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Regulation of calcium signalling and gene expression by glutathione

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Abstract

The glutathione redox couple is an information-rich redox buffer that interacts with numerous cellular components. To explore the role of glutathione in redox signalling, leaf contents were increased either chemically, by feeding reduced glutathione (GSH), or genetically, by over-expressing the first enzyme of the GSH biosynthetic pathway, γ-glutamylcysteine synthetase (γ-ECS). Leaf discs were also fed glutathione disulphide (GSSG), leading to increases in both GSH and GSSG. The effects of increases in GSH were compared with non-specific changes in leaf thiol status induced by feeding dithiothreitol (DTT) or the monothiol β-mercaptoethanol (β-ME). Photosynthesis measurements showed that none of the feeding treatments greatly disrupted leaf physiology. Transgenic plants expressing aequorin were used to analyse calcium signatures during the feeding treatments. Calcium release occurred soon after the onset of GSH or GSSG feeding, but was unaffected by DTT or β-ME. Pathogenesis-related protein 1 (PR-1) was induced both in the γ-ECS overexpressors and by feeding GSH, but not GSSG. Feeding DTT also induced PR-1. Key transcripts encoding antioxidative enzymes were much less affected, although glutathione synthetase was suppressed by feeding thiols or GSSG. It is concluded that modulation of glutathione contents transmits information through diverse signalling mechanisms, including (i) the establishment of an appropriate redox potential for thiol/disulphide exchange and (ii) the release of calcium to the cytosol.

Key words: Calcium signalling, cytosol, dithiothreitol, gene expression, glutathione, β-mercaptoethanol, regulation.

Introduction

Glutathione has many functions in plant biology, including sulphur metabolism, regulation of growth and development, cell defence, redox signalling, and regulation of gene expression. These functions depend on the concentration and/or redox state of the leaf glutathione pools (Noctor and Foyer, 1998*a*; May *et al.*, 1998). Changes in intracellular glutathione status may, therefore, be expected to have important consequences for the cell, through modification of the metabolic functions associated with glutathione-regulated genes. In animal cells, redox regulation of the transcription factor NFκB involves glutathione. This regulation is important for T cell function since glutathione augments the activity of these cells (Suthanthiran *et al.*, 1990). The application of exogenous glutathione can elicit changes in the transcription of genes encoding cytosolic Cu,Zn superoxide dismutase, glutathione reductase, and 2-cys peroxiredoxins (Hérouart *et al.*, 1993; Wingsle and Karpinski, 1996; Baier and Dietz, 1997). Glutathione-inducible hypersensitive elements have been identified in the proximal region of the chalcone synthase (CHS) promoter (Dron *et al.*, 1988). GSH is also a physiological regulator of many thiol-disulphide exchange reactions, including those implicated in chloroplast transcription.

The glutathione redox couple is also considered to be a key player in homeostatic adjustment of the cellular redox potential. In animal cells, substantial evidence implicates redox potential as an important factor determining cell fate, and the glutathione redox couple is the key player

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(Schafer and Buettner, 2001). Unlike many other redox couples such as ascorbate/dehydroascorbate and NADPH/NADP, the glutathione redox potential depends on both GSH/GSSG and absolute glutathione concentration. The concentration-dependent term of the Nernst equation is second-order with respect to GSH, but first-order with respect to GSSG, hence the accumulation of GSH can offset the change in redox potential caused by decreases in the GSH/GSSG ratio.

It has long been known that defence genes are among those induced by GSH (Wingate *et al.*, 1988; Dron *et al.*, 1988). In particular, thiol-disulphide status appears to be crucial for the expression of pathogenesis-related (PR) proteins, which are induced by salicylic acid (SA) and are involved in systemic acquired resistance (SAR). Increases in SA trigger the reduction of disulphide bonds located on both the regulatory protein NPR1 and on certain TGA transcription factors with which NPR1 interacts (Mou *et al.*, 2003; Després *et al.*, 2003). This reduction stimulates both the translocation of NPR1 from the cytosol to the nucleus and the physical interaction of NPR1–TGA1 that is necessary for the activation of PR gene transcription (Mou *et al.*, 2003; Després *et al.*, 2003). The redox dependence of the pathway suggests that any biotic or abiotic stimulus that can perturb the cellular redox state will up-regulate the same set of defence genes via the NPR1 pathway (Mou *et al.*, 2003). Redox-linked effects explain, for example, PR gene expression in response to UV-B exposure (Green and Fluhr, 1995) or in catalase-deficient mutants (Willekens *et al.*, 1997) where extensive, specific oxidation of the glutathione pool and greatly enhanced glutathione accumulation occurs in certain conditions (Noctor *et al.*, 2000; May and Leaver, 1993). Indeed, it is noteworthy that oxidation of the leaf glutathione pool followed by enhanced glutathione synthesis is also seen in plant–pathogen interactions (Vanacker *et al.*, 2000; Mou *et al.*, 2003). In this situation SAR induction involves an early burst of ROS and a transient or more sustained increase in cellular redox state (more oxidized), followed by a sharp decrease in cellular redox potential (more reduced) as a result of the accumulation of antioxidants such as GSH.

