Expression of senescence-enhanced genes in response to oxidative stress

Saeid Navabpour¹, Karl Morris¹, Rebecca Allen¹, Elizabeth Harrison¹, Soheila A-H-Mackerness² and Vicky Buchanan-Wollaston¹,*

¹ Horticulture Research International, Wellesbourne, Warwick CV35 9EF, UK
² Department for Environment Food and Rural Affairs, Room 701, Cromwell House, Dean Stanley Street, London SW1P 3JH, UK

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Abstract

Expression of the LSC54 gene, encoding a metallothionein protein, has been shown previously to increase during leaf senescence and cell death. Evidence is presented in this paper to indicate that the extent of LSC54 expression is related to levels of oxidative stress in the tissues. Treatment of Arabidopsis cotyledon and leaf tissues with the catalase inhibitor, 3-amino-1,2,4-triazole, or with silver nitrate result in the enhanced expression of LSC54. Combined treatments with quenchers of reactive oxygen species (ROS), such as ascorbate, tiron and benzoic acid indicated that this induced expression was due to increased levels of ROS. The expression of many other senescence-enhanced genes was also found to be inducible by the increase in ROS. Treatment of plant tissue with 3-amino-1,2,4-triazole, followed by silver nitrate, resulted in protection from the severe damage caused by the silver nitrate treatment and reduced expression of many of the genes examined. However, one gene, encoding a lipid hydroperoxide-dependent glutathione peroxidase, showed increased expression in the protected tissue, which may indicate a role for this enzyme in the protection of plant tissue from oxidative stress. ROS-enhanced expression of at least one of the genes investigated required the presence of the salicylic acid signalling pathway, which was not required for the expression of LSC54.

Key words: Arabidopsis, expression, ROS, senescence-related genes.

Introduction

During growth and development, a plant has to cope with a range of different internal and external stresses and the ability to adapt to metabolic and environmental changes is essential for survival. The production of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), superoxide O₂⁻ and its more toxic derivatives, hydroxyl radicals (OH) and singlet oxygen (¹O₂), occurs at all times during plant growth and development, but is increased when plants are exposed to various biotic and abiotic stresses (Elstner, 1982; Asada, 1994; Dat et al., 2000). These toxic ROS oxidize proteins, unsaturated fatty acids and DNA, resulting in cellular damage and cell death.

Plants have a number of different defence mechanisms by which they respond to oxidative stress. These include the production of both non-enzymatic antioxidants such as ascorbate and glutathione and enzymatic antioxidants such as catalase, superoxide dismutase and ascorbate peroxidase. When these defences fail to protect the plant from the ROS, cell death will result. In many cases the plant exhibits symptoms similar to those seen during leaf senescence before cell death occurs. For example, plants that were exposed to stresses such as ozone or UV-B irradiation, each resulting in increased levels of ROS, responded by showing symptoms of premature senescence followed by necrotic cell death (Pell et al., 1997; Jansen et al., 1998). Also, infection by pathogens can induce premature senescence and localized cell death (Morel and Dangl, 1997).

Changes in gene expression measured during these stress responses were found to be similar to some of the changes which occur during leaf senescence (Miller
et al., 1999; John et al., 2001; Hanfrey et al., 1996; Butt et al., 1998). Senescence in plants is defined as the final stage of development. It culminates in death of the senescing cells and tissues after much of the nutrient content has been remobilized to developing tissues or seeds (Noodén, 1988). Leaf senescence involves the degradation of protein, lipid, chlorophyll, and nucleic acids and these processes result in considerable metabolic changes. These degradative processes, particularly for lipids, often result in increased production of ROS (Thompson et al., 1998) and the plant responds to this by senescence-enhanced production of certain antioxidant enzymes (Buchanan-Wollaston, 1997; Jimenez et al., 1998). However, in general, the antioxidant status of the leaf is reduced during senescence; levels of ROS are enhanced, many antioxidant enzymes show reduced activity and lipid peroxidation levels increase (Del Rio et al., 1998; Jimenez et al., 1998; Thompson et al., 1998; John et al., 2001; Prochazkova et al., 2001).

In this paper, the potential links between oxidative stress responses and leaf senescence have been investigated by studying the signals that lead to increased expression of senescence-enhanced genes. The expression of the senescence-enhanced promoter for the LSC54 metallothionein gene, is enhanced in cells undergoing various types of cell death, such as during the hypersensitive pathogen response and in necrotic cell death (Butt et al., 1998). The question of what signalling molecule or molecules, might be common to all these types of cell death is addressed. In addition, the expression patterns of other senescence-enhanced genes in response to the signals that induce LSC54 expression are examined.

