# Development of novel genetic strategy for the identification of genes regulating Arabidopsis stress responses

Summary of Ph.D. thesis

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## INTRODUCTION

As sessile organisms, plants encounter a great range of environmental changes during their life cycle. Drought, high salinity and low temperature are the most common environmental stress conditions that influence plant growth and development. Higher plants have evolved sophisticated mechanisms to cope with the external insults. Perception of stress initiates a network of stress-induced responses, which is controlled by a complex web of signalling pathways and results in gene expression changes. Most of our knowledge of these regulatory mechanisms has been derived from molecular genetic analysis of the model plant *Arabidopsis thaliana*.

Receptor molecules involved in primary perception of abiotic stress stimuli are at present largely unknown in higher plants, with the exception of AtHK1, a putative two-component hybrid histidine kinase osmosensor, which is implicated in osmosensing under salt stress (Urao et al. 1999, *Plant Cell* **11**: 1743). The transmission of primary stress signals, sensed by receptors to downstream effector molecules is far for being understood. Numerous downstream signalling components have been identified including hydrogen peroxide, phospholipid secondary messengers, and cytosolic $Ca^{2+}$ oscillation, which control the activity of CDPK, SnRK and MAPK-type protein kinase phosphorylation cascades.

Abscisic acid (ABA) phytohormone plays a central role in the control of abiotic stress responses. ABA controls gas exchange and photosynthesis by triggering stomatal closure, induces the synthesis of seed storage proteins and lipids, promotes seed desiccation tolerance and dormancy, inhibits transition from embryonic stage to germination, and influences other aspects of plant development as well. ABA stimulates the expression of many drought and

osmotic stress-induced genes through complex signalling mechanisms.

Different genetic strategies have been developed for the identification of plant genes controlling environmental stress responses. A number of examples demonstrate that functional characterization of Arabidopsis genes is greatly facilitated by insertion, deletion or point mutation, most of which result in loss-of-function phenotype. Such knockout mutations provide information about gene functions through the phenotype generated by the absence of the functional gene product. One of the limitations of the knockout or knockdown mutations is that knocking out those genes that are redundant usually does not produce detectable phenotype. In the plant genome many genes are duplicated or arranged in gene families with at least partially overlapping functions. Although many duplicated genes show some degree of transcriptional divergence, most of them share common expression pattern and similar function. Essential genes represent a different problem, as their mutations often lead to embryo lethality therefore their functional analysis is difficult or impossible (Errampali et al. 1991, *Plant Cell*, **3**:149).

Gain-of-function mutations can be generated by point mutagenesis leading to alteration in protein function, or by enhanced expression of the mutated gene. Activation tagging using specially engineered T-DNA vectors containing strong, multiple constitutive enhancers has been applied for generation of dominant mutations that may reveal the functions of gene family members (Nakazawa et al. 2003, *Plant J*, **34**: 741). Upon integration into the vicinity of a gene, the enhancer elements can increase the transcription level of the target gene, which may result in dominant, constitutively altered phenotype. In order to activate a plant gene with a foreign enhancer or promoter, the T-DNA has to be inserted in a proper orientation into the vicinity, but not in the coding region of the target gene, which could affect its activity. However, the enhancer sequences may affect transcription of several genes located in the vicinity of the insert, leading to complex altered phenotype. Another approach is to generate

gain-of-function alleles by genetic transformation of the selected plants using transformation competent cDNA library. Constitutive over-expression of the randomly inserted cDNA clones may lead to dominant phenotypes, which can be identified in appropriate genetic screens. However uncontrolled over-expression of the introduced cDNA may complicate the analysis of the affected gene if high transcriptional activity leads to reduced plant viability, pollen sterility or embryo lethality. Constitutive activation of stress regulatory genes for example, can reduce cell proliferation and result in dwarf, sterile plants (Kasuga et al. 1999, *Nat Biotechnol*, **17**: 287; Gilmour et al. 2000, *Plant Physiol*, 124:1854)

We have developed a novel genetic strategy, which is suitable for the identification of unknown genes regulating special stress responses. We have constructed a controlled cDNA over-expression system by Gateway cloning of an Arabidopsis cDNA library into the chemically inducible pER8-GW plant transformation vector (Zuo et al. 2000. *Plant J*, **24**: 265). The cDNA library was introduced into Arabidopsis plants containing *ADH1-LUC* reporter, to screen for activation of the *ADH1* (stress-responsive alcohol dehydrogenase) promoter.