From the above observations, it appears that induction of glutathione accumulation has at least two effects. Firstly, it acts as a mechanism to offset stress-initiated oxidation of glutathione and, secondly, it can cause changes in gene transcription either directly or indirectly via interaction with regulatory proteins and/or transcription factors. The aim of the present study was to explore the mechanisms whereby GSH, GSSG, and thiol compounds modulate gene expression in the absence of stress. Photosynthetic CO_2 assimilation rates were used as a physiological marker to verify that effects on gene expression and calcium signalling induced by thiol feeding were independent of adverse effects on major leaf functions.

Materials and methods

Plant material and growth conditions

Tobacco seeds (*Nicotiana tabacum* cv. Samsung) were germinated on moistened filter paper. After 10 d, the seedlings were transferred to compost and grown in the glasshouse at 22 °C. Young fully expanded leaves were harvested from 45-d-old plants and used for thiol treatments, glutathione determinations, calcium signalling, and gene expression analysis.

Tobacco transformation for overexpression of maize γ-glutamylcysteine synthetase (γ-ECS)

A maize γ-ECS cDNA was cloned and sequenced as described in Gomez *et al.* (2004). The γ-ECS cDNA was subcloned in the *Sac*I restriction site of pp5ln (derived from pUC19; Pignocchi *et al.*, 2003) by standard recombination techniques. Targeting to the chloroplast was achieved by inserting a RuBisCO small subunit transit peptide from soybean as a *Xho*I–*Nco*I fragment upstream of the coding sequence for γ-ECS, originating the 2x35S-γ-ECS expression cassettes targeted to the cytosol and chloroplast. These expression cassettes were ligated into cj102, a derivative plasmid of pGPTV, as a *Hin*dIII–*Sac*I fragment into cj102 in replacement of the 2×35S-β-*glucuronidase* cassette (Pignocchi *et al.*, 2003). The cj102-γ-ECS and cj102-*transit peptide*-γ-ECS constructs obtained were transformed into *A. tumefaciens* LBA4404 by electroporation.

Sterile cultures of *Nicotiana tabacum* L. (cv. Petit Havana, mutant SRI) were transformed with the constructs described above by *A. tumefaciens* leaf disc infection (Gallois and Marinho, 1995). Transgenic and wild-type plants were grown in compost (Petersfield products, Leicester, UK) at 22 °C day/night in controlled environment chambers supplying an irradiance of 250 μmol m^{-2} s^{-1} at plant height as a 16 h photoperiod. T_1 seeds from primary transformants were germinated in Petri dishes containing 1.5% agar in distilled water (non-selective medium), supplied with 100 mg l^{-1} of kanamycin (selective medium). T_2 seeds were obtained from individual T_1 plants and used to generate T_2 transgenic progeny that were analysed for glutathione contents and gene expression analysis.

Thiol feeding experiments to intact leaves

Leaves were excised under water from 45-d-old plants and the following solutions were supplied through the petiole for 16 h in darkness: 10 mM 3-(*N*-morpholino)-propanesulphonic acid buffer (MOPS), pH 6.0 with either 0, 5 mM or 20 mM GSH; 5 mM GSSG; 5 mM β-ME; or 5 mM DTT. Immediately after the feeding experiments, treated leaves were either used for photosynthesis measurements or RT-PCR as described below or were frozen in liquid N_2 for metabolite analysis. Reduced and oxidized glutathione were extracted and assayed as described by Noctor and Foyer (1998*b*). Spectrophotometric determinations of chlorophyll were performed on 80% (v/v) acetone extracts. The total chlorophyll (*a*+*b*) content of leaves (g^{-1} fresh weight) and leaf discs (m^{-2}) was calculated from the absorbances at 645 nm and 663 nm. The chlorophyll concentration in acetone solution (mg l^{-1}) was calculated as $20.2A_{645}$ plus $8.02\ A_{663}$.

Measurements of CO_2 exchange, chlorophyll a *fluorescence and metabolite assays*

Measurements of CO_2 and H_2O exchange under steady-state conditions and sampling for metabolite analysis were performed as described in Novitskaya *et al.* (2002). All experiments were conducted at 22 °C and 50% relative humidity. Irradiance was provided by overhead lamps and adjusted by neutral density sheeting. Gas composition was controlled by a gas mixer supplying CO_2 and O_2 to the stated partial pressures, with balanced N_2. Respiratory CO_2 release was monitored for 20 min in the dark, then plants were illuminated until a steady-state rate of CO_2 uptake was attained. To

analyse the dependence of net CO_2 uptake on irradiance, photosynthesis was induced at 360 μbar CO_2 at the lowest irradiance (100 μmol m^{-2} s^{-1}) as shown in Fig. 2 of the Results. Light was then increased step-wise from 100 μmol m^{-2} s^{-1} to 1600 μmol m^{-2} s^{-1}, and measurements were taken on attainment of the steady-state rate at each irradiance. Chlorophyll *a* fluorescence analysis was conducted using a PAM fluorimeter (Veljovic-Jovanovic *et al.*, 2001).

