DHAR contributes to the protection against the oxidative stress in plants cells (Yamasaki *et al.*, 1999; Kwon *et al.*, 2003; Ushimaru *et al.*, 2006). A cDNA encoding the rice DHAR was cloned (Urano *et al.*, 2000) and used in the generation of transgenic *Arabidopsis thaliana* expressing DHAR in the cytosol (Ushimaru *et al.*, 2006). This DHAR gene was used in the current work.

The second gene chosen for this investigation was *E. coli gor* encoding GR. From previous studies it seemed clear that GR of bacterial origin could function in plant chloroplasts to increase both the GSH/GSSG ratio and the total glutathione pool (Noctor *et al.*, 1998) and glutathione itself is an attractive target for engineering stress tolerance in plants because of its multiple roles in plant defences against both biotic and abiotic stresses (Foyer *et al.*, 1997). Moreover, this same gene has been inserted in the plastome of tobacco plants and its expression resulted in increases in the enzyme activity and total glutathione levels (Poage *et al.*, submitted). GR is also a particularly attractive candidate for combined expression with DHAR, since the two enzymes catalyse consecutive steps in the ascorbate-glutathione cycle in chloroplasts (Mittler, 2002).

The final target enzyme in this study was a glutathione-S-transferase. These are evolutionarily conserved detoxification enzymes with the ability to conjugate a broad range of potentially harmful xenobiotics to glutathione, thereby rendering them more susceptible to removal from the cell (Burns *et al.*, 2005). Some GSTs have been shown to function as glutathione peroxidases to detoxify directly the products of oxidative stress (Bartling *et al.*, 1993). An *E. coli* GST gene was used in this work, which has been shown to exhibit a GSH-dependent peroxidase activity against cumene hydroperoxide (Nishida *et al.*, 1994) and proved to be important for bacterial resistance to oxidative stress generated by hydrogen peroxide (Kanai *et al.*, 2006). As well as having a potential impact in its own right, this enzyme, combined with GR could significantly influence glutathione homeostasis, further affecting the ROS scavenging capacity of the chloroplasts. The aim of this work was to produce transplastomic tobacco plants that express these important enzymes of the antioxidant pathway in the plastome both singly and in pairwise combinations and to analyse their influences on enzyme activity, key metabolite (glutathione and ascorbate) contents and plant tolerance to oxidative stresses.

Results

Construction of the chloroplast expression vectors and plant transformation

The plasmid pZS-197 contains the chimeric aminoglycoside 3′adenylyltransferase (*aad*A) gene that confers resistance to spectinomycin/streptomycin and integrates gene(s) of interest into the large single copy region of the tobacco chloroplast genome in between the *rbc*L and *acc*D genes (Svab and Maliga, 1993). The *aad*A and gene(s) of interest are driven by the constitutive plastid rRNA operon promoter (*Prrn*), and transcription is terminated by *psb*A3′ untranslated region. This vector was modified to contain an *AscI/PacI* cloning site upstream of the *aad*A gene where the transgene(s) were cloned (Figure 1). All four constructs pZS-DHAR, pZS-GST, pZS-DHAR:GR and pZS-GST:GR were introduced into tobacco by biolistic bombardment (Svab and Maliga, 1993). Bombarded leaves were placed on RMOP medium with no antibiotic selection pressure for 2 days, then cut into 10 mm^2 pieces and transferred to RMOP containing 500 mg/L spectinomycin. Between 20 and 27 putative transformants were obtained from 24 bombarded leaves with each of the different constructs.

Foreign gene(s) integration

Transplastomic plants were initially confirmed by PCR using transgene-specific primers and external primers designed to confirm the correct incorporation of the construct into the chloroplast genome (data not shown). Four and three independently generated lines per single (pZS-DHAR and pZS-GST) and double gene (pZS-DHAR:GR and pZS-GST:GR) constructs, respectively, were characterised in detail. The lines are referred to subsequently as DHAR 3,4,6,15; GST 4,5,8,19; DG 2,3,4 and GG 15,16,18.

Chloroplast transformants were subjected to two additional regeneration cycles under antibiotic selection to obtain homoplasmic plants. Successful chloroplast transformation was then confirmed by Southern blot analysis. In the chloroplast genome, *EcoRI* and *EcoRV* sites flank the chloroplast border sequence 5′ of *rbc*L and 3′ of *acc*D respectively (Figure 1) which generates a 3.2 kb fragment when digested with these two restriction enzymes. When the chloroplast genome is transformed with the cassette from pZS-DHAR or pZS-GST, its insertion between *rbc*L and *acc*D increases the size of the *EcoRI/EcoRV* digested fragment to 5.2 kb; and to 6.4 kb when the chloroplast genome is transformed with the cassette pZS-DHAR:GR or pZS-GST:GR (Figure 1). Total DNA from each clone and WT tobacco was digested with *EcoRI* and *EcoRV* and two blots with two different probes were made for each type of transformant. Blots shown in Figure 2 were probed with a Digoxigenin (DIG) labelled *rbc*L fragment designed to hybridize at the 3′end of *rbc*L present in both the WT and transformed plastomes. The probe hybridized to a 3.2 kb fragment in the WT as expected, and to a 5.2 kb (Figure 2a) or 6.4 kb (Figure 2b) fragment, confirming the correct integration of the transgene(s) in the

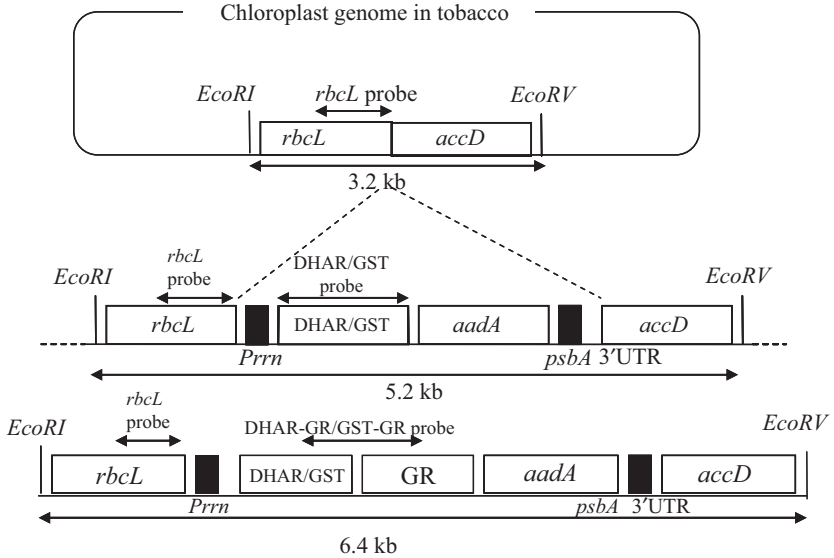

Figure 1 Diagram of expression cassettes and integration site used for plastid transformants (not to scale). The site of integration of pZS-dehydroascorbate reductase (DHAR), glutathione-S-transferase (GST), GST:glutathione reductase (GR) and DHAR:GR constructs is represented by dotted lines. The location of the different probes used for Southern blot analysis and the size of the *EcoRI/EcoRV* fragment generated in each case are indicated.

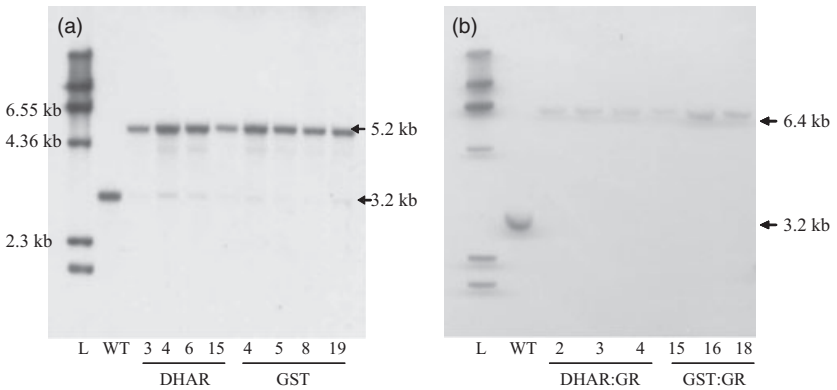

Figure 2 Southern blot analysis of T1 generation of dehydroascorbate reductase (DHAR) and glutathione-S-transferase (GST) (a), DHAR:glutathione reductase (GR) and GST:GR (b) transformed plants using a *rbcL* probe. Hybridisation of *EcoRI/EcoRV* digested total genomic DNA with the DIG labelled *rbcL* probe. The *rbcL* probe hybridises to a 3.2 kb fragment in WT, a 5.2 kb fragment in both DHAR and GST lines (a) and a 6.4 kb fragment in both DHAR:GR and GST:GR lines (b). L: DIG labelled Marker weight II (Roche).

chloroplast genome, for all of the tested transgenics. DIG labelled gene specific probes designed to hybridize to the entire DHAR and GST genes, and the middle part of the DHAR:GR and GST:GR transgenes showed no hybridizing fragment to the WT, while all the transformed lanes showed presence of the transgene(s) (Figure 3). In addition to the strong band for the transplastomic fragment, a faint hybridization signal that corresponds in size to the restriction fragment from the WT genome can be seen in Figure 2a. Persistence of wild-type like hybridization signal even after multiple rounds of regeneration is often seen in transplastomic lines and is usually caused not by true heteroplasmy of the chloroplast transformants but rather by the presence of promiscuous plastid DNA in one of the other two genomes of the plant cell (Ruf *et al.*, 2001; Wurbs *et al.*, 2007).

