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Synthesis and properties of glutathione reductase in stressed peas

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Abstract. We have subjected peas (*Pisum sativum* L.) to four different oxidative stresses: cold conditions (4 °C) in conjunction with light, treatment with paraquat, fumigation with ozone, and illumination of etiolated seedlings (greening). In crude extracts of leaves from stressed plants, an increase (up to twofold) in activity of glutathione reductase (GR) was observed which was consistent with previous reports from several laboratories. In all cases, except for ozone fumigation, the increase in activity was not due to an elevation in the steady-state levels of GR protein. None of the applied stresses had any effect on steady-state levels of GR mRNA. In contrast to the small increase in GR activity, the K_m of GR for glutathione disulphide showed a marked decrease when determined for extracts of stressed leaves, compared with that from unstressed plants. This indicates that GR from stressed plants has an increased affinity for glutathione disulphide. The profile of GR activity bands fractionated on non-denaturing acrylamide gels varied for extracts from differently stressed leaves and when compared with GR from unstressed plants. The changes in GR-band profiles and the alteration in the kinetic properties are best explained as changes in the isoform population of pea GR in response to stress.

Key words: Glutathione reductase (properties, isoforms) – mRNA (glutathione reductase) – Oxidative stress – *Pisum* (glutathione reductase)

Introduction

Glutathione (γ-glutamyl-cysteinyl glycine) is the major low-molecular-weight thiol compound in most plants and is considered to be essential for the normal functioning of several cellular processes (for a review, see Alscher 1989). Glutathione has major roles in sulphur transport, as a protein-disulphide reductant (Zeigler 1985), in the detoxification of xenobiotics (e.g. herbicides; Timmerman 1989), in regulating gene expression in response to environmental stress and pathogen attack (Dron et al. 1988; Wingate et al. 1988), and as a donor of reducing equivalents to ascorbate as part of a system for the protection of chloroplasts and other cellular compartments from oxidative damage (the ascorbate-glutathione cycle; Foyer and Halliwell 1976; Nakano and Asada 1980; Bielawski and Joy 1986; Dalton et al. 1986; Klapheck et al. 1990).

Most of the functions described above, especially its antioxidative role, result in the oxidation of glutathione to glutathione disulphide (GSSG). In all organisms so far studied, the reduction of GSSG to glutathione is carried out by the flavoprotein oxidoreductase glutathione reductase (GR; E.C.1.6.4.2) in an NADPH-dependent reaction.

The activities of GR, and of other enzymes associated with the removal of oxyradical species in plants, have been reportedly influenced by various environmental factors. In peas and spinach, exposure to ozone or to a mixture of ozone, SO_2 and NO_2, caused a twofold increase in GR activity (Mehlhorn et al. 1987; Tanaka et al. 1988). More recently however, GR activity was not found to be induced in pea by ozone fumigation (Madamanchi et al. 1992). Treatment of bean leaves with biphenyl-ether herbicide caused a slight elevation of GR activity (Schmidt and Kunert 1987); the combination of high light and magnesium deficiency in French bean leaves resulted in a sevenfold increase in GR activity (Cakmak and Marschner 1992); high atmospheric O_2 content increased GR activity by approximately threefold in maize and cotton (Foster and Hess 1980, 1982); chilling of apple callus in culture or of *Zea diploperennis* seedlings resulted in GR-activity increases of no more than twofold (Janhke et al. 1991; Kuroda et al. 1991). A more dramatic 40- to 122-fold increase in GR activity in nee-

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Abbreviations: GR = glutathione reductase; GSSG = glutathione disulphide; Rubisco = Ribulose-1,5-bisphosphate carboxylase-oxygenase; RNase A/T1 = ribonucleases A and T1;

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dles of eastern white pine was correlated with the severity of the winter season and is consistent with the requirement for increased protection against photo-oxidative injury under conditions of low temperature and high light (Anderson et al. 1992).