Thiol feeding to leaf discs

Leaf discs (2.5 cm diameter) were excised under water from leaves of 45-d-old plants and incubated in Petri dishes containing 10 mM 3-(*N*-morpholino)-propanesulphonic acid buffer (MOPS), pH 6.0, with either 0, 5 mM, or 20 mM GSH; 5 mM GSSG; 5 mM β-ME; or 5 mM DTT. Incubations were carried out for 16 h at room temperature in the dark. Discs were then used either for expression analysis or metabolite analysis.

RNA extraction and RT-PCR analysis

Total RNA was extracted from five leaves using Trizol reagent (GibcoBRL) according to the manufacturer's recommendations. DNA contamination was removed from the RNA samples using DNase I (Invitrogen) and checked using actin-specific primers giving differential PCR products in the presence of genomic DNA (Pignocchi *et al.*, 2003). One microgram of total RNA was reverse transcribed using 0.5 μg Oligo$(dT)_{12–18}$ (GibcoBRL), 0.5 mM dNTP, 10 mM DTT, and 200 U of Superscript II reverse transcriptase (GibcoBRL). PCR conditions were standardized using gene-specific primers for actin content. Linear amplification for semi-quantitative RT-PCR was obtained with 28 cycles and PCR conditions were 5 min at 94 °C, cycle of 40 s at 94 °C, 40 s at 52 °C, and 1 min at 72 °C, followed by 10 min at 72 °C, using 1 μl of the reverse transcriptase reaction. Primer sequences for actin, PR-1, tAPX, sAPX, cAPX, AO, and GLDH were as published in Pignocchi *et al.* (2003) and GSH-S primers were as published in Gomez *et al.* (2004).

Calcium imaging

Leaf discs from plants expressing aequorin were excised and incubated in thiols as described above. Aequorin imaging was performed using an intensified CCD camera (model EDC-02), with camera control unit (HRPCS-2) and image acquisition and processing software (IFS216), all from Photek (St Leonards-on-Sea, UK) as described previously (Knight *et al.*, 1999).

Results

Leaf glutathione contents are greatly increased by GSH or GSSG feeding

Tobacco leaf total glutathione contents were increased from below 0.5 μmol mg^{-1} chlorophyll to about 6.0 μmol and 11.0 μmol mg^{-1} chlorophyll after feeding with either 5 mM or 20 mM GSH, respectively, for 16 h (Fig. 1A). GSH feeding had less effect on the leaf GSH/GSSG ratios, which were about 11 in control leaves, about 17 in leaves supplied with 5 mM GSH, and about 9 in leaves supplied with 20 mM GSH (Fig. 1B). Feeding with 20 mM GSSG also increased leaf total glutathione contents to 6.5 μmol mg^{-1} chlorophyll (Fig. 1A), but decreased GSH/GSSG (Fig. 1B). Despite the significant decrease in GSH/GSSG, this ratio remained at around 4, i.e. most of the 12-fold increase in total glutathione induced by feeding GSSG was due to accumulation in the form of GSH (Fig. 1, compare A and B). Feeding 5 mM DTT

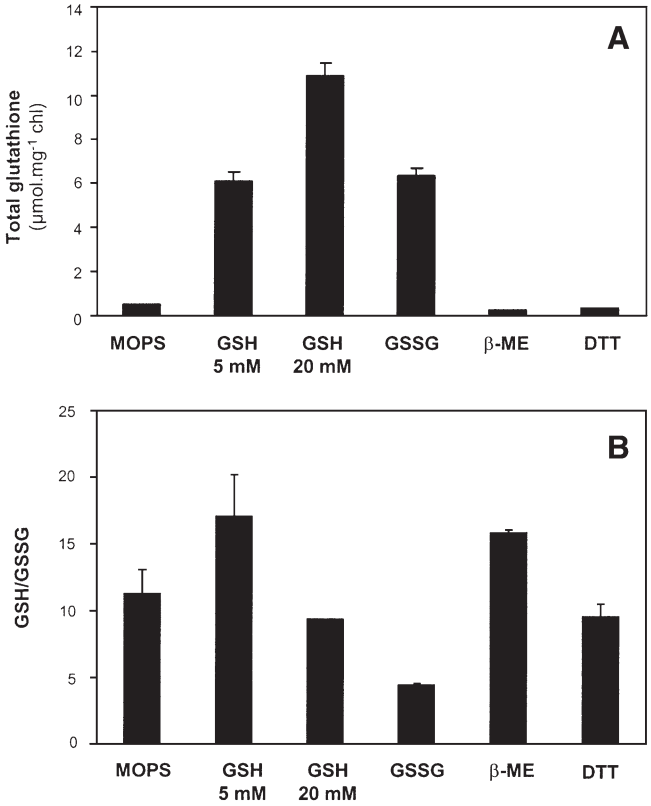

Fig. 1. Total glutathione content (A) and reduced glutathione (GSH)/glutathione disulphide (GSSG) ratios (B) in tobacco leaves in response to feeding thiols or GSSG. Values are the means plus SE of five separate leaf samples in one representative experiment. Each experiment was repeated three times.

or β-ME did not significantly increase leaf glutathione compared with leaves supplied with buffer alone (Fig. 1A) and had relatively small effects on GSH/GSSG (Fig. 1B). A small increase was observed in leaves fed β-ME (Fig. 1B). In conclusion, total glutathione could be increased by feeding GSH or GSSG, but not DTT or β-ME. GSH/GSSG was only appreciably affected by supplying GSSG, which caused the ratio to decrease. Similar changes in glutathione to those observed in leaves were found when leaf discs were treated with equivalent solutions (data not shown).

