

NOVEL DROUGHT-INDUCED GENES FROM *GLYCINE LATIFOLIA*

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Abstract

Wild relatives of crop species have proven a useful genetic resource for improving the ability of crops to overcome a range of biotic and abiotic stresses. This study looks at *Glycine latifolia*, a drought-tolerant wild relative of soybean, with a view to understanding mechanisms of drought tolerance and ultimately improving the drought tolerance of soybean. It is hoped that by gaining an understanding of the genes that confer drought resistance to *G. latifolia* it may be possible to screen for homologues of these genes in soybean and develop gene-specific markers for breeding. The cDNA from a number of drought-induced genes have been isolated via a subtracted cDNA library and the expression patterns of three novel sequences have been characterised. Two of the genes appear to be regulated by abscisic acid (ABA) while the third is expressed independently of ABA. The first ABA-dependent gene has very low homology with other sequences in the Genbank database but has some similarity to chaperone proteins. The second ABA-dependent gene appears to be a dehydrin while the ABA-independent gene may be a chitinase.

Introduction

Soybean is the world's most important oil seed and grain legume (Ming and Lianzheng 1999; Sonka 1999). The harvested seed has the largest useable protein content of all cultivated legumes and is also highly valued because of its oil (Hymowitz 1990), yet the crop is generally not cultivated in dryer environments (Lawn and Imrie 1991). Among the major grain legumes, soybean is the most sensitive to water stress (Sinclair and Ludlow 1986). However, other species within the genus *Glycine* and in particular *Glycine latifolia* are highly tolerant of water stress (James 2000).

Glycine max (soybean) and *Glycine latifolia* are physically very different plants. *G. latifolia* is a perennial plant with a trailing or twining stem up to 1 m or more long, its leaves are trifoliolate with leaflets 2-5.8cm X 1-3.3cm (Hacker 1990). *G. max* is an erect annual plant up to 1.75m tall, its leaves are also trifoliolate but with leaflets 3-14cm X 2.5-10cm (Hacker 1990). The genetic difference between these two species is evidenced by their incompatibility for breeding (Marshall and Broué 1981) despite both species having diploid genomes with $2n = 40$ (Newell et al 1987).

In this study we have used suppression subtractive hybridisation (SSH) to create a cDNA library from *G. latifolia* that was enriched for drought-induced transcripts. The library was screened using differential screening with probes made from the drought-induced cDNA enriched library and a library with drought-induced cDNAs removed. Real time PCR was used to confirm that the putative drought-induced cDNAs were in fact more abundant when leaf relative water content (RWC) decreased.

Many drought-inducible genes have been identified in a variety of plant species. These genes can be roughly grouped according to the functions of their products, e.g. compatible solutes, scavenging reactive intermediates, cellular protectors and regulatory genes. Osmotic stress can occur when depletion of soil water results in an increased solute concentration in the soil and a corresponding reduced water potential. As a result, plants under this stress will lose turgor unless they can restrict

water loss from leaves and other plant parts. In the first instance this occurs via stomatal closure. As the stress increases, the plants may maintain water uptake via osmotic adjustment, which lowers the water potential of the leaf and maintains an osmotic gradient into the leaf. During osmotic adjustment plants actively accumulate compatible solutes such as glycerol, mannitol, proline, sucrose, trehalose and quaternary ammonium compounds (Legaria et al 1998).

Reactive oxygen intermediates (ROIs) occur as a result of the inhibition of photosynthesis brought about by limited water availability (Holmberg and Bülow Leif 1998). A number of enzymes (and to a lesser extent some metabolites such as ascorbate, glutathione and tocopherol) assist in the reduction of ROIs (Schwanz and Polle 2001). These metabolites reduce H_2O_2 to water and the reactions are catalysed by peroxidases (Polle 2001). Catalases can also convert H_2O_2 to H_2O and O_2 . Other enzymes called superoxide dismutases (SODs) can convert the highly toxic O_2^- to the less toxic H_2O_2 and O_2 (Polle 2001).

Cellular protectors refer to the group of genes that code for certain late embryogenesis abundant (LEA) proteins, more commonly called dehydrins (Close 1996). The exact function of these proteins is still not clear but numerous studies have demonstrated the induction of dehydrins by drought stress (Maitra and Cushman 1994; Giordani et al 1999; Thompson and Corlett 1995). Dehydrins are amorphous or highly disorganised proteins which may enable them to conform to the shape of other proteins or membranes and provide a cohesive layer while the hydrated state of the dehydrin may impart greater stability (Close 1996; Kermode 1997). Heat shock proteins (HSPs) are another group of proteins involved in cellular protection. Their function is also not clear but some HSPs are induced by water stress and they play a role in preventing the aggregation of denatured proteins (Campbell et al 2001) (Horwitz 1992).