Seed assays were performed to confirm homoplasmy of all transplastomic lines. There was no segregation of antibiotic resistance in the T1 generation, confirming homoplasmy (data not shown).

Protein expression and enzyme activities

To confirm transgene(s) expression, total soluble protein obtained from transformed and non-transformed leaves was subjected to Western blot analysis. In all the transplastomic plants, the gene or combination of genes introduced was found to express immunoreactive protein (Figure 4). Densitometric analysis of DHAR blots compared to those of various concentrations of purified protein (not shown) gave estimated recombinant protein levels of 0.79% total soluble protein (TSP), while a similar analysis of TSP blots gave an estimated recombinant protein level of 0.75% TSP. Band intensities of other proteins, suggested expression levels of similar magnitude. The expression of the different introduced genes resulted in increased specific activity of their respective enzymes (Table 1).

In leaves from the DHAR plants, the level of DHAR activity increased between 1.8 and 2.7 fold when compared to WT. This increase was found to be significant ($P < 0.001$). In leaves from the DHAR:GR plants, this increase was found to be greater, between 3.2 and 4 fold. Leaves of the GST plants exhibited an increase in GST activity of approximately 2-fold, while this increase was greater, at 2.9- to 3.7-fold in the GST:GR double transformants. As some GST enzymes can exhibit a GPX activity, and the GST from *E. coli* was shown to have GPX specific activity against cumene hydroperoxide (Nishida *et al.*, 1994), the GPX assay was performed on leaves of WT

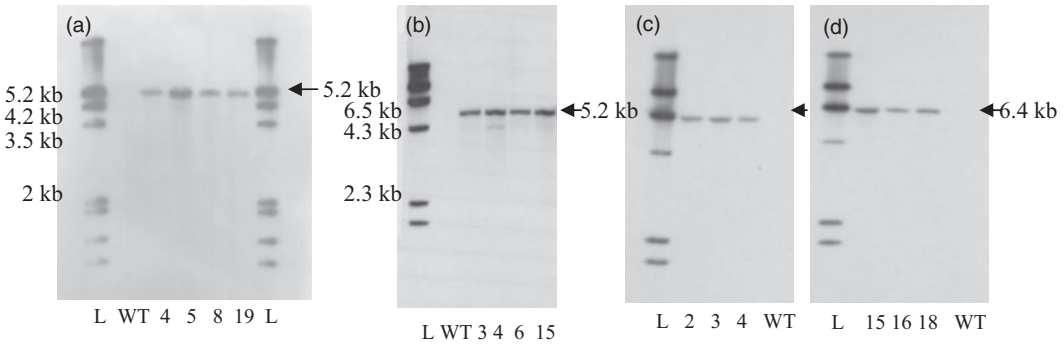

Figure 3 Southern Blot analysis of T1 generation of transformed plants using gene specific probes. Hybridization of *EcoRI*/*EcoRV*-digested total genomic DNA with the DIG-labelled gene-specific probe. No hybridization occurs in the WT lanes. (a) Glutathione-S-transferase (GST) lines (as numbered). L: DIG labelled Marker Weight III (Roche). (b) Dehydroascorbate reductase (DHAR) lines (as numbered). L: DIG-labelled Marker Weight II (Roche). (c) DHAR:glutathione reductase (GR) lines (as numbered). L: DIG-labelled Marker Weight II (Roche). (d) GST:GR lines (as numbered). L: DIG-labelled Marker Weight II (Roche).

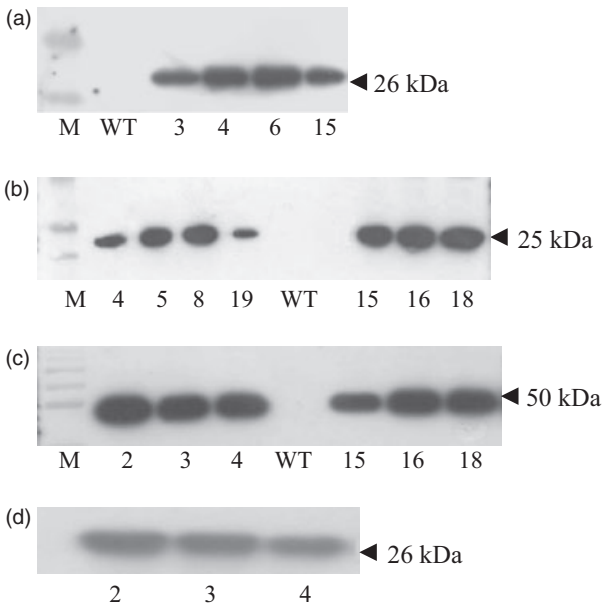

Figure 4 Western Blot analysis of transplastomic leaf extracts. (a) Dehydroascorbate reductase (DHAR) protein 26 kDa detected in DHAR clones 3,4,6 and 15. (b) Glutathione-S-transferase (GST) protein 25 kDa detected in GST clones 4,5,8,19 and GST:glutathione reductase (GR) clones 15,16,18. (c) GR protein 50 kDa detected in DHAR:GR clones 2,3,4 and GST:GR clones 15,16,18. (d) DHAR protein 26 kDa detected in DHAR:GR clones 2,3,4.

and GST as well as GST:GR. The levels of GPX were slightly increased (1.7 fold on average) in GST lines compared to WT. In the GST:GR lines, this increase was somewhat higher, at 2.6 fold (Table 1).

GR activity was measured in leaves of WT, DHAR:GR and GST:GR lines but also in GST and DHAR lines as both GST and DHAR enzymes use reduced glutathione as a substrate. An enhanced DHAR or GST activity might have an impact on the GR activity. The GR level in leaf extracts of the DHAR:GR and GST:GR plants was higher than in the WT with an increase estimated between 3.6- and 4.7-fold for the DHAR:GR lines and between 4.1- and 4.5-fold for the GST:GR lines. No significant difference in terms of GR levels was found between the DHAR:GR and the GST:GR lines. In the DHAR and GST lines, the GR level was slightly increased compared to the WT. These levels were significantly lower than those determined for the DHAR:GR and the GST:GR plants ($P < 0.001$) but the difference with the WT was still found to be significant ($P < 0.05$).

Metabolite contents

The levels of reduced and oxidized ascorbate were measured in WT, DHAR and DHAR:GR lines, while the levels of reduced and oxidized glutathione were measured in fully expanded leaves of all transplastomic lines. As expected, the levels of reduced ascorbate (AsA) in the leaves of DHAR lines increased up to 1.6-fold over the WT, while the levels of oxidized ascorbate (DHA) decreased by 40% overall (Figure 5a). Consequently, the ratio [AsA]/[DHA], increased markedly in the leaves of DHAR overexpressing plants. The total ascorbate content was also found to be slightly increased in the DHAR plants. Both these effects became significantly enhanced in the DHAR:GR double transformants.

The level of total glutathione was slightly increased in the DHAR and GST transformed lines when compared to the WT. This increase was mostly due to an increase in the levels of oxidized glutathione (GSSG). As a consequence, the ratio [GSH]/[GSSG], was decreased significantly in the transgenic lines by comparison with the WT (Figure 5b).

In the case of the DHAR: GR and GST:GR transformants (Figure 5c), the total content of glutathione compared to the WT, was significantly elevated by 2.4-fold for both transformed lines. This increase was explained by similar increases in both oxidized and reduced glutathione. As a consequence, the ratio [GSH]/[GSSG] was little changed in the transformants.

Analysis of stress tolerance in transplastomic lines

Chilling stress

Growth of transplastomic and WT seedlings under non-stressful conditions (24 °C) was similar. When incubated at 15 °C, all the seeds germinated but the growth rate of the seedlings was considerably reduced by the low temperature, especially for the WT seedlings which also exhibited yellow or pale green cotyledons suggesting a delay in chlorophyll development. After 12 days all the seedlings exhibited reduced growth at 15 °C,

Table 1 Specific activities of DHAR, GST, GPX and GR in protein extracts from WT and transplastomic tobacco plants

Plant line	DHAR activity	GR activity	Plant line	GST activity	GPX activity	GR activity
WT	27.9 ± 4	20.7 ± 2	WT	4.5 ± 0.5	20.1 ± 0.5	20.7 ± 2
DHAR3	75.8 ± 9	34.2 ± 4.1	GST4	9.5 ± 1.5	30.9 ± 5	29.4 ± 4.5
DHAR4	68.4 ± 6	28.1 ± 2.3	GST5	10.2 ± 2.3	28.7 ± 1.9	24 ± 1
DHAR6	50 ± 3	30 ± 1	GST8	8 ± 0.7	38.5 ± 1.2	32.3 ± 3
DHAR15	74.2 ± 2.7	40 ± 3.6	GST19	11 ± 1.6	40.2 ± 2	33 ± 0.9
DG2	100 ± 3	75 ± 8.1	GG15	13 ± 0.6	50.6 ± 4.3	85 ± 6.2
DG3	89.3 ± 8.1	89 ± 5.6	GG16	16.7 ± 1.3	57.3 ± 1.6	94 ± 5
DG4	113 ± 6	97 ± 4.1	GG18	15.5 ± 2.1	50 ± 1.2	86 ± 4.3

DHAR, dehydroascorbate reductase; GPX, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase.
DHAR, GPX and GR values are expressed in nmol/min per milligram protein. GST values are expressed in μmol/min per milligram protein. Data are means ± SD of specific activities from three separate assays on three different plants per line. Samples for the assay were collected from nodes 3 and 4 of 6 weeks-old soil grown plants. The differences between WT and transgenic plants are statistically significant ($P < 0.05$) as determined by ANOVA.

but the reduction was much more pronounced in the WT seedlings compared to all the transplastomic lines (Figure 6a) and the DHAR: GR and GST:GR seedlings displayed a further improvement in their tolerance, in comparison with the single transformants. Furthermore, WT seeds didn't germinate when incubated at 4 °C for up to 50 days, while all transgenic seeds germinated albeit slowly (data not shown).

