SZENT ISTVÁN UNIVERSITY

Analysis of function and regulation of the drought-specific *DS2* gene in *Solanaceae* species

PhD thesis

Róbert Dóczi

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PhD school:	**Plant Sciences**
Field of science:	Plant cultivation and horticulture
Head of school:	**Prof. Dr. Ferenc Virányi DSc** Szent István University Department of Plant Protection
Sci Secretary	**Dr. Gábor Gyulai, PhD** Associate Professor. PhD Biology SZIU, Department of Genetics and Plant Breeding Gödöllő
PhD program:	Plant Breeding by Genetic and Biotechnological Methods
Head of program:	Prof. Dr. László Heszky DSc academician Szent István University Department of Genetics and Plant Breeding
Supervisor:	Dr. Zsófia Bánfalvi PhD Agricultural Biotechnology Center

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Approved by the head of school Approved by the head of program

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Approved by the supervisor

BACKGROUND, AIMS

Drought is one of the most systematic plagues affecting agriculture. It is estimated that global crop losses due to drought exceed $10 billion annually. Hungarian agriculture also frequently suffers from droughts. Meanwhile our knowledge about the biology of drought tolerance rapidly expands.

Plants have evolved an array of physiological and biochemical responses to drought and other environmental stimuli, which are generated by significant changes in gene expression. In the last 10-15 years several drought-inducible genes were described from different species. Products of these genes have the following main functions: [1] Non-enzymatic proteins and low molecular weight compounds that stabilise membranes and other macromolecules and enhance the water binding capacity of the cell (LEAs, chaperones, protease inhibitors, osmolites). [2] Binding of excess ions (LEAs). [3] Enhancing water uptake (aquaporins). [4] Removal of unnecessary or damaged proteins (ubiquitins, proteases).

Adaptation processes triggered by different environmental stresses are achieved through reprogramming of the gene expression pattern. This

is controlled by several signal transduction pathways. Analysis of stress- and hormone-inducible genes revealed that different environmental stimuli often induce the same genes. On the other hand analysis of signal transduction pathways revealed that the same signalling elements can participate in the signalling of different stimuli. Thus, these pathways are not separate units but parts of a complex regulatory network, where specificity is rather an exception than a rule. This enables a fine-tuning of adaptive responses and signalling of many possible environmental conditions becomes possible by the combination of relatively few signalling elements. Of course overlaps in gene expression can also be explained by the fact that adaptation to different kinds of stresses often requires the activity of the same genes. Cross-talk between different signalling pathways has become a subject of intense research in recent years. Most drought-induced genes are also inducible by cold or by ABA treatment, and many elements of this kind of regulation are well known. However, neither *cis*-elements nor transcription factors and further upstream signalling elements are known of the pathway that regulates the *erd1* gene of *Arabidopsis* independently of both ABA and cold.

Enhanced drought tolerance can be engineered either by introduction of foreign genes or by optimising regulatory processes. Both approaches have already produced transgenic plants with significant increases in stress-tolerance. By these means not only yield quantity but as well quality can be improved. Reduced irrigation requirement is also important from an aspect of environmental protection. Thereby potentials

of traditional plant breeding for drought-tolerance can be significantly expanded by biotechnological means, which provides an effective way to utilise the limited water resources of Earth.

Research on the molecular biology of water-loss in the Agricultural Biotechnology Center, Gödöllő, Hungary was initiated by the isolation of the *DS2* gene by Dániel Silhavy in the research gruop led by Dr. Zsófia Bánfalvi. The DS2 cDNA isolated from the wild potato species, *S. chacoense* showed high homology to members of the *ASR* gene family. The function of *ASR*-homologous genes is unknown, however their characteristic feature is that they are induced by ABA, stress and ripening. Similarly to other ASR genes, *DS2* is also strongly induced by drought, however, it is not inducible by ABA. As the production of stress-tolerant crops requires stress-inducible promoters besides the genes conferring tolerance, the drought-specific nature of *DS2* induction turned our attention to the regulation of this gene.

