The glutaredoxin gene family in wheat functions beyond redox homeostasis regulation

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INTRODUCTION

The main function of glutathione (GSH) in the cell is to act as a redox buffer against irreversible damage to cell machinery caused by a build up of reactive oxygen species (ROS). Other functions of glutathione in the cell include xenobiotic and heavy metal detoxification, signalling in plant-pathogen interactions as well as plant developmental processes and cell cycle control[1]. Because glutathione can form bonds to thiols of proteins, and can be enzymatically transferred and removed, it provides an interesting way by which protein activity can be modulated as has been shown for NF-κB in humans[2] and NPR1 in *Arabidopsis*[3].

Glutaredoxins (GRXs) are glutathione dependant oxidoreductase enzymes which mediate the reversible reduction of disulphide bonds of proteins and protein-glutathione mixed disulphides and are found in all free-living organisms[4,2]. Apart from their obvious role in the regulation of redox balance, plant glutaredoxins have been shown to bind iron sulphur centres[5], regulate defensin expression[6] and be crucial for flower development[7,8].

Glutathionylation is now being seen as a reversible post translational modification analogous to phosphorylation and inherently linked to cellular redox potential. In order to obtain a greater understanding of the role of glutathionylation, it is necessary to study redox active protein families such as glutathione S-transferases (GSTs) and GRXs. This paper describes aspects of GRX gene expression in wheat and the potential role of three GRX genes in inflorescence development.

MATERIALS AND METHODS

Expression analyses

Wheat GRX gene models used for this analysis were first described by Ziemann et al[9]. The relevant wheat ESTs were searched in the TIGR plant TA database (http://plantta.tigr.org/) and information was downloaded into tables for analysis. Rice and *Arabidopsis thaliana* GRX gene models are those supplied by Xing et al.[10]. EST information for those organisms was obtained through genome browsers of the relevant organisms (http://www.phytozome.net/index.php), or though the TIGR plant TA database. Sorghum GRX gene loci were obtained by using annotated rice GRX peptide sequences as queries in the Phytozome TBLASTN search engine. Sorghum genes were classed into their active site groups and the number of ESTs belonging to each gene model was found through the genome browser. Microarray information from *Arabidopsis* was obtained for GRX genes described by Xing et al[10]. The Meta-Analyser function of Genevestigator (https://www.genevestigator.ethz.ch/) "Plant Organs" dataset values for "seedling", "rosette", inflorescence" and "root" were used for overall expression of the three GRX groups and was compared to values from "callus" and "cell suspension"[11]. Probes were found for all annotated GRX genes except At2g47870 (CCMC), At2g21460 (CCMS), At4g15670 (CCMS) and At4g28730 (CSYC) (Data tables and sequences will be provided on request).

CLONING OF TaGRX-CCMC9, 11 AND 12 FROM CDNA

RNA was isolated from wheat cv. Cranbrook inflorescences using TRIsure reagent (Bioline) and first strand cDNA was produced with Bioscript (Bioline) according to the manufacturer's recommendations. 5ng of cDNA was used in a separate control PCR reaction with intron based primers to exclude the chance of amplifying from genomic DNA. *ROXY-like* GRX genes were amplified with the following primers 5' ATGCAGTACGGCGCGGCGGCCGAGCAG 3' and 5' CTAGAGCCAGAGCGCGCCGGCCTCCTTGAG3'.
The amplified products were excised from an agarose gel and purified (Eppendorf Perfectprep Gel Cleanup Kit) prior to cloning using the pGEM-T Easy Cloning System (Promega). Plasmid DNA was isolated using CsCl based minicolumn procedure (SV-Plasmid mini-prep kit, Promega) and the plasmid was subjected to digestion with restriction enzymes *Ngo*MIV and *Eco*RI and in a separate reaction *Eco*RI and *Bgl*I and run on an agarose gel to determine the specific identity of the wheat *ROXY-like* sequence.