Feeding GSH or GSSG does not disrupt major leaf function

The light-response curves of photosynthetic CO_2 uptake were similar in *N. tabacum* leaves supplied for 16 h with either GSH (5 mM and 20 mM), GSSG (20 mM), β-ME (5 mM), or DTT (5 mM), steady-state rates of photosynthesis being comparable on a leaf area basis, at saturating light intensities (Fig. 2A). Thus, leaf glutathione contents were greatly increased by GSH or GSSG feeding (Fig. 1A) without detrimental effects on photosynthetic CO_2 assimilation rates (Fig. 2A). The dark-adapted ratio of variable (F_v) to maximal (F_m) chlorophyll *a* fluorescence was not greatly decreased by feeding 5 mM GSH or GSSG (Fig. 2B). The

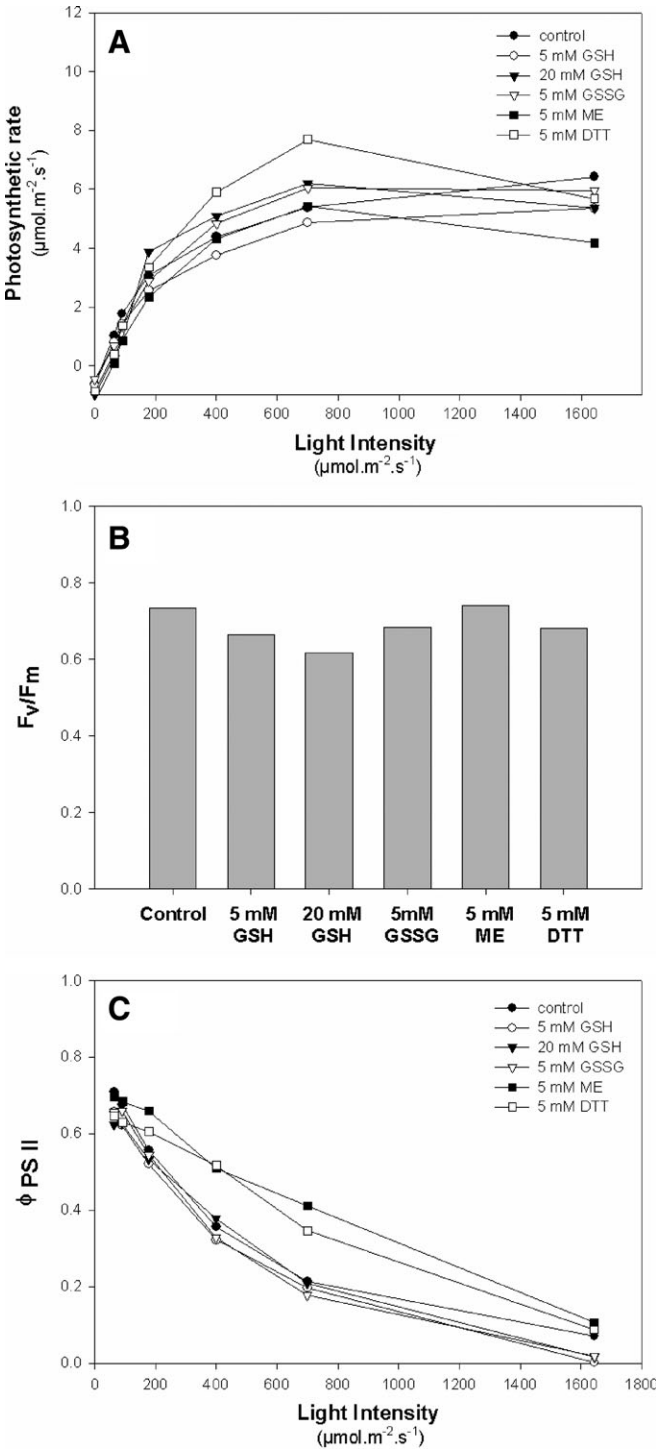

Fig. 2. Effects of feeding thiols and GSSG on photosynthetic competence. (A) Photosynthetic CO_2 assimilation rates. (B) Ratios of variable (F_v) to maximal (F_m) chlorophyll *a* fluorescence. (C) Apparent quantum efficiency of photosystem II (ϕ_{PSII}). The data are taken from single leaves supplied with thiols, GSSG or buffer alone (control) through the petiole at the same time and measured simultaneously after 16 h feeding in one representative experiment. Each experiment was repeated three times.

quantum efficiency of photosystem II (ϕ_{PSII}) decreased with increasing irradiance in a similar manner in leaves supplied with GSH or GSSG to those of controls treated with buffer alone (Fig. 2C). By contrast, leaves supplied with either β-ME or DTT showed smaller decreases in this parameter with increasing light availability at all but the highest irradiances (Fig. 2C). The slower decrease in ϕ_{PSII} at intermediate light intensities caused by these thiols may be related to a decrease in non-photochemical quenching via inhibition of violaxanthin de-epoxidation (Noctor *et al.*, 1991).