Based on the above our research work had a double aim:

[1.] To verify a role for *DS2* in water stress by its constitutive expression and by antisense inhibition in transgenic potato plants.

[2.] Dissection of the regulation of *DS2* by analysis of its inducibilty by different stresses related to water stress, and by isolation and characterisation of the promoter of the gene.

MATERIALS AND METHODS

Living materials

Escherichia coli DH5α or JM109 strains were used for molecular cloning. *Agrobacterium tumefaciens* C58C1 strain with pGV2260 was used for plant transformations and transient expression assays. The following plant species and cultivars were used for our experiments: *Solanum tuberosum* cv. White Lady and cv. Désirée, *Solanum brevidens*, *Lycopersicum esculentum* cv. K252 and cv. Korall, *Nicotiana benthamiana*. Plants were cultivated in pots either in greenhouse or in phytotron. *In vitro* plant materials were propagated from cuttings on MS media with 16/8 h light/dark periods and at 24°C temperature. Treatments of plants with different environmental stimuli and chemicals were carried out following procedures available in the literature.

Molecular biology methods

Isolation and manipulation of nucleic acids (nucleic acid preparation, restriction endonuclese cleavage, electrophoretic separation, cloning into vectors, polymerase chain reaction, hybridisation techniques, sequencing, plant transformation, transient gene expression assay) were

carried out following techniques commonly used in molecular biology as well as by following guidelines provided by the suppliers.

RESULTS

Detection of presence and copy number of DS2-*homologous genes in solanaceous species*

The DS2 cDNA clone was isolated in our group from the wild potato species, *S. chacoense*. Southern hybridisation was applied to test the presence of sequences homologous to *DS2* in other related species. Using either *Eco*RI, *Hin*dIII or *Xba*I digested genomic DNA one major hybridising band was detected in *S. tuberosum* cv. White Lady, *S. brevidens* and in the tomato cultivars Korall and K252. In the case of *S. tuberosum* cv. Désirée one major hybridising band was detected in *Xba*I digested genomic DNA and 3 hybridising bands with equal, however, weaker intensity appeared when the DNA was digested with either *Eco*RI or *Hin*dIII. In the most distantly related relative, *N. benthamiana*, only a barely visible, very weak band was detected. Consequently, genes with high homology to *DS2* – with the exception of tobacco - are present in these species.

Experiments to determine the function of DS2

Transgenic potato plants were generated with constructs that either

constitutively overexpress the gene or inhibit *DS2* expression by producing antisense RNA. If the DS2 protein has an essential function in desiccation tolerance then to some extent the overproducing lines should have an increased while the antisense lines should have a decreased drought-tolerance. Northern hybridisations revealed that of 15 sense lines 12 showed a very high basal *DS2* expression, while drought-induced *DS2* expression was significantly reduced in 4 of 25 antisense lines. However, no alterations in the stress-tolerance of the transgenic lines could be detected. This is most probably due to the high redundancy of stress-induced proteins in plants.

Response of DS2 *expression to different environmental stimuli*

Expression of *DS2* in potato and tomato was studied by northern hybridisation. We demonstrated that the gene in the cultivated potato and tomato is drought-inducible, and this induction is independent of ABA, as it could not be achieved by exogenous ABA treatment. High levels of *DS2* expression were detected in the ABA-deficient mutant tomato line, *sitiens*. This further demonstrates that regulation of the gene is ABA-independent. Thus, even though sequence homology suggests similar functions for *DS2* and the other *ASR*-homologous genes, there is a remarkable difference between their regulations.

There are several connections between signal transduction mechanisms of different environmental stimuli; therefore we investigated the effect of the most important abiotic stresses on *DS2* expression. We

found that *DS2* expression is drought-specific, as the gene was not inducible by salt, cold, heat, hypoxic or oxidative stresses. This is a rather unique phenomenon, considering the intense communications between the different signalling pathways. The ABA-mediated or cold-inducible drought signalling pathways are well characterised in *Arabidopsis*, however, little is known about pathways that act independently. These results prompted us for further characterisation of *DS2* expression by isolation and analysis of its promoter.

