

**Genetic screening for the mutations affecting *ERF3* expression  
in *Arabidopsis thaliana***

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## INTRODUCTION

Plants are sessile and therefore have to cope with different environmental stresses. One such stress is the salinity, which affects crop production world-widely (Bohnert et al., 1995). To deal with environmental stimuli, plants have to carry out various physiological and metabolic responses (Bartels and Nelson, 1994; Thomashow, 1994; Bohnert et al., 1995). Much progress has been made toward understanding the molecular basis of these responses. Particularly, many genes important for stress tolerance have been cloned in different plant species. These genes could be of high significance in Agriculture because they could be used to genetically improve stress tolerance in crops. Although a number of signaling pathways have also been assigned to various stress responses, how plants perceive and transduce stress signals and how different signaling pathways cross-talk are still largely remained to be elucidated (Chinnusamy et al., 2002).

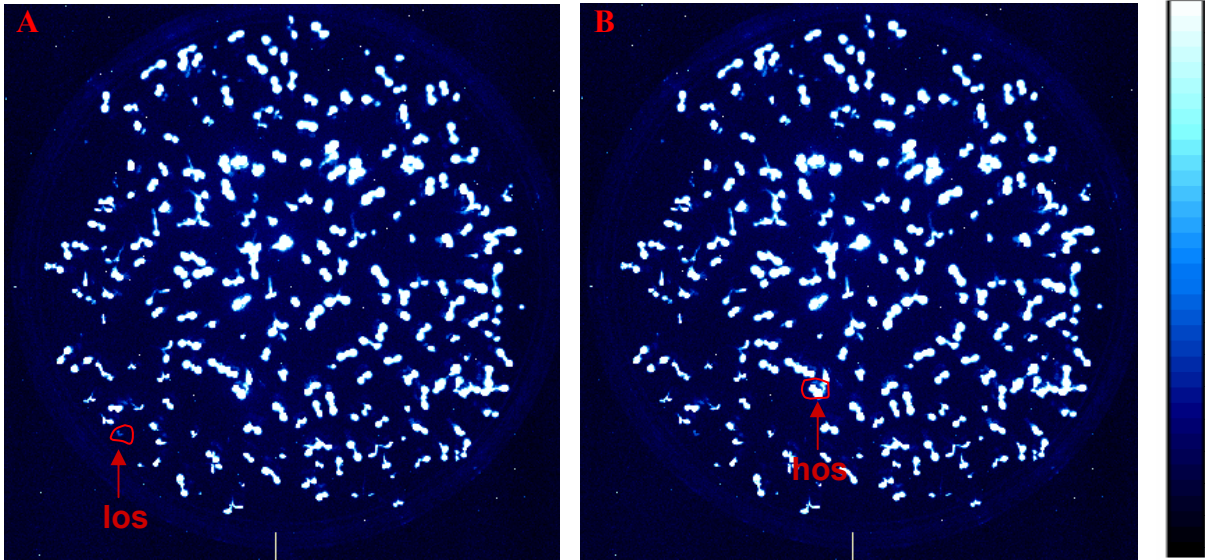
Genetic screening for mutations, affecting signaling control of gene expression in response to environmental stresses, have largely contributed to our understanding in plant abiotic stress response. For example, an elegant mutant screening system using luciferase as a reporter gene fused with stress-inducible promoter of RD29A gene was employed to identify signaling components that control RD29A gene expression in response to abiotic stress and hormone ABA (Ishitani et al., 1997). Many genes that either positively or negatively regulate stress-responsive gene expression have been isolated and functionally characterized by employing this genetic system combined with other molecular research tools (Xiong et al., 2002). This high throughput mutant screening method has been valued as an efficient and versatile mean to identify signaling components controlling gene expression (Chinnusamy et al., 2002).

