Plant Molecular Stress Physiology (PMSP) Lab Protocol

Analysis of Gene Expression of drought-induced Genes in Response to Different Enviornmental Conditions in *Craterostigma plantagineum*, *Arabidopsis thaliana*, and *Eutrema salsugineum* and the Screening of Mutants in an *Arabidopsis thaliana* popoulation

Presented to

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Experiment 1: Plant model systems: *Craterostigma plantagineum* as desiccation tolerant plant and *Eutrema salsugineum* as salt tolerant plant

1.1. Desiccation tolerance in *Craterostigma plantagineum*

To conduct this experiment plants were recovered from their substrate and dehydrated under five different conditions: at room temperature in the light, at room temperature in the dark, at 37°C, at 65°C, and at room temperature in the light for 48 hours and afterwards incubated at 65°C. Three weight measurements (fresh, dehydrated, and hydrated weights) were recorded every 0.5, 1, 2, 4, 24, and 48 hours for dehydration and at 48.5, 49, 50, 52, 72 hours for hydration. The Relative Water Content (RWC) was evaluated by substituting the obtained values (not shown) in the following equation:

$$\mathrm{RWC} = \frac{\text{fresh weight} - \text{dry weight}}{\text{turgid weight} - \text{dry weight}} \times 100 \quad \text{(Pieczynski et al. 2013)}$$

Afterwards, pheynotypic changes showing the response of stress tolerance in *C. plantagineum* to different physiological conditions were recorded (figure 1.1).

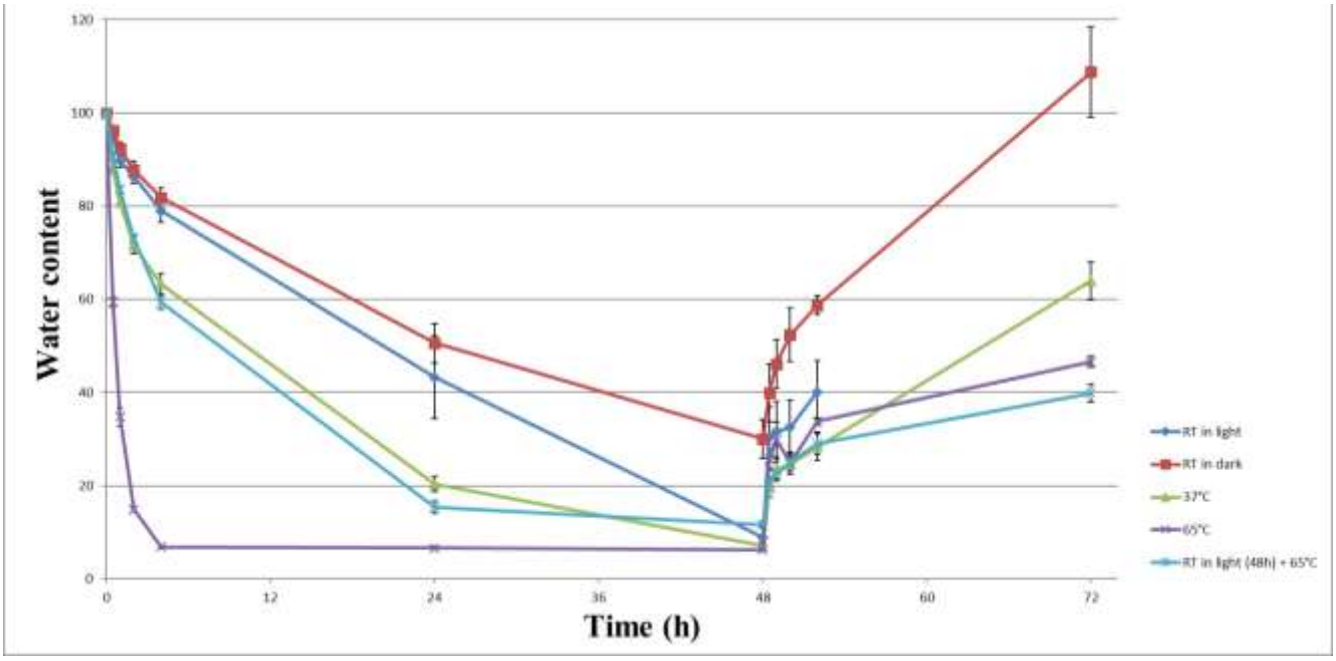


Figure 1.1. The variation of the RWC of *C. plantagineum* in response to five conditions. Dark blue: room temperature in light, Red: room temperature in the dark, Green: at 37°C, Purple: at 65°C, and Light blue: room temperature in the dark and then at 65°C. Measurements were taken at these time intervals: 0.5, 1, 2, 4, 24, and 48 hours (during dehydration) then 48.5, 49, 50, 52, and 72 (rehydration).

1.1.1. Response of *C. plantagineum* to dehydration

From figure 1.1, it can be determined that plant acclimation to dehydration is time-dependent. Using time-dependncy can not be explained alone from this figure unless it was coupled to another condition. For instance, the rate of water loss in room temperature in the dark and light were less than that of 37°C at the same time interval (4 hours) and water loss was even more extreme in plants exposed to 65°C, indicating that the *C. plantagenium* needs more time to produce drought-induced proteins if they were exposed to temperatures of 37°C. If *C. plantagineum* was exposed to 65°C the protein complexes and enzymes including LEA and LEA-like proteins would break down as well as the enforced loss of water through the leaves will cause the acclimation process to be indefinitely halted, eventually leading to the death of the plant cells. Principally, the death of plants at 65°C was due to the cessation of the production of chemicals and compounds such as anthocyanin, sucrose, and diminished antioxidant enzyme activity. Under most conditions, *C. plantagineum* required 48 hours to fully acclimate to dehydration and lose most of its cellular water content, except for 65°C in which 4 hours of exposure was sufficient to kill the plants.

Furthermore, the reposne of *C. plantagineum* is light-depenednet. The impact of dehydration on water loss was more severe in plants grown in the light (9% RWC) as compared to plants grown in the dark (30%). This shows that plants lose most of their cellular water content and acclimate faster to dehydration if they were exposed to sun light. The milder impact of expoure to dark on acclimation to drought stress implicate several light-regulated genes associated with drought stress in the process of drought acclimation. Moreover, the production of sevral compounds and secondary metabolites such as those involved in the chemical protection of subcellular organelles also appear to be light regulated (figure 1.1). Nevertheless, unlike drying in the dark, drying in the light resulted in loss of viability after rehydration. Plants recovered more water in dark-exposed conditions. Yet, the lag in regaining the lost water content in light-exposed plants may be due to the fact that plants need more time to recover from dehydration which was much more severe in light exposure as compared to dark's.

One of the anomalies in our experiment was that after growing *C. plantagineum* at room temperature in the light and then expose them to high temperature (65°C), the pattern of water loss was abnormal and the rehydration phase showed that the plants recovered normally. This is unexpected because after exposure to 65°C we would expect the plants to die and never recover again. To provide better results, this experiment is ought to be repeated again.

1.1.2. Morphological Changes in *C. plantagineum* due to dehydration

1.1.2.1. Morphological changes in leaves and stems

During dehydration and under all conditions, *C. plantagineum* undergoes the curling of both their leaves and their stem segments (figure 1.2). The leaves particularly fold inward into the center of the plant and move closer to one another, partially closing the leaf face. Farrant et al. (2003) has proposed that the folding process minimizes the exposure of the desiccated leaf to light in angiosperm species.

Furthermore, drying of *Craterostigma wilmsii* and *Myrothamnus flabellifolius* shades chlorophyll to avoid Reactive Oxygen Species (ROS). A change in color from green to brown has been detected as well. This is beacues under dehydration conditions, the excitation energy harnessed by chlorophyll can lead to the formation of reactive oxygen species (ROS). The excitation energy harnessed by chlorophyll cannot be dissipated via photosynthesis (Halliwell 1987) and can lead to the formation of reactive oxygen species (ROS) (Farrant, 2000), which if left unquenched can instigate extensive damage at the subcellular level (Halliwell 1987). Resurrection plants minimizes the formation of ROS by limiting the light–chlorophyll interactions and quenching them via antioxidants. Poikilochlorohyllous species such as X*erophyta humilis* and *C. plantagineum* break down chlorophyll to avoid the formation of ROS (Farrant, 2000).