Thiol feeding modulates gene expression

Using marker transcripts (PR-1, AO, GLDH, tAPX, sAPX, cAPX) the role of thiols in co-ordinating gene expression linked to plant–pathogen interactions and crosstalk between the glutathione and ascorbate redox systems was examined. Two approaches were employed. First, transcripts were analysed in leaves supplied with GSH, GSSG, β-ME, or DTT. Second, transcripts were analysed in tobacco transformed to accumulate GSH.

Feeding leaves or leaf discs with 5 mM or 20 mM GSH resulted in a large increase in the abundance of transcripts encoding PR-1 (Fig. 3). This effect was also observed in

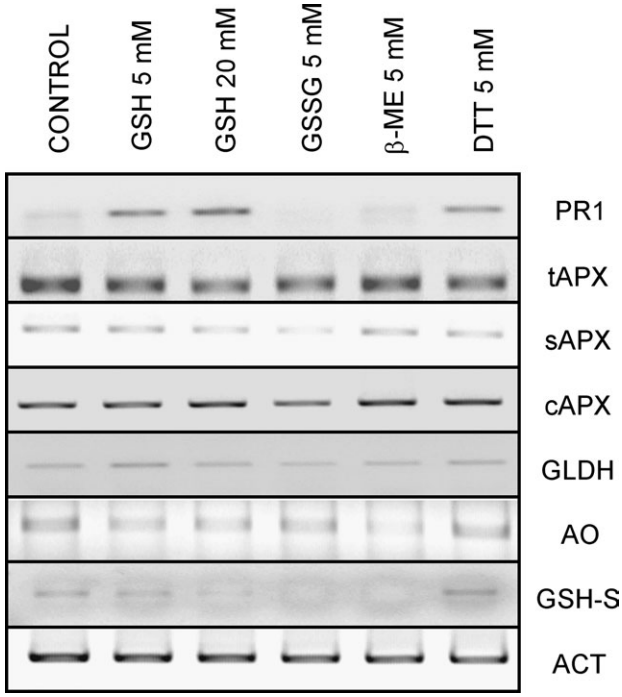

Fig. 3. Modulation of transcript abundance, as estimated by semi-quantitative RT-PCR, in tobacco leaves by thiols or GSSG. The transcript data (lower figures) are taken from single leaves supplied with thiols. GSSG, or buffer alone (control) through the petiole at the same time and measured simultaneously after 16 h feeding in one representative experiment. Each experiment was repeated three times. Reduced glutathione (GSH); glutathione disulphide (GSSG); β-mercaptoethanol (β-ME); dithiothreitol (DTT); pathogenesis-related protein 1 (PR1); thylakoid (t), stromal (s), cytosolic (c) ascorbate peroxidase (APX); L-galactono-1,4-lactone dehydrogenase (GLDH); ascorbate oxidase (AO); glutathione synthetase (GSH-S); actin (ACT).

transgenic tobacco plants overexpressing the maize γ-ECS in the chloroplast or cytosol where leaf glutathione was increased by 50–70% (Fig. 4). Unlike DTT, which also increased the level of PR-1 mRNA accumulation, neither GSSG nor β-ME had any effect compared with the control supplied with buffer alone (Fig. 3). None of the added thiols had any marked effect on the abundance of transcripts encoding the tAPX, sAPX, or cAPX except that cAPX mRNA was lower in leaves supplied with 5 mM GSSG (Fig. 3). GLDH, AO, and GSH-S mRNAs were slightly decreased following feeding with either 20 mM GSH, 5 mM β-ME, or 5 mM GSSG (Fig. 3). Levels of AO transcripts were also lower in transformed tobacco plants expressing maize γ-ECS in the chloroplast, but not the cytosol (Fig. 4), whereas GLDH transcript abundance was increased in leaves supplied with GSH (Fig. 3) and in plants expressing maize γ-ECS in the cytosol (Fig. 4), but similar to controls in transformed tobacco plants expressing maize γ-ECS in the chloroplast (Fig. 3).

GSH and GSSG modulate calcium signalling

The level of aequorin luminescence was changed in tobacco leaf discs supplied with GSH or GSSG. Luminescence increased rapidly in leaf discs supplied with 20 mM GSH, reaching a peak within the first 2 h of incubation. Calcium release into the cytoplasm was transient, however, luminescence falling back to a low level within 3 h of the onset of feeding (Fig. 5). A similar transient increase in aequorin luminescence was observed upon GSSG feeding. In this case, however, the peak was reached later and the duration of calcium release was much longer than that observed with 20 mm GSH. Since GSSG feeding led to a marked increase in leaf disc GSH, it is likely that the transient and the kinetic are caused by conversion of added GSSG to internal GSH, which then stimulates calcium release. Moreover, supplying a lower concentration of GSH (5 mM) caused a similar, but somewhat later, transient increase in aequorin luminescence that was also broader than that observed with 20 mm GSH. By contrast, neither β-ME nor DTT caused any change in the basal level of aequorin luminescence (Fig. 5).