Materials and methods

Plant material
Arabidopsis thaliana, accession Columbia, seedlings and plants were grown in growth chambers at a temperature of 22/16 °C (day/night), and 30 W m⁻² s⁻¹ with a 12 h photoperiod. NahG transgenic and jar1 mutant plants were grown under the same conditions. Expression of the LSC54 gene was assessed using a transgenic Arabidopsis line (627/2/9) carrying the B. napus LSC54 promoter fused to the GUS gene (Butt et al., 1998). Seedlings were grown for 7 d before treatment as described in (Butt et al., 1998). For the TBARM measurements and the RNA extraction and northern hybridization experiments, plants were grown for 5 weeks before treatment. At this time leaves were at the mature green stage (MG). Plants were left for a further 5 weeks until flowering was well advanced and senescing, partially chlorotic, leaves were collected for the S2 stage (senescence stage 2, Butt et al., 1998).

Chemical treatments
Spray treatments were applied until liquid dripped from the cotyledons or leaves. The effects of chemical treatments on the expression of LSC54 were carried out using 7 d seedlings. Seedlings were sprayed with the different chemicals. A range of concentrations of each treatment was tested initially to identify the optimum level to use. Final experiments were carried out using 1 mM silver nitrate, 20 mM 3-AT, 10 mM sodium ascorbate, 25 mM Dabco, 2.5 mM Tiron, 5 mM benzoic acid, catalase at 4 K units, and superoxide dismutase at 4 K units. All chemicals were obtained from Sigma-Aldrich. For the combined treatments, the first treatment was sprayed onto the plants, followed 2 h later by the second treatment. Each experiment was repeated at least three times with similar results. For the RNA extractions, similar spray treatments were carried out on 5-week-old plants.

Measurement of GUS expression
Histochemical analysis and enzyme activity quantification was carried out as described in Butt et al. (1998). All experiments were repeated at least three times.

Measurement of thiobarbituric acid reactive material (TBARM)
TBARM were measured as described in Page et al. (2001). Arabidopsis plants were sprayed with water, 3-AT, silver nitrate, and the mixture of silver nitrate+3-AT. Leaves were harvested 2 d after treatment, frozen in liquid nitrogen and assayed for TBARM levels.

RNA isolation and RNA blot analysis
RNA was isolated and northern hybridization was carried out as previously described (Buchanan-Wollaston and Ainsworth, 1997). Probes for the hybridization experiments were derived from Arabidopsis. Most of the genes used were originally identified as senescence-enhanced genes in Brassica and the Arabidopsis homologues of these have been identified using genomic sequence and EST databases. EST clones were obtained from the Arabidopsis Biological Resource Center, Ohio.

Results

Chemical treatments that induce expression of LSC54
The expression of the LSC54 promoter is induced during developmental senescence, wounding, HR-induced cell death, and necrotic cell death (Butt et al., 1998). The aim was to identify a chemical treatment that could induce LSC54 expression, and use this to characterize the common signals that cause gene expression under these different conditions. Transgenic Arabidopsis seedlings carrying the LSC54 promoter:GUS fusion were used to test a variety of treatments including copper sulphate, aluminium sulphate and UV-B irradiation and, although these treatments did result in some increase in expression (data not shown), the most consistent enhancement of expression was obtained when the seedling tissue was treated with either silver nitrate (1 mM) or 3-amino-1,2,4-triazole (3-AT) (20 mM). Quantitative comparisons using a fluorometric assay for GUS activity indicated that treatment with silver nitrate or 3-AT significantly increased LSC54 expression (Fig. 1A). The physical damage caused by these treatments is shown in Fig. 1B. The treatment with silver nitrate caused extensive cell death in the treated seedlings while the effects of the 3-AT treatment were much less severe.
Combined treatment with 3-AT reduces the toxic effects of silver nitrate

In these studies, in order to identify a consistent treatment for the induction of the LSC54 gene, a number of double treatments were investigated. Interestingly, instead of showing a combined enhancement, treatment of the plant seedlings with both silver nitrate and 3-AT together resulted in a significantly lower expression of the LSC54 gene than that obtained with silver nitrate alone. When seedlings were sprayed with 20 mM 3-AT followed 2 h later by silver nitrate, the LSC54 expression measured was at a level similar to that induced by the 3-AT treatment alone (Fig. 1A). This was reflected by the physical damage to the seedlings. Those treated with both 3-AT and silver nitrate appeared considerably healthier than those treated with silver nitrate alone (Fig. 1B). Thus, pre-treatment with 3-AT may result in the induction of protective mechanisms which enable the plant to cope with the severe stress caused by the treatment with silver nitrate.