Abscisic acid (ABA) is a key regulatory hormone and numerous studies have demonstrated its role in regulating gene expression during drought response (Chandler and Robertson 1994). It is now hypothesised that at least four independent signalling pathways function in the activation of stress-inducible genes under dehydration conditions: two are ABA-dependent and two are ABA-independent (Shinozaki et al 1998). Different transcription factors and other pathway components (including protein kinases) are required in all of the signal transduction pathways for expression of the drought-inducible genes. Some of these have been characterised (Mikami et al 1998; Shinozaki et al. 1998; Soderman et al 1996; Urao et al 1994), but further molecular analysis could provide better understanding of how they interact and possibly reveal new regulatory genes. This information should suggest how modulation of these signalling pathways could improve plant resistance to drought stress, and hence provide options for plant improvement.

Results and Discussion

To determine the best time to collect RNA from *G. latifolia* in order to isolate drought-induced transcripts, expression studies of homologues of known drought-induced cDNAs were carried out. This study indicated that RNA from leaves with a RWC of 40-60% were suitable for isolating the maximum number of different drought-induced cDNAs, i.e. those induced early in the drought response and those induced late. Once RNA was extracted and converted to cDNA it was further enriched for drought-induced cDNA via suppression subtractive hybridisation, whereby cDNAs present in the sample but not induced by drought were removed. The remaining cDNA was amplified by PCR step so that cDNA from low abundance transcripts was more easily isolated.

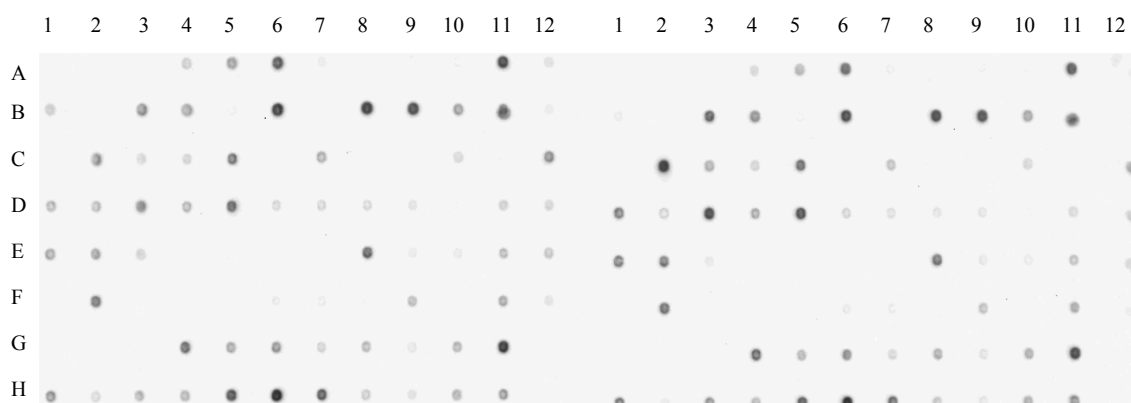
The subtracted cDNAs were cloned into *E. coli* and then screened by macroarray (**Fig. 1**), and putative drought-induced cDNAs were sequenced to assist their identification (**Table 1**). The

expression levels of these cDNAs were measured as RWC decreased to confirm that they are induced by drought. More detailed studies were carried out on three of these confirmed drought-induced genes coding for: (a) a putative chaperone protein (GLUP003B09), (b) a putative dehydrin (GLUP003F11), and (c) a putative chitinase (GLUP004D08).

Expression of GLUP003B09

Expression of GLUP003B09 was unaffected by mild stress (75-85%) but increased as RWC decreases to 40-50% (**Fig. 2a**) and was expressed predominantly in the leaf with very little expression detected in the roots. The transcript levels in the leaves from “wet” plants were increased by a factor of 3 in response to ABA and by a factor of 6 in response to salt treatment. In both ABA and salt responses transcript levels begin to decline after 12 hours of exposure. This pattern is consistent with expression that is controlled by the ABA-dependent pathway. The deduced amino acid sequence of GLUP003B09 has 75.5% identity with an “expressed protein” from *Arabidopsis thaliana* with no assigned function. The deduced protein sequence also contains a region with part of a conserved motif found in molecular chaperone proteins. In chaperone proteins this region is involved with the binding of unfolded polypeptides but the exact function of this domain is still unclear.