1.1.2.2. Morphological changes in the roots

Moreover, a change in color from white to red or light brown was detected in the roots of dehydrated plants (figure 1.2). this may be due to the absence of water, which prompt the hardening of the roots (Franco et al., 2006). The influence of drought stress on root activity and development has not been extensively studied, because securing safe access to observe roots and studying root system dynamics is tremendously difficult since they require successive as well as non-destructive measurements (Franco and Abrisqueta, 1997; Franco et al., 2002a). Nevertheless, recent research have shown that the change in color may be associated with the suberisation of the exodermis and may reflect a metacutisation process. The latter was correlated with the capacity to grow under drought conditions of *Limonium cossonianum* (Franco et al., 2002b), *Lotus creticus* (Franco et al., 2001), and *Silene vulgaris* plants (Franco et al., 2008).

Deep rooting is a critical factor influencing the ability of the plant to absorb water from the deeper layers of the soil (Franco et al., 2006; 2011). Also, a greater percentage of fine roots, capable of penetrating smaller soil pores, presumably optimises the exploratory capabilities of the root system as a whole, and

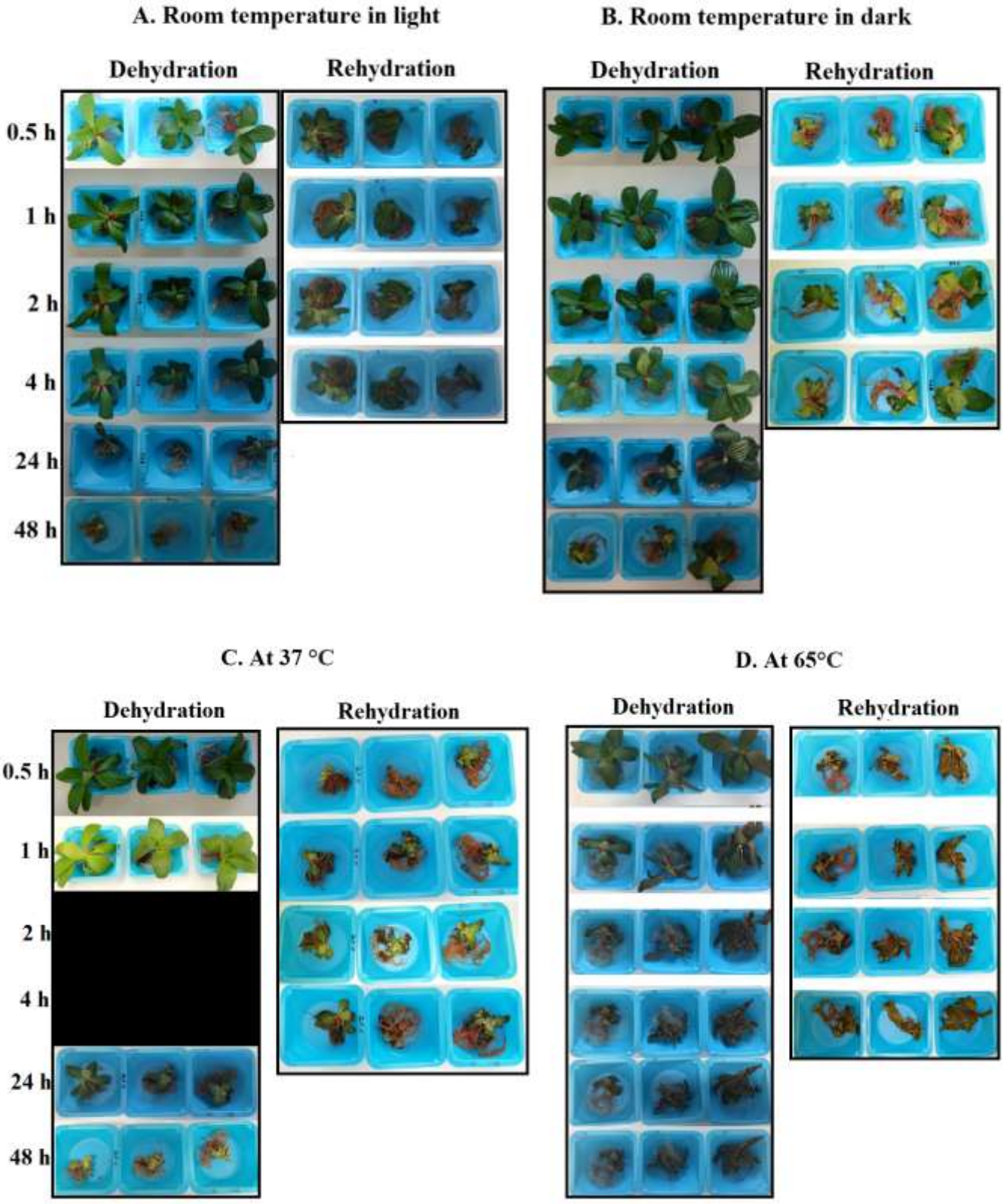
may have an important role for survival of plants to drought stress. The behaviour of plants' roots to dehydration is dynamic, they can either develop thinner roots as in drought-stressed *Silene vulgaris* (Franco et al., 2008) or thicker roots as in *Myrtus communis and Nerium oleander* plants (Banon et al., 2002; Banon 2006). However, it could not be concluded from our data wether water-deficit slightly largened or reduced the size or area of the roots.

1.1.3. Response of *C. plantagineum* to rehydration

Regarding the hydration phase, some of the data was missing for plants grown at 37°C. Nevertheless, it could be clearly seen that plants grown in the dark were not affected by dehydaration as much as were those gown in the light, and 7 hours after hydration, plants grown in the dark (59% RWC) recovered much faster than those grow in the light (40%) implying that the plants needs more time to acclimate itself to irrigation if it was exposed to light.

C. plantagineum plants exposed to 37°C (figure 1.2 C) didn't recover well even though it would be expected that they will retrieve some of their lost cellular water content because the temperature was not severe. High humidity, due to the placement of plants on a high shelf, may be the reason why plants exposed to 37°C didn't retrieve their lost water content (figure 1.2). This experiment should be repeated again and more caution should be taken regarding the placement of the plants.

These results do conform with the relative water content experiment. However, plants grown at 65°C and plants grown at room temperature then 65°C both didn't recover at all (figure 1.2). Unlike relative water content experiment, plants plants grown at room temperature then 65°C appears to have recovered, maybe the dateset that we collected for this sample was contaminated or mixed up with other datasets.


A. Room temperature in light
Dehydration
Rehydration
0.5 h
1 h
2 h
4 h
24 h
48 h
B. Room temperature in dark
Dehydration
Rehydration
C. At 37 °C
Dehydration
Rehydration
0.5 h
1 h
2 h
4 h
24 h
48 h
D. At 65°C
Dehydration
Rehydration

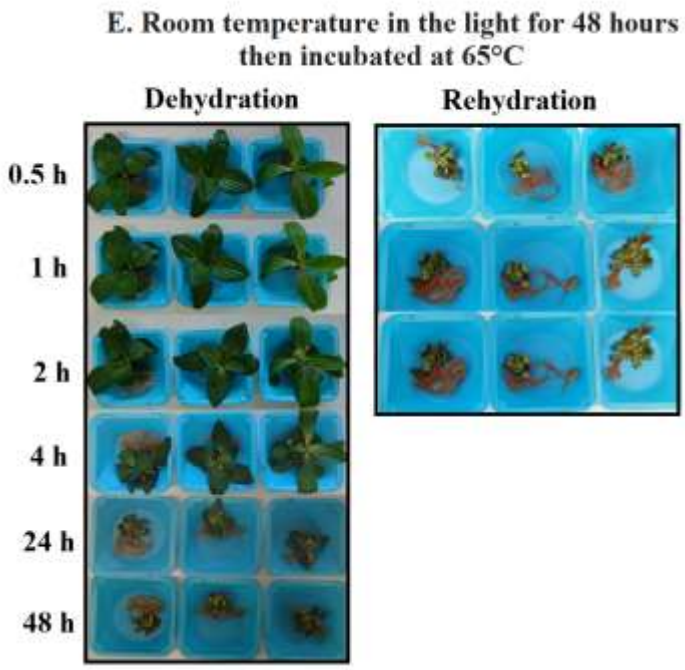


Figure 1.2. The response of *C. plantagineum* to dehydration and rehydration. *C. plantagineum* plants were exposed to five conditions: (A) room temperature in light, (B) room temperature in the dark, (C) at 37°C, (D) at 65°C, and (E) room temperature in the light and then at 65°C. Measurements were taken at these time intervals: 0.5, 1, 2, 4, 24, and 48 hours (during dehydration) then 48.5, 49, 50, 52, and 72 (rehydration).