Ethylene responsive element binding factors (ERFs) are part of a family of transcription factors, that to date, have only been found in higher plants (Fujimoto et al., 2000). The ERF domain, a highly conserved DNA binding domain (Hao et al., 1998), is a unique feature of this protein family. Five different ERF proteins (*AtERF1* to *AtERF5*) encoded by Arabidopsis genes were identified,

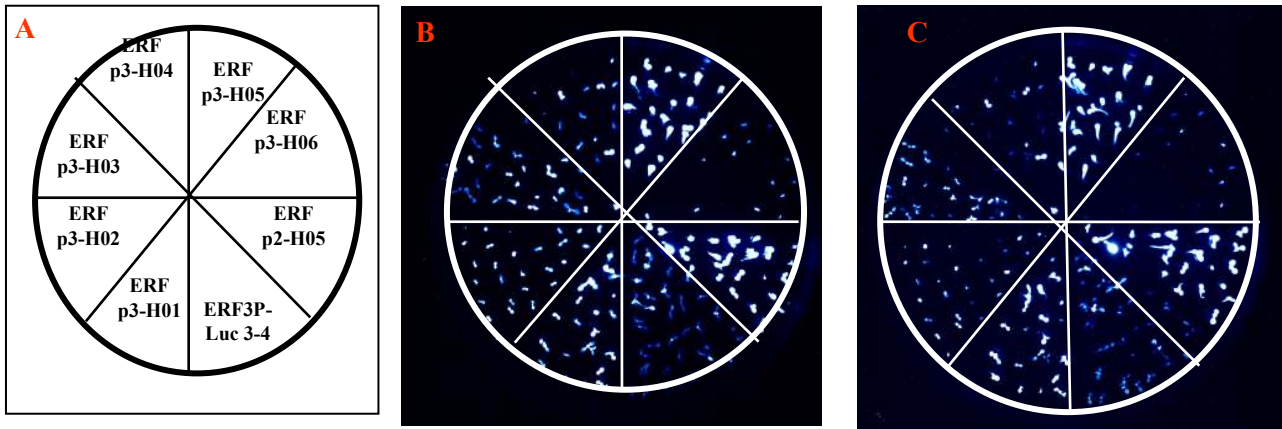
analyzed and placed into three classes based on the amino acid identity within the ERF domain. According to Fujimoto et al. (2000), the *AtERFs* play a role in regulating responses to both abiotic stresses and ethylene. The class II proteins *AtERF3* and *AtERF4* act as transcriptional repressors and use an active repression mechanism. The expression of *AtERF3* gene is moderately induced by salinity and drought stress (Fujimoto et al., 2000). Interestingly, the salt-induced expression of *AtERF3* is partly abolished by the mutation in *EIN2* gene that encodes an essential signaling component in ethylene response (Fujimoto et al., 2000), suggesting a signaling cross-talk between salt stress and ethylene response.

## **PRELIMINARY DATA**

To identify the signaling components controlling *AtERF3* gene expression, we employed the genetic screening method as described by Chinnusamy et al. (2002). The promoter of *AtERF3* was fused with a firefly luciferase reporter gene (*AtERF3P-LUC*). The construct was introduced into *Arabidopsis thaliana* (ecotype Col-0) via *Agrobacterium*-mediated floral-dip transformation method. Homologous lines of transgenic plants harboring the chimeric gene construct were generated. The expression of the luciferase gene driven by the *AtERF3* promoter in these transgenic plants is also induced by NaCl treatment. By using a highly sensitive, low light CCD imaging system, we are able to monitor the luciferase expression level in the transgenic plants *in vivo* through bioluminescence imaging. For genetic screening of mutants with altered NaCl-induced luciferase expression, we generated a M<sub>2</sub> population of ethyl methanesulfonate (EMS)-mutagenized seeds consisting of 20 pools with the genetic background of homozygous *AtERF3P-LUC* transgene. We have completed the mutant screening by identifying seedlings with higher or lower luciferase expression level using the luciferase imaging system: as shown in Figure 1, and 2. The genetic screen identified both negative and positive regulators of *AtERF3*.



**Figure 1.** Luminescence image of the seedlings after a 3 hr 200mM NaCl treatment. The putative mutants are circled. *los*, low expression of osmotically responsive genes; and *hos*, high expression of osmotically responsive genes. The contrast of the image is adjusted to locate *los* (A) and *hos* (B) mutants. The bar on the right of the images shows luminescence intensity from lowest (black) to highest (white).