Oxidative stress results in an increase in damage to macromolecules such as lipids, nucleic acids and carbohydrates and the extent of this can be examined by assaying TBARM (thiobarbituric acid-reactive materials) levels (Hodges et al., 1999). This assay measures levels of malondialdehyde (MDA), which is a secondary end-product of polyunsaturated fatty acid oxidation. Increases in MDA levels are indicative of an increase in lipid peroxidation, especially in green leaves which have relatively high levels of polyunsaturated fatty acids with three or more double bonds. However, the TBARM assay is not specific and will also measure other degradation products from nucleic acids and carbohydrates.

The silver nitrate and 3-AT treatments both resulted in a significant increase in TBARM levels (Fig. 2) indicating that the level of lipid peroxidation and other damage was enhanced by these treatments. The TBARM level was considerably higher after silver nitrate treatment than after 3-AT treatment and was attenuated by the combined treatment with 3-AT. This result confirms that the 3-AT treatment is reducing the damage caused by the silver nitrate. The level of oxidative damage found after the different treatments mirrors the level of GUS expression from the LSC54 promoter in the same tissues.

**Oxidative stress caused by silver nitrate treatment**

It was hypothesized that the treatment with silver nitrate, that is resulting in increased lipid peroxidation and extensive cellular damage, is causing a rise in ROS levels in the treated tissues. The level of LSC54 expression appears to parallel the level of damage experienced by the treated tissues and thus increased ROS may be the signal leading to the expression of the LSC54 gene. If this is the case, it should be possible to interfere with LSC54...
expression by pretreatment with substances that quench ROS, such as ascorbate (Knox and Dodge, 1985). Plant tissue was treated with ascorbate and then with silver nitrate 2 h later and the level of GUS expression was measured after 2 d (Fig. 1A). A significantly lower level of LSC54 expression was detected in this treated tissue, approximately 6–10-fold less than that obtained in tissue treated with silver nitrate alone. Ascorbate treatment also attenuated the detrimental effects of the silver nitrate on the plant tissue, considerably reducing the necrotic lesions (Fig. 1B). These results indicated that the increased expression of the LSC54 gene and the extensive cell death caused by treatment with silver nitrate is due to the enhanced levels of ROS in the treated tissue.

Ascorbate is an efficient quencher of a number of different ROS. To determine which particular species were produced in the plant tissue following silver nitrate treatment and also to identify the species that have a role in LSC54 activation, a number of other antioxidant treatments more specific to particular ROS were tested. DABCO quenches singlet oxygen (\(^{1}\text{O}_2\)) (Knox and Dodge, 1985), Tiron quenches superoxide (\(\text{O}_2^-\)) (Shimazaki et al., 1980) and benzoic acid detoxifies hydroxyl radicals (OH). All the antioxidants, when used in combined treatments with silver nitrate, had some effect on expression of LSC54 (Fig. 3), significantly decreasing the level obtained with silver nitrate treatment alone. However, none was as effective as ascorbate. These results indicate that silver nitrate treatment of plant tissue causes the generation of a mixture of ROS, either directly or after interconversion via a number of different pathways.

Expression of other senescence-enhanced genes is induced by oxidative stress

The metallothionein gene encoded by LSC54 is expressed at very low levels in healthy green leaves and this expression increases considerably during senescence and starting quite early in senescence, well before signs of chlorosis or cell death (Butt et al., 1998). The observations described above indicate that LSC54 expression is induced by ROS and there are many reports in the literature to indicate that ROS levels increase in senescing leaves (Thompson et al., 1998; Del Rio et al., 1998). Therefore, it was of interest to determine whether any other genes that have been identified as senescence-enhanced show an expression profile similar to LSC54 in response to increased ROS. RNA was isolated from mature green leaves that had been sprayed with water, 3-AT or silver nitrate and RNA blot analysis carried out with a range of senescence-enhanced genes (Fig. 4). In addition, RNA was isolated from leaves that had been exposed to the combined treatment of 3-AT followed by silver nitrate 2 h later and a comparison of expression levels in green and senescent leaves is included (Fig. 4).