Glutathione reductase has been purified from four plant species and its properties studied: maize (Mahan and Burke 1987), pea (Kalt-Torres et al. 1984; Connell and Mullet 1986; Bielawski and Joy 1986; Edwards et al. 1990; Madamanchi et al. 1992), spinach (Halliwell and Foyer 1978) and pine (Anderson et al. 1990). Pea leaf GR was resolved into a single band of M_r 55 kDa on SDS-polyacrylamide gels (PAGE). However, on two dimensional gels up to eight isoforms were detected with isoelectric points ranging from 5.6 to 6.5 (Edwards et al. 1990). Two subsets of these isoforms were immunodetected in chloroplast and mitochondrial fractions (Edwards et al. 1990). These data have been confirmed recently with the detection of at least six charge-separable isoforms of pea GR by a chromatofocussing procedure (Madamanchi et al. 1992). Several possible post-translational modifications such as changes in oxidation states, glycosylation or phosphorylation, which could have accounted for such isoforms, were ruled out (Edwards et al. 1990). Evidence for multiple isoforms of GR has been found in spinach (Guy and Carter 1984), eastern white pine (Anderson et al. 1990) and tobacco (Foyer et al. 1991).

The availability of an antiserum to GR (Edwards et al. 1990) and cDNAs encoding the putative plastidial form of GR from peas (Creissen et al. 1992) has provided the opportunity for us to examine the steady-state levels of GR protein and mRNA in relation to the properties and activity of GR in this species when subjected to four different environmental stresses likely to lead to oxidative damage.

Materials and methods

Growth of plants and exposure to stress conditions. Peas (*Pisum sativum* L.) cv. Birte or Feltham First, sown in trays of vermiculite, were grown in controlled-environment chambers at a temperature of 20° C/15° C (light/dark) with a daily light period of 18 h and photon fluence rate of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Externally applied stress conditions were as follows:

Low temperature: Peas (cv. Birte) were germinated and grown for 14 d post-germination under normal conditions, then half the population was transferred to the same growth conditions but with an ambient temperature of 4° C. Leaf samples were taken one and five months after transfer to the lower temperature.

Paraquat treatment: The leaves of 14-d-old pea plants (cv. Birte) were painted with 3 mM paraquat at daily intervals for 8 d. Leaf samples were taken daily, from 1 to 8 d after beginning the treatment. Control plants were painted with water.

Greening: Peas (cv. Birte) were germinated and grown in the dark for 9 d and then exposed to the same light level as the normal growth conditions. Leaves were sampled 5 h, 24 h and 6 d after transfer to the light.

Ozone fumigation: Ozone fumigations were carried out on 7-d-old pea plants (cv. Feltham First) as described by Mehlhorn and Welburn (1987). Plants were supplied with unfiltered ambient air containing 200 $nl \cdot l^{-1}$ ozone at a rate of 2 $m^3 \cdot min^{-1}$ for 7 h per day, commencing 3 h after the beginning of the photoperiod. Fumigation was continued for 5 d per week for two weeks. Ozone was produced by a silent electrical-discharge ozone generator (Wallace and Tierman Ltd., Tonbridge, UK) bubbled through deionised water (changed daily) and monitored continuously with a Dasibi 1008 ozone analyser (Analysis Automated, Oxford, UK). Control plants were supplied with unfiltered ambient air as above but with no ozone.

Enzyme assays and immunodetection of GR. Immediately upon sampling, leaves were frozen in liquid nitrogen and a crude cleared homogenate was produced as described previously (Edwards et al. 1990). For the comparison of GR activities in different extracts, the assay of Smith et al. (1988) was employed on homogenates containing 100 µg of total protein. The protein concentration in the extracts was determined colorimetrically (Bradford 1976). For the determination of K_m values of GR for GSSG in crude protein extracts, the assay measuring the GSSG-dependent oxidation of NADPH was employed as previously described (Edwards et al. 1990).

Immunodetection of GR in crude pea leaf extracts after separation on denaturing SDS-containing polyacrylamide gels (SDS–PAGE; Laemmli 1970) by Western blotting using anti-GR immunoglobulin (IgG) has been described previously (Edwards et al. 1990).

Activity gels for GR. Crude extracts (equivalent to 200 µg protein) were fractionated on 7.5% (w/v) non-denaturing polyacrylamide gels (activity gels). The samples and gels were prepared in the same way as for SDS–PAGE, except that the SDS and mercaptoethanol were omitted from all buffers and the samples were not boiled prior to loading onto the gel. The GR-activity-staining method of Halliwell and Foyer (1978) was used. Duplicate control gels were stained in the absence of GSSG.