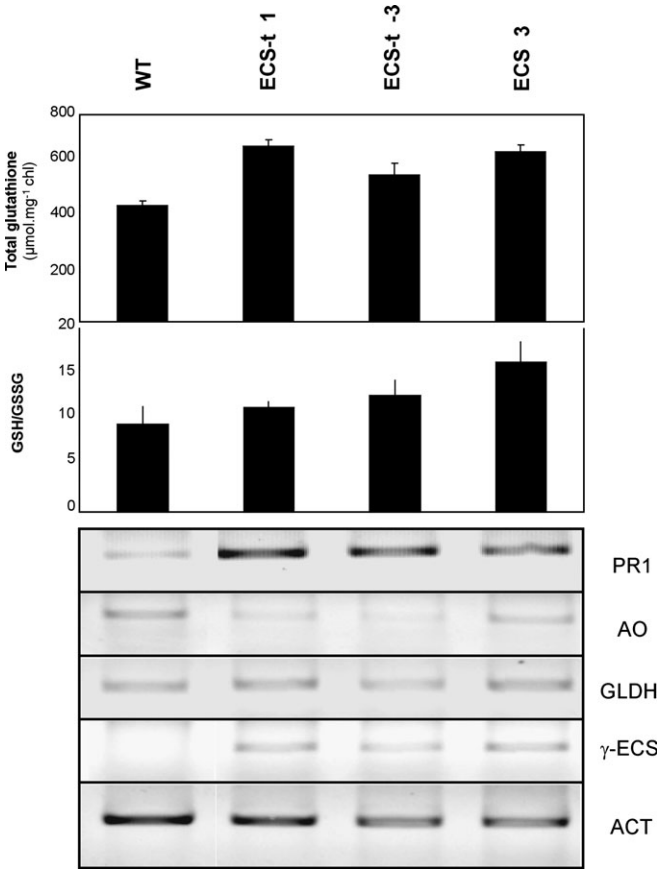

Fig. 4. Modulation of transcript abundance, as estimated by semi-quantitative RT-PCR, in transformed tobacco leaves expressing a maize γ-glutamylcysteine synthetase cDNA in the chloroplast (ECS-t) or cytosol (ECS). Other abbreviations are as in the legend to Fig. 3.

Discussion

Like all other aerobic organisms, plants are able to maintain cytoplasmic thiols in the reduced (-SH) state because of the low thiol-disulphide redox potential imposed by millimolar amounts of glutathione, which acts as a thiol buffer. Although transient disulphide bonds do occur during the catalytic cycle of some enzymes, stable protein disulphide bonds are relatively rare except in quiescent tissues such as seeds. The multiple roles of GSH within the cell, together with the stability of GSSG, may make this redox couple ideally suited to information transduction, and it is becoming increasingly clear that glutathione status is important in signalling. Glutathione strongly interacts with two key signalling factors: H_2O_2 and, as has been shown here, Ca^{2+}.

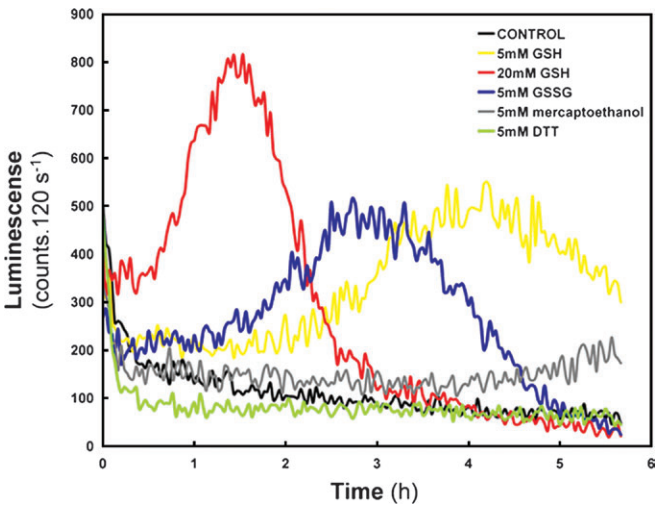

Fig. 5. Changes in free calcium in transgenic tobacco leaf discs expressing aequorin measured via luminescence imaging. Leaf discs were placed in solutions of different thiols at time 0. Reduced glutathione (GSH); glutathione disulphide (GSSG); β-mercaptoethanol (β-ME); dithiothreitol (DTT).

Reversible thiol-disulphide exchange has long been recognized as an important mechanism of modulating protein function (Kunert and Foyer, 1993; Demple, 1998). It is only relatively recently, however, that this mechanism has been shown to have a crucial signalling role in plant stress responses (Mou *et al.*, 2003; Després *et al.*, 2003). In addition, reversible protein thiolation protects essential thiol groups on key proteins from irreversible inactivation during oxidative stress and also plays an important regulatory role in controlling metabolism, protein turnover and gene transcription (Foyer and Noctor, 2001).

The present data suggest that glutathione-associated signalling may be complex and diverse, and this could reflect the various parameters associated with the glutathione redox couple. The glutathione redox potential is related to $[GSH]^2$/GSSG, and so depends on both GSH/GSSG and the total concentration of the glutathione pool in any given compartment (Schafer and Buettner, 2001). Thus, accumulation of glutathione can make the redox potential more negative even if GSH/GSSG does not change. Conversely, GSSG can accumulate without changes in the redox potential, if there is a compensatory increase in the total concentration. It is therefore interesting to examine which parameters are most important in controlling glutathione-associated gene expression and calcium signalling.