In the leaf disc tests, the Fv/Fm values taken at 48 h and 72 h time points were expressed as a percentage against the values obtained before the stress treatment. Before the stress, the Fv/Fm values were quite similar for WT and transformed plants. After 48 h incubation at 8 °C, the maximal Photosystem II (PSII) activity of WT, GST and DHAR leaf discs all showed the same tendency, a decrease to approximately 88% of the pre-stress level. Only after the 72 h treatment, was a difference observed between the leaf discs, the maximal PSII activity of the WT leaf discs dropped to 72%, while in GST and DHAR leaf discs it remained higher, at between 75% and 83% (Figure 6b). With DHAR:GR and GST:GR leaf discs, a difference could already be seen after 48 h of chilling stress, as these maintained a higher maximal PSII activity than the WT, with values between 90% and 94%. After 72 h treatment, the maximal photosynthetic capacity of the transgenic leaf discs was still higher than WT, between 83% and 87% compared to 72% for the WT.

To determine whether overexpressing DHAR, GST and GR had any influence on H_2O_2 accumulation during chilling stress exposure, the amount of H_2O_2 was also measured in control and transgenic leaf discs, in the presence or absence of stress. There was no difference in H_2O_2 content of WT and transgenic leaf discs without stress treatment. The H_2O_2 content increased markedly in all lines when they were incubated at 8 °C for 72 h but remained significantly lower in transplastomic leaf discs than in WT. DHAR:GR and GST:GR leaf discs were more efficient at reducing H_2O_2 levels than were DHAR or GST alone (Figure 6c).

Salt and heavy metal stress

To test their salt tolerance capacity, seeds were germinated on germination medium supplemented with high concentrations of NaCl: 100, 150, or 200 mM, and seedling growth was recorded after 14 days. On 100 or 150 mM NaCl medium, seedlings grew more slowly than on the control plates, but no differences in growth between WT and transplastomic seedlings were observed. However, in 200 mM NaCl medium the growth of WT seedlings was strongly inhibited in comparison with transplastomic seedlings. The mean reduction in growth of WT seedlings grown in 200 mM NaCl was 83% in length while DHAR and GST seedlings were reduced by only 57% and 62% respectively and DHAR:GR and GST:GR seedlings by 65% and 63% respectively (Figure 7). No significant difference in salt tolerance was observed between the transplastomic lines.

Heavy metal tolerance was analysed by germinating seeds on germination medium containing several concentrations of $CdCl_2$ (0.25, 0.5, 0.75 and 1 mM) or $ZnSO_4$ (0.5, 1, 2 and 5 mM). The percentage germination was determined after 7 days, however, no difference in sensitivity to these two heavy metals was detected between WT and any of the transplastomic lines (data not shown).

Effect of methyl viologen (MV) on leaf bleaching and oxidative damage to lipids

Evaluation of the response to oxidative stress caused by MV involved visual observations on leaf discs, chlorophyll determinations, membrane damage as assessed by conductivity measurements, and lipid peroxidation as determined by malondialdehyde measurements. None of these measurements revealed any differences between the DHAR or GST (expressed singly) lines and WT, so the data presented herein are restricted to the double transformant (DHAR:GR and GST:GR) lines, all of which exhibited marked differences from WT for all four of these parameters.

The level of bleaching in the WT leaf discs was greater than in the DHAR:GR and GST:GR leaf discs for the 0.5 and 1 μM MV treatments, which indicated that WT leaf discs were undergoing more severe damage. At 5 μM, both lines suffered severe damage and no obvious difference between them was seen (Figure 8a). These differences are confirmed in comparisons of the percentage of chlorophyll retained after exposure to the different MV solutions (Figure 8b) which show much greater chlorophyll retention in the double transformants, compared to WT, at the two lower levels of MV.

The membrane damage in MV treated, WT and transgenic leaf discs was measured by electrolyte leakage. A protective effect was again detected in the double transplastomic leaf discs after exposure to 0.5 and 1 μM MV, which showed a far

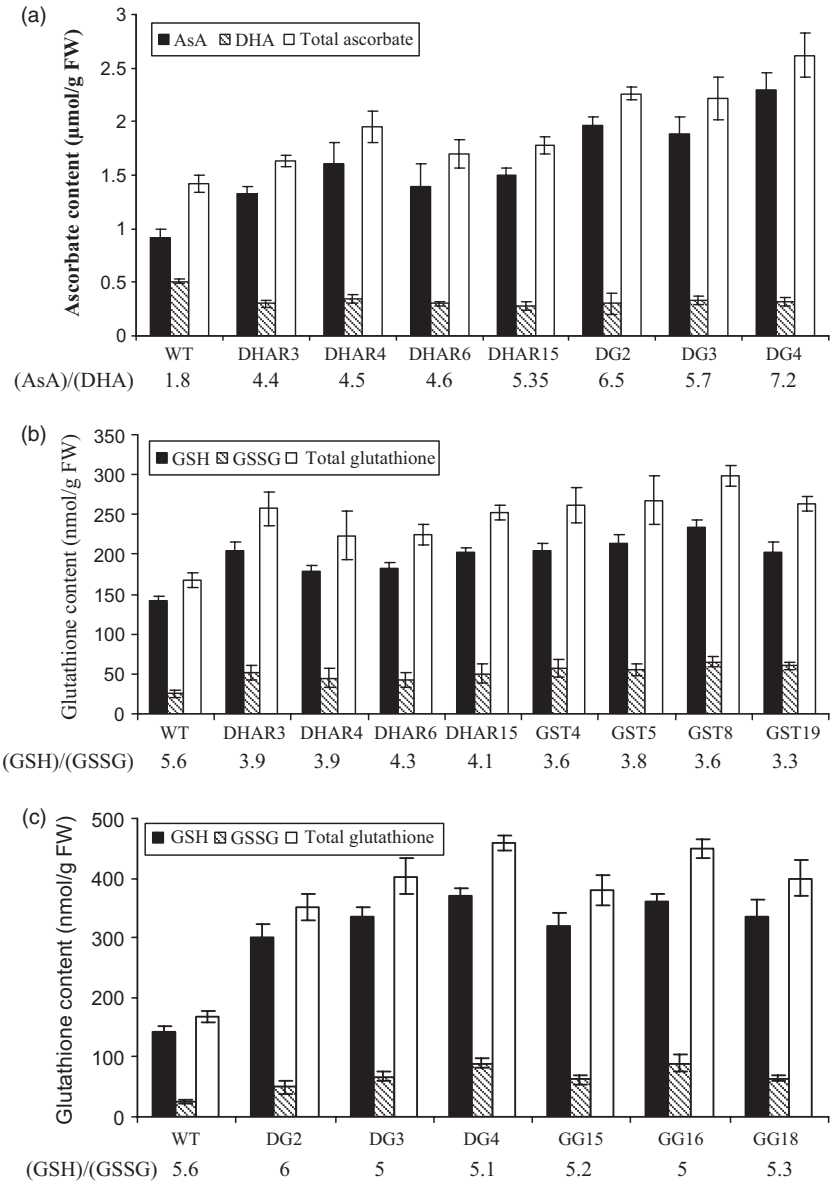

Figure 5 Biochemical characterization of transplastomic and WT tobacco plants. Samples for the assay were taken from leaves (nodes 3 and 4) of 6 weeks-old soil-grown plants. Results are expressed in µmol ascorbate/g FW and in nmol glutathione/g FW. Shown here are the means ± SD of three independent experiments on three different plants per line. In all cases the differences between WT and transgenic plants are statistically significant ($P < 0.05$) as determined by ANOVA. (a) Levels of total, reduced (AsA) and oxidized (DHA) ascorbate in WT and dehydroascorbate reductase (DHAR) and DHAR:glutathione reductase (GR) transgenic plants. (b) Levels of total, reduced (GSH) and oxidized (GSSG) glutathione in WT, DHAR and glutathione-S-transferase (GST) transgenic plants. (c) Levels of total, reduced (GSH) and oxidized (GSSG) glutathione in WT, DHAR:GR and GST:GR transgenic plants. The ratio of AsA to DHA or GSH to GSSG in each line is indicated below the histograms.

lower percentage electrolyte leakage compared to WT (Figure 8c). Furthermore, these lines also showed a significantly lower level of lipid peroxidation (as assessed by malondialdehyde levels) at the lower MV concentrations (Figure 8d).