Electroelution from protein gels. The GR-staining bands in the activity gels were excised and placed in a dialysis bag, containing 1 ml 0.2 × electrophoresis buffer (1 × electrophoresis buffer was 0.025 M Tris, 0.129 M glycine, pH 8.3–8.6). The dialysis bag was placed in a horizontal electrophoresis chamber and the protein released from the gel by electroelution at 100 V for 18 h at 4° C. The eluted protein was concentrated by freeze-drying and was subjected to SDS–PAGE, and GR immunodetected by Western blotting.

Preparation of RNA and detection of GR transcripts. Total RNA was prepared from stressed and unstressed pea leaves (0.5–5 g fresh weight) as described by Creissen et al. (1992). Detection of GR transcripts in 20 µg of total RNA was done using ribonucleases A and T1 (RNase A/T1) protection assay (Zinn et al. 1983; Melton et al. 1984). For the assay a uniformly ^{32}P-labelled RNA (riboprobe) was synthesised in an in-vitro transcription reaction using the plasmid pGR201 (Creissen et al. 1992) linearised with the restriction endonuclease StuI (at coordinate 1788 in the sequence of the GR cDNA; Creissen et al. 1992). The transcription reaction was carried out as described by Mullineaux et al. (1993) except that α-[^{32}P]CTP was used and the transcription reaction was carried out at room temperature. After 60 min, the reaction was treated with 75 units of RNase-free deoxyribonuclease I (DNase I; Pharmacia, Hounslow, Middlesex, UK) at 37° C for 15 min and then applied to a 3-ml column of Sephadex G50 (Pharmacia) equilibrated in 10 mM Tris-HCl (pH 8), 0.1 mM EDTA. The riboprobe was collected in the void-volume fractions.

The riboprobe encoded the antisense strand of the GR cDNA, spanning coordinates 2030 to 1788 (Creissen et al. 1992), and was designed to protect the 3′ end of the GR mRNA.

The RNase A/T1-resistant fragments were separated on a 6% (w/v) polyacrylamide-8 M UREA sequencing gel (Mullineaux et al. 1993) and detected by autoradiography.

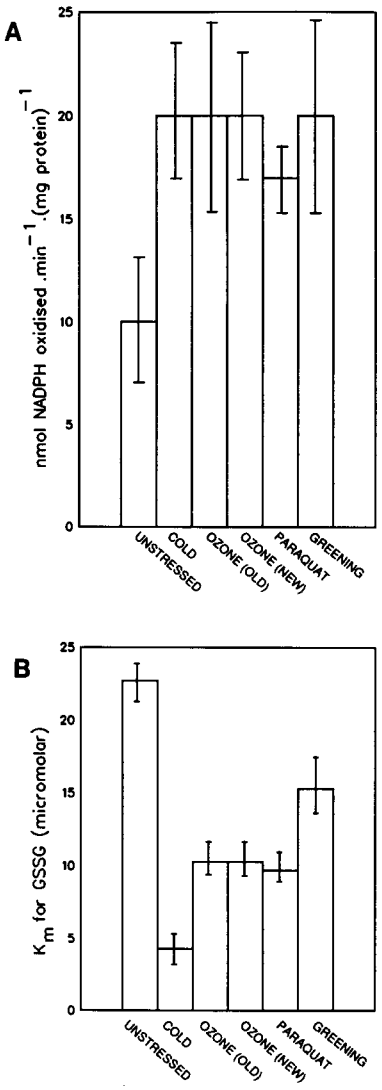

Fig. 1. **A** Specific activities (means of three experiments) of GR in extracts from pea leaves grown under normal conditions (see *Materials and methods*) and subjected to chilling (4° C for one month), ozone fumigation (200 nl · l^{-1} ozone for 7 h per day, 5 d per week, samples harvested after two weeks), paraquat treatment (leaves painted with 3 mM paraquat and harvested 24 h later) and greening of 9-d-old etiolated seedlings, placed in the light for 5 h. In the control and ozone-fumigated samples, "old" and "new" refer respectively to leaves present prior to fumigation, which subsequently showed damage, and "conditioned growth" showing no visible damage (Mehlhorn and Wellburn 1987). For full details of each set of stress conditions see *Materials and methods*. **B** The K_m values of GR for GSSG (means of three experiments). The determinations were carried out on the leaf extracts from stressed and normal plants described in **A**