1.2. Vitality test

By using razor blades and styrofoam, leaves and roots from *C. plantagieum* were cut and subsequently stained with fluoresceine-diacetate (FDA) to identify whether they had intact and living cells (figure 1.3). the dye imparts a yellow-green color on living cells. Control samples used as positive controls in both leaves and roots showed that cells were living. Some of the cells were left intact in the leaves of room temperature plants grown in the light (Figure 1.3; B and H) as compared to many intact cells in the roots. Concerning plants gwon at room temperature in the dark (figure 1.3; C and I)and 37°C (figure 1.3; D and J), living cells were detected in the roots and leaves. These results conform with the RWC and morphological analyses for plants grown in the dark, but for plants grown in the light, cells did not remain viable after dehydration. While plants grown at 65°C (figure 1.3; E and K) and at room remperature in lightd (figure 1.3; F and L) did not survive. The results in figure 1.3 E and K are not in accordance with the RWC measurments.

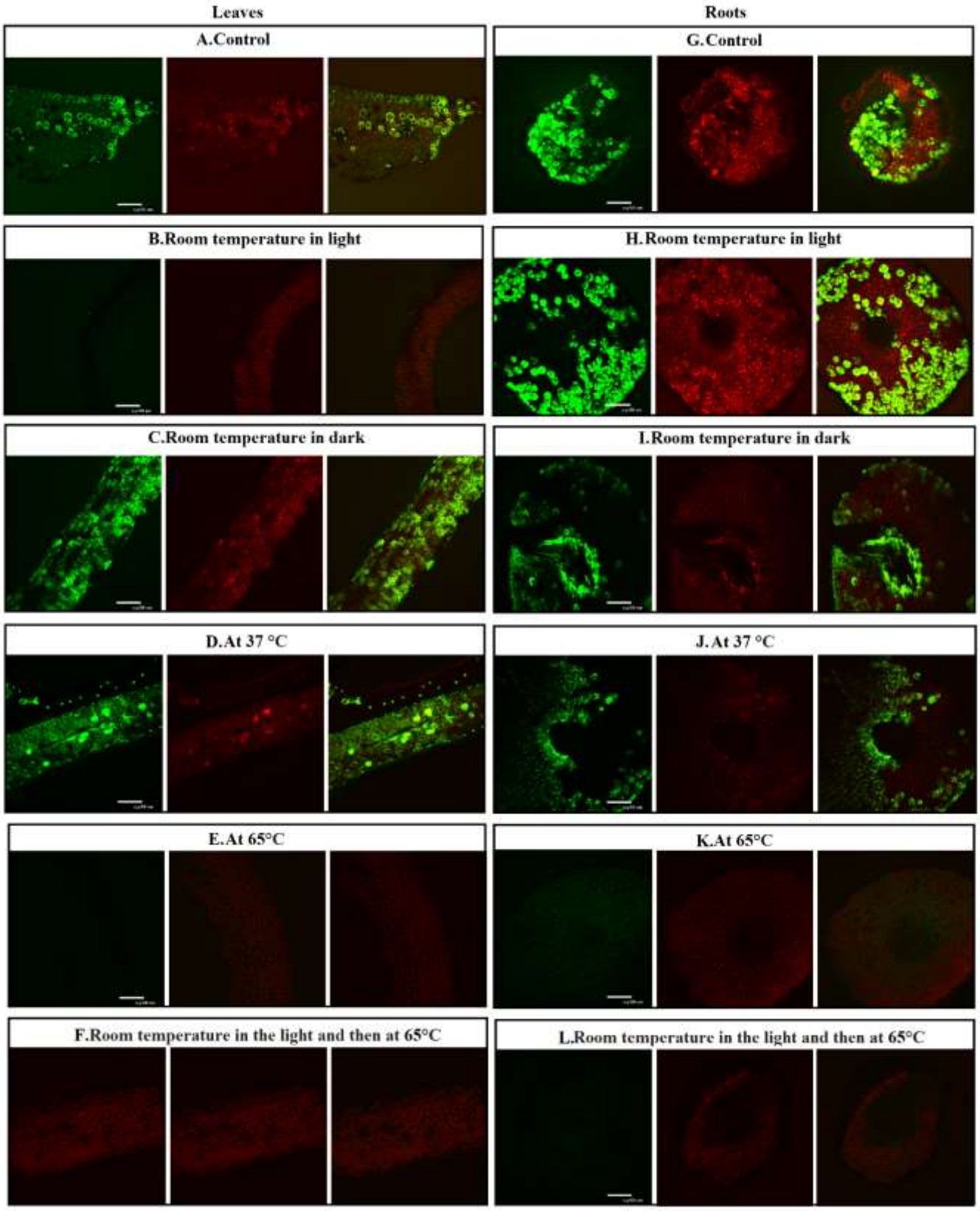

Leaves
Roots
A.Control
G.Control
B.Room temperature in light
H.Room temperature in light
C.Room temperature in dark
I.Room temperature in dark
D.At 37 °C
J.At 37 °C
E.At 65°C
K.At 65°C
F.Room temperature in the light and then at 65°C
L.Room temperature in the light and then at 65°C

Figure 1.3. FDA staining of leaf and root tissues of *C. plantagineum*. Images (A) to (F) shows stained leaves whereas images (G) to (L) shows stained roots. (A) and (G) show control samples for leaves and roots respectively. Plants were treated under different conditions before staining leaves and roots with FDA: (B and H) room temperature in light, (C and I) room temperature in the dark, (D and J) at 37°C, (E and K) at 65°C, and (F and L) room temperature in the light and then at 65°C.

Nonetheless, plants were expected to grow in a healthy manner when they are exposed to light and dark at room tepmperature as well as for those grown at 37°C. however, high temperatures (65°C) is expected to kill of the plants and destroy the cells. FDA was not stained in E, K, F, and I of figure 1.3, confirming our expectations.

1.3. Comparative analyses of salt tolerance in *Eutrema salsugineum* and the model plant *Arabidopsis thaliana*

Eutrema salsugineum and the model plant *Arabidopsis thaliana* were cultivated till they were mature and incubated for 5 days in tap water supplemented with 0 mM NaCl, 100 mM NaCl, 200 mM NaCl, 300 mM NaCl, and 600 mM NaCl (figure 1.4). Measurements of electrical conductivity using io-leakage test were taken after three and five days consequently (figure 1.5).

1.3.1. Morphological changes in *Eutrema salsugineum* and *Arabidopsis thaliana*

Despite the fact that both sodium (Na^+) and chloride (Cl^-) ions are accumulated in most of the plants, their accumualtion at high concentrations may hamper the growth of affected plants. Cl^- is and an essential co-factor in photosynthesis as well as an important micronutrient that regulates cytoplasmic enzyme activities. Cl^- also act as a counter anion to stabilize membrane potential, and is involved in the regulation of turgor pressure and pH (Xu et al., 2000).

As dehydration experiment advances from 1 to day 3, no differences could be observed in *E. salsugineum* plant treated with 0 mM, 100 mM, and 200 mM Nacl whereas as the concentraton increased to 300 mM and 600 mM NaCl, the leaves of E. salsugineum started to shrink in size. Likewise, the leaves of *A. thaliana* shrank as twice as much as *E. salsugineum* as the concentration increased from 0 mM to 600 mM NaCl. E. On the fifth day of salt treatment, *E. salsugineum* plants maintained healthy and steady growth at the following concentrations: 0 mM, 100 mM, 200 mM, and 300 mM NaCl. This is evident because of

the process of homeostasis of Na^+ and Cl^- which constitute an important mechanism that aids higher plants in reducing NaCl stress (Hong et al., 2009). This shows that genes that are only expressed upon salt stress are upregulated in *E. salsugineum* whereas they are not regualted in *A. thaliana*, maybe because there are no homologs of salinity-induced genes in *A. thaliana*.