Discussion

This study approaches stress research from a novel perspective by applying chloroplast transformation technology to the engineering of oxidative stress resistance. Plastid transformation has proved to be an effective tool for enhancing tolerance to several abiotic stresses including drought (Lee *et al.*, 2003; Zhang *et al.*, 2008), salt (Kumar *et al.*, 2004) and chilling (Craig *et al.*, 2008). Even though these are important advances in the application of plastid engineering to the stress research field, engineering for specific stresses fails to address overall mechanisms which may confer resistance to a broader range of abiotic stresses plants encounter in the environment. The work presented herein demonstrates a capacity to modify particular aspects of the endogenous antioxidant pathways via plastid transformation, creating plants more resistant to several stresses.

Four types of transplastomic tobacco plants were generated. In two of them one single gene was introduced in the plastome encoding DHAR or GST. In the other two, a combination of two genes was inserted in the plastome, DHAR or GST in combination with GR. In all lines, the expression of the introduced genes resulted in substantial increases in the total leaf specific activity of their respective enzymes. Measurements were not made on isolated chloroplasts, which would have supported the chloroplast location of the elevated enzyme activity. The increase however is consistent with the high level of immunologically detected recombinant protein, which should be restricted to the plastids, on the basis of plastid specific promoters, and the absence of any known mechanism for protein export from chloroplasts. A presumption of chloroplast location (without supporting data) is also a common feature of reports on nuclear transformants with recombinant ROS scavenging enzymes targeted to the chloroplasts through transit sequences

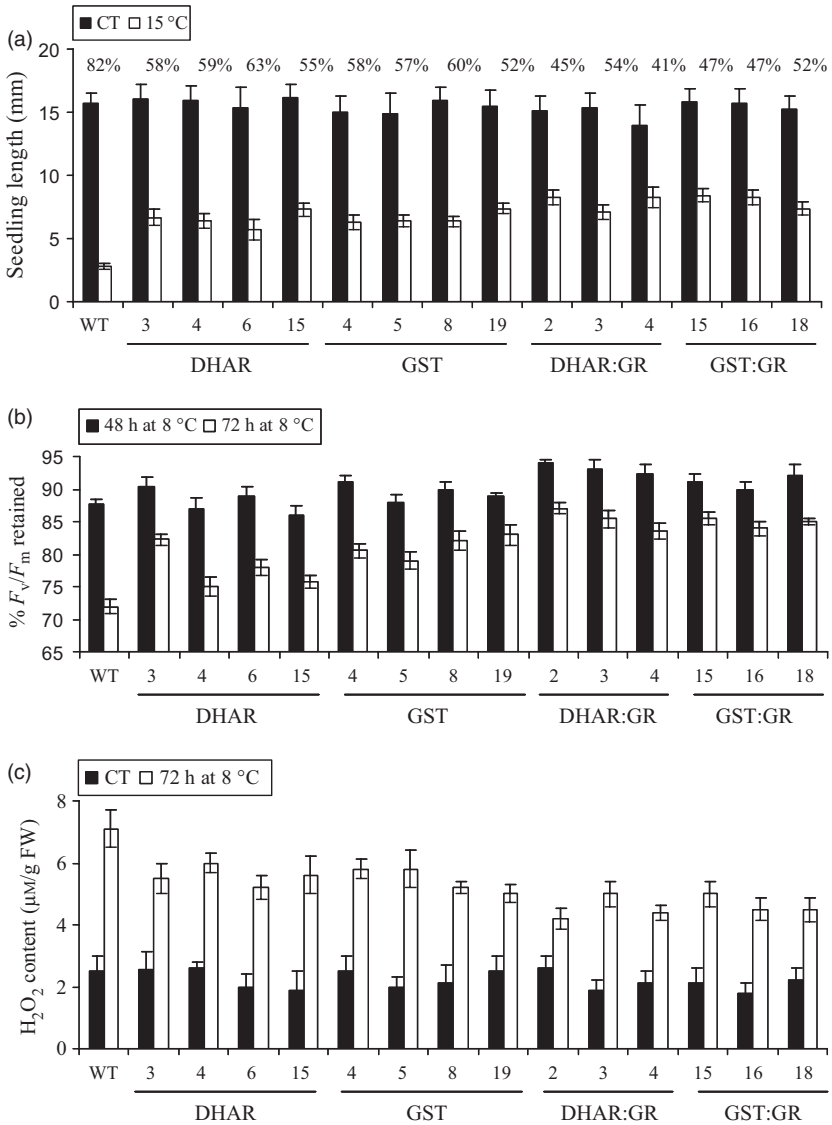

Figure 6 Chilling tolerance in transplastomic seedlings and plants. (a) Transplastomic glutathione-S-transferase (GST), dehydroascorbate reductase (DHAR), DHAR:glutathione reductase (GR), GST:GR, as well as WT seedlings were grown at 15 °C or at 24 °C on germination medium. Measurements were taken after 12 days on 25 randomly selected seedlings per line. The experiment was repeated three times. Shown here are the means ± SD. The differences between WT and all transgenic plant lines are statistically significant ($P < 0.05$) as determined by ANOVA. The decrease in length of the seedlings grown under chilling stress compared to those grown under control condition (24 °C) is indicated as a percentage. (b) Effect of chilling on the relative variable fluorescence of WT, GST, DHAR, DHAR:GR and GST:GR transgenic leaf discs. The minimal fluorescence (Fo), and maximal fluorescence (Fm) were measured on dark-adapted samples before stress, and after 48 h and 72 h incubation at 8 °C, light intensity of 80 μmol/m^2 per second. Maximal photosynthetic efficiency of PSII was measured as the relative variable fluorescence Fv/Fm where Fv = Fm–Fo. Shown here are the means ± SD of three independent experiments on six leaf discs taken from two plants per line. The differences between WT and all transgenic plant lines are statistically significant ($P < 0.05$) as determined by ANOVA (c) Effect of chilling on H_2O_2 accumulation in leaf discs of WT, DHAR,GST, DHAR:GR and GST:GR. H_2O_2 content expressed as μmol/g FW was determined in non-treated leaf discs (control) and after the leaf discs had been incubated 72 h at 8 °C with moderate light (intensity 80 μmol/m^2 per second). Data represent the means ± SD of three independent experiments on six leaf discs taken from two plants per line. As determined by ANOVA ($P < 0.05$) there were no statistical differences between lines for unstressed plants, but WT was different from all transplastomic lines, after chilling treatment. In all cases, CT, control (unstressed) plants.

(e.g. Foyer *et al.*, 1995; Kwon *et al.*, 2001, 2003; Lee *et al.*, 2007; Ahmad *et al.*, 2010).

Some comparisons can be made with expression levels obtained with comparable nuclear transformants, while recognising that different gene sources, plant species and age of plant material might have been used in the different studies. The current DHAR expressing plants had increases in total DHAR activity (as calculated from the data in Table 1) ranging from 1.8- to 2.7-fold, over wild type, in single transformants, and 3.2- to 4.0-fold, in double transformants (with GR). These increases are considerably greater than those achieved by nuclear transformation in the most comparable study (Ushimaru *et al.*, 2006), in which the increases in DHAR activity ranged from 1.2 to 1.5, which proved sufficient to significantly enhance salt tolerance in

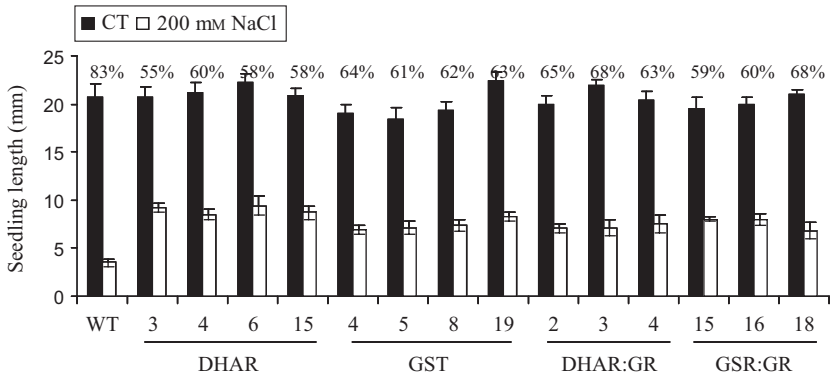

Figure 7 Salt tolerance in transplastomic seedlings. Dehydroascorbate reductase (DHAR), glutathione-S-transferase (GST), DHAR:glutathione reductase (GR), GST:GR and WT seedlings were grown on germination medium containing 200 mM NaCl and on germination medium without added salt, as a control. The length of 25 seedlings per line was measured after 14 days and the experiment was repeated three times. Data are the means ± SD. As determined by ANOVA ($P < 0.05$) there were no statistical differences between lines for unstressed seedlings, but under salt stress WT was different from all transplastomic lines. The decrease in length of the seedlings grown under salt stress compared to those grown under control conditions is indicated as a percentage on top of the histograms. CT, control (unstressed) plants.

seed germination tests. Our results are however, similar to those for nuclear tobacco transformants expressing a human DHAR (Kwon *et al.*, 2003), in which an increase over wild type of 2.29-fold was reported. Interestingly, it was observed in the plants expressing a combination of two genes, DHAR or GST/GPX activity was higher than when the genes encoding these were expressed singly. This could be a consequence of improved translation of the dicistronic message. However it may also be due to the complex interplay between antioxidants leading to enhanced endogenous expression levels. A similar result was obtained in a study of nuclear transgenic plants expressing several antioxidant enzymes simultaneously. The APX activity in plants overexpressing CuZnSOD and APX was higher than that of plants expressing APX alone (Kwon *et al.*, 2002). GST levels in our single transformants were 1.8–2.4-fold higher than wild type, which was similar to levels reported for nuclear transformants of tobacco (Roxas *et al.*, 2000; Yu *et al.*, 2003), although in neither of these reports was the recombinant enzyme targeted to the chloroplasts. All three studies show similar increases in GPX, as well as GST activity. In the present work, both these enzyme activities were further increased when GST was expressed together with GR (GG plants). GR levels in our double transformants (with DHAR or GST) were 3.6- to 4.7-fold higher than wild type. This compares favourably with nuclear transformants expressing GR in tobacco chloroplasts; 2.2-fold (Broadbent *et al.*, 1995) or 3-fold (Aono *et al.*, 1993), although a remarkable 1000-fold increase in chloroplast-targeted GR was reported in transgenic poplar trees (Foyer *et al.*, 1995).