Results

Growth of peas was severely retarded at 5° C and, after five months, stem elongation tended to be horizontal. Control peas grown under identical conditions at 20° C senesced after five to six weeks. After 2d, paraquat-treated leaves displayed necrotic spots which covered all treated leaves by day 8. Etiolated peas underwent rapid greening upon exposure to light and within 12 h the chlorophyll content of the leaves was visually determined to be equivalent to that of control pea leaves. After ozone fumigation, the only visible leaf injury was a slight curling of the older leaves (labelled "old" samples in Figs. 1–3), while leaf growth which had occurred after commencing fumigation, here termed "new" growth, was phenotypically normal. This is the "conditioned" response described by Mehlhorn and Wellburn (1987).

The level of GR activity was never found to increase more than twofold in response to any of the applied stresses (Fig. 1A) and all changes noted had occurred by the first sampling point (data not shown). However, determinations of the K_m of GR for GSSG revealed that, in all cases, it had been reduced (Fig. 1B). The K_m for NADPH could not be determined accurately in crude extracts because oxidation of this substrate in the absence of GSSG also changed in a concentration-dependent manner.

In contrast to the changes in the kinetic properties of GR, alterations in the steady-state levels of GR protein, as determined by Western blotting of total leaf protein extracts, revealed an increase of approximately twofold for ozone-fumigated plants and no increase for the other applied stresses (Fig. 2). Lower-molecular-weight bands observed in some of the samples have not been characterised. However, changes in the amounts of other proteins were apparent in the corresponding Coomassie-stained gels (Fig. 2). The changes noted were the induction of the small and large subunits of ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) in the greening plants; the decline of the Rubisco subunits during cold stress and paraquat treatment; and the induction of polypeptides (in all treatments but paraquat) of molecular weights in the 30- to 35-kDa region of the SDS–PAGE gels. No changes were detected in the steady-state levels of GR mRNA for any of the stress conditions, as measured using the RNase A/T1 procedure (Fig. 3).

Electrophoretic analysis of extracts from control and stressed leaves on non-denaturing polyacrylamide gels stained for GR activity revealed a total of six bands. The pattern of activity stains varied for extracts from differently stressed leaves and two of the bands (numbers 5 and 6) were unique to stressed leaves (Fig. 4A). Extracts from ozone-fumigated and paraquat-treated leaves also displayed alteration in the pattern of GR-staining bands (data not shown). Proteins from the marked bands (Fig. 4A) were eluted and Western blot analysis was used to confirm that they reacted with anti-GR antibodies (Fig. 4B).

Discussion

We have examined the activity, synthesis and properties of GR from leaves of pea plants subjected to cold, ozone fumigation, treatment with paraquat and greening of etiolated seedlings. We noted small but reproducible rises in the activity of GR in crude extracts from stressed leaves (Fig. 1A). This is consistent with previous reports for other plant species subjected to a range of stresses