Many of the senescence-enhanced genes tested showed induced expression in the treated tissue. The expression pattern with the LSC54 gene mirrored the result obtained with the fluorometric assays for GUS expression (Fig. 1A) with more expression of this gene after silver nitrate treatment than after treatment with 3-AT and a reduced expression with the combined treatment. Other genes that showed a similar expression pattern included LSC94 which encodes the pathogenesis related protein PR1a, LSC222 which encodes a chitinase (both in Hanfrey et al., 1996) and LSC760 and LSC790 which encode an aspartic protease and a cysteine protease respectively (Buchanan-Wollaston and Ainsworth, 1997). The levels of expression of the photosynthetic gene ribulose biphosphate carboxylase small subunit (RBCS) were severely repressed by the silver nitrate treatment, and less so by the 3AT treatment. The combined treatment resulted in a similar level of RBCS expression as that seen in the tissue treated with 3-AT alone.

The expression pattern of LSC803, a senescence-enhanced gene which encodes a lipid hydroperoxide-dependent glutathione peroxidase (Page et al., 2001) was slightly different. This gene showed induced expression after both silver nitrate and 3-AT treatment, but its expression level was higher in the tissue exposed to the combined treatment.

The observation that many senescence-enhanced genes were induced in tissue treated with silver nitrate, together with the reduced expression of the RBCS gene, may indicate that this treated tissue is entering a phase of senescence before cell death occurs. However, many genes that showed induced expression during senescence were

![Fig. 3. GUS expression levels in cotyledons treated with different antioxidants. GUS activity was measured by a fluorometric assay in cotyledon tissue of the LSC54 promoter-GUS transgenic, harvested 2 d after different spray treatments. Each result is the average of three independent experiments. Statistical analysis of the data showed that all the combined treatments resulted in a significant difference (P=0.05) to the treatment with silver nitrate alone.](http://jxb.oxfordjournals.org/)

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not expressed in this tissue (Fig. 4). For example, expression of SAG12 was not induced. This gene encodes another cysteine protease and has been defined as a senescence-specific gene; it is only expressed in senescing leaves that are showing signs of chlorosis (Lohman et al., 1994; Noh and Amasino, 1999). Also, expression of LSC460 and LSC30, encoding cytosolic glutamine synthetase and ferritin, respectively (Buchanan-Wollaston and Ainsworth, 1997), was not induced following silver nitrate treatment. Overall, about half of the senescence-enhanced genes that were tested showed induced expression in tissue treated with silver nitrate (additional data not shown).

**Signalling pathways leading to gene expression in response to ROS**

Gene expression during plant senescence appears to be controlled by a number of different signalling pathways. Stress-response pathways such as those involving salicylic acid (SA) and jasmonic acid (JA) have been shown to have a role in controlling gene expression during plant senescence (Morris et al., 2000; He et al., 2002). In this study, the importance of these pathways in controlling the expression of two genes LSC54 and LSC94 (PR1a) in response to oxidative stress was investigated. Firstly, the effect of ROS quenchers on the expression of the two genes was compared. Northern hybridization analysis was carried out using RNA isolated from treated green leaves from mature plants. Silver nitrate-induced expression of both genes was reduced considerably by pretreatment with ascorbate, Tiron, and benzoate (Fig. 5A). This confirmed the previous fluorometric data which indicated that LSC54 expression was due to ROS and showed that expression of LSC94 was also due to enhanced levels of ROS in the treated tissues. Transgenic NahG plants, which are defective in the SA pathway which is required for the expression of PR1a during pathogen responses (Gaffney et al., 1993), and jar1 mutant plants which are defective in...
the jasmonate (JA) signalling pathway (Staswick et al., 1992) were used to investigate the role of these two pathways in gene expression in response to silver nitrate. Figure 5B shows that expression of LSC54 is independent of both pathways whereas LSC94 requires the SA pathway for expression. Therefore, taking the results shown in Fig. 5A and B together, the increased levels of ROS caused by the silver nitrate treatment result in induction of the SA pathway which is required for LSC94 expression. Expression of LSC54 is via a different pathway and is independent of SA. The JA pathway was not involved in the expression of either of these genes following silver nitrate treatment.