Overexpression of a specific antioxidant gene has been shown to influence the expression of other antioxidant genes; indeed transgenic GST expressing seedlings were shown to have a higher APX activity and MDHAR activity compared to non-transformed seedlings (Roxas *et al.*, 2000). Tobacco plants expressing a human DHAR gene showed an increase in GR activity (Kwon *et al.*, 2001). The GR activity of the DHAR and GST transformant lines generated in this work also exhibited a small increase in GR activity compared to the WT. Indeed glutathione reductase activity was increased in all the transplastomic lines, irrespective of whether or not they included a *gor* transgene, although it was further enhanced by the inclusion of the latter.

The analysis of the glutathione and ascorbate pools revealed that manipulating the activity of ROS scavenging enzymes affects the antioxidant content of the transformed plants. The levels of reduced ascorbate (AsA) were increased in all transgenic lines overexpressing DHAR and as the levels of DHA were markedly decreased, the ratio AsA/DHA was significantly increased in these lines. This effect was much more pronounced than in the nuclear transformants of Arabidopsis (Ushimaru *et al.*, 2006), which also showed far greater inter-line variation. Nuclear transformants of tobacco expressing human DHAR (Kwon *et al.*, 2003) exhibited a greater than doubling of the AsA/DHA ratio, similar to the current findings, although in contrast to our results, and those of Ushimaru *et al.* (2006), they did not find an increase in total ascorbate levels. Other reports have also shown that plants overexpressing DHAR contained larger quantities of AsA (Kwon *et al.*, 2001; Chen and Gallie, 2004). In the current work the increase in AsA/DHA ratio was even more pronounced in the DHAR:GR lines. Ding *et al.* (2009) have previously highlighted the fact that the capacity for reduction of the glutathione pool by GR plays an important role in maintaining the ascorbate pool and redox state.

In the DHAR and GST overexpressing plants, an altered ratio of oxidized and reduced glutathione levels was observed. This might be explained by the fact that both GST and DHAR depend on GSH as an electron donor. Indeed, it was noticed that the amount of GSH was slightly lower than in WT plants, whereas the amount of GSSG was higher; consequently the ratio of GSH/GSSG was significantly decreased in the transgenic plants. The double transgenic lines DHAR:GR and GST:GR were characterized by a substantial increase in total glutathione. Mullineaux *et al.* (1994) reported that overexpression of GR in the tobacco chloroplast, but not in the cytosol, increased both the reduced state and total pool of glutathione. Overexpression of GR in the chloroplast in poplar trees also resulted in an increase in both foliar GSH and the GSH/GSSG ratio (Foyer *et al.*, 1995). However, in the present work, the ratio GSH/GSSG of the double transgenic plants remained similar to the WT. This might be explained by an increase in both GSH and GSSG probably due to the increased DHAR and GST activity. The reduced glutathione generated is used by DHAR and

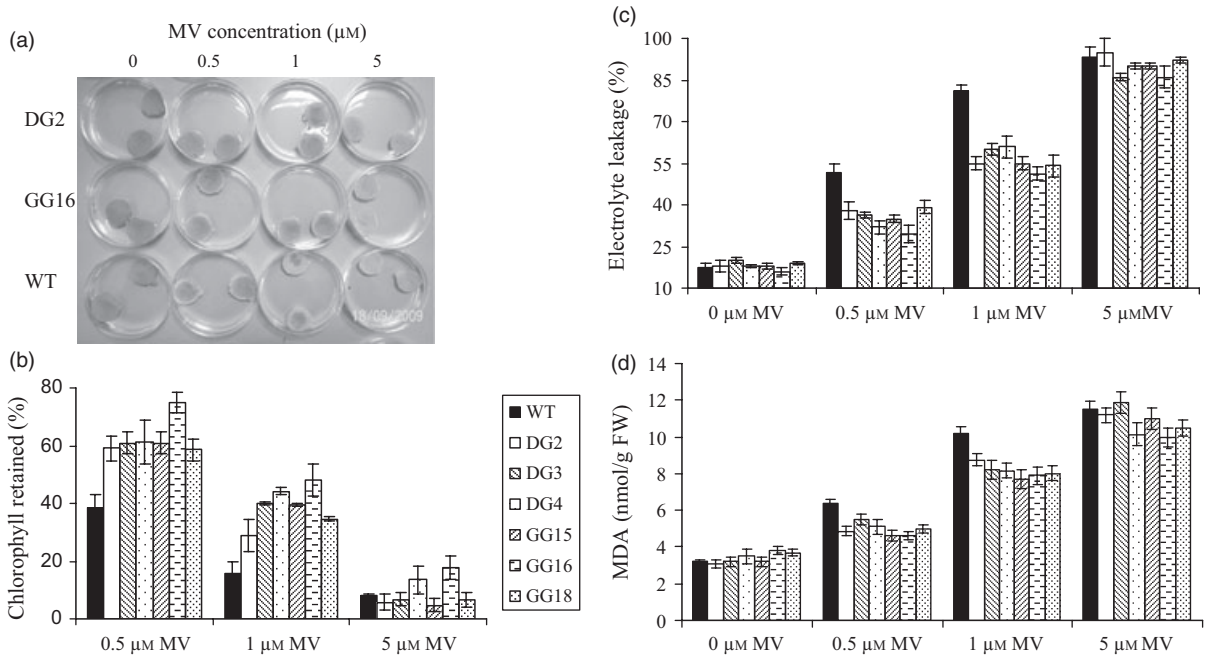

Figure 8 Effect of methyl viologen (MV) on leaf discs of dehydroascorbate reductase (DHAR):GR, glutathione-S-transferase (GST):glutathione reductase (GR) and WT tobacco plants. (a) Representative pictures showing phenotypic differences of transgenic DHAR:GR2 (DG2), GST:GR16 (GG16) and WT leaf discs floated on water, 0.5, 1 or 5 μM MV solution. Leaf discs were floated on the MV solution, pre-incubated in the dark for 12 h and then exposed to light (150 μmol/m^2 per second) for 24 h. (b) Effect of MV on the chlorophyll content of leaf discs. The percentage chlorophyll retained was calculated as follows: the chlorophyll content was measured in each leaf disc after paraquat treatment and compared to the chlorophyll content of leaf discs floated on water only. The experiments were repeated three times on three different plants per line. (c) Analysis of membrane damage as measured by electrolyte leakage. Conductivity of the different solutions was measured and expressed as percentages of the conductivity values obtained after total cell lysis by autoclaving the samples. The experiments were repeated three times on three different plants per line. (d) Effect of MV on the content of malondialdehyde in leaf discs from WT and transgenic lines DHAR:GR and GST:GR. Malondialdehyde (MDA) content expressed as nmol/g FW was determined in non-treated leaf discs (control H_2O) and after the different MV treatments. Data represent the means ± SD of 3 independent experiments on six leaf discs taken from two plants per line. In (b), (c) and (d) WT was significantly different from all transplastomic lines ($P < 0.05$), as determined by ANOVA, at 0.5 or 1 μM MV, but not at 0 or 5 μM MV.

GST enzymes; a balance might be established between the recycling of GSH by GR and its oxidation by DHAR or GST.