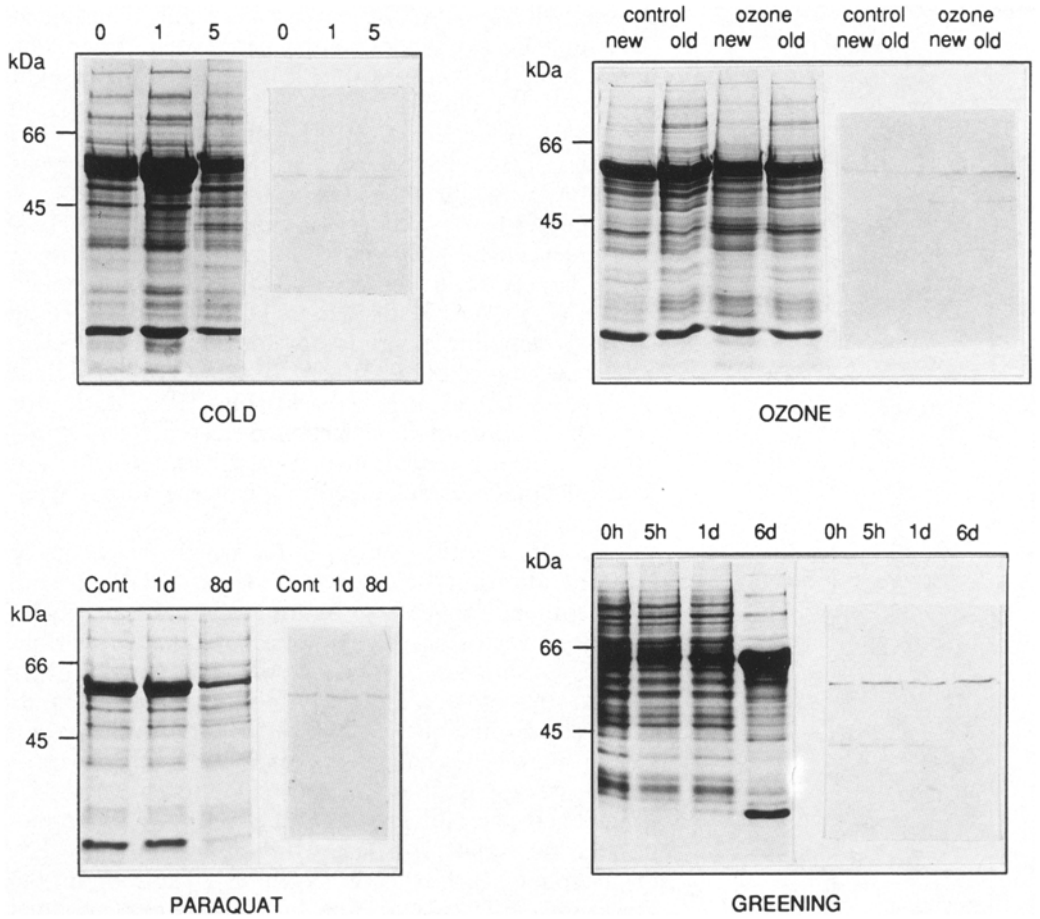

Fig. 2. Total protein profile (Coomassie-stained gels; *left half of each panel*) and immunodetectable GR (Western blots; *right half of each panel*). The stress conditions referred to are the same as described in the legend of Fig. 1. In the cold (chilling) panel, *tracks 0, 1* and *5* are the number of months pea plants were subjected to 4° C. In the paraquat panel, *tracks 1d* and *8d* refer to the days of harvesting after daily treatment with 3 mM paraquat. The control *(Cont)* leaves were painted daily with water for 8 d. In the greening panel, *tracks 0h, 5h, 1d* and *5d* refer to the number of hours (h) or days (d) of exposure of etiolated seedlings to light prior to harvesting. In the ozone-fumigated samples, "old" and "new" are described in the legend of Fig. 1. A 100-μg sample of protein extract was used for each track. Glutathione reductase was immunodetected using anti-GR IgG at 80 pg · ml^{-1} (Edwards et al. 1990) The two most-abundant Coomassie-stained proteins are the subunits of Rubisco (at ca. 60 kDa and 15 kDa)

(Mehlhorn et al. 1987; Tanaka et al. 1988; Foster and Hess 1980; 1982; Janhke et al. 1991; Kuroda et al. 1991; Cakmak and Marschner 1992; Schmidt and Kunert 1987; Pastori and Trippi 1992). There is relatively little information on GR synthesis under stress conditions. However, our data are consistent with those of Tanaka et al. (1988) showing a small elevation in the steady-state levels of GR protein in spinach subjected to ozone fumigation, but contrast with results obtained with ozone-fumigated peas, where no elevation of immunodetectable GR was found (Madamanchi et al. 1992). There are no data from any other source regarding transcription of GR genes in response to stress.

