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Homoglutathione synthetase and glutathione synthetase in drought-stressed cowpea leaves: Expression patterns and accumulation of low-molecular-weight thiols ☆

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ABSTRACT

Glutathione (GSH) is an abundant metabolite and a major antioxidant in plant cells. However, in the Leguminosae, homoglutathione (hGSH) may replace glutathione (GSH) partially or completely. To date, cowpea (*Vigna unguiculata*) has been considered a non-hGSH-producing species, and no hGSHS cDNA has been isolated. Here we report on the cloning of a full-length cDNA coding for a hGSHS (EC 6.3.2.23) and the cloning of a partial cDNA coding for a putative glutathione synthetase (GSHS; EC 6.3.2.3) in cowpea leaf extracts. These cDNAs possess, respectively, the leucine/proline hGSHS signature and the alanine/alanine GSHS signature at the 3′ end. Expression analysis showed a significant up-regulation of hGSHS during progressive drought stress that could be directly related to the drought tolerance of the cowpea cultivar used, while GSHS was mainly constitutively expressed. Nevertheless, quantification of low-molecular-weight thiols confirmed the previous findings that cowpea is essentially a GSH producing plant, as no hGSH was detected in the leaves. These findings raise new questions regarding the function, activity and substrate specificity of the cloned hGSHS cDNA. These questions are discussed.

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Introduction

Glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is the most abundant low-molecular-weight (LMW) thiol present in most eukaryotic organisms, where it performs multiple functions (Noctor et al., 2002; Maughan and Foyer, 2006). These include storage and transport of sulphur (Macnicol and Bergmann, 1984) and control of the redox status. GSH is a strong reductant that can scavenge toxic reactive oxygen species (ROS) directly (Moran et al., 2000) or in cooperation with other antioxidants and ROS-processing enzymes (Noctor and Foyer, 1998; Szalai et al., 2009). GSH is also involved in the detoxification of xenobiotics (May et al., 1998) and, as a precursor of phytochelatins, in the sequestration of heavy metals (Noctor et al., 1998). GSH is also a substrate for glutathione reductase (GR) and glutathione peroxidase, two ROS scavenging enzymes (Szalai et al., 2009).

Enhanced ROS production in plants is an inevitable consequence of environmental stress (De Kok and Stulen, 1993), including drought stress. However, evidence is now emerging to suggest that ROS are more than deleterious by-products of stress, and are likely to be important secondary messengers that trigger adaptation responses to the changing environment (Dat et al., 2000; Cruz de Carvalho, 2008; Foyer and Noctor, 2009). Hydrogen peroxide (H_2O_2) is one of the candidate ROS most likely to act as secondary messenger, and the cellular antioxidant system plays a key role in the regulation of this signal (Foyer et al., 2009).

In the Leguminosae family, homoglutathione (hGSH, γ-glutamyl-cysteinyl-β-alanine) replaces GSH either completely or partially (Matamoros et al., 1999). For example, hGSH was the most abundant tripeptide thiol found in the nodules of *Glycine max* (Klapheck, 1988), *Pisum sativum* and *Phaseolus vulgaris* (Matamoros et al., 1999). GSH and hGSH appear to be essential for the proper development of the root nodules resulting from the symbiotic interaction (Frendo et al., 2005). *Lotus japonicus* has also

Abbreviations: GR, glutathione reductase; GSH, reduced glutathione; GSHS, glutathione synthetase; GSSG, glutathione disulphide; hGR, homoglutathione reductase; hGSH, homoglutathione; hGSHS, homoglutathione synthetase; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction

☆ The nucleotide sequence data are registered in GenBank under the accession numbers EU164853 (*VuhGSHS*) and GU046514 (*VuGSHS*).

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been shown to be mainly a hGSH-producing species (Matamoros et al., 2003), in which hGSH was detected in nodules, roots and leaves. In the case of *Medicago truncatula*, the opposite was found and GSH was the most abundant tripeptide in the nodules, leaves and flowers of mature plants (Frendo et al., 1999). Many of the roles ascribed to GSH are also performed by hGSH (Frendo et al., 2001), particularly the control of the cellular redox status and ROS scavenging (Dalton et al., 1986). The synthesis of GSH and hGSH proceeds through two ATP-dependent steps that are similar in plants, micro-organisms and animals. The first reaction common to the synthesis of both tripeptide thiols is catalyzed by γ-glutamyl-cysteine synthetase (EC 6.3.2.2), and the second step is catalyzed by specific enzymes, glutathione synthetase (GSHS; EC 6.3.2.3) for GSH and homoglutathione synthetase (hGSHS; EC 6.3.2.23) for hGSH. It has been suggested that separate genes encode GSHS and hGSHS, the divergence in specificity arising from gene duplication between the Fabales, the Solanales and the Brassicales in the course of evolution. Of these taxa, hGSH has been exclusively detected in Fabales (Frendo et al., 2001).