However, at a concentration of 600 mM NaCl, the plant started to be severely affected by the accumulation of salts in the soil. High salinity induces reduction in growth as a consequence of water-deficit induced stress aslong with with the effects of excess concentrations of Na^+ and Cl^- ions (Munns and Tester, 2008) capable of hindering critical biochemical processe. Neverhtless, it is expected from *E. salsugineum* to survive if treated with 600 mM NaCl, therefore, we propose that while rewatering plants between time intervals, the salty water covered the leaves hindering some of the biological process in them. Thus, salt didn't accumulate only in the soil but aslo on the leaves. Rajendran et al. (2009) has said that Early at the onset of salt stress events, leaf appearance can be delayed, leaf expansion can be retarded, and leaf senescence, prior to the large accumulation of toxic ions, can be promoted.

It should be noted that Cl^- is and an essential co-factor in photosynthesis as well as an important micronutrient that regulates cytoplasmic enzyme activities. Cl^- also act as a counter anion to stabilize membrane potential, and is involved in noth the regulation of turgor and pH (Xu et al., 2000). However, the retardation of growth in our our are prompted by high Cl^- concentrations which induce chlorotic toxicity symptoms by reducing the photosynthetic capacity and quantum yield due to chlorophyll degradation. Additionally, Na^+ is a toxic ion at high concentrations because it affects the uptake of K^+ and interrupt stomatal regulation which results in a reduction in the photosynthetic capacity and growth (Tavakkoli et al., 2010). After 5 days of salt treatment, the leaves of *A. thaliana* started to wither and eventually at 600 mM NaCl concentration they showed early signs of death (e.g., hindered growth, smaller leaves, etc…).

Tavakkoli et al. (2010) have proposed that it is better to study the impact of Na^+ and Cl^- individually instead of collectively (in the form of NaCl) on growth because they have shown that Na^+ and Cl^- are limiting growth through different mechanisms but simultaneously. Conducting this kind of experiments on salt tolerant and salt sensitive species may shed the light on the mechanisms by which salt tolerance is acquired in poikilochlorohyllous species.

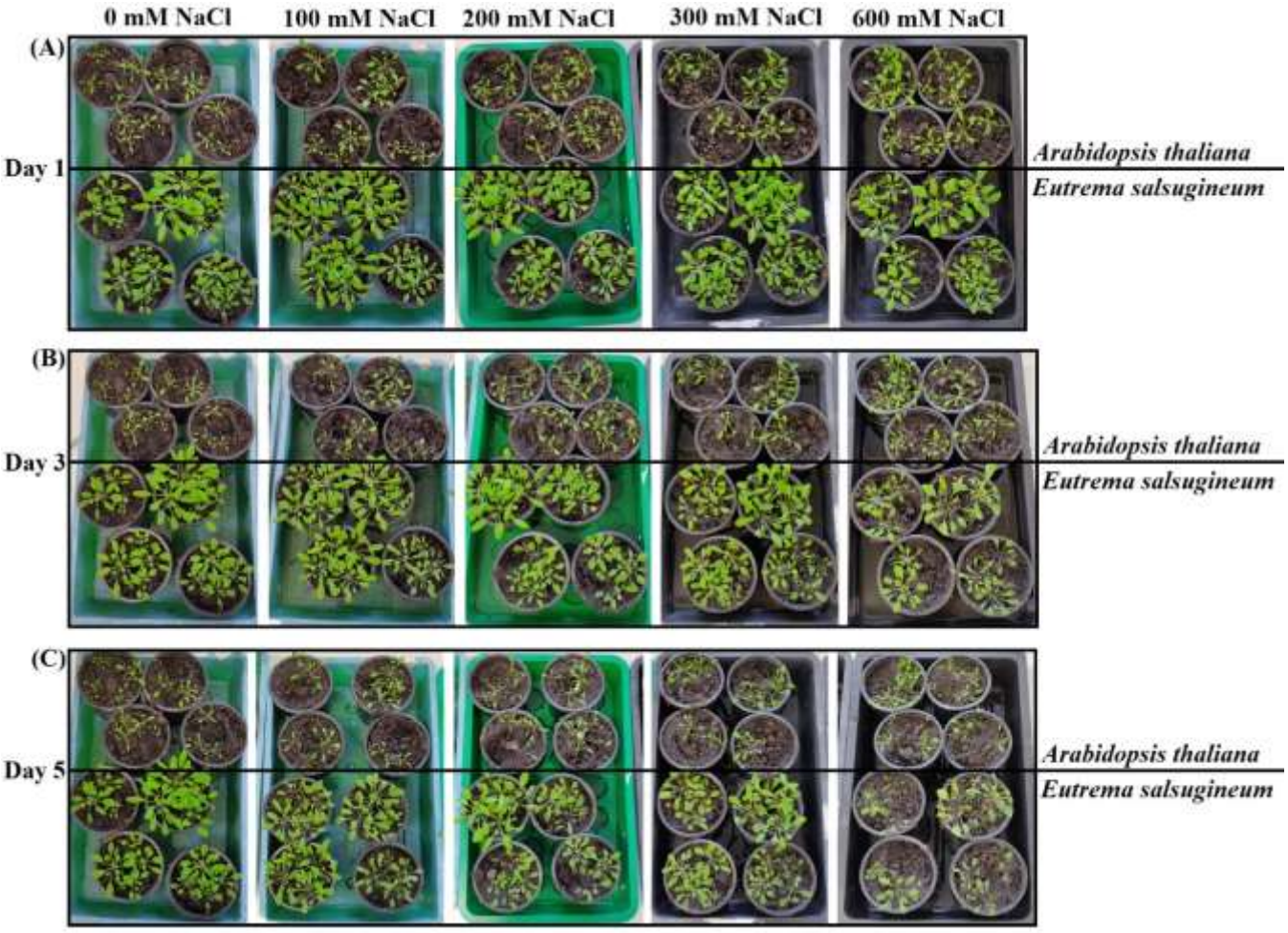


Figure 1.4. Dehydration experiment by applying salt treatment of varying concentrations (0 mM NaCl, 100 mM NaCl, 200 mM NaCl, 300 mM NaCl, and 600 mM NaCl) on *A. thaliana and E. salsugineum*. (A) Day 1 of treatment. (B) Day 3 of treatment. (C) Day 5 of treatment.

1.3.2. Determining the viablity of cells via the ion-leakage test

The following experiment aims to identify the effect of salt treatment on the leakage of ions from cellular membranes in *A. thaliana* and *E. salsugineum*. Results (appendix 1 in supplementary data) showed that after 3 days of salt treatment, the relative ion leakge of E. salsugineum increased from 15% (0 mM NaCl) to 22% (600 mM NaCl) as compared to *A. thaliana's* which increased from 5% (0 mM NaCl) to 59% (600 mM NaCl).

This prompts us to suggest that after 3 days of treatment, *E. salsugineum* displayed steady maintenance of tolerance as compared to *A. thaliana*. Preserving a relative ion leakage percentage less than 22%

implies that pathways involved in cellular hemeostasis are activated. By combining these results with morphological changes, it can be concluded that there must be salinity-induced genes in *E. salsugineum* that are protecting the plants from withering and dying, suggesting a role of NaCl in a signal transduction pathway responsible for the activation of the aformentioned genes. While *A. thaliana* lacks these signalling pathways and probably the genes as well, therefore they shrank in size and died.

On the other hand, the impact of salt stress was much more severe after treating A. thaliana and *E. salsugineum*, having relative ion leakage percentages of 99% and 75% respectively. By combining these results with morpholigcal changes, it could be proposed that *E. salsugineum* is more tolerant of salt stress than *A. thaliana*. A small peak (85%) was detected at 300 mM NaCl concetration for *E. salsugineum*. This may be caused by a delay in the production or expression of salinity-induced genes. However, at 600 mM NaCl concentration, the relative ion leakage decreased to 75%.