**Fig.2A.** Arrangement of the control (ERF3P – Luc 3-4) and putative mutant plants.

**Fig.2B.** Re-screening of putative mutants without NaCl treatment.

**Fig.2C.** Re-screening of putative mutants after a 3h-200mM NaCl treatment.

**Figure 2.** In this step, the false positives were eliminated. Roughly estimating there were approximately 400-500 plants that were re-screened and the true mutant seedlings were transferred to soil.

## OBJECTIVES AND EXPERIMENTAL PLANS

### I. OBJECTIVES

The ultimate goal of this project is to understand the signaling control of *AtERF3* gene expression in response to salt stress and signaling cross-talk between salt stress and ethylene response in Arabidopsis. **The objectives of this project include: 1) to identify genetic mutants displaying altered (higher or lower) luciferase expression driven by the *AtERF3* promoter; 2) to characterize the mutants physiologically and molecularly, which will include the examination of morphological phenotypes, determination of physiological response of the mutants to various abiotic stresses and plant hormones, and molecular determination of the expression of the native *AtERF3* gene and other stress-responsive genes; and 3) to carry out genetic crosses for further mutation analysis.**

### II. EXPERIMENTAL PLANS

#### Mutant Isolation

Genetic screening of the 20 pools of *AtERF3P – LUC* M<sub>2</sub> EMS seeds will be carried out as described below. ~0.2 gram of the M<sub>2</sub> seeds homologous in *AtERF3-LUC* transgene from each pool were sterilized as described by Chinnusamy et al. (2002) and planted in 150 X 15 mm plates containing ½ Murashige and Skoog salts (Murashige and Skoog, 1962), 1.5% sucrose, and 0.7% agar, pH 5.7. To identify the *hos* and *los* mutants, 7-day old seedlings will be subjected to a 200mM NaCl treatment for three hours. The seedlings will then be sprayed with 1mM luciferin, a substrate of luciferase. The luciferase expression in the seedlings catalyses the adenylation of luciferin to produce luciferyl adenylate: which then reacts with molecular oxygen to form photon-emitting oxyluciferin. After spraying the luciferin on the seedlings, the seedlings will be kept in the dark for five minutes to allow the full penetration of luciferin into the cells. This dark period will also promote the decay of the chlorophyll fluorescence that occurs in the green seedlings; to prevent interference with the luciferin fluorescence. Bioluminescence imaging of the seedlings in each plate will then be carried out by

using a luciferase imaging system containing a dark box and a high sensitive CCD camera (Andor). Once the image is taken, the plate with the seedlings is superimposed on the luminescence image on the computer monitor. The individual plants with abnormally low or high luminescence levels, when compared to the other plants (plants showing similar luminescence intensity in the plate), are marked as putative *los* and *hos* mutants, respectively. These putative mutants are then transferred from the plates to soil (Chinnusamy et al., 2002). The seeds will then be harvested from each putative mutant.

### **Re-screening and morphological and developmental phenotyping**

The putative mutants will be re-tested by luciferase imaging and the false positives will be eliminated. Roughly estimating there will be approximately 400-500 plants that will be re-screened and the true mutant seedlings will be transferred to soil. Genomic DNA will be isolated from each mutant line and the genetic background will be verified by PCR-based confirmation of the *AtERF3P-LUC* transgene. Intensive notes on the growth and development of each individual mutant lines will be taken.

### **Genetic Crosses**

Each mutant line will be crossed with Arabidopsis wild type Col-0. Genetic analysis of the F1 and F2 plants will be carried out to determine whether the mutant phenotype is recessive or dominant. Mutant plants will also be crossed with Arabidopsis ecotype Ler. F2 seeds of the crosses will be obtained for further mapping-based gene cloning.

### **Mutant characterization**

Physiological and molecular characterization will be conducted according to the regular procedures in our lab. Response of the mutants to salt, cold, freezing, drought, osmotic stress, and different plant hormones will be tested. Expression of the native *AtERF3* genes and other stress related genes will be detected by Northern blotting.

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