Furthermore, we demonstrated that expression of *DS2* can be blocked by application of cycloheximide - a potent protein synthesis inhibitor -, so *DS2* induction requires *de novo* synthesized protein factors.

Isolation and analysis of DS2 genomic clones

In order to characterise the regulatory region we isolated the *DS2* gene and analysed its sequence. White Lady potato genomic library was screened with a *DS2*-specific probe, by plaque hybridisation method. Three fragments containing different lengths of the putative promoter region were cloned into plasmid vectors. They contained 234 bp (pDS2G clone), 498 bp (pDS2H) and 1140 bp (pDS2J) upstream of the translational start codon. The 1140 putative promoter region is not homologous to any known sequences (accession number: AJ320154).

Generation of StDS2 promoter::GUS fusion constructs

In order to verify the transcriptional activity of the isolated sequence the three promoter fragments were fused to the *GUS::NosT* reporter construct. The different promoter fragments of the clones DS2G-H-J were amplified by a PCR primer specific to the –1 - –20 sequence together with a vector-specific primer. They were fused upstream to the *GUS*::*NosT* cassette in a plant transformation vector. The resulting clones were designated according to the lenght of their promoter fragments: pGGUS (234 bp), pHGUS (498 bp) and pJGUS (1140 bp).

Detection of promoter activity in transgenic potatoes

Transgenic potato lines carrying either GGUS, HGUS or JGUS construct and the kanamycin selection marker gene *npt*II, were generated via *Agrobacterium*-mediated leaf transformation. Probably due to technical reasons no shoots were regenerated with the GGUS construct, while 18 JGUS and 97 HGUS kanamycin-resistant transgenic potato lines were obtained. Activity of the *DS2* promoter was individually tested in each line by histochemical staining of PEG-treated detached leaves. 2 JGUS and 2 HGUS lines with good histochemical staining were used in further experiments.

The plants were propagated *in vitro* then transferred to pots in the greenhouse and watered regularly. After dehydration achieved either by withdrawing water from the plants for one week or by PEG treatment of detached leaves, GUS activity of all four *StDS2* promoter-containing

transgenic lines increased markedly. This indicates that both fragments carry the cis-elements required for drought-inducible transcriptional activity. On the other hand, GUS activity did not change after ABA or cold treatments. Thus both promoter fragments are sufficient to maintain the specific nature of *StDS2* regulation.

Detection of promoter activity in tobacco

A transient gene expression assay was applied to test the activity of the 498 bp promoter region in another species. *N. benthamiana*, also belonging to the *Solanaceae* family, was known to be especially suitable for an *Agrobacterium* infiltration assay and in its genome we could only detect a very weak cross-hybridisation with *DS2*. *Agrobacterium* susupension carrying the HGUS construct was infiltrated into *N. benthamiana* leaves. The samples were then either dried or well watered, and the activity of the GUS gene was visualised by X-Gluc staining. The dried leaves showed much more intense blue staining than the negative controls.

The signal transduction mechanism regulating *DS2* expression is therefore probably ubiquitous in the *Solanaceae* family, independently of the presence of the *DS2* gene. Thus, we assume the existance of other genes, which are regulated by this mechanism.

Novel results

1. Genomic Southern hybridisation was applied to test the presence of *DS2* homologous genes in different species. We concluded that the gene is present in the cultivated potato, in a wild tuberless potato species, *S. brevidens* and in tomato, meanwhile only a very weak cross-hybridisation was detected in tobacco. *DS2* is probably a one or low copy number gene.
2. Function of the gene was tested by altering its expression level. Transgenic potato lines were generated that either constitutively overexpress the gene or inhibit *DS2* expression by producing antisense RNA. However, alterations in expression level did not result in an altered phenotype during drought-stress, therefore the function of the gene remains obscure.
3. Regulation of the gene was first studied by Northern analysis. We found that water loss – irrespectively of whether it is a result of drought or of PEG treatment – induced a very high *DS2* expression. This induction is independent of ABA and of other abiotic stresses, such as salt, cold, heat, oxidative stress or hypoxia. Cycloheximide treatments revealed that the induction of the gene requires de novo synthesised protein factor(s). *DS2* has an identical expression pattern in potato and tomato.
4. For further analysis of the regulation of the gene we isolated its genomic copy from *S. tuberosum*. The *StDS2* gene contains an intron of 263 bp, and compared to the 5´ truncated *S. chacoense*

cDNA there are 14 additional bases up to the translation initiation site. There is 99,35% homology between the two genes at the nucleic acid level, which results two substitutions at the amino acid level. 1140 bp of putative promoter region was isolated, that is not homologous to any known sequences.