Discussion

The herbicide 3-AT acts as an inhibitor of catalase, which degrades hydrogen peroxide. This treatment has been used previously to induce oxidative stress in plant tissues and has been shown to increase the cellular levels of hydrogen peroxide (Amory et al., 1992; Prasad et al., 1994). Silver nitrate treatment has been shown to increase levels of phytoalexins and to induce expression of defence-related genes such as thionin (Epple et al., 1995). Silver salts have been used to induce heavy metal stress in plants (Wettlaufer et al., 1991) and excess concentrations of heavy metals have been shown to lead to increased levels of ROS and hence cause oxidative stress (Clijsters et al., 1999; Schutzendubel and Polle, 2002). Therefore, treatment of Arabidopsis tissue, either cotyledons or mature green leaves, with silver nitrate or 3-AT solution is likely to cause an increase in ROS in the treated tissue. An increase in ROS would result in increased damage to macromolecules such as lipids. An increase in TBARM levels following silver nitrate or 3-AT treatments is indicative, although not a quantifiable measure, of increased lipid peroxidation and oxidative damage (Fig. 2). Consistent with this, treatment with ascorbate and other quenchers of ROS was shown to protect the plant from the toxic effects of silver nitrate (Figs 1, 3). Ascorbate is a natural component of the plant’s antioxidant defence system and will react with superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen (reviewed by Smirnoff and Wheeler, 2000).

Many other senescence-enhanced genes were also induced in response to both silver nitrate and 3-AT treatments (Fig. 4) and the expression of these genes was reduced when the silver nitrate-treated tissue was also treated with ascorbate (Fig. 5A and data not shown). Therefore, ROS may be a general signal that induces the expression of a group of genes during senescence. However, many senescence-enhanced genes did not show enhanced expression in response to ROS indicating that the silver nitrate treated tissue was not undergoing true senescence.

The treatment with 3-AT, resulting in protection from subsequent silver nitrate treatment, is comparable to previous reports that have indicated that exposure of plants to a mild oxidative stress confers some resistance to further stress treatments (reviewed in Dat et al., 2000). High levels of ROS lead to phytotoxicity while relatively low levels can be used for acclimatory signalling. Pretreatment of Arabidopsis plants with H2O2 protected them from oxidative damage caused by exposure to high light intensities (Karpinski et al., 1999). H2O2 was also implicated as a regulatory factor in the acclimation to chilling stress in maize seedlings (Prasad et al., 1994). Similarly, in the experiments described here, tissue pretreated with 3-AT showed more resistance to the toxic effects of silver nitrate. Most of the genes examined showed reduced expression in the tissue subjected to the combined treatment, the exception being the gene LSC805. This gene encodes a phospholipid hydroperoxide glutathione peroxidase, an enzyme involved in the detoxification of fatty acid hydroperoxides (Beeor-Tzahar et al., 1995). The activity of this protein might be expected to reduce the endogenous hydroperoxide content of the tissue and thus reduce the level of ROS production. Vranova et al. (2002) showed that increased expression of a glutathione peroxidase gene occurred in ROS-acclimated tobacco leaves and these authors postulated that this protein plays a role in enhancing tolerance to oxidative stress.

Expression of both the LSC54 and the LSC94 genes is induced in response to an increase in ROS. However, from previous work (Morris et al., 2000), it was known that the senescence-enhanced expression of LSC94 (PR1α) depends on the presence of the salicylic acid (SA) pathway while the expression of LSC54 is independent of this pathway. It has now been shown that ROS-induced expression of LSC94 depends on the presence of the SA pathway, while LSC54 expression does not. Therefore, several different signalling pathways may be activated by the increase in ROS. Epple et al., (1995) showed that treatment with silver nitrate caused increased expression of a thionin gene and expression of this gene in Arabidopsis has been shown to depend on the octadecanoid pathway which leads to the synthesis of jasmonic acid (JA) (Bohlmann et al., 1998). Therefore, some of the genes showing induced expression in response to silver nitrate treatment may be induced via a pathway involving JA although neither LSC54 nor LSC94 required this pathway for ROS-induced expression (Fig. 5B).

In summary, the treatment of Arabidopsis leaf tissue with silver nitrate results in the initiation of an oxidative stress and it is likely that a variety of different signalling pathways lead to gene expression in response to this stress (Neill et al., 2002). Similarly, gene expression following pathogen infection and during pathogen-induced cell death also requires a combination of different pathways (Gaffney et al., 1993; Penninckx et al., 1998). It has been shown that
many genes showing enhanced expression during leaf senescence also show induced expression in response to increased oxidative stress. This implicates ROS as a common signal that could have a role in controlling the expression of certain genes during senescence and cell death. Finally, the LSC54 gene could be a useful marker for the detection of oxidative stress in plants.

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