Diverse pathways of glutathione-regulated gene expression

Table 1 shows a simple summary of the principal data presented in this manuscript. Increasing leaf GSH contents either by direct feeding to leaves or leaf discs or by constitutive overexpression of γ-ECS led to the accumulation of PR-1 transcripts, as did supplying DTT. Significantly, β-ME was without effect, as was GSSG, even though the latter treatment caused GSH to increase (Fig. 1, compare A and B). Although it is worth noting that glutathionylation between GSH and reduced protein thiols can occur by enzymatic mechanisms (Starke *et al.*, 2003), the failure of GSSG feeding to induce PR-1 transcripts suggests that GSSG-dependent glutathionylation is not involved in the regulation of PR-1 expression. The data are consistent with recent findings that the reduction of disulphides on NPR1 and interacting transcription factors is crucial in the link between SA and PR gene expression (Mou *et al.*, 2003; Després *et al.*, 2003). Thus, GSH and DTT are able to mimic this part of the pathogen response, presumably by facilitating the disulphide reduction of NPR1 and TGA, which is a process downstream of salicylic acid. This would be coherent with the notion that reduction of NPR1 and TGA is accomplished by protein disulphide reductases (thioredoxins or glutaredoxins). The active sites of these proteins can be reduced efficiently by DTT but much less effectively by the monothiol β-ME. The effect of the monothiol GSH on PR-1 expression may reflect the establishment of redox potentials sufficiently negative to increase the reduction status of key thioredoxins or glutaredoxins. When GSSG is supplied, the resulting decrease in GSH/GSSG means that such high redox potentials are not reached, despite the increase in overall glutathione concentration, and this may explain the differential effects of the two forms of glutathione (Table 1). Interestingly, recent data with *Arabidopsis* shows that a specific cytosolic thioredoxin is co-expressed with PR-1 in response to stress, including pathogen attack (Laloi *et al.*, 2004).

Table 1. *Summary of effects of glutathione and thiols on gene expression and calcium release*

Arrows indicate appreciable changes in parameters. Upwards arrow, increase in parameter; equals sign, no change in parameter; downwards arrow, decrease in parameter. GSH treatment refers to 5 mM glutathione. GSSG, oxidized glutathione; β-ME, β-mercaptoethanol; DTT, dithiothreitol; γ-ECS, overexpression of γ-glutamylcysteine synthetase; n.m., Not measured.

Parameter	Treatment				
	GSH	GSSG	β-ME	DTT	γ-ECS
Total glutathione content	↑	↑	=	=	↑
GSH/GSSG	=	↓	=	=	=
PR-1 expression	↑	=	=	↑	↑
Calcium	↑	↑	=	=	n.m.

Pathogen-induced increases in the intracellular GSH concentration and GSH-dependent induction of phenylalanine ammonia lyase and chalcone synthase (CHS) have been demonstrated previously (Dron *et al.*, 1988; Wingate *et al.*, 1988; Vanacker *et al.*, 2000). However, using an artificial precursor of GSH biosynthesis, L-oxothiazolidine-4-carboxylate, to increase intracellular thiol concentrations, Edwards *et al.* (1991) showed that enhanced intracellular GSH concentrations were not sufficient to induce PAL induction and phytoalexin synthesis. They concluded that changes in the intracellular glutathione concentration in response to pathogen attack were too slow to be consistent with the initiation of the elicitation response. However, it has recently been suggested that the simultaneous presence of pro- and anti-oxidants may be important in the NPR1/TGA interaction (Mou *et al.*, 2003; Després *et al.*, 2003). Indeed, a complex response of the glutathione couple is well documented in plant–pathogen interactions, where an oxidative burst and rapid induction of glutathione accumulation precede the maximal induction of transcripts encoding phenylpropanoid metabolism enzymes (Zhang *et al.*, 1997; Vanacker *et al.*, 2000).

GSH feeding induced PR-1 expression without any inhibition of photosynthesis or visible changes in leaf phenotype. Moreover, the transformed plants expressing maize γ-ECS in either the cytosol or chloroplasts were morphologically comparable to controls (data not shown). A similar type of thiol-disulphide regulation may control the activities of the two abscisic acid-signalling cascade phosphatases, ABI1 and ABI2, since oxidation of component protein cysteines inactivates these two stress signalling

components (Meinhard and Grill, 2001; Meinhard *et al.*, 2002). The signalling cascade leading to GSH-S, APX, AO, and GLDH expression is not known, but may involve modulation of these protein phosphatases.