The transplastomic plants developed in this work were evaluated for protection against oxidative stresses generated by cold, salt, heavy metals and MV. The results presented herein demonstrate that the transplastomic tobacco seedlings, expressing ROS scavenging enzymes, were less sensitive to low temperatures and salt stress but no advantage was conferred to heavy metal stress. In both chilling and salt stresses, the protective effect could be observed when DHAR or GST levels were enhanced independently and in the case of chilling stress, a further improvement was observed when these were combined with increased GR activity. The most comparable study utilizing nuclear transformation to increase DHAR levels in tobacco chloroplasts (Kwon *et al.*, 2003) showed a similar protective effect on seedling growth against cold and salinity, but chlorophyll fluorescence of established plants was not assessed. Ushumura *et al.* (2006) showed a protective effect of DHAR expression in Arabidopsis on seed germination, but in both their study and that of Kwon *et al.* (2003) only 100 mM NaCl was used. The present study found a greatly improved seedling growth, in comparison with wild type, at double this concentration. Direct comparisons with published data are hampered by differences in stress treatments and methods of analysis, although broad inferences can be drawn. For example, nuclear transformants of tobacco expressing GST (Roxas *et al.*, 2000) exhibited qualitatively similar enhancement of tolerance to cold and salinity, to those presented here, but they used lower salt concentration (100 mM) and lower temperature (10 °C) than were employed in the present investigation. Also, they did not monitor chlorophyll fluorescence under stress, but did explore a different parameter: metabolic activity, as assessed by calorimetric analysis. Furthermore, their investigation did not involve targeting of the recombinant protein to the chloroplasts, and they do not comment on its probable cellular location. The present study extended the investigation to explore H_2O_2 levels in cold-stressed plants and showed they were reduced in the transplastomic lines, suggesting the improved tolerance is due to enhanced levels of ROS-scavenging enzymes, siphoning off excess H_2O_2, reducing the risk of its reduction to the toxic $^{\cdot}OH$ radical. Both the reduction of H_2O_2 and the improvement of chilling tolerance were more pronounced in the double transformants than in the lines expressing either DHAR or GST. In the DHAR:GR lines this is probably due to improved operation of the ascorbate-glutathione cycle, through the increase of two consecutive enzymes in it, while the GST:GR lines combine one of these enzymes (GR) with the peroxidase (GPX) activity of GST, which will contribute to the removal of H_2O_2.

The effect of expression in the chloroplasts on resistance to oxidative stress caused by methyl viologen application was also explored through the analysis of chlorophyll retention, electrolyte leakage and lipid peroxidation. Expression of ROS scavenging

enzymes directly at the site of ROS production does enhance protection from MV induced stress. However this result is not systematic. DHAR and GST overexpressing plants did not differ from WT in their tolerance to MV, while DHAR: GR and GST: GR did. In other studies using nuclear transformation, overexpression of GR in transgenic plants conferred increased tolerance to MV (Aono *et al.*, 1993; Broadbent *et al.*, 1995). However, in previous studies done in our laboratory (Poage *et al.*, submitted) transplastomic tobacco plants expressing the bacterial GR on its own didn't show any increased resistance to MV. The MV treatment was repeated on these same GR plants along with WT, GST, DHAR, DHAR: GR and GST: GR plants as a control experiment in the current study. This confirmed the previous results. No difference was observed between WT and GR leaf discs in terms of electrolyte leakage and chlorophyll analysis after MV exposure. The improved resistance to MV is clearly dependent on combining increased GR activity with that of DHAR or GST.

These different results illustrate the fact that in some cases, overexpression of a single antioxidant enzyme does not provide protection against oxidative stress and that simultaneous expression of multiple antioxidant enzymes is more effective than single expression for enhancing tolerance to environmental stresses. Kwon *et al.* (2002) demonstrated that overexpression of both CuZnSOD and APX (CA plants) genes in tobacco chloroplasts resulted in enhanced tolerance to MV induced oxidative stress compared to expression of either of these genes alone. They suggested that the combined increases in SOD and APX were complementary because they could increase the capacity for superoxide and H_2O_2 scavenging and also protect both the native and transgene-derived CuZnSOD from deactivation during stress. In a later study, tobacco plants expressing CuZnSOD, APX and DHAR (CAD plants) targeted to the chloroplast showed again elevated protection against MV and salt stress in comparison with the CA plants (Lee *et al.*, 2007).

In the work presented herein, the improved protection against MV and chilling could be explained by synergistic effects of DHAR with GR or GST with GR. The expression of these combinations of transgenes would increase the regeneration of reduced ascorbate and glutathione and participate in a more rapid scavenging of superoxide radicals and hydrogen peroxide prior to their interaction with target molecules.

There is considerable potential for engineering-improved stress tolerance by improvement of the antioxidant defence of the chloroplasts, as chloroplasts appear to be the main location affected by conditions of stress in plant cells (Tang *et al.*, 2006). The work presented herein confirms that through chloroplast transformation one or several antioxidant enzymes can be successfully expressed in the chloroplasts, leading to a significant enhancement in the ability of the plant to withstand adverse environmental conditions. The method used combines the advantages of a single step transformation procedure with the expression of the genes directly in the chloroplast, and can provide new insights into the role of antioxidant and their relationship within the ROS scavenging pathway in this organelle.

Experimental procedures

Vector construction and plant transformation

The plastid transformation vector pZS197 (Svab and Maliga, 1993) was modified to contain an *AscI/PacI* site upstream of the *aminoacid dA* (spectinomycin resistance) marker gene. The coding region of rice dehydroascorbate reductase (DHAR AB037970.1) gene (Urano *et al.*, 2000; Ushimaru *et al.*, 2006), kindly provided by Dr T. Ushimaru, Shizuoka University, Japan, and *E. coli* GST (D38497.1) gene were first cloned by PCR using high fidelity Platinum Taq Pfx (Invitrogen Paisley, Scotland, UK) and ligated into pCR2.1-TOPO (Invitrogen). After sequencing, they were integrated into the modified pZS197 as *AscI/PacI* fragments to obtain the vectors pZS-DHAR and pZS-GST. The expression vectors pZS-DHAR:GR and pZS-GST:GR were generated by inserting *E. coli gor*, encoding glutathione reductase (GR M13141.1), as a *PacI/PacI* fragment downstream of the DHAR and the GST genes in the pZS-DHAR and pZS-GST vectors, respectively (Figure 1).

Generation of chloroplast transgenic tobacco (*Nicotiana tabacum* var. Petit Havana) was carried out by the biolistic method. Young leaves were bombarded with plasmid DNA coated 0.6 μm tungsten particles using a gene gun (PDS 1000He; Bio-Rad, Perth, Scotland, UK). Primary spectinomycin resistant tobacco lines were selected on RMOP regeneration medium containing 500 mg/L spectinomycin (Svab and Maliga, 1993). Green calli and shoot formation were observed after 3–4 weeks of selection. Shoots were transferred to MS (Murashige and Skoog, 1962) pots for rooting. Transplastomic lines were subjected to two additional rounds of regeneration on RMOP with spectinomycin to obtain homoplasmic tissue. Plants were then moved to soil and seeds were collected from self-pollinated transgenic plants and used for further analysis.

Molecular analysis of transformation

Integration of foreign genes into the chloroplast genome was confirmed by Southern Blot analysis. Genomic DNA was extracted from leaves using the method described by Frey (1999). Four micrograms of total DNA per sample was digested with *EcoRI* and *EcoRV*, separated in 0.8% (w/v) agarose gel (6 h, 70 V) and transferred to Nylon membrane (Hybond N+; Amersham, Little Chalfont, Bucks, UK). Southern hybridization was performed using a non-radioactive DNA labelling and detection protocol (McCabe *et al.*, 1997). An *rbcL* probe and genes specific probes DHAR, GST, DHAR:GR and GST:GR were synthesized and labelled with DIG-dUTP (Roche, Burgess Hill, West Sussex, UK) by PCR. Following hybridization and membrane washing, the hybridized probe was detected using an anti-DIG antibody (Roche) and a chemiluminescent substrate (CDP star; Roche). Signals were visualized by exposure to Kodak X-ray film for 1–5 min.

Immunoblot analysis

Expression of the different transgenes in leaf tissue was confirmed by Western blot analysis. Total proteins were extracted from 100 mg of leaf material homogenized in extraction buffer (50 mM HEPES, 1 mM EDTA, 10 mM potassium acetate, 5 mM magnesium acetate and 2 mM phenylmethylsulphonylfluoride) and centrifuged at 18 000 ***g*** for 15 min at 4 °C. Protein concentrations were determined using the Bradford assay with BSA as a protein standard (Bradford, 1976). Twenty micrograms of total soluble protein was loaded per lane and electrophoresed in a 12% SDS-PAGE gel. Proteins were transferred to a nylon membrane and incubated with rice DHAR antibody (kindly provided by Dr T. Ushimaru, Shizuoka University, Japan), GR polyclonal antibody (Lab Frontier, Seoul, Korea) or GST antibody (GeneTel Lab, Madison, WI). Alkaline phosphatase conjugated

anti-rabbit IgG (Sigma, Arklow, Wicklow, Ireland) for DHAR and GR, and anti-chicken IgG (Sigma) for GST were used as secondary antibodies. Recombinant protein was quantified from DHAR and GST lines by running protein samples and purified protein, at various concentrations, on the same gel and analysing the image of the Western blot by densitometry using the Scion Image programme.

Biochemical analysis

Enzyme activities and metabolite contents were assayed in leaf samples taken from nodes 3 and 4 of 6 week-old soil grown plants. Samples for activity assays were prepared from 200 mg of leaf tissue ground in liquid nitrogen and homogenized in extraction buffer (50 mM potassium phosphate buffer, pH 7), centrifuged at 18 000 ***g***, for 15 min at 4 °C. DHAR activity was determined by the increase in absorbance at 295 nm due to GSH dependent production of ascorbate using an extinction coefficient of 14/mM per centimetre (Nakano and Asada, 1981). GST assay was carried out using the universal substrate 1-chloro 2,4-dinitrobenzene CDNB (Sigma) and activity was determined spectrophotometrically by the change in absorbance at 340 nm (Veal *et al.*, 2002). GR activity was determined following the procedure outlined by Kwon *et al.* (2001). The reaction was measured by the decrease in absorbance at 340 nm using an extinction coefficient of 6.22/mM per centimetre. GPX activity was assayed using cumene hydroperoxide as substrate. The reaction rate was measured spectrophotometrically by following the oxidation of NADPH at 340 nm (Veal *et al.*, 2002). Protein concentrations were determined using the dye-binding assay of Bradford (1976). Results were expressed as unit per mg of protein.