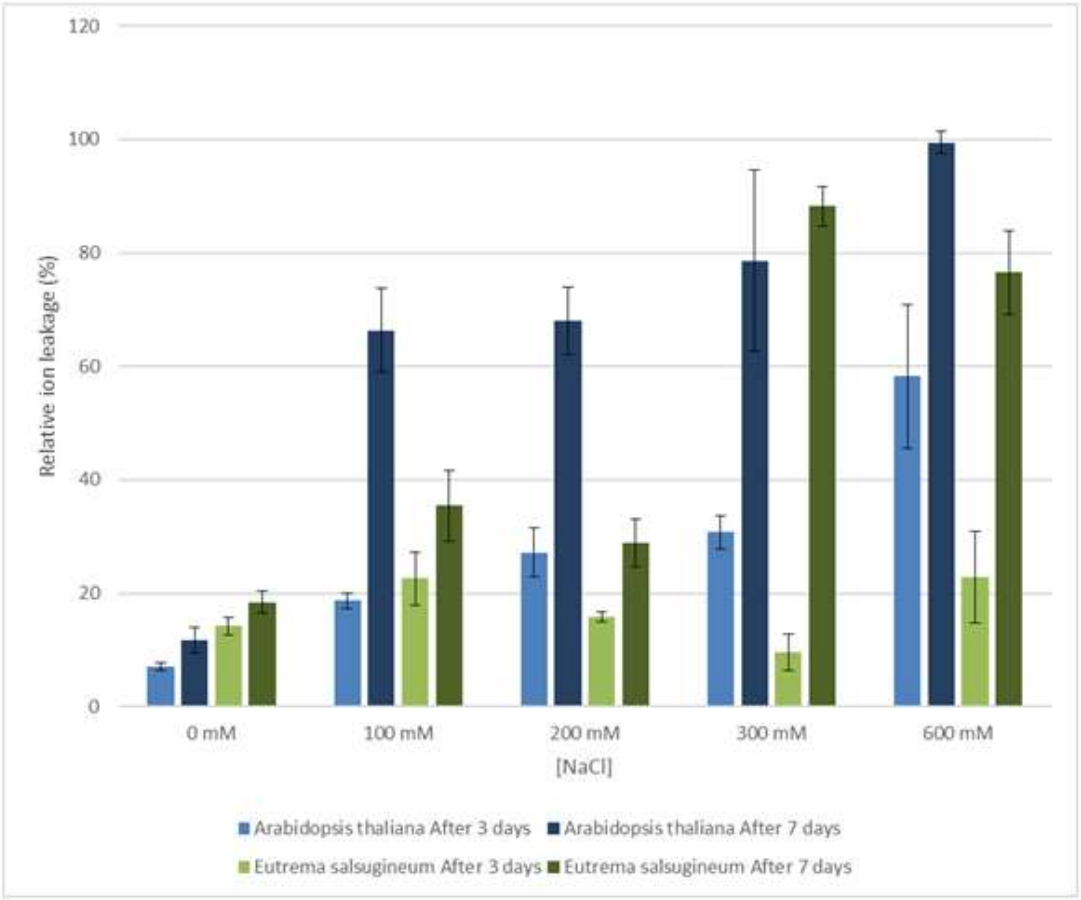


Figure 1.4. Ion leakage test. Varying concentrations of salt treatment (0 mM NaCl, 100 mM NaCl, 200 mM NaCl, 300 mM NaCl, and 600 mM NaCl) were used to evaluate the relative ion leakage of cellular membranes, thereby determining the viability of the cells.

Experiment 2: Gene expression under abiotic stress conditions in *Craterostigma plantagineum*

Molecular studies have shown that angiosperms cope with dehydration by using constitutive expression and several repair mechanisms which are induced upon rehydration. Yet, upon dehydration, several pathways are induced to preserve the physiological and molecular integrity of the plant cell before it becomes fully dehydrated. The latter facilitates the recovery processes of the plant upon rehydration. Performing transcriptome analysis allows the monitoring of the coordinated expression of plant processes involved in halting development and growth as well as acclimation in response to drought stress at the transcript and protein levels.

A plethora of pathways are activated in responses in dehydration including pathways involved in the synthesis of antioxidant defense proteins, enzymes related to carbohydrate metabolism, mechanical stress protection proteins, protective proteins such as late embryogenesis abundant (LEA) proteins, regulatory proteins (e.g., transcription factors and kinases), and signaling molecules (Rodriguez et al., 2010; VanBuren et al., 2015).

Nonetheless, LEA-like proteins are going to be the subject of this experiment. LEA proteins along with hydrophillins are ubiquitously synthesized creating a water hydration shell to certain proteins and macromolecules (Olvera-Carrillo et al., 2010). They play an important role in protecting cellular structures from dehydration-associated damaging effect (Wise and Tunnacliffe 2004; Reyes et al. 2005). This protecting function has been also found to be exerted by LEA-like dehydrin gene from the moss *Physcomitrella* (Saavedra et al., 2006). In addition, LEA proteins act as chaperones, facilitating the prevention of protein aggregation as a result of desiccation (Goyal et al., 2005).

The LEA2 protein CDeT6-19 and the LEA-like protein CDeT11-24 are investigated in this experiment at the levels of transcripts and proteins. On the basis of sequence similarity, it has been found that CDeT6-19 belongs to the group 2 LEA proteins. This group is characterized by a Ser-rich sequence and one or more lysine-rich segments (Close, 1996). CDeT6-19 is mainly localized to the cytosol and undergoes reversible phosphorylation (van den Dries et al., 2011). On the other hand, it has been found the CDeT11-24 protein has specific sequence motifs that are present in other LEA proteins, such as a highly conserved lysine-rich domain (Velasco et al., 1998). CDeT11-24 is localized to the cytoplasm and a potentially lipid binding LEA protein (van den Dries et al., 2011). Nevertheless, CDeT11-24 has not been yet classified to any established group of LEA proteins. It has been proposed that CDeT11-24 protein is in involved in pathways which senses the water status of the plant (Velasco et al., 1998).

In *C. plantagineum,* the transcription factor CpMYB10 from the Myb family binds to and regulates its own promoter along with the promoter of CDeT11-24 whereas the leucine zipper protein CpHB-7, a drought-responsive a transcriptional regulator, is a negative modulator of ABA and targets the desiccation-inducible LEA-like protein CDeT6-19 (Deng et al., 2006). The activation and deactivation of LEA and LEA-like proteins depends largely on post-translational modifications. Rohrig et al. (2006) indicated that phosphorylation and dephosphorylation of CDeT11-24 and CDeT6-19 proteins are part of the desiccation tolerance mechanism of *C. plantagineum*.

In this experiment, the regulation of The LEA2 protein CDeT6-19 and the LEA-like protein CDeT11-24 has been investigated at two levels in *C. plantagineum*. The regulation of CDeT11-24 and CDeT6-19 is only studied at the transcriptional and translational levels. The post-translational modifications have been elaborately investigated by Rohrig et al. (2006). Regulatory mechanisms of the desiccation-related genes CDeT11-24 and CDeT6-19 are compared to investigate the nature of regulation during transcrtion and translation. The obtained data provide more insight into the expression patterns of CDeT11-24 and CDeT6-19 during dessication tolerance after exposure to dehydration conditions as well as sorbitol and NaCl treatments.

2.1. Treatment of C. plantagineum plants

C. plantagineum plants were grown inside the lab. Plants were divided into two groups: treated and unterated. For dehydration treatments plants were left to dehydrate for 4 and 24 hours consecutively. For chemical treatments, leaves were independently treated with a 150 mM Sodium-Chloride solution overnight and 150 mM Sorbitol solution overnight.

2.1. Gene expression analysis on the RNA level: Analyses of mRNA accumulation

2.1.1. Isolation of total RNA

Total RNA was isolated from untreated and treated plants using the method outlined by Valenzuela-Avendaño et al. (2005). The OD of isolated RNA was not measured for. Afterwards, the RNA was prepared by adding sample buffer and run on agarose gel (see figure 2.1).

To monitor the quality and ensure the equal loading of RNAs, ethidium bromide-stained agarose gels showing ribosomal RNA bands were used (see figure 2.1). In untreated leaves of *C. plantagineum* and for 4 hours and 24 hours dehydration treatments as well as for NaCl, relatively high levels of total RNA were detected. Whereas low levels of total RNA were spotted after sorbitol treatment. The bands were clear

and distinct implying that there were no visible contaminations.the upper band shows the heavy 28S rRNA while the lower band shows the light 18S rRNA. In 4 hours, 24 hours, and NaCl treatments a light band below 18S rRNA can be observed, these bands represent tRNA.