## OBJECTIVES

The aim of my work was to develop a novel genetic strategy, which is suitable for the identification of transcriptional regulation of known stress response genes. The system is composed of two components. One component is the promoter of a stress response gene fused to a reporter gene; the other is a controlled cDNA expression library.

- Construction of transformation competent Arabidopsis random cDNA library;

- To clone the *ADH1* promoter and fuse to firefly luciferase gene;
- To generate transformant Arabidopsis plants containing the *ADH1-LUC* reporter;
- To transform ADH-LUC Arabidopsis line with the cDNA library;
- To identify cDNA clones, which when over-expressed induce the activity of *ADH1-LUC* reporter;
- Characterization of at least one of the factor inducing the *ADH1* gene.

## METHODS

- *In vitro* and *in vivo* culture of *Arabidopsis thaliana*;
- Agrobacterium mediated genetic transformation of Arabidopsis, selection and maintainance of transgenic plants;
- Molecular cloning techniques based on enzymatic manipulation of DNA;
- Plant genomic DNA and total RNA extraction;
- Gene expression studies: semiquantative and Real-time RT-PCR;
- Measurements of *in vivo* luciferase enzyme activity in intact seedlings;
- Histochemical staining (GUS reporter gene activity, alcohol dehydrogenase enzyme activity).

## RESULTS

1. An Arabidopsis cDNA library was constructed and cloned into the estradiol inducible pER8-GW plant expression vector. Subsequently, the pER-GW/cDNA library was transformed into competent Agrobacterium cells and was used for genetic transformation of Arabidopsis plants. After

sequencing randomly selected cDNAs we concluded, that more than half of the cDNA population are full-length.

2. To investigate the regulation of specific stress signaling events, the *ADH1-LUC* reporter gene was constructed, in which the promoter of the Arabidopsis *alcohol dehydrogenase 1* (*ADH1*) was fused to the firefly luciferase gene. Transgenic Arabidopsis lines were generated carrying single locus insertions of *ADH1-LUC* reporter construct, and their reporter gene activity was tested based on ABA treatment. The line number 16 was selected, in which the basal *ADH1-LUC* activity was low, under standard culture conditions, whereas the treatment of seedlings with 20μM ABA, 200mM NaCl, 400mM sucrose, 300mM mannitol or 10mM hydrogen peroxide resulted in reproducible, high level induction of light emission due to the activation of *ADH1-LUC* reporter. The ADH-LUC line showed a similar pattern of luciferase expression in response to ABA and during stress conditions, which started with transient increase in bioluminescence, followed by gradual decrease in *ADH1-LUC* expression within 3 to 4 hours. Interestingly, the ABA treatment caused faster increase in reporter gene activity than other stress conditions.

3. To perform genetic screens for the identification of novel factors involved in transcriptional regulation of *ADH1* gene, the ADH-LUC line was transformed with the inducible cDNA library. 20,000 hygromycin resistant T1 seedlings were screened and 11 plants were identified displaying enhanced luciferase activity. Further, testing of these lines confirmed, that 2 of them had estradiol dependent changes in bioluminescence intensity. One of the 2 lines, ADH/121 was chosen for further characterization. ADH/121 line showed gradual increase in bioluminescence after estradiol treatment alone. However, combined with

20μM ABA caused sustained reporter gene activity for at least 24 hours. In a similar experiment, in the presence of estradiol, transfer of ADH/121 seedlings to culture media supplemented with 200mM NaCl or 300mM mannitol displayed persistent maintenance of high-level luciferase expression for at least 16 hours. However, the parental ADH1-LUC seedlings showed only transient increase in bioluminescence within 3 to 4 hours followed by gradual decrease of *ADH1-LUC* expression in response to combined treatment with either 20μM ABA and estradiol or 200mM NaCl and estradiol.