a. Probe made from Dry – Wet library



b. Probe made from Wet – Dry library

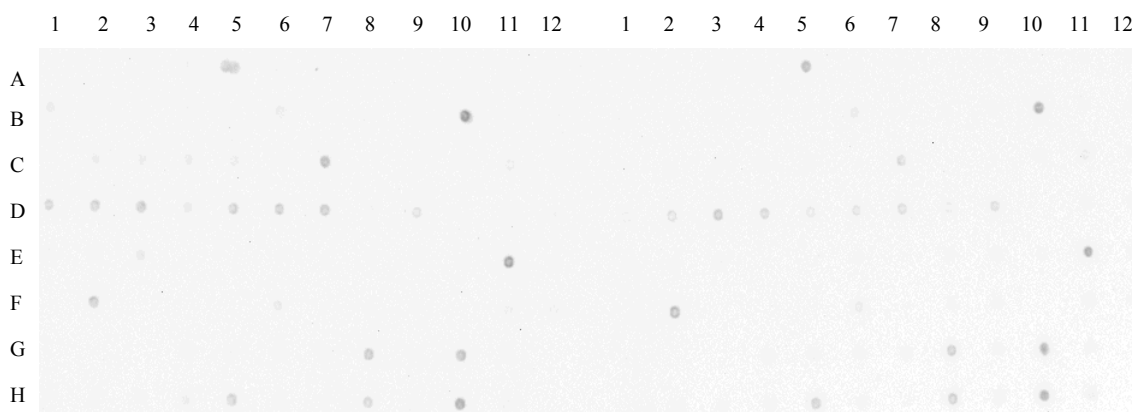


Figure 1. Differential screening of putative drought-induced cDNAs from the drought-induced enriched library with probes made from (a) the Dry – Wet library and (b) the Wet – Dry library. Clones have been spotted in duplicate in a 96-well format (A1-H12). Control PCR reactions with pCRII TOPO vector as template were used as negative controls in positions H11 and H12. These negative controls were used to correct for background signal when calculating the difference in expression between the two probes.

Table 1. cDNAs isolated by PCR suppression subtractive hybridisation from *Glycine latifolia* subjected to drought conditions.

Genbank ID	Clone homology
GLUP001B10	maturation-associated protein
GLUP001D08	putative serine carboxypeptidase II
GLUP002A12	early light-inducible protein precursor
GLUP002D07	metallothionein-like protein class II
GLUP003B02	adenosylhomocysteinase
GLUP003B05	putative lipid transfer protein
GLUP003B09	cold_stressed_Glycine_clandestina
GLUP003D02	unknown protein
GLUP003F11	unknown EST
GLUP003F12	Nonspecific lipid-transfer protein precursor
GLUP003H05	plastidic aldolase
GLUP004A02	mutator-like transposase
GLUP004C03	Glu-tRNA(Gln) amidotransferase subunit C
GLUP004D08	RuBisCO-associated protein
GLUP004F11	s-adenosylmethionine synthetase 2
GLUP005G05	unknown protein
GLUP005G09	No significant ID
GLUP005H05	unknown EST
GLUP005H09	CPRD86 [<i>Vigna unguiculata</i>] - stress inducible
GLUP006F06	Soybean proline-rich cell wall protein gene
GLUP006H10	putative casein kinase I [<i>Oryza sativa</i>]
GLUP007F04	chlorophyll a/b-binding protein
GLUP009B10	early light-inducible protein precursor
GLUP007A08	chloroplast precursor
GLUP007F06	Fructose bisphosphate aldolase
GLUP009F02	cold stress protein SRC1
GLUP11D04	putative transcription factor

Expression of *GLUP003F11*

The expression of *GLUP003F11* was analysed in the same manner as that for *GLUP003B09*. No significant increase in transcript levels were detected at 75-85% RWC but increased significantly at 40-50% and increased further at 30-40% (**Fig. 2b**). Expression under well-watered conditions was similar for leaf and stem tissues, but when water was withheld, only leaf tissue displayed a significant increase in transcript levels. Expression was induced by salt and ABA and both reached a maximum after 12 hours of approximately 4-fold above control levels. Sequence alignments show *GLUP003F11* has 68% identity with a dehydrin from *Retama raetam*. In common with other dehydrins it has a highly conserved 15 amino acid Lys-rich K segment and an S segment consisting of a tract of Ser residues at the amino terminus (Close 1996). It is expressed mainly in the leaf and also appears to be regulated by ABA-dependent pathways.