For glutathione and ascorbate determination, tissue samples were frozen in liquid nitrogen, ground with 2.5 M $HClO_4$ and centrifuged for 15 min at 18 000 ***g***. The supernatant was neutralized with 1.25 M Na_2CO_3 to obtain pH values between 4–5 and 6–7 for determination of ascorbate and glutathione, respectively. Glutathione pools (total, reduced and oxidized) were determined according to the method described in Roxas *et al.* (1997). Total glutathione was determined in a 200 μL reaction containing 20 μL of the crude extract, and 180 μL of the assay buffer made of 0.1 M potassium phosphate buffer (KPB) pH 6.6, 0.6 mM DTNB (5, 5,-Dithio-bis (2-nitrobenzoic acid)) (Sigma), 1 U/mL GR (Sigma) and 0.2 mM NADPH. The samples and assay buffer were loaded in triplicates on a 96 well plate.

The change in absorbance at 412 nm was monitored for 3 min at timed intervals. The amount of glutathione was determined by reference to a glutathione standard curve. To determine the amount of oxidized glutathione (GSSG), the samples were first incubated with 2 μL/100 μL of extract of 2-vinylpyridine at 25 °C for 1 h, after which the procedure was the same as described above. Reduced glutathione (GSH) was calculated as the difference between the total glutathione and the oxidized glutathione, the results were expressed as nmol Glu/g fresh weight (FW).

Analysis of ascorbate pools (total, reduced and oxidized) was carried out as described in Foyer *et al.* (1983). The assay buffer contained 0.1 M KPB pH5.6 and 0.25 unit/mL of ascorbate oxidase (Sigma). Twenty microlitre of crude extract and 180 μL of assay buffer were loaded per well in triplicate, this first measure giving the amount of reduced ascorbate (AsA) determined by comparison to a standard curve. The total amount of ascorbate (AsA + DHA) was measured by the same method following reduction to ascorbic acid in a reaction mixture containing 20 mM DTT (Sigma) and 50 mM HEPES-KOH buffer pH 7 and incubated for 30 min at room temperature. The amount of DHA was finally determined as the difference between these two assays. The results were expressed in nmol Ascorbate/g FW.

Seedling stress analysis

T1 seeds from the different transplastomic lines were analysed together with control WT seed lines. For low temperature treatment, seeds were germinated on germination medium containing plates and incubated in growth chambers at either 4 °C or 15 °C. For salt treatment, seeds were placed on germination medium with added concentrations of salt: 100, 150 and 200 mM NaCl and incubated in a growth chamber at 24 °C. For these two experiments, seedling growth was evaluated after 12 or 14 days by length measurements of 25 randomly selected seedlings per line. For heavy metal treatment, germination medium plates contained 0.25 mM, 0.5 mM, 0.75 mM and 1 mM of $CdCl_2$, or 0.5 mM, 1 mM, 2 mM and 5 mM of $ZnCl_2$. The seeds were placed on the plates and after 1–2 weeks, seeds were scored for percentage germination.

MV treatment

Leaf discs (11 mm in diameter) were cut out from leaves of nodes 3–4 of soil grown plants and floated, abaxial surface down, on 10 mL of water or solutions containing different concentrations of MV (0.5 μM, 1 μM and 5 μM) in 60 mm Petri dishes. Plates were incubated in the dark for 12 h to allow diffusion of the MV into the leaf disc and then placed under light (intensity 150 μmol/m^2 per second) for 24 h. Triplicates plates were prepared for each line of plant treated, to measure chlorophyll content, malondialdehyde (MDA) content and electrolyte leakage of the leaf discs.

Chilling stress on leaf discs

Leaf discs (11 mm in diameter) were punched out from leaves of node 3 and 4 of 6 week-old plants grown in soil, and were subjected to chilling treatment. Per plate, 60 mm Petri dishes, two leaf discs were floated on 10 mL of distilled water and incubated at 8 °C for up to 72 h with moderate light intensity (80 μmol/m^2 per second). Chlorophyll fluorescence was measured after 48 h and 72 h and H_2O_2 content was measured after 72 h treatment. For each line, three plants were tested, and the experiment was repeated three different times.

Measurements of chlorophyll content

Chlorophyll was extracted by soaking one leaf disc from the MV treatment, in 1 mL of 95% ethanol for 2 h at 80 °C. The absorbance of supernatant was read at 649 and 664 nm. Chlorophyll content was determined using the equations from Lichtenthaler (1987).

Determination of oxidative damage to lipids

Oxidative damage to lipids was determined by measuring the content of MDA according to the method described by Heath and Packer (1968). Following control and MV treatment, two leaf discs/samples were frozen in liquid nitrogen, ground in 0.2 mL of distilled water and homogenized with an equal volume of 0.5% (w/v) thiobarbituric acid in 20% (v/v) trichloroacetic acid (TCA). Samples were heated at 95 °C for 30 min and quickly cooled on ice for 15 min. After 30 min centrifugation at

13 000 rpm, at 4 °C, the supernatant was loaded in triplicate in a 96-well plate and the absorbance read at 532 nm and 600 nm. The value for non-specific absorption at 600 nm was subtracted from the 532 nm value. The MDA content was calculated using its absorption coefficient of 155 nmol/cm and then expressed as nmol MDA per gram fresh weight.

Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide was measured spectrophotometrically after reaction with potassium iodide (KI) following the method described in Alexieva *et al.* (2001). After the control and cold treatment, three leaf discs/samples were ground in liquid nitrogen and homogenized in 0.1% (w/v) cold TCA. The homogenate was centrifuged at 13 000 rpm for 25 min, 4 °C. The supernatant was kept for determination of H_2O_2 content. The reaction mixture consisted of 50 μL of 0.1% cold TCA leaf extract supernatant, 50 μL of 0.1 M KPB pH6.8 and 200 μL of reagent KI (1 M KI w/v in dH_2O). The 300 μL reaction was loaded in triplicate in a 96 well plate. The reaction was developed for 1 h in the dark and the absorbance measured at 390 nm. The amount of H_2O_2 was calculated using a standard curve prepared with known concentration of H_2O_2. The H_2O_2 content was then expressed as μmol H_2O_2 per gram fresh weight.

Electrolyte leakage analysis

The electrolyte leakage into the solutions used for floating the leaf discs in the MV experiment was determined using the EC215 conductivity meter (Hanna Instrument). The electrical conductance was measured first after each experiment (EC initial), then the bathing solutions containing the leaf discs were autoclaved at 120 °C for 20 min and the electrical conductance was measured again to get the total ion leakage (ECmax). The relative electrolyte leakage was then calculated as a percentage:

$$\text{EC relative} = (\text{EC initial}/\text{EC max}) \times 100$$

Measurements of chlorophyll fluorescence

The photosynthetic activity of the leaf discs subjected to chilling stress was estimated by chlorophyll fluorescence determination of photochemical yield (Fv/Fm) which represented the maximum yield of the photochemical reaction in photosystem II. Chlorophyll fluorescence was measured with the Handy PEA chlorophyll fluorimeter (Hansatech Instruments, Norfolk, UK). Samples were dark adapted for 15 min prior to each measurement and Fv/Fm was determined.

Statistical analyses

All data are presented as mean and standard deviation of the mean. Statistical analysis was carried out using one way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

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References

Ahmad, R., Kim, Y.-H., Kim, M.-D., Kwon, S.-Y., Cho, K., Lee, H.-S. and Kwak, S.-S. (2010) Simultaneous expression of choline oxidase, superoxide dismutase and ascorbate peroxidase in potato plant chloroplasts provides synergistically enhanced protection against various abiotic stresses. *Physiol. Plant.* **138**, 520–533.

Alexieva, V., Sergiev, I., Mapelli, S. and Karanov, E. (2001) The effects of drought and ultraviolet radiation on growth and stress markers in pea and wheat. *Plant Cell Environ.* **24**, 1337–1344.

Aono, M., Kubo, A., Saji, H., Tanaka, K. and Kondo, N. (1993) Enhanced tolerance to photooxidative stress of transgenic *Nicotiana tabacum* with high chloroplastic glutathione reductase activity. *Plant Cell Physiol.* **34**, 129–135.

Badawi, G.H., Kawano, N., Yamauchi, Y., Shimada, E., Sasaki, R., Kubo, A. and Tanaka, K. (2004) Overexpression of ascorbate peroxidase in tobacco chloroplasts enhances the tolerance to salt stress and water deficit. *Physiol. Plant.* **121**, 231–238.

Bartling, D., Radzio, R., Steiner, U. and Weiler, E.W. (1993) A glutathione-S-transferase with glutathione-peroxidase activity from *Arabidopsis thaliana*. Molecular cloning and functional characterization. *Eur. J. Biochem.* **216**, 579–586.

Bock, R. (2001) Transgenic plastids in basic research and plant biotechnology. *J. Mol. Biol.* **312**, 425–438.

Bock, R. and Khan, M.S. (2004) Taming plastids for a green future. *Trends Biotechnol.* **22**, 311–318.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principal of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Broadbent, P., Creissen, G.P., Kular, B. and Wellburn, A.R. (1995) Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. *Plant J.* **8**, 247–255.