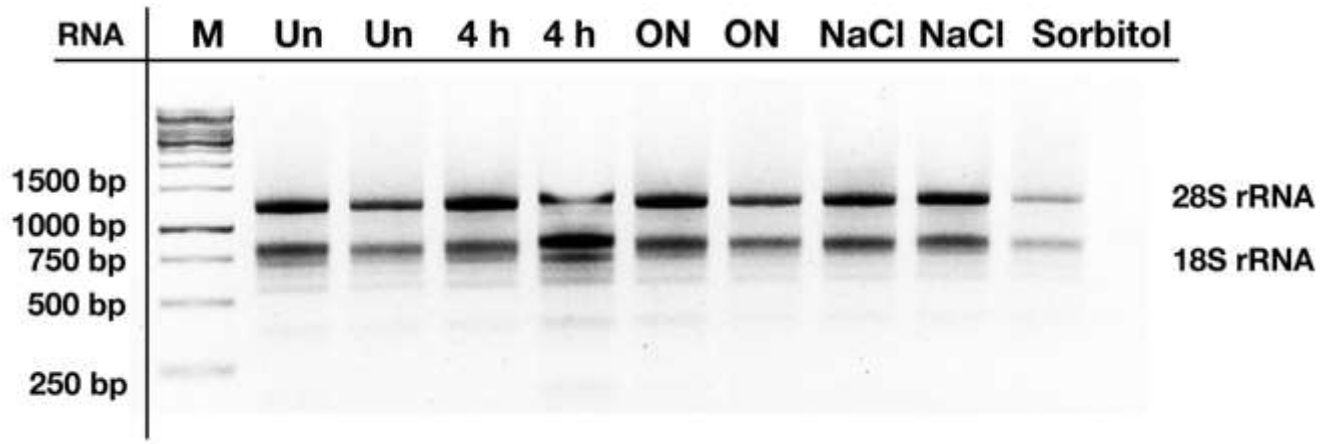


Figure 2.1. Total RNA isolation of untreated (Un) plants as well as 4 hours dehydration (4h), overnight dehydration (ON), Sodium-Chloride treatment (NaCl), and Sorbitol treatment.

2.1.2. Reverse trascriptase PCR

After monitoring the quality of, one microgram of isolated total RNA from each sample (4 hours and 24 hours dehydration, NaCl treatment, sorbitol treatment) was used to synthesize first-strand cDNA. Then, the samples are treated with RNase-free DNase to prevent the occurance of any genomic DNA amplification which could contaminate the extracted cDNA. Three genes were amplified in the previously treated samples: EF1ά, CDeT11-24 and CDeT6-19 using specific primers shown in figure 2.2.

Genes	Forward primer (5'→3')	Reverse primer (5'→3')
CDeT6-19	ACACCGACGAGTACGGAAACC	CGCCGGGAAGCTTCTCTTTG
CDeT11-24	TCGGAAGACGAGCCTAAGAA	ACAGCGCCTTGTCTTCATCT
EF1α	AGTCAAGTCCGTCGAAATGC	CACTTGGCACCCTTCTTAGC

Figure 2.2. Gene specific forward and reverse primers used to amplify three genes: a constitutitively expressed house keeping gene EF1ά which is stable in dehydration and hydration conditions, a gene encoding LEA-like protein CDeT11-24, and a gene encoding LEA2 protein CDeT6-19.

Products of PCR reactions were then separated by using agarose gel electrophoresis. Transcript levels of CDeT1124 and CDeT6-19 were normalized to the transcript levels of the reference gene EF1ά which encodes for a gene termed Elongation Factor 1 ά belonging to a large family of elongation factors in *C. plantagineum* and is stable during dehydration (Obtained relative expression values are not shown). Figure 2.2 shows transcript abundance of cDNA in the presence of reverse transcriptase. Strong and clear bands were detected in untreated and all treatment conditions.

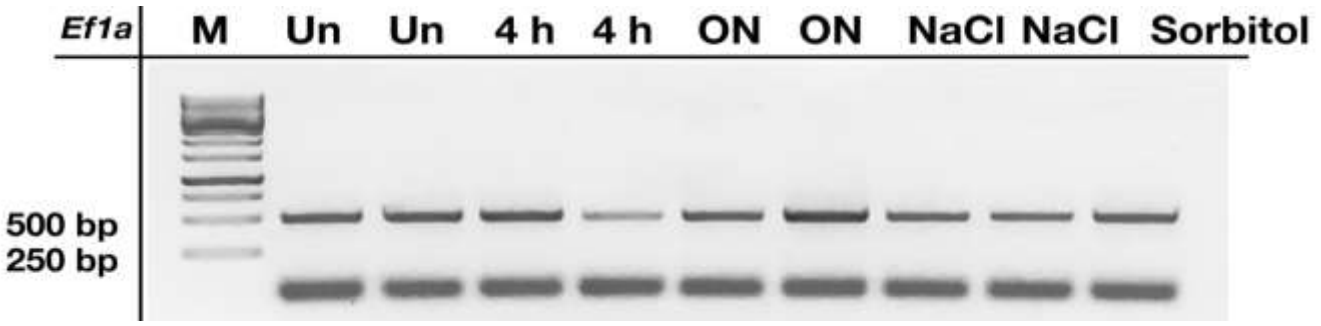


Figure 2.2. Transcript abundance of gene encoding EF1ά in *C. plantagineum* subjected to different treatments. Reverse-transcriptase was used to reverse transcribe RNA transcripts into cDNA. Transcript levels of genes encoding EF1ά were used as positive controls. Un corresponds tountreated, 4h corresponds to 4 hours dehydration (4h), ON corresponds to overnight dehydration, NaCl corrseponds to Sodium-Chloride treatment (NaCl), and Sorbitol corresponds to Sorbitol treatment. M corresponds to marker.

Figure 2.3 shows transcript abundance of cDNA in the absence of reverse transcriptase. No bands were detected in untreated and all treatment conditions.

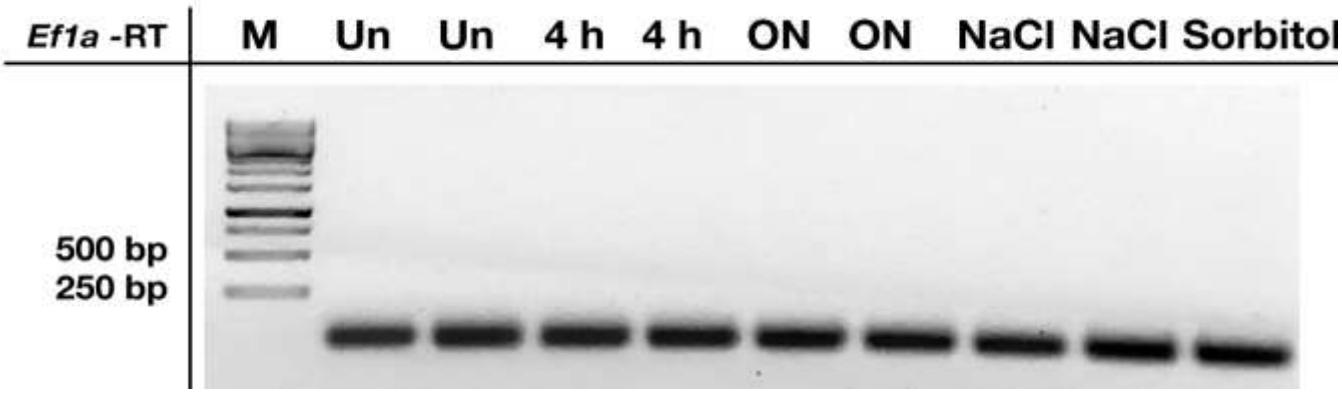


Figure 2.3. Transcript abundance of gene encoding EF1ά in *C. plantagineum* subjected to different treatments. Reverse-transcriptase was not used to reverse transcribe RNA transcripts into cDNA. The figure was used as negative control. No cDNA tanscripts were detected.

In untreated leaves low levels of CDeT11-24 transcrips were detected in *C. plantagineum* while in CDeT6-19 transcripts were almost two-fold the amount of CDeT11-24. The presence of transcripts in the untreated leaves indicate that the palnts are prepared for dehydration even before exposure to drought or salt stress. Nonetheless, after 4 hours of dehydration, more CDeT11-24 and CDeT6-19 transcripts accumulated in the total RNA pool. The intensity of the band increased one-fold in the case of CDeT11-24 whereas it increased two folds in the case of CDeT6-19. This demonstrates that *C. plantagenium* responds rapidly to dehydration, inducing the activation of several pathways which increase the production of trnascripts under drought stress conditions. As duration of dehydration increases to 24 hours, production of transcripts reaches high levels for CDeT11-24 and CDeT6-19 and a one-fold increase in the intensity of the band is registered for both (see figure 2.4).

