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Cloning and characterisation of glutathione reductase cDNAs and identification of two genes encoding the tobacco enzyme

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Abstract. We have isolated 4 cDNA clones (GRT1-4) encoding glutathione reductase (GR) from a tobacco (*Nicotiana tabacum* L.) leaf cDNA library. The cDNAs were almost identical: GRT1, GRT3 and GRT4 represented the same gene, differing only in that GRT4 contained an intron within the C-terminal part of the coding sequence. Failure to splice out this intron resulted in a substitution of the final 13 amino acids of the deduced amino acid sequence. A second gene was represented by GRT2. Southern blots indicated that there were two related GR genes in tobacco. The presence of multiple isoforms of GR in tobacco may be explained in part by the expression of a small gene family. In addition, alternative isoforms may result from translation of different mRNAs derived from the same gene by intron skipping during the splicing of nascent GR mRNAs.

Key words: Glutathione reductase (cDNAs) – Intron skipping – *Nicotiana*

The thiol tripeptide, glutathione (GSH; γ-L-glutamyl-L-cysteinyl glycine), is the major low-molecular-weight thiol in most plants. In addition to its role in sulphur transport, GSH is involved in a number of metabolic and regulatory processes, including acting as a protein-disulphide reductant (Ziegler 1985), in the regulation of gene expression in response to pathogen attack (Dronn et al. 1988) and to environmental stress (Wingate et al. 1988), and as a donor of reducing equivalents to ascorbate as part of the oxidative-damage protection mechanism, primarily in chloroplasts, but also in other cell compartments (the ascorbate-GSH cycle; Foyer and Halliwell 1976; Nakano and Asada 1980; Dalton et al. 1986)

The tobacco GR cDNA sequences have been lodged in the EMBL database; accession numbers X76455 (GRT1), X76293 (GRT2) and X76533 (GRT4)

Abbreviations: bp=base pairs; GR=glutathione reductase; RACE=rapid amplification of cDNA ends

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Most of its metabolic functions result in the oxidation of GSH to glutathione disulphide which is reduced to GSH in all organisms so far examined by glutathione reductase (GR; EC1.6.4.2). The importance of GR in the ascorbate-GSH pathway, and its consequent role in the protection against oxidative damage, has resulted in a considerable amount of research into the properties of the enzyme. Multiple isoforms of GR have been identified in a number of species including pea (Edwards et al. 1990; Madamanchi et al. 1992) and tobacco (Foyer et al. 1991). The mechanism by which multiple isoforms are generated, and their potential significance in stress responses, is of considerable interest. We have previously isolated cDNAs for plastidial GR from a pea leaf cDNA library (Creissen et al. 1992). All of the cDNAs appeared to represent a single gene, which suggested that plastidial GR was not represented by a multigene family in this species and this has subsequently been confirmed by analysis of genomic clones and by restriction fragment length polymorphism (RFLP) mapping (Creissen et al. 1994; and data not shown).

The use of tobacco as a model species for studying the effects of altered levels of enzymes involved in protection against oxidative stress by overexpression of foreign genes in transgenic plants has meant that a better knowledge is required of these enzymes and of their regulation. In this paper we describe the isolation of cDNAs for GR from tobacco and discuss the relationship between genes, mRNAs and GR isoforms in this species.

Preparation of nucleic acids and cDNA cloning. Tobacco leaves were frozen in liquid nitrogen and ground to a powder. Nucleic acids were extracted by allowing the powder to thaw in 50 mM Tris-HCl, (pH 9.0), 150 mM LiCl, 5 mM EDTA, 5% (w/v) SDS, followed by three extractions with phenol:chloroform:*iso*amyl alcohol (25:24:1, by vol.). RNA was precipitated by the addition of an equal volume of 4 M LiCl. The DNA remained in the supernatant and was precipitated by the addition of 2.5 volumes of ethanol.