The meaning of these relatively small increases in GR activity is difficult to assess when compared with the induction of synthesis and activity of other stress-associated and defence-related enzymes, such as heat-shock proteins (Rochester et al. 1986), sucrose synthase (Marana et al. 1990) phenylalanine ammonia-lyase (PAL; Bolwell et al. 1985) and "pathogenesis related" proteins (Kauffmann et al. 1987). In the same experiments we observed the appearance of several proteins in the 30- to 35-kDa range (Fig. 2) whose synthesis may have been induced in response to the applied stresses at levels greater than any GR synthesis. It may be of more relevance that GR protein appears to have remained constant in stressed leaves, unlike more abundant proteins such as the Rubisco subunits which displayed greater variations in their levels (Fig. 2). Thus, the observed small changes in GR activities may reflect the greater relative stability of this enzyme compared with other proteins.

The one exception is the increase in GR protein levels, but not its transcript, in leaves of ozone-fumigated peas. In addition to the above arguments, this observation could also be explained by an increase in the translation of GR mRNA. Interestingly, increases in translation of GR mRNAs have been suggested to account for increased GR specific activities in a drought-tolerant maize variety (Pastori and Trippi 1992).

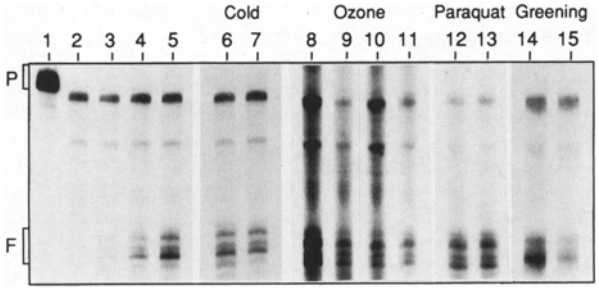

Fig. 3. Ribonuclease A/T1 protection assay for GR transcripts using a riboprobe (*P; track 1;* 5000 cpm loaded) of opposite polarity (see *Materials and methods*) to protect the 3′ end of GR mRNA(s) in total RNA samples prepared from stressed leaves of pea. The fragment of the riboprobe resistant to nuclease treatment is shown (*F*). *Tracks 2–5* are the result of carrying out a protection assay on 0, 5, 10 and 20 μg total RNA, respectively. The stress conditions for the leaves from which total RNA was prepared are described in the legend of Fig. 1. *Tracks 6, 8, 10, 12* and *14* are the results of protection assays carried out on 20 μg of total RNA prepared from stressed leaves as indicated above the panel. *Tracks 7, 9, 11, 13* and *15* are the same assays carried out on 20 μg total RNA from control plants grown under equivalent conditions but without the applied stress. Approximately 500 000 cpm of riboprobe was used for each assay

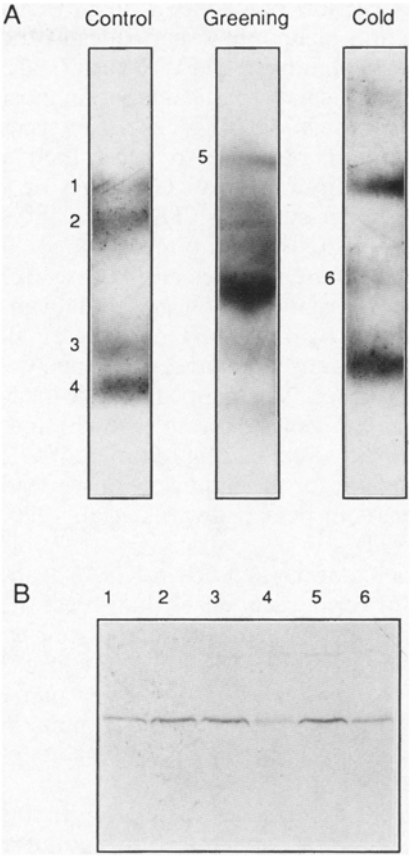

Fig. 4. A Native 7.5% (w/v) polyacrylamide gel stained for GR by the method of Halliwell and Foyer (1978). A 200-μg sample of protein extract, prepared from leaves subjected to the stresses indicated above the panel and as described in the legend of Fig. 1, was used for each track. The region of the gel containing GR-specific bands is amplified to discern the different band types (numbered 1–6). Non-specifically staining bands on control gels incubated without GSSG migrated faster and were not present in this region of the gel (data not shown). **B** Western blot of the different GR bands numbered 1–6 in **A.** The bands were identified on the native gel, eluted and re-fractionated by SDS–PAGE prior to blotting. 80 pg · ml^{-1} anti-GR IgG was used

The lack of any major induction of GR in the plant species cited so far, should be contrasted with the 40- to 122-fold elevation of GR activity in needles of eastern white pine over two seasons which correlated with the severity of the winter season (Anderson et al. 1992). Glutathione reductase and other protective enzymes may be induced in some species as a long-term adaptive response to stress rather than an immediate reaction at the onset of stress. However, even after one month at low temperature, pea leaves failed to show a greater than twofold elevation in GR activity (Fig. 1A) and there was no further elevation after five months (data not shown).

