

Gene mining in halophytes: functional identification of stress tolerance genes in *Lepidium crassifolium*

Summary of PhD thesis

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INTRODUCTION

Extreme environmental conditions limit plant growth and impose abiotic stress to plants. Land degradation, including desertification, drought and salinity affects around one third of the global land surface (Jarraud 2005). Adaptation of plants to suboptimal conditions requires extensive physiological and molecular reprogramming, leading to major changes in metabolic, proteomic and transcript profiles.

Research on model organisms such as *Arabidopsis thaliana* and application of system biology approaches has identified a number of genes and regulatory hubs which control the networks linking stress perception and metabolic or developmental responses (Ahuja et al. 2010). However, study of a stress sensitive model has limitations in understanding tolerance to harsh environments. Extremophile plants, such as xerophytes and halophytes can grow in arid regions or on saline soils, which are otherwise lethal to nonadapted species. Halophytes represent 1% of all plant species; can optimally thrive in the presence of 50–250mM NaCl, whilst some withstand salt concentrations up to 600mM NaCl (Flowers and Colmer 2008). While the physiology of halophytes has been extensively studied, molecular regulation of the extremophile character still remains to be understood. *Eutrema salsugineum* (previously called *Thellungiella salsuginea*) is a salt tolerant relative of *Arabidopsis*, which has been used in a number of comparative studies to reveal the genetic and molecular basis of halophytism (Amtmann 2009). Natural genetic variability of extremophiles is an attractive genetic resource to improve tolerance of crops to adverse environments (Nevo and Chen 2010). Transfer of tolerance traits to other species is however usually hampered by incompatibility. Transformation of genomic or cDNA libraries can facilitate random gene transfer between different species. Examples include a cDNA library of *E. salsugineum*, expressed in *Arabidopsis*, leading to the identification of several *Eutrema* genes which improved salt tolerance (Du et al. 2008). A binary bacterial artificial chromosome library was used to transfer large genomic fragments of *E. salsugineum* to *Arabidopsis* and screen for salt tolerance (Wang et al. 2010).

Here, we describe the novel version of the Conditional cDNA Overexpressing System (COS), which was developed to randomly transfer and express cDNA clones in *Arabidopsis* under the control of a chemically inducible promoter system (Papdi et al. 2008; Rigó et al. 2012). The cDNA library was derived from the less-known halophyte of the *Brassicaceae* family

Lepidium crassifolium, which naturally grows on salty-sodic soils in Central Europe and Asia. Random transfer and overexpression of *L. crassifolium* cDNA in *Arabidopsis* could facilitate the identification of novel tolerance genes. Here, we demonstrate that regulated expression of several *L. crassifolium* cDNA could enhance salt, osmotic or oxidative stress tolerance of *Arabidopsis*. The COS system is therefore suitable for interspecific gene transfer and can be employed to identify valuable genes from less-known wild species.

AIMS

General aim of this project was to identify novel genes from a poorly known salt and drought tolerant plant species *Lepidium crassifolium* and to elevate stress tolerance of a sensitive species with specific transfer of *Lepidium* genes. *Arabidopsis thaliana* a widely known model plant was used as salt sensitive species, and the salt and drought tolerant *Lepidium crassifolium*, was our gene source.

Our particular aims were:

To modify the previously available Conditional cDNA Overexpression System (COS), to allow random transfer of *Lepidium* genes into *Arabidopsis*.

To develop novel screening systems to identify transgenic *Arabidopsis* plants with improved tolerance to osmotic, ionic or oxidative stresses.

To isolate and identify *Lepidium* cDNAs from transgenic *Arabidopsis* plants showing elevated stress tolerance.

To verify the capacity of the identified *Lepidium* cDNAs to enhance stress tolerance in independent transgenic *Arabidopsis* lines, which overexpress the cloned cDNAs under the control of constitutive pCAMV35S or stress inducible RD29A promoters.

METHODS

- Growing *A.thaliana*, *E. salsugineum* and *L. crassifolium* plants.
- Generation of transgenic *Arabidopsis* plants by *Agrobacterium*-mediated transformation.
- Analysis of abiotic stress tolerance *in vitro* (salinity, osmotic, oxidative stress) and in greenhouse.
- Analysis of chlorophyll fluorescence by Image PAM.
- Molecular cloning of *Lepidium* cDNAs.
- Isolation of plant genomic DNA and total RNA.
- Investigation of gene expression levels with Northern blot analysis.
- Measurements of chlorophyll fluorescence and chlorophyll content.
- Measurements of growth rate and rosette area with PlantSize software.
- Identification of T DNA insertion sites with TAIL-PCR technique.

RESULTS

Extremophile plants are valuable gene sources of tolerance traits that have different important roles in stress tolerance. Through the application of these genes, we can elevate the stress tolerance of crop plants. We have employed the modified Conditional cDNA Overexpression System (COS) to transfer a cDNA library from *Lepidium crassifolium* to the glycophyte *Arabidopsis thaliana*.

Development of the gene identification system

We characterized salt and drought tolerance of *Lepidium crassifolium* plants and we compared it with closely related *Eutrema salsugineum* and *Arabidopsis thaliana* in controlled conditions. We examined the changes in root length, in photosynthetic activity and in proline content of the plants, during different salt treatments. Results could confirm extremophile nature of *L. crassifolium*, suggesting that this species can be a valuable source of stress tolerance genes.