A cDNA library was constructed in bacteriophage λgt11 (Amersham International plc, Amersham, Bucks., UK) using poly(A)RNA obtained by oligo(dT) affinity chromatography of total RNA. The GR cDNAs were identified by screening the library (10^6 recombinants) with the pea GR cDNA clone pGR201 (Creis-

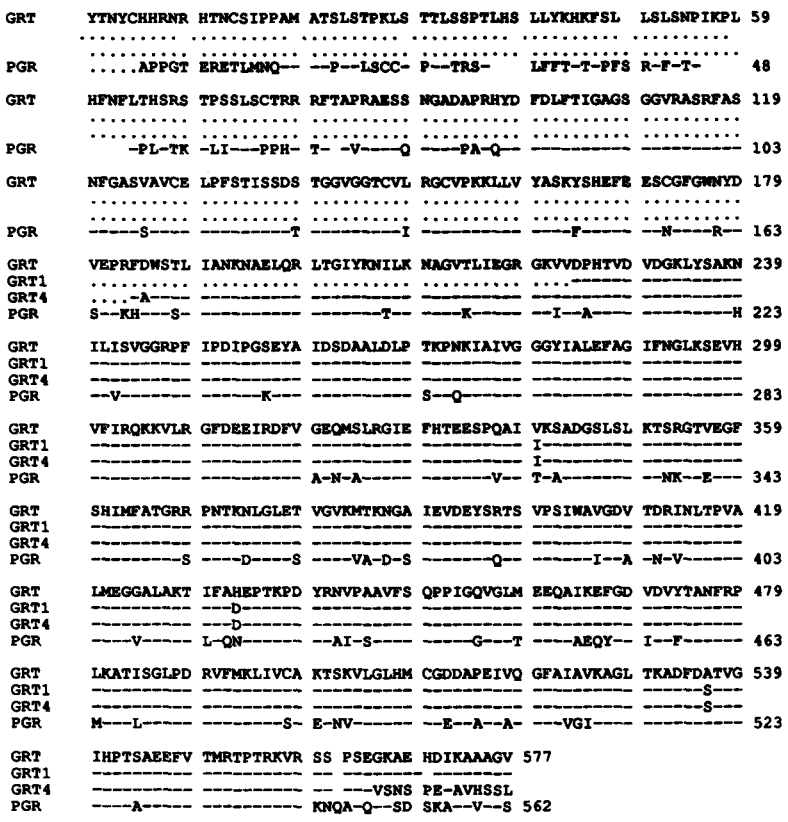

Fig. 1. Alignment of the deduced amino acid sequences of tobacco (GRT, GRT1 and GRT4) and pea (PGR) glutathione reductases. The GRT sequence represents the consensus generated from GRT2 and the 5′RACE clone. The first residue encoded by GRT2 is at position 21. *Dashes* indicate that the residue is the same as that found in GRT. Differences from the GRT sequence are indicated. The mature tobacco GR polypeptide commences at approximately position 87. *Spaces* represent positions where gaps have been introduced to make the alignment

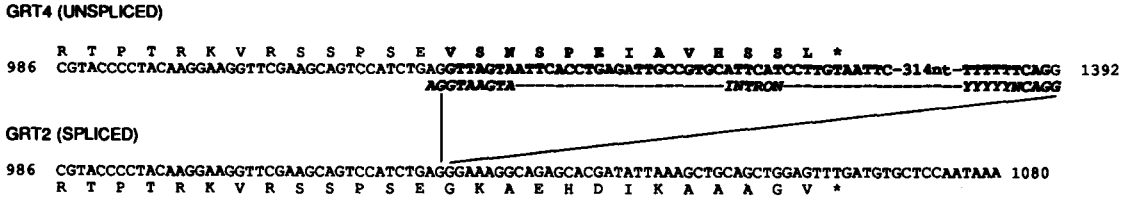

Fig. 2. The cDNA sequence and predicted amino acid sequence comparison of the C-terminal regions of the polypeptides encoded by the unspliced and spliced GR RNAs (GRT4 and GRT1, respectively). The position of the intron and the consensus 5′ donor and 3′ acceptor sites are indicated in *italics*. The intron-encoded terminal 13 amino acids are indicated in *bold type*

sen et al. 1992). After subcloning of cDNAs into pBluescript II SK+ (Stratagene, Cambridge, UK), GR inserts were sequenced on both strands of double-stranded templates (Sanger et al. 1977).