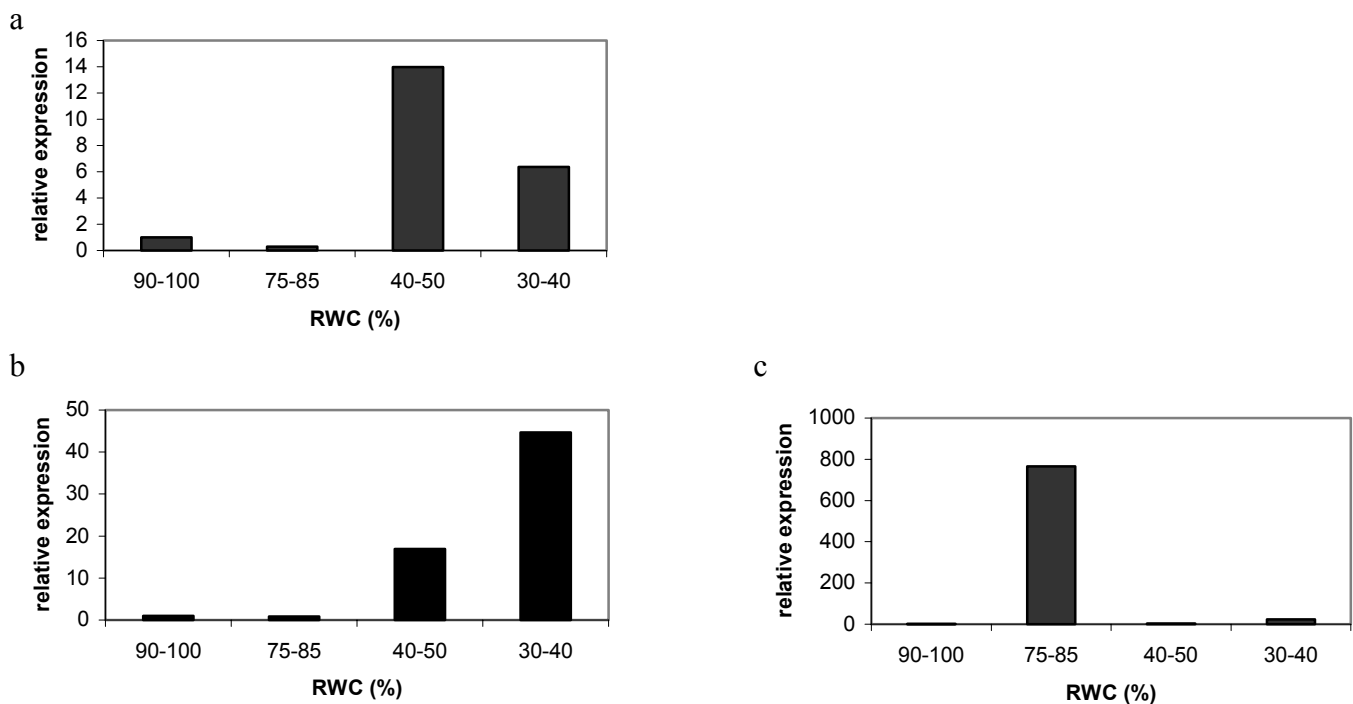


Figure 2. Results of transcript levels against different RWC ranges for (a) *GLUP003B09*, (b) *GLUP003F11* and (c) *GLUP004D08*. Each range consists of cDNA from leaves of at least 5 plants within that RWC range. Results are normalised to 26S rRNA transcript levels and relative to the control range (90-100% RWC) which is given a value of 1.

Expression of *GLUP004D08*

GLUP004D08 transcripts were close to undetectable at 90-100% RWC but at 75-85% there was a drastic increase followed by a return to nearly undetectable levels at 40-50% (**Fig. 2c**). Even at its highest level, *GLUP004D08* transcripts were relatively low in abundance. This expression pattern may indicate that *GLUP004D08* plays a role in signalling during the early drought response. The levels of transcripts present in the seed and in the leaf and stem under well-watered conditions were similar. When water was withheld there is a marked increase in the leaf and a smaller but significant increase in the stem too. Treatment with salt and ABA did not appear to affect the levels of *GLUP004D08* transcripts, which suggests that it acts independently of ABA and may be involved in a non-specific stress response. Sequence analysis of the deduced amino acid sequence revealed a region of significant similarity (E-value of $2e-05$) to the Glycosyl hydrolases family 18 conserved domain. This family of enzymes includes chitinases (Perrakis et al 1994).

Conclusion

This study has proved an effective means of isolating novel drought-induced genes. The challenge remains to demonstrate which (if any) of these genes has the potential improve the drought tolerance of plants, or alternatively if they can be used to develop useful markers for drought resistance in the soybean breeding program. Over-expressing and silencing of the genes via transgenic techniques provides a convenient means for testing the function of these genes. This work is currently in progress together with experiments to test for differential expression of homologues of these genes in different cultivars of soybean that vary in their resistance to drought.

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