40 000 transgenic *Arabidopsis* lines were generated with the COS system, which carried random cDNAs of *L. crassifolium*. The *Lepidium* cDNA overexpressing *Arabidopsis* plants were screened for tolerance to oxidative, salt and high osmotic stress. The screens were done *in vitro*

and growth rate or chlorophyll fluorescence of plants were used as screening criteria. In order to identify new genes, we developed a high throughput and non-invasive new screening system based on chlorophyll fluorescence imaging. The maximal PSII quantum yield (Fv/Fm) and the effective PSII quantum yield (Φ PSII) were the most characteristic parameters for chlorophyll fluorescence based screening. Changes in rosette size or color were quantified with our own developed computer application called PlantSize, a MatLab based computer software. We screened all the 40000 transgenic lines, and identified 20 lines with elevated salt, osmotic or oxidative stress tolerance. cDNA clones were amplified from selected lines and the identity of the clones were determined by DNA sequencing. The 82% of inserted DNA were full length with full open reading frame, and in 18% they have missed the 5' end of cDNA. Sequence identity of the predicted *Arabidopsis* and *Lepidium* amino acid sequences was over 90%, confirming close phylogenetic relationship between these species. Extensive testing was performed to confirm the tolerance characteristics associated with the identified genes. We introduced the identified cDNA sequences into plant expression vectors, which overexpressed the cDNAs under the control of constitutive pCaMV35S or stress-induced pRD29A promoters. We transformed wild type *Arabidopsis* plants with these constructs in order to confirm the responsibility of cDNAs in elevated stress tolerance of *in vitro* and greenhouse grown plants.

Examples of gene identification

Lines with estradiol-dependent stress tolerance.

In line PL127P4 the identified full length cDNA encoded a protein which shows high homology to an *Arabidopsis* Acyl CoA binding protein (ACBP6). The *Arabidopsis* ACBP proteins were shown to regulate phosphatidylcholine and phosphatidic acid levels, modulate phospholipase D and ABA signaling. The PL127P4 line showed elevated stress tolerance and had higher Fv/Fm value during osmotic stress treatment only in the presence of estradiol. The predicted *Lepidium* protein has a typical ACBP domain. Such proteins are involved in phosphatidylcholine and Acyl-CoA binding, protecting acyl-CoAs from degradation by microsomal acyl-hydrolases and functioning as intracellular carriers of Acyl-CoA esters. ACBPs were implicated in post-stress membrane repair mechanisms as well.

The PL542Na1 line was derived from a plant, which grew better on high salt medium. PL542Na1 plants were more tolerant to salt stress than Col-0 in the presence of estradiol, but were similar to wild type in the absence of the inducer. Based on the inserted cDNA, the predicted amino acid sequence showed the highest similarity to the GDSL-like lipase/acylhydrolase family protein MVP1/GOLD36/ERMO3, encoded by *ATIG54030* in *Arabidopsis* and were named LcMVP1. The AtMVP1 is implicated in maintenance of endoplasmic reticulum integrity, protein trafficking and endoplasmic reticulum-related defences.

In several lines tolerance to the selected trait was not dependent on estradiol induction of the inserted cDNA. In such lines either T-DNA insertion or an independent mutation can be responsible for the observed phenotype.

The line PL304Na01 was identified by screening for enhanced growth on high salt medium. The line showed enhanced tolerance to salt, sorbitol and paraquat under *in vitro* conditions. To identify the T-DNA insertion site in the *Arabidopsis* genome, the flanking genomic region was amplified by TAIL-PCR, sequenced and the insertion mapped by sequence homology search. The T-DNA insert in this line was localized into the 1st intron of the 5' UTR region of *ATIG31830* gene, 135bp upstream from the ATG. *ATIG31830* encodes PUT2, Polyamine uptake transporter 2, an amino acid permease family protein. Interestingly, the protein was recently described as PARAQUAT RESISTANT 1 (PAR1), which was shown to be involved in paraquat transport. Mutation of PAR1 reduced paraquat sensitivity, while overexpression has enhanced it.

Chlorophyll fluorescence of salt-stressed PL803Na3 plants was less affected than wild type. While Fv/Fm values of Col-0 plants gradually decreased during salt irrigation, Fv/Fm of PL803Na3 was only slightly reduced. Mapping the T-DNA insertion by sequencing the flanking genomic region revealed that it took place in the 3' region of the gene *AT2G39010*. The gene encodes the Plasma membrane intrinsic protein 2E (PIP2E, AtPIP2,6), which has water channel activity.

CONCLUSION

We demonstrated that the properly designed COS system is suitable to explore natural variability of wild species, facilitate interspecific gene transfer and contribute to our efforts to understand molecular bases of drought and salt tolerance. Identified genes can further be utilized as molecular tools to improve stress tolerance of crops.

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PUBLICATION LIST

This PhD thesis is based on these articles:

Rigó G. és Valkai I., Faragó D., Kiss E., Van Houdt, S., Van de Steene, N., Hannah, M., Szabados L. (2016). Gene mining in halophytes: functional identification of stress tolerance genes in *Lepidium crassifolium*. *Plant Cell Environ.* 39:2074–2084. doi.10.1111/pce.12768. IF: 6,960

Faragó D., Sass L., Valkai I., Andrási N., Szabados L. (2018) PlantSize offers an affordable, non-destructive method to measure plant size and color in vitro. *Frontiers in Plant Science*, 9:219. <https://doi.org/10.3389/fpls.2018.00219>, IF:4,298

Other publications

Baba, A. I., Rigó G., Ayaydin F., Rehman A., Andrási, N., Zsigmond L., Valkai I., Urbancsok J., Vass I., Pasternak T., Palme K., Szabados L., Cséplő Á. (2018) Functional analysis of the Arabidopsis thaliana CDPK-related kinase family: AtCRK1 regulates responses to continuous light. *Int. J. Mol. Sci.* 2018, 19(5), 1282; doi:10.3390/ijms19051282, IF: 3.226

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