5. In order to study the activity of *StDS2* promoter 3 plant transformation vector constructions were generated. These contained 234 bp (pGGUS), 498 bp (pHGUS), or 1140 bp (pJGUS) of *DS2* promoter in front of the *GUS* reporter gene.
6. Following *A. tumefaciens* mediated plant transformation 2-2 transgenic lines were isolated that carried the HGUS or JGUS constructs, and GUS activity of these lines could be elevated by drought. This induction maintained specificity as, it was unaffected by ABA or cold, just as in the case of the *DS2* gene. Therefore 498 bp of the *StDS2* promoter carries the *cis*-elements required for *DS2*-like regulation.
7. An *Agrobacterium* infiltration assay revealed that this 498 bp of the *DS2* promoter region is also functional and drought-induced in tobacco.

CONCLUSIONS AND SUGGESTIONS

Previously in our lab a gene was isolated from the wild potato species, *S. chacoense*, designated *DS2*, that is strongly induced by drought in an ABA-independent fashion. The aim of our work was to analyse the function and regulation of this gene.

Function of *DS2* was studied by altering the expression level of the gene in transgenic potato plants. This is a widely applied method to test genes with unknown function. However, altering the expression level in the case of *DS2* did not result in any phenotypic changes. It is possible that due to the high redundancy of stress-proteins any effect caused by changes in gene expression is masked by the effects of other genes. Nevertheless, sequence data and expression pattern serve as indirect evidence for the function of *DS2*. Its role as an actual stress-protectant could perhaps be verified by alternative experimental approaches, such as expression of the gene in yeast. However, considering the stress-protectant mechanisms already existing in yeast, it is also possible that the presence of the DS2 protein would not result in a detectable improvement. This could be overcome by expression of *DS2* in stress signalling mutants (e.g. *SNF*, *Mig*, *Hog*) with impaired stress-gene expression.

We demonstrated that in potato and tomato *DS2* is significantly induced by drought, independently of ABA and other stress factors. The *DS2* gene and 1140 bp of its promoter region (*StDS2*) was isolated from the genome of *S. tuberosum*. Furthermore, by using a GUS reporter gene, it was demonstrated that 498 bp of this sequence carries the *cis*-elements

responsible for specific expression. Several transgenic plants with improved stress-tolerance have been produced by overexpression of osmolites and transcription factors. At the same time these plants generally manifest significant growth retardation in comparison with the wild type plants. The reason for this is the application of constitutive promoters, as these plants waste resources for transgene expression and osmolite synthesis even if it is not required. Biotechnological developments of this kind therefore necessitate the application of stress-inducible promoters. By the predicted spread of GM crops a demand for stress-tolerant varieties will possibly emerge. Although a number of stress-inducible promoters have been isolated, the *StDS2* promoter has such a high degree of specificity, that could prove to be a real advantage. Another good reason for searching for alternative solutions is the fact that the isolated sequences are often patent protected. Means for practical application of the *DS2* promoter are already under investigation in our group. The yeast gene *TPS1*, responsible for trehalose biosynthesis, was fused to the *DS2* promoter for generating transgenic plants with this construct.

Besides practical applications further studies on *StDS2* promoter could contribute to dissection of drought signalling. Elements of the ABA-mediated and cold inducible signalling pathways are well described. On the other hand neither *cis*- nor *trans*-elements are known of the mechanism(s) that regulate genes independently of these factors. One possible approach to identify *cis*- and *trans*-elements regulating *DS2*

could be the application of band-shift, protein footprint and then yeast one-hybrid techniques.

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