It has been shown that the glutathione level was increased in response to drought stress in sunflower seedlings (Sgherri and Navari-Izzo, 1995) and wheat flag leaves (Herbinger et al., 2002) in response to a 24 h period of imposed drought stress in wheat leaves (Bartoli et al., 1999) and in response to desiccation in detached poplar leaves (Morabito and Guerrier, 2000). Contrasting results were found by Loggini et al. (1999), where a decline in total glutathione level was observed in two wheat cultivars in response to drought, and this was independent of their tolerance level. On the other hand, in alfalfa nodules, drought stress was shown to have no significant effect on the levels of GSH and hGSH (Naya et al., 2007). In a previous study, it was shown that drought stress leads to different activities and expression levels of GR (Contour-Ansel et al., 2006; Torres-Franklin et al., 2008) in cowpea and common bean cultivars differing in their degree of drought tolerance. Considering the importance of GSH and/or hGSH in sulphur and antioxidant metabolism, we focused on the synthesis pathway of these molecules. In the present study, we investigated whether GSHS and/or hGSHS expression was modulated by drought stress in cowpea leaves and if the expression patterns could be related to drought tolerance. We examined progressive drought stress of whole plants and desiccation stress of detached leaves in parallel in order to investigate whether the responses differed between these two types of water stress. We also measured drought stress-induced (h)GSH accumulation and redox state to gain further insights into the metabolism of low-molecular-weight thiols in cowpea in response to drought.

Materials and methods

Plant culture and sample treatments

Two *Vigna unguiculata* (L.) Walp (Vu) cultivars, one tolerant to drought ("EPACE-1") and the other drought-sensitive ("1183"), were grown under greenhouse conditions as described by d'Arcy-Lameta et al. (2006). Progressive drought stress was applied to 5-week-old plants by withholding irrigation. Leaf water potential was measured daily after 4 h of illumination with a pressure bomb (PMS instrument, Corvallis, USA) according to Scholander et al. (1964). Well-developed second and third leaves from the top were harvested when plants reached leaf water potentials of $\Psi_w = -1.0 \pm 0.1$ MPa (S1 plants), $\Psi_w = -1.5 \pm 0.2$ MPa (S2 plants) and $\Psi_w = -2.0 \pm 0.2$ MPa (S3 plants), referred to as "mild", "moderate" and "severe water stress". In addition, detached leaves were air-dried (desiccation treatment) at 24 °C at dim light for 30 min, 2 h and 5 h. Leaves from control plants ($\Psi_w = -0.5 \pm 0.1$ MPa) were harvested prior to drought or desiccation treatments. All sampled leaves were flash frozen in liquid nitrogen and kept at –80 °C until use.

Total RNA isolation

Frozen leaf material was ground with a mortar and pestle in liquid nitrogen. About 100 mg fresh material was used for total RNA extraction, using the RNEasy Plant Minikit (Qiagen, France) according to the manufacturer's instructions. Total RNAs were quantified with a Nanodrop ND-1000 spectrophotometer (Starlab, USA) at 260 nm.

Cloning of hGSHS and GSHS cDNAs

Legume hGSHS and GSHS protein sequences obtained from GenBank™ (http://www.ncbi.nlm.nih.gov) were aligned using the CLUSTALW program (http://pbil.ibcp.fr/htm/index.php) (Thompson et al., 1994). Oligonucleotides were designed from consensus regions and used as primers in reverse transcription-polymerase chain reaction (RT-PCR) amplification of cowpea hGSHS and GSHS cDNA fragments (Table 1).

RT-PCR was performed on control "1183" leaf total RNAs. The RT-PCR assay contained 100 ng total RNA, 15 pmol each oligonucleotide, dNTP mix (10 mM each), 1 unit of a mix of reverse transcriptase and Taq Pol enzymes (one-step Reverse Transcription-PCR system, Qiagen, France) and the manufacturer's buffer in a total volume of 25 μl. RT-PCR was performed using a thermal cycler (Mastercycler Gradient, Eppendorf AG, Germany), with a common first step (50 °C for 30 min, 95 °C for 15 min) followed by 35 cycles (denaturation step at 95 °C for 30 s, annealing at 50.7 °C for 30 s, extension at 72 °C for 80 s) for hGSHS and 30 cycles (denaturation step at 95 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 80 s) for GSHS. After the last cycle, a final extension was carried out at 72 °C for 7 min. Amplified cDNA fragments were visualized after separation on 1% (w/v) agarose gels, using a UV light transilluminator (Snapshot, Syngene, France) and purified (Wizard PCR Prep, Promega, France).

Cloning was performed using the pGEM-T Easy Vector Plasmid System I (Promega, France) following the manufacturer's instructions and used to transform *Escherichia coli* strain GT869 competent cells by heat shock. After selection of the recombinant bacteria, plasmid DNA was isolated using Wizard® Plus SV Minipreps DNA Purification System kit (Promega, France). Sequencing was performed by Genoscreen (Lille, France).

Rapid amplification of 5′ and 3′ cDNA ends (RACE)

PCR amplification of the 5′ and 3′ regions of hGSHS cDNA and of the 3′ region of GSHS cDNA was achieved by the use of the 5′/3′

Table 1
Heterologous primers designed from consensus regions of leguminous GSHS and hGSHS protein sequences and specific primers designed from cowpea cDNA sequences. (1) cloning; (2) expression. F=forward primer; R=reverse primer.

Sense		Primer sequence	T_m
hGSHS			
(1)	F	5′-CAA ATA GAG ATG AAC ACT ATT-3′	50.7 °C
	R	5′-GAC ACC ATT CAG TAG GAA AAG-3′	
(2)	F	5′-GAA AGT GGC TAT ATG GTG CG-3′	57.0 °C
	R	5′-GAC ACC ATT CAG TAG GAA AAG C-3′	
GSHS			
(1)	F	5′-AMT WTK TCT GCW GCA GTT-3′	52.0 °C
	R	5′-AGC WGC AAC BCC RCC TTC-3′	
(2)	F	5′-ATC TGA TGA AGG AGG GGT TG-3′	59 °C
	R	5′-ATG TGG GTT ACA ATC CCG AA-3′	

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