The aforementioned results could be corroborated by comparing the results to those obtained by Juszaczaak and Bartels (2017) shown in figure 2.5. the expression of EF1ά did not vary among the samples whereas at time 0 (T0) levels of CDeT11-24 and high levels CDeT6-19 were detected. More transcritps were produced as the duration of dehydation treatment increased from 0 hours to 24 hours, reaching its peak production after 48 hours.

Likewise, NaCl treatment resulted in the induction of transcript production of CDeT11-24 and CDeT6-19, yet more CDeT6-19 trasncripts were produced as compared to the low amounts of CDeT11-24. Treatment with sorbitol yielded similar results to NaCl treatment.

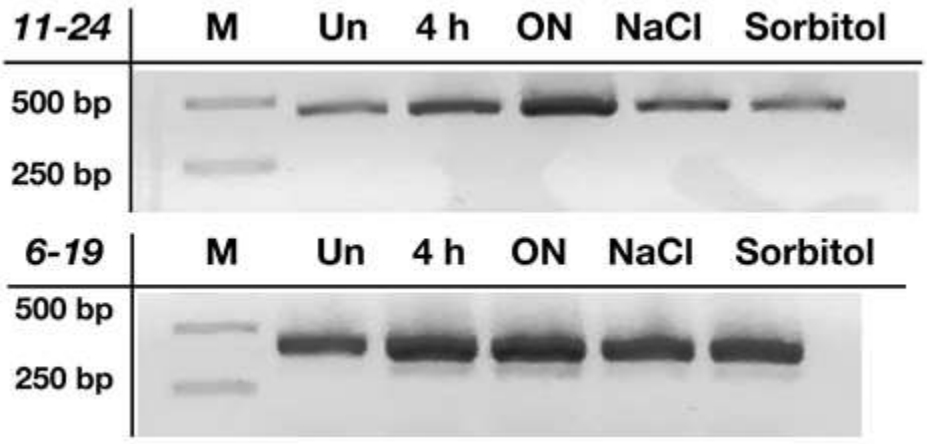


Figure 2.4. Transcript abundance of gene encoding CDeT11-24 and CDeT6-19 in *C. plantagineum* subjected to different treatments. Reverse-transcriptase was used to reverse transcribe RNA transcripts into cDNA.

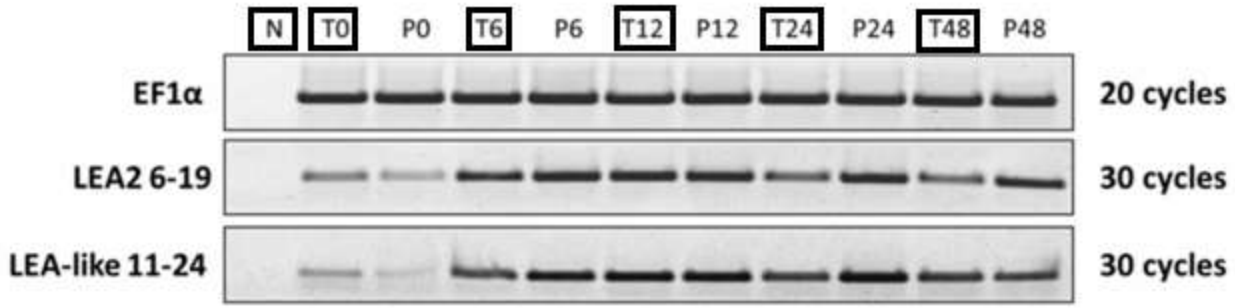


Figure 2.5. Transcript abundance of genes encoding CDeT6-19 and CDeT11-24 in C. plantagineum subjected to dehydration at different time frames. Transcript level of genes encoding EF1ά were used as positive control. T corresponds to total (T) RNA isolated from C. platagineum subjected to dehydration for 0 (T0), 6 hours (T6), 12 hours (T12), 24 hours (T24), and 48 hours (T48).

2.1.3. Quantification of relative expression of CDeT11-24 and CDeT6-19

The transcripts of isolated total RNA for genes encoding CDeT6-19 and CDeT11-24 were quantified (figure 2.6). The transcritp levels were normalized to the respective transcript level of EF1ά. To quantify the intensity of the bands ImageJ software was used (Juszaczaak and Bartels; 2017). The relative expression of CDeT11-24 increased from 60% in untreated plants to 180% afer overnight dehydration treatment while the relative expression of CDeT11-24 increased from 140% to 250 percent after 24 hours of dehydration. The transcript accumulation patterns in relative quantification is in accordance with the analysis performed on the gels shown in figure 2.4.

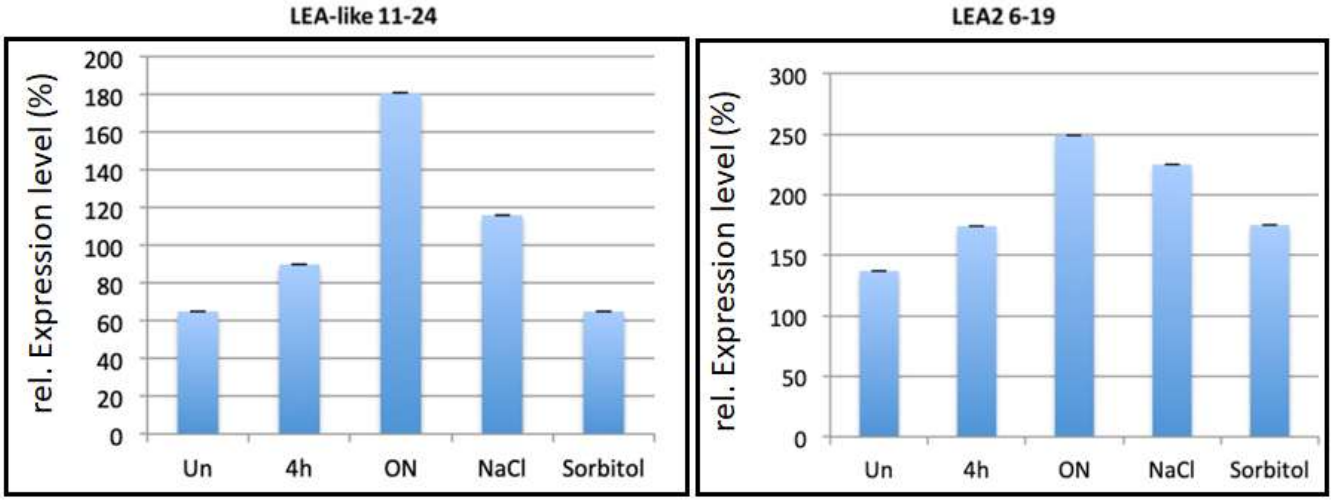


Figure 2.6. Quantification of transcript levels of genes encoding CDeT6-19 and CDeT11-24 in total RNA extracted from C. plantagineum plants subjected to dehydration for 0, 4, and 24 hours as well as treated with NaCl and sorbitol.

2.2. Gene expression analysis on the protein level: Analyses of protein accumulation by immunodetection

Total proteins were extracted from the leaves of untreated, partially and fully dehydrated, and chemically treated *C. plantagineum* plants. The extracted proteins were analysed by two-dimensional PAGE. Four gels were prepared. One was stained with Coomassie stain, the second was stained with silver staining, and the third and fourth gels were reserved for western blotting. Following wester blotting, the proteins on the two gels were stainined with Ponceau staining and subsequently immunodetected with an antibody stain using a peroxidase-linked enzme reaction.

As expected, the expression of Rubisco in total protein extractions was detected in all lanes (between 45 and 66 kDa) in Coomassie staining and silver staining (figure 2.7). No strong background signal was identified and no unspecific binding was detected implying that the staining process in both gels was good. In both gels CDeT11-24 was detected at 71 kDa and CDeT6-19 was detected at less than 10 kDa. The experimental molecular mass is not the same as the theoratical or expected molecular mass since the proteins migrate higher than their expected molecular mass.

Velasco et al. (1998) suggested that this abnoraml behavior of migration on the gel could be czaused by the hydrophilic nature of the proteins. The predicted molecular mass of CDeT11-24 and CDeT6-19 is 45 kDa and 16 kDa respectively. As compared to CDeT6-19, CDeT11-24 protein has a higher molecular mass and migrate slower on the gel.