Southern hybridisation. Total DNA (10 μg) from tobacco leaves was digested with *Nsi*I and electrophoresed on a 0.8% agarose gel. After transfer of the DNA to nitrocellulose, genomic GR DNA fragments were detected by probing with radiolabelled probes (Feinberg and Vogelstein, 1983) at high stringency (0.1×SSC, 0.5% (w/v) SDS, 65°C; 1×SSC is 150 mM NaCL, 15 mM tri-sodium citrate) followed by autoradiography.

Rapid amplification of cDNA ends (RACE) The procedure described by Frohman et al. (1988) was employed to amplify cDNAs representing the 5′ end of the GR mRNA. A gene-specific oligonucleotide primer (5′-GCTAGTCTTTAAAGACAGTG-3′), corresponding to co-ordinates 997–978 of the GRT2 sequence, was used for the reverse transcription of GR mRNA to generate the first cDNA strand. The cDNA was tailed with dATP and the resulting product was amplified by the polymerse chain reaction (PCR). Amplified products were cloned into the *Eco*RV site of pBluescript II SK+ and sequenced.

Four independent clones representing different lengths of GR transcript were isolated from a λgt11 cDNA library by hybridising to the pea GR cDNA, pGR201 (Creissen et al. 1992). The longest of these (GRT2) contained an insert of 2211 base pairs (bp) . The remaining three clones were shorter, having inserts of 2.0 kb (GRT4), 1.8 kb (GRT1) and 1.6 kb (GRT3). The clones GRT3 and GRT1 were identical over their regions of overlap.

The longest cDNA encoded a polypeptide which lacked an initiation methionine. In order to determine the position of a potential initiation codon, we cloned a 5′RACE PCR product. An alignment of the deduced amino acid sequences of a consensus GR sequence (generated by combining the GRT2 and 5′RACE product sequences), GRT1, GRT4 and the deduced sequence for the pea polypeptide is shown in Fig. 1. The predicted tobacco GR amino acid sequences were very similar to each other with only a small number of conservative substitutions and they shared considerable amino acid

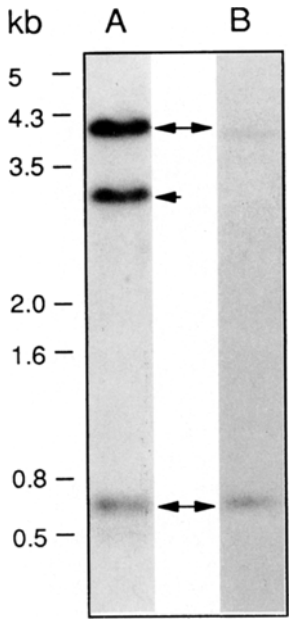

Fig. 3. Southern blot of tobacco GR genes. Tobacco DNA (10 μg) was digested with *Nsi*I, resolved on a 0.8% agarose gel, and blotted onto nitrocellulose. The blot was probed with GRT1 (*lane A*) or with an intron-specific probe (*lane B*). Intron-specific hybridising bands are indicated by the double *arrowheads*

sequence identity with the pea GR polypeptide. The N-terminal domain of the consensus sequence (GRT; Fig. 1) is rich in serine residues, which is typical of chloroplast transit-peptide sequences, and analysis of the deduced amino acid sequence (Gavel and von Heijne 1990) indicates that cleavage of the preprotein would occur between residues 86–87.