4. To compare the induction of endogenous *ADH1* gene with activation of the *ADH1-LUC* reporter construct, the ABA and estradiol induced changes were monitored in ADH1 mRNA levels by quantitative RT-PCR, in the ADH/121 line. Combination of estradiol with ABA treatment resulted in increased ADH1 mRNA levels as seen on ABA induction, but the transcript levels failed to decline even 24 hours after the induction. Histochemical staining based on alcohol dehydrogenase activity showed that the induced cDNA could enhance the endogenous ADH enzyme activity.

5. PCR amplification and sequence analysis revealed that a single pER8-GW T- DNA insert in the ADH/121 line carried a full-length cDNA of the *At1g53910* gene encoding RAP2.12, a yet uncharacterized member of AP2/ERF (ethylene responsive element binding factor) transcription factor family.

6. To confirm that the estradiol-inducible activation of RAP2.12 transcription was indeed responsible for the activation of the ADH1-LUC reporter in the ADH/121 line, the isolated cDNA was recloned into the

pER8-GW vector and transformed into the parental ADH1-LUC line again. Most of the ADH1-LUC transformants (10 out of 13) carrying the pER8GW-RAP2.12 construct showed estradiol-inducible luciferase activity, which was similar to the activity of the ADH/121 line.

7. To determine whether RAP2.12 expression correlates with the induction of ADH1-LUC reporter, RAP2.12 mRNA levels were monitored by RT-PCR in the parental ADH1-LUC and the cDNA containing ADH/121 lines. High levels of RAP2.12 transcript were detected only in the estradiol-treated ADH/121 seedlings.

8. To investigate the interaction between of the *ADH1* promoter and the RAP2.12 transcription factor, transgenic Arabidopsis lines carrying point mutated or deleted promoter-binding sites containing *ADH1-GUS* constructs were crossed with the ADH/121 plants. Estradiol induction was able to activate the *ADH1* promoter regulated GUS enzyme activity in the absence of GT, GC, G-Box promoter elements, respectively, suggesting that none of these promoter elements are responsible for RAP2.12 mediated activation of *ADH1*.

9. cDNA molecules encoding the transcription factors RAP2.2 and RAP2.3 showing high sequence similarity to RAP2.12 were cloned into pER8-GW expression vector and transformed into ADH-LUC plants. Both of them could induce the ADH1-LUC activity upon estradiol treatment. This indicates that the biological function of the members of this gene subfamily can be similar or overlapping.

10. Microarray data published in the Genevestigator database were used for comparing the gene expression patterns of *RAP2.2*, *RAP2.3* and *RAP2.12*

in the different organs, tissues and developmental stages of Arabidopsis plants. *RAP2.3* has low expression level in different organs and tissues at any developmental stages. However, the expression pattern of *RAP2.2* and *RAP2.12* is similar in organ and tissue level, mainly expressing in the matured and germinating seeds, hypocotyl and leaf stem. Interestingly, ethylene treatment induces the expression of *RAP2.2* and *RAP2.3* genes but not the RAP2.12.

Our data show that the AP2/ERF transcription factors RAP2.2, RAP2.3 and *RAP2.12* are positive regulators of *ADH1* transcription, but probably act independently of ABA signal transduction.

## LIST OF PUBLICATIONS

**Papdi C**, Ábrahám E, Joseph MP, Popescu C, Koncz C, Szabados L (2008) Functional identification of Arabidopsis stress regulatory genes using the Controlled cDNA Overexpression System. *Plant Physiol*, 147: 528–542.

**Papdi C,** Joseph MP, Pérez Salamó I, Vidal S, Szabados L (2009) Genetic technologies for the identification of plant genes controlling environmental stress responses. *Functional Plant Biology*, 36: 696-720