Glutathione reductase from cold-acclimated spinach was found to differ from that from non-hardened plants in several characteristics, e.g. electrophoretic mobility in non-denaturing polyacrylamide gels and thermal inactivation (Guy and Carter 1984). Similarily, the most notable difference in GR from the different plant extracts is the lower K_ms for GSSG under stress conditions. These differences could be due to the presence of low-molecular-weight effectors in the extracts. However, changes in the composition of the protein bands stained for GR activity prepared from total extracts of stressed leaves (Fig. 4) indicated that changes in the population of GR isoforms occurred in response to stress. The changes in the kinetic properties of GR from the different stressed leaves are also consistent with this view. A change in the composition of the GR isoform population, whose individual members have different characteristics, could bring about a net change in the measurable properties of the total GR activity, such as the lowering of the K_m for GSSG described here. A similar phenomenon has been observed in the induction of bean PAL isoforms in response to fungal elicitor (Bolwell et al. 1985); the induced PAL isoforms had lower K_m values than the isoforms present in uninduced cell cultures. This induction of PAL isoforms with a higher affinity for their substrate has been suggested to result in the channeling of more phenylalanine into the phenylpropanoid pathway (Bolwell et al. 1985). Similarily, we suggest that the appearance of GR isoforms with a higher affinity for at least one of the enzyme's substrates may result in an increased recycling of glutathione during periods of oxidative stress. In addition, several of the stress conditions studied are likely to result in a depletion of NADPH and in changes in the glutathione pool, so that a simple increase in the amount of GR activity may not be as important to the stressed plant as changes in the ability of GR to function under the new conditions.

The reason why GR protein levels should increase in response to ozone fumigation but not chilling, greening and paraquat treatment is not clear. However, it is worth noting that the primary site of ozone-induced oxidative stress is probably not the chloroplast. Rather, the stress

may result from the reaction of ozone with ethylene, leading to the production of lipophilic peroxides at the cell wall and membranes (Mehlhorn and Wellburn 1987).

The data presented in this paper indicate that, in most cases, changes in the composition of GR isoform types may be more important in response to stress than a significant induction of transcription of GR genes or a net increase in GR protein synthesis. There are three ways in which changes to the isoform population could occur, none of which are mutually exclusive. These are:

(i) Differential expression of a multigene family, similar to that of glutamine synthetase (Gebhardt et al. 1986), PAL (Dixon and Lamb 1990) and chalcone synthase (Harker et al. 1990). We have mapped a single locus in peas containing one GR gene (data not shown) and, therefore, differential expression leading to different GR forms is unlikely to account for the eight isoforms spread over three compartments in peas (Edwards et al. 1990; Madamanchi et al. 1992).

(ii) Differential splicing of nascent GR mRNAs, leading to the generation of coding sequences that direct the synthesis of different forms of GR. Differential splicing has not been commonly encountered in plants, but has been observed in the expression of the P gene of maize (Grotewald et al. 1991) and some transcription units in plant DNA viruses (Accotto et al. 1989; Mullineaux et al. 1990; Dekker et al. 1991).

(iii) Post-translational processing of GR preproteins may occur. Since the GR transcript codes for a putative plastidial targeting sequence (Creissen et al. 1992), the processing during import into organelles may generate isoforms which are specific to some compartments. Post-translational modifications, such as phosphorylation, glycosylation and generation of different oxidation states may be unlikely (Edwards et al. 1990), but other changes, such as removal of C-terminal residues, similar to that which occurs during the processing of α-amylase preprotein in barley aleurone cells (Sogaard et al. 1991), or different conformational states of the protein (Gething and Sambrook, 1992), have not been discounted.

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