Glutathione triggers calcium release

The results presented here (Fig. 5) demonstrate that feeding GSH rapidly and specifically triggers calcium release. Unlike PR-1 induction, calcium release is triggered by both GSH and GSSG (Table 1). Measurements of the leaf GSH/GSSG ratio suggest that most of the GSSG absorbed by the leaf is converted to GSH. The more rapid induction of calcium release by 5 mM GSSG than 5 mM GSH can be explained by the higher affinity of the plasma-membrane glutathione transporter for GSSG than GSH (Foyer *et al.*, 2001). Moreover, the rapidity of the response to calcium is more or less correlated with total GSH supplied to the leaf (Fig. 5), 5 mM GSSG being potentially equivalent to 10 mM GSH. Thus, it is possible that calcium release responds to the total GSH concentration, rather than glutathione redox potential, and this could explain the different effects of GSSG on PR-1 expression and on calcium signatures. Mou *et al.* (2003) suggest that a GSH/GSSG ratio of about 15 is required for transfer of NPR1 from the cytosol to the nucleus. While, this value may vary according to experimental conditions, the data presented here clearly show that when the GSH/GSSG ratio falls to a low value (below 5), as occurs upon GSSG feeding (Fig. 1), PR-1 is not expressed, even though total glutathione is much enhanced and calcium release occurs (Table 1).

Control of cellular glutathione status

The increases in glutathione obtained by overexpressing the maize γ-ECS in tobacco confirm numerous earlier studies that this enzyme is a limiting factor in glutathione accumulation in plants (Rüegsegger and Brunold, 1992; Noctor *et al.*, 1996; Cobbett *et al.*, 1998; Xiang *et al.*, 2001). Overexpression of an *E. coli* γ-ECS, but not GSH-S, in poplar or tobacco substantially increases leaf glutathione contents (Noctor *et al.*, 1996, 1998; Creissen *et al.*, 1999), as does homologous overexpression of the *Arabidopsis* γ-ECS (Xiang *et al.*, 2001). The marked chlorotic phenotype produced by chloroplastic *E. coli* γ-ECS overexpression in tobacco was not observed when maize γ-ECS was expressed in tobacco, but leaf GSH contents were increased and PR-1 protein expression was observed in both cases (Fig. 4; Creissen *et al.*, 1999). The results presented here (Fig. 3) show that supplying leaves with either 20 mM GSH or 5 mM GSSG decreases the abundance of GSH-S transcripts in tobacco leaves, perhaps implying a type of feedback control on GSH-S expression or GSH-S transcript stability via the total glutathione pool size. Unfortunately, γ-ECS transcripts were below the level of detection in wild-type tobacco plants under the conditions used in Fig. 3.

In addition to γ-ECS activity, the most important factor controlling plant glutathione is the availability of cysteine (Noctor *et al.*, 1997), and these two factors are likely co-ordinated. *In vivo* activity of γ-ECS is determined by control at multiple levels. The 5′ untranslated region (5′ UTR) of the *gsh1* gene encoding γ-ECS was found to interact with a repressor-binding protein that was released upon the addition of H_2O_2 or decreases in the GSH/GSSG ratio (Xiang and Bertrand, 2000). H_2O_2 increases tissue glutathione contents, whereas jasmonic acid increases the transcript abundance of the enzymes of GSH synthesis but does not affect GSH concentration (Xiang and Oliver, 1998). A redox-sensitive 5′ UTR-binding complex is thus suggested to control γ-ECS mRNA translation and hence GSH synthesis in *A. thaliana* (Xiang and Bertrand, 2000). The pro-oxidative events linked to NPR1 activation may, therefore, not only be required for monomerization of the NPR1 protein as suggested by Mou *et al.* (2003) but may also be required for activation of γ-ECS mRNA translation. Consistent with this notion is the observation that oxidation of the glutathione pool precedes glutathione accumulation in catalase mutants (Noctor *et al.*, 2000), in maize subject to cold stress (Gomez *et al.*, 2004), and in barley undergoing a pathogen-induced oxidative burst (Vanacker *et al.*, 2000). The nature of the link between redox state perturbation and enhanced glutathione accumulation is therefore complex and remains unresolved. However, current literature data suggest at least two possibilities with regard to effects on GSH synthesis. Firstly, the activity of adenylylsulphate reductase, a key enzyme in sulphate assimilation, may be activated by decreases in GSH/GSSG (Bick *et al.*, 2001). Secondly, as noted above, H_2O_2 or associated decreases in GSH/GSSG ratios kickstart γ-ECS translation (Xiang and Oliver, 1998).

Conclusions

The glutathione redox couple interacts with numerous cellular components. Glutathione status is probably involved in the transmission of oxidative stress signals, for example, through GSSG-driven or enzyme-catalysed protein glutathionylation, reactions that may be reversed (and therefore, regulated) by specific enzymes such as glutaredoxins (Johansson *et al.*, 2004; Lemaire, 2004). On the other hand, decreases in glutathione redox potential ('over-reduction'), linked to an enhanced GSH/GSSG ratio, increases in total glutathione, or both, may be an obligatory event in the modulation of redox potential necessary to allow the efficient reduction of disulphides on regulatory proteins. Lastly, glutathione acts to stimulate calcium release into the cytosol, an effect that appears to depend on total glutathione concentration. As previously discussed in depth (Noctor and Foyer, 1998*a*), the accumulation of glutathione occurs in response to various stresses, and the attendant release of calcium reported here may mean that

glutathione accumulation is equally as important in signal transduction as in chemical defence against reactive oxygen. By triggering the activation of calcium-dependent protein kinases, increases in glutathione concentration may participate in early signal transduction events and function in the integration of multiple abiotic and biotic stimuli.

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