The 3′ (non-translated) regions of the different cDNAs contained a number of base changes and small insertions (1–5 bp), indicating that they were encoded by two distinct genes, GRT1, GRT3 and GRT4 being transcribed from one gene while GRT2 was transcribed from a second gene. In addition, the transcripts from the GRT1/3/4 class could be separated further into two subclasses by the insertion in GRT4 of an additional 468 bp near to the 3′ end of the coding region. The insertion point is flanked by the sequence 5′-AGGTTAGTA at the 5′ end and 5′-TTTTTTCAGG at the 3′end, which are in good agreement with the consensus sequences for intron-splice donor and acceptor sites (Sharp et al. 1987). Part of the intron is in frame with the coding sequence and consequently the presence of the intron would result in the substitution of the final 13 amino acids of the deduced polypeptide sequence (Fig. 2).

An attempt was made to gain some further information on the probable number of GR genes in the *N. tabacum* genome. The unspliced (i.e. intron-containing) GRT4 cDNA contained an *Nsi*I restriction enzyme site which was absent from the spliced cDNA (GRT1) and from GRT2. This difference was exploited in Southern blot analysis of the GR genes. Duplicate blots of *Nsi*I-digested tobacco DNA were probed either with the GRT1 cDNA (which would hybridise to genes showing homology to any of the cDNAs) or with the intron alone. The intron probe hybridised to two fragments of 0.65 and 4.2 kb (Fig. 3, panel B). The GRT1 probe hybridised to a major band of 3.3 kb in addition to the two fragments recognised by the intron probe (Fig. 3, panel A). The hybridisation of the intron-specific probe to two genomic bands was predicted from the cDNA sequence and confirms that the GRT1/3/4 class is encoded by a single gene. The evidence from cDNA sequencing, which classified GRT2 as being encoded by a second gene, and the presence of only one addition hybridising band on the Southern blot strongly supports the cDNA evidence for the presence of no more than two closely related GR genes in tobacco. However, there may still be more GR genes, for instance encoding the cytosolic isoform(s) which differ significantly at the nucleotide sequence level and are therefore not detectable with existing GR DNA probes.

As is the case for pea, the number of genes encoding GR in tobacco contrasts sharply with the number of isoforms of the enzyme (Foyer et al. 1991). In pea, a single nuclear gene encoding organellar GR has been identified. In transgenic plants the product of this gene is targeted to both chloroplasts and mitochondria simultaneously (Creissen et al 1995). It is possible that a similar situation exists in tobacco. We have previously examined the effects of various stresses in pea and have proposed two possible mechanisms which could result in changes to the GR isoform population. These were differential splicing of nascent mRNAs and post-translational processing of GR preproteins (Edwards et al. 1994). The data presented here support the possibility that a form of differential splicing (intron skipping) may play a role in isoform generation. However, RNase A/T1 protection analysis revealed that the unspliced mRNA represented only a small percentage of the total GR transcripts (data not shown) which would be unlikely to account for any of the major GR isoforms. Intron skipping, resulting in heterogeneity in the C-terminus of a maize phospholipid-transfer protein has been reported, and it was suggested that such a mechanism could have a function in subcellular targeting (Arondel et al.1991). Furthermore, there is evidence that some environmental stresses can result in splicing failure (Winter et al. 1988; Ortiz and Strommer 1990), and it may be that unspliced GR RNA accumulates in response to the presence or absence of an environmental cue. In addition, it is possible that different, as yet unidentified, GR genes may be induced under certain environmental conditions.

The identification of two genes encoding plastidial GR is consistent with the allotetraploid state of tobacco (Smith 1968). The possible function of the substitution of the terminal 13 residues in the deduced amino acid sequence encoded by GRT1/GRT4 is unclear. It is interesting to note, however, that all GR sequences from plants contain a C-terminal extension of unknown function of 22–26 amino acids compared to the C-terminus of the human *Escherichia coli,* and *Pseudomonas aeruginosa* enzymes (Creissen et al. 1992; Kubo et al. 1993) and it is possible that this domain is in part responsible for the

appearance of different isoforms of the enzyme in plants exposed to a variety of environmental stresses.

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