Complementation studies with wheat genes TaGRX-CCMC9, 11 AND 12

Primers (5' TCTAGAATGCAGTACGGCGCGGCGGCCGAGCAG 3' and 5' TCATGACTAGAGCCAGAGCGCGCCGGCCTCCTTGAG 3') were used on gDNA from wheat cv. Cranbrook to amplify *ROXY-like* genes. Cloning into the pGEM-T Easy and plasmid isolation was followed by BDT3.1 sequencing by the T7 primer. pGEM-T Easy plasmid DNA was digested with *Xba*I and *Bsp*HI and loaded on

an agarose gel; insert DNA was isolated with a gel cleanup procedure. pGSA1252 plasmid containing the 4.6kbp *ROXY1* locus was digested with *Xba*I and *Nco*I, followed by CIAP treatment (Fermentas), agarose gel separation and gel cleanup and finally ligation to the insert DNA. Transformation vectors containing wheat *ROXY-like* genes were digested with *Xba*I and *Nco*I to confirm the presence of the specific insert. Recombinant plasmids were transformed into competent *A. tumefaciens* strain GV3101 cells with electroporation and subsequently introduced into the *roxy1-3* mutant background using the floral infiltration method[12]. T1 transformants were obtained by BASTA selection and phenotyped upon flowering.

RESULTS AND DISCUSSION

Expression of wheat GRX genes

There are three GRX subgroups in plants, each characterised by an active site motif: CPYC, CGFS and the CC-type. There are two major catalytic mechanisms for glutaredoxins; a dithiol and a monothiol reaction, the dithiol reaction mediates the reduction of intramolecular disulphide bridges, while the monothiol mechanism mediates the deglutathionylation of protein-GSH mixed disulphides. GRXs with the active site CXXC are able to catalyse both reactions but GRXs with a CXXS active site are restricted to the monothiol mechanism.

Previous work identified 104 expressed GRXs from wheat EST databases, of which 43 were from the CPYC group, 27 were from the CGFS group and 34 were from the CC-type group, making the wheat GRX family the largest described so far[9]. The number of ESTs in the database per gene for each group was calculated (Table 1).

	CC-type	CGFS	CPYC
Rice	11.7	63.2	95.4
Wheat	2.5	5.1	7.5
A. thaliana	22.7	59.3	92.0

Table 1 - The calculation of ESTs per gene of GRXs in three selected plants shows that CC-type genes are generally expressed to a lesser extent than CPYC or CGFS genes.

The number of ESTs in the database gives a rough guide to the extent of expression of each gene. The result indicates that each CC-type GRX gene is expressed to a lesser degree than the CPYC or CGFS types in all higher plants examined. This observation is consistent with findings using the *Arabidopsis* microarray database, which has an average expression score of 460 for CC-type genes compared to 2352 and 4739 for CGFS and CPYC type genes respectively.

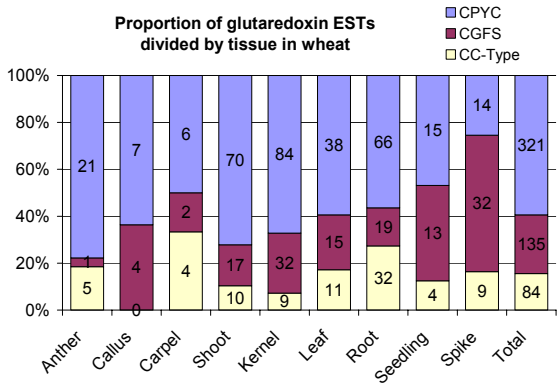

Figure 1 - Distribution of wheat GRX ESTs by tissue of origin.

Using all available wheat GRX ESTs (544), an overall EST count found that CGFS GRXs represented 25% of ESTs, whereas CC-type and CPYC GRXs represented 15% and 60% of ESTs, respectively. The distribution of expression was observed in various tissues (Figure 1). CPYC ESTs are over-represented in shoots and anthers but under-represented in spike tissue. CGFS ESTs are over-represented in spike but under-represented in anther tissue while CC-type ESTs are somewhat over-represented in carpel and root tissue but under-represented in callus. The observation of CC-type genes being down-regulated in callus tissue is consistent with findings from *Arabidopsis* microarray databases, which indicate a decrease of 54% in CC-type gene expression in cell suspension and callus (data not shown). It is not known why such a decrease in CC-type expression is observed in suspension cultures, but it may be due to the tissue specific nature of expression, the direct effect of hormones such as 2,4-D, kinetin and gibberellic acid or the fact that expression of CC-type genes is required for cell differentiation.

Characterisation of ROXY-like genes from wheat

Previous work on the isolation of wheat *ROXY-like* genes found the presence of three distinct genes (*TaGRX-CCMC9*, *11* and *12*) which displayed 61% amino acid identity with *ROXY1*[9]. PCR on cDNA from inflorescences of wheat cv. Cranbrook was performed and the amplified products were cloned into the pGEM-T Easy plasmid. Restriction digestion was performed on 14 plasmid clones, that analysis found that the majority of clones were shown to be *TaGRX-CCMC12*, with *CCMC11* and *CCMC9* expressed to a lesser extent (Figure 2).