Burns, C., Geraghty, R., Neville, C., Murphy, A., Kavanagh, K. and Doyle, S. (2005) Identification, cloning, and functional expression of three glutathione transferase genes from *Aspergillus fumigatus*. *Fungal Genet. Biol.* **42**, 319–327.

Chen, Z. and Gallie, D.R. (2004) The ascorbic acid redox state controls guard cell signalling and stomatal movement. *Plant Cell*, **16**, 1143–1162.

Craig, W., Lenzi, P., Scotti, N., De Palma, M., Saggese, P., Carbone, V., McGrath Curran, N., Magee, AM., Medgyesy, P., Kavanagh, T.A., Dix, P.J., Grillo, S. and Cardi, T. (2008) Transplastomic tobacco plants expressing a fatty acid desaturase gene exhibit altered fatty acid profiles and improved cold tolerance. *Transgenic Res.* **17**, 769–782.

Daniell, H. (2007) Transgene containment by maternal inheritance: effective or elusive? *Proc. Natl Acad. Sci. USA*, **104**, 6879–6880.

Daniell, H., Ruiz, O.N. and Dhingra, A. (2005) Chloroplast genetic engineering to improve agronomic traits. *Methods Mol. Biol.* **286**, 111–138.

Ding, S., Lu, Q., Zhang, Y., Yang, Z., Wen, X., Zhang, L. and Lu, C. (2009) Enhanced sensitivity to oxidative stress in transgenic tobacco plants with decreased glutathione reductase activity leads to a decrease in ascorbate pool and ascorbate redox state. *Plant Mol. Biol.* **69**, 577–592.

Foyer, C.H., Rowell, J. and Walker, D. (1983) Measurement of the ascorbate content of spinach leaf protoplast and chloroplasts during illumination. *Planta*, **157**, 239–244.

Foyer, C.H., Descourvieres, P. and Kunert, K.J. (1994) Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. *Plant Cell Environ.* **17**, 507–523.

Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Pruvost, C. and Jouanin, L. (1995) Overexpression of glutathione reductase but not glutathione synthase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiol.* **109**, 1047–1057.

Foyer, C.H., LopezDelgado, H., Dat, J.F. and Scott, I.M. (1997) Hydrogen peroxide and glutathione associated mechanisms of acclamatory stress tolerance and signaling. *Physiol. Plant.* **100**, 241–254.

Frey, J.E. (1999) Genetic flexibility of plant chloroplasts. *Nature*, **398**, 115–116.

Heath, R.L. and Packer, L. (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch. Biochem. Biophysiol.* **125**, 180–198.

Kanai, T., Takahashi, K. and Inoue, H. (2006) Three distinct-type glutathione S-transferases from *Escherichia coli* important for defense against oxidative stress. *J. Biochem.* **140**, 703–711.

Kumar, S., Dhingra, A. and Daniell, H. (2004) Plastid expressed betaine aldehyde dehydrognease gene in carrot cultured cells, roots and leaves confers enhanced salt tolerance. *Plant Physiol.* **136**, 2843–2854.

Kwon, S.Y., Ahn, Y.O., Lee, H.S. and Kwak, S.S. (2001) Biochemical characterization of transgenic tobacco plants expressing a human dehydroascorbate reductase. *J. Biochem. Mol. Biol.* **34**, 316–321.

Kwon, S.Y., Jeong, Y.J, Lee, H.S., Kim, J.S., Cho, K.Y., Allen, R.D. and Kwak, S.S. (2002) Enhanced tolerances of transgenic tobacco plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against methyl viologen mediated oxidative stress. *Plant Cell Environ.* **25**, 873–882.

Kwon, S.Y., Choi, S.M., Ahn, Y.O., Lee, H.S., Lee, H.B., Park, Y.M. and Kwak, S.S. (2003) Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *J. Plant Physiol.* **160**, 347–353.

Lee, S.B., Kwon, H.B., Kwon, S.J., Park, S.C., Jeong, M.J., Han, S.E., Byun, M.O. and Daniell, H. (2003) Accumulation of trehalose within transgenic chloroplasts confers drought tolerance. *Mol. Breeding*, **11**, 1–13.

Lee, Y.P., Kim, S.H., Bang, J.W., Lee, H.S., Kwak, S.S. and Kwon, S.Y. (2007) Enhanced tolerance to oxidative stress in transgenic tobacco plants expressing three antioxidant enzymes in chloroplasts. *Plant Cell Rep.* **26**, 591–598.

Lichtenthaler, H.H. (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods Enzymol.* **148**, 350–382.

McCabe, M.S., Power, J.B., de Laat, A.M. and Davey, M.R. (1997) Detection of single copy genes in DNA from transgenic plants by nonradioactive Southern blot analysis. *Mol. Biotechnol.* **7**, 79–84.

Mittler, R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* **7**, 405–410.

Mittler, R., Vanderauwera, S., Gollery, M. and van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci.* **9**, 490–498.

Mullineaux, P., Creissen, G., Broadbent, P., Reynolds, H., Kular, B. and Wellburn, A. (1994) Elucidation of the role of glutathione reductase using transgenic plants. *Biochem. Soc. Trans.* **22**, 931–936.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Nakano, Y. and Asada, K. (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplasts. *Plant Cell Physiol.* **22**, 867–880.

Nishida, M., Kong, K.-H., Inoue, H. and Takahashi, K. (1994) Molecular cloning and sitedirected mutagenesis of glutathione *S*-transferase from *Escherichia coli*. The conserved tyrosyl residue near the N terminus is not essential for catalysis. *J. Biol. Chem.* **269**, 32536–32541.

Noctor, G. and Foyer, C.H. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49**, 249–279.

Noctor, G., Arisi, A., Jouanin, L., Kunert, K., Rennenberg, H. and Foyer, C.H. (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *J. Exp. Bot.* **49**, 623–647.

Roxas, V.P., Smith, R.K., Allen, E.R. and Allen, R.D. (1997) Overexpression of glutathione-S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotechnol.* **15**, 988–991.

Roxas, V.P., Lodhi, S.A., Garrett, D.K., Mahan, J.R. and Allen, R.D. (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase. *Plant Cell Physiol.* **41**, 1229–1234.

Ruf, S., Hermann, M., Berger, I.J., Carrer, H. and Bock, R. (2001) Stable genetic transformation of tomato plastids and expression of a foreign protein in fruit. *Nat. Biotechnol.* **19**, 870–875.

Sen Gupta, A., Webb, R.P., Holaday, A.S. and Allen, R.D. (1993) Overexpression of superoxide dismutase protects plant from oxidative stress. *Plant Physiol.* **103**, 1067–1073.

Svab, Z. and Maliga, P. (1993) High frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. *Proc. Natl Acad. Sci. USA*, **90**, 913–917.

Tang, L., Kwon, S.Y., Kim, S.H., Kim, J.S., Choi, J.S., Cho, K.Y., Sung, C.K., Kwak, S.S. and Lee, H.S. (2006) Enhanced tolerance of transgenic potato plants expressing both superoxide dismutase and ascorbate peroxidase in chloroplasts against oxidative stress and high temperature. *Plant Cell Rep.* **25**, 1380–1386.

Urano, J., Nakagawa, T., Maki, Y., Masumura, T., Tanaka, K., Murata, N. and Ushimaru, T. (2000) Molecular cloning and characterization of a rice dehydroascorbate reductase. *FEBS Lett.* **466**, 107–111.

Ushimaru, T., Nakagawa, T., Fujioka, Y., Daicho, K., Naito, M., Yamauchi, Y., Nonaka, H., Amako, K., Yamawaki, Y. and Murata, N. (2006) Transgenic Arabidopsis plants expressing the rice dehydroascorbate reductase gene are resistant to salt stress. *J. Plant Physiol.* **163**, 1179–1184.

Veal, E.A., Toone, W.M., Jones, N. and Morgan, B.A. (2002) Distinct roles for glutathione S-transferases in the oxidative stress response in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **277**, 35523–35531.

Wurbs, D., Ruf, S. and Bock, R. (2007) Contained metabolic engineering in tomatoes by expression of carotenoid biosynthesis genes from the plastid genome. *Plant J.* **49**, 276–288.

Yamasaki, H., Takahashi, S. and Heshiki, R. (1999) The tropical fig *Ficus microcarpa* L.f. cv. Golden leaves lacks heat-stable dehydroascorbate reductase activity. *Plant Cell Physiol.* **40**, 640–646.

Yoshimura, K., Miyako, K., Gaber, A., Takeda, T., Kanaboshi, H., Miyasaka, H. and Shigeoka, S. (2004) Enhancement of stress tolerance in transgenic tobacco plants overexpressing *Chlamydomonas* glutathione peroxidase in chloroplasts or cytosol. *Plant J.* **37**, 21–33.

Yu, T., Li, Y.S., Chen, X.F., Hu, J., Chang, X. and Zhu, Y.G. (2003) transgenic tobacco plants overexpressing cotton glutathione S-transferase (GST) show enhanced resistance to methyl viologen. *J. Plant Physiol.* **160**, 1305–1311.

Zhang, J., Tan, W., Yang, X.H. and Zhang, H.X. (2008) Plastid-expressed choline monoaxygenase gene improves salt and drought tolerance through accumulation of glycine betaine in tobacco. *Plant Cell Rep.* **27**, 1113–1124.