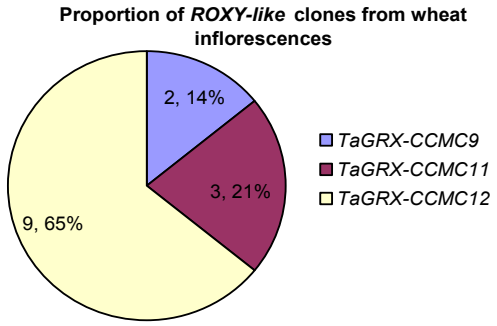

Figure 2 - The proportion of ROXY-like clones in the cDNA library as determined by restriction digestion.

To determine whether the wheat *ROXY-like* coding sequences can functionally replace that of *ROXY1* in *A. thaliana*, the three wheat genes were cloned into a plasmid containing the *ROXY1* flanking regions consisting of 3616bp upstream of the ATG codon and 449bp downstream of the TGA stop codon[7]. The wheat *ROXY-like* genes were inserted into the plasmid with *Xba*I and *Nco*I to replace the *ROXY1* coding sequence as shown Figure 3.

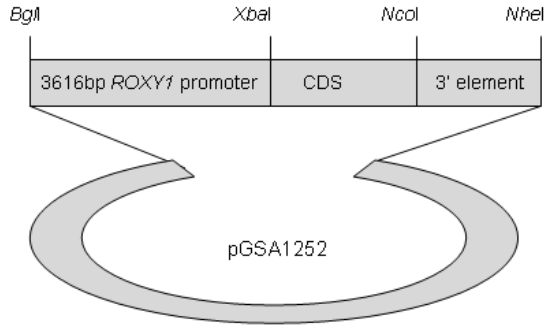

Figure 3 - Cloning of the wheat *ROXY-like* gene coding sequence (CDS) into the pGSA1252 transformation vector.

The three plasmids were then transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into the *roxy1-2* mutant background. Transgenic plants resistant for BASTA were phenotyped according to petal number and morphology. The *roxy1-2* mutants displayed an average of 2.5 petals per flower while all three wheat *ROXY-like* genes produced wild type phenotypes with 4 petals per flower and normal morphology. Presence of the transgene was confirmed by PCR with gene-specific primers on genomic DNA. The results indicate that when the coding sequence of *ROXY1* is replaced with *TaGRX-CCMC9*, *11* or *12,* full complementation of the *roxy1-2* mutant phenotype is achieved, thereby strongly implicating these three wheat genes in inflorescence development.

The task remains to determine how these *ROXY*-like genes exert their function; i.e. what their *in vivo* targets are. Furthermore, fully quantitative reverse transcriptase PCR on different tissues and developmental stages is required to further detail the regulation of these genes to determine their possible roles in formation of specific organs in the wheat inflorescence.

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REFERENCES

1. Rouhier, N., Lemaire, S.D. & Jacquot, J.P. (2008). *Annu Rev Plant Biol.* 59:143-166.
2. Shelton, M.D., Kern, T.S & Mieyal, J.J. (2007*). J Biol Chem.* 27;282(17):12467-74.
3. Mou. Z., Fan, W. & Dong, X. (2003). *Cell*. 113; 935-944.
4. Fernandes, A.P. & Holmgren, A. (2004). *Antioxid. Redox Signal.* 6:63-74.
5. Feng, Y., Zhong, N., Rouhier, N., Hase, T., Kusunoki, M., Jacquot, J.P., Jin, C. & Xia, B. (2006). *Biochemistry*. 45:7998-8008.
6. Ndamukong, I., Abdallat, A.A., Thurow, C., Fode, B., Zander, M., Weigel, R. & Gatz, C. (2007) *Plant J.* 50:128-39.
7. Xing, S., Rosso, M.G. & Zachgo, S. (2005). *Development*. 132:1555-65.
8. Xing, S. & Zachgo, S. (2008) *Plant J.* 53:790-801.
9. Ziemann, M., Lauri, A., Xing, S., Bhave, M. & Zachgo, S. (2007). *Proceedings of the 57th Australian Cereal Chemistry Conference*, Royal Australian Chemical Institute, Victoria, pp. 72-75.
10. Xing, S., Lauri, A. & Zachgo, S. (2006). *Plant Biol* (Stuttg) 8:547-55.
11. Zimmermann, P., Hennig, L. & Gruissem, W. (2005). *Plant Sciences* 10:407–409.
12. Clough, S. J. & Bent, A. F. (1998). Plant J.16, 735-743.