The results indicate that both CDeT11-24 and CDeT6-19 accumulate regularly before dehydration treatment (Un), at a very early (4h) and late (ON) stages of dehydration, and after treatment with NaCl and sorbitol. These results conform with those obtained from RT-PCR. Nevertheless, it should be noted that the intensity of the bands in overnight (ON) treatment is much higher than in other treatments, implying that dehydration overnight causes the production of even more LEA and LEA-like proteins.

Additionally, two membranes were blotted using western blotting. The blotted nitrocellulose membranes were then stained with Ponceau to evaluate the protein transfer and mark protein standards and protein

lanes. Clear and distinct bands were detected throughtout the two gels and the Rubisco bands (black boxes, figure 2.8) were plainly visible. After making sure that the protein bans were clearly visible, the proteins on the two nitrocellulose membranes were immunodeteced with antibody stain using a peroxidase-linked enzyme reaction.

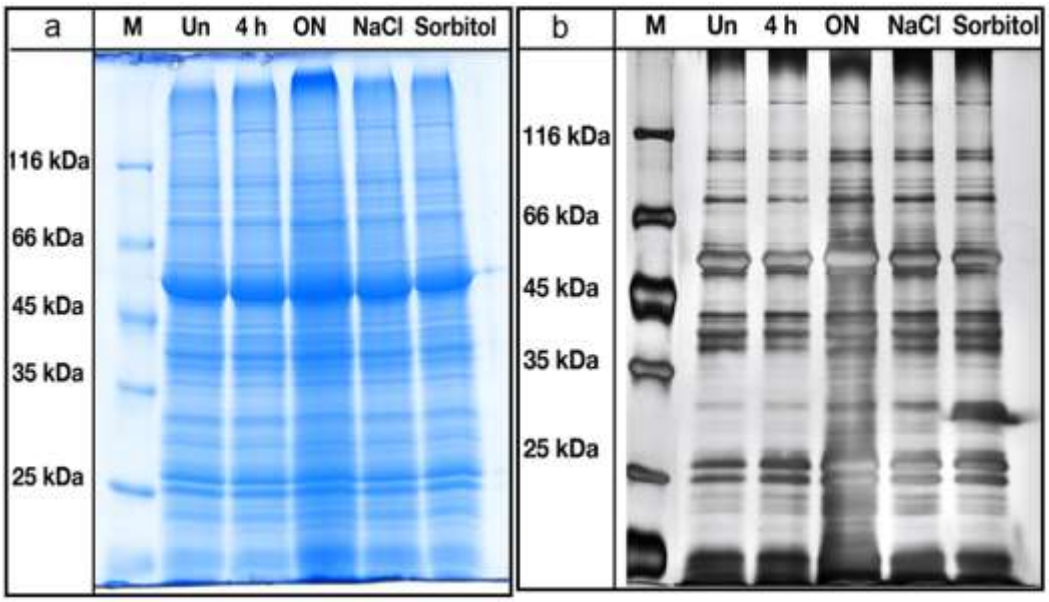


Figure 2.7. Coomassie stained SDS-PAGE gel of samples containing CDeT6-19 and CDeT11-24 proteins. It contains M = marker (5 µl) and 20µl of total protein was loaded into each wel. (a) Coomassie staining. (b) silver staining.

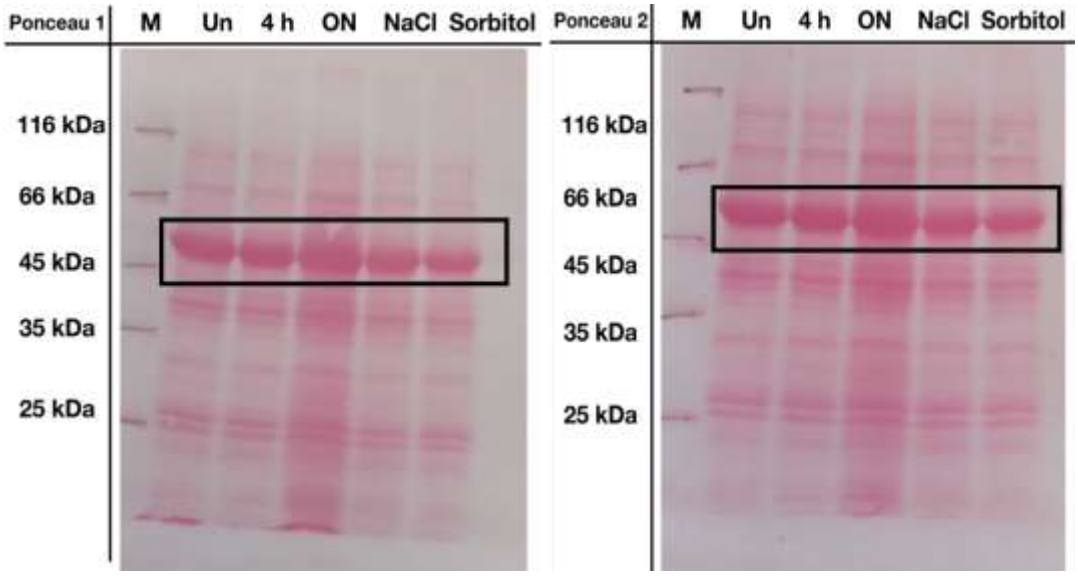


Figure 2.8. Ponceau S staining of nitrocellulose membrane containing the blotted total proteins. Ponceau 1 and Ponceau 2 represent the two blotted nitrocellulose membranes. Rubisco bands are marked with black rectangles.

As shown in figure 2.9, no accumulation of CDeT-11-24 was detected in untreated tissues as well as in response to sorbitol treatment. However, two faint bands were detected after 4 hours of dehydration and NaCl treatment. The highest accumulation of CDeT11-24 was detected after 24 hours of dehydration. The presence of two bands in 4 hours and NaCl and the presence of many bands in the overnight treatment is caused by the existence of many isoforms of CDeT11-24 which could be detected by using the provided polyclonal anitbodies. Unlike mono-clonanl ntibodies, the provided antibodies were poly-clonal which are charaterized by the selection of all isoforms of the targeted protein found in the extracted total protein samples. These soforms are highly homologous to the targeted CDeT11-24.

Nonetheless, by comparing the protein gels (figure 2.9) with transcript levels (figure 2.4), huge discrepencies could be seen. Unlike the transcripts of CDeT11-24 which were detected in untreated and treated plants, proteins were only translated after 4 hours and 24 hours of dehydration and after NaCl tratment. This indicates that CDeT11-24 is consituitively expressed under all conditions and it accumulates at high levels when plant tissue is exposed dehydration stress. Proteins also accumulate much more in dehydrated tissues. Furthermore, NaCl treatment exeerted the same effect as overnight treatment and transcripts were detected in RT-PCR. Yet, even though CDeT11-24 proteins accumulated after NaCl treatment, the accumulation was two-fold less than overnight treatment. Similar to untreated tissue, treatment with sorbitol didn't induce the translation of proteins even though the transcripts were detected in RT-PCR.

This indicates that the expression of CDeT11-24 protein is not regulated at the transcript level but on the translational level. CDet11-24 is not expressed because the transcripts were not translated into proteins in untreated tissue and under sorbitol treatment. Therefore, NaCl and dehydration may induce several pathways which induce the translation of CDeT11-24. More research should be conducted to identify the underlying pathways which are responsible for this divergence in the expression of proteins.

From figures number 2.4 and 2.9 the expression patterns of CDeT6-19 could be inferred. All transcripts in figure 2.4 were detected in untreated plants and under different treatment conditions. Nevertheless, unlike CDeT11-24 proteins which were only expressed after 4 hours and 24 hours of dehydration and under NaCl treatment, CDeT6-19 proteins were expressed in untreated tissue and under all treatment conditions. In the overnight treatment, plenty of isoforms were detected due to the use of poly-clonal antibodies, yet all the other bands found at the top could be classified as background. The lower bands had a molecular mass of 16 kDa matching the predicted molecular mass of CDeT6-19.

This implies that the expression of CDeT6-19 protein is neither regulated at the transcript level nor on the translational level. CDeT6-19 is expressed because it was translated under all conditions. Suggesting that, NaCl treatment, sorbitol treatment, and dehydration may activate several pathways which induce the translation of CDeT6-19.

Though CDeT11-24 is regulated at the translational level, Rohrig et al. (2006) showed that both CDeT11-24 and CDeT6-19 are also regulated by post-translational modifictions via phosphorylation which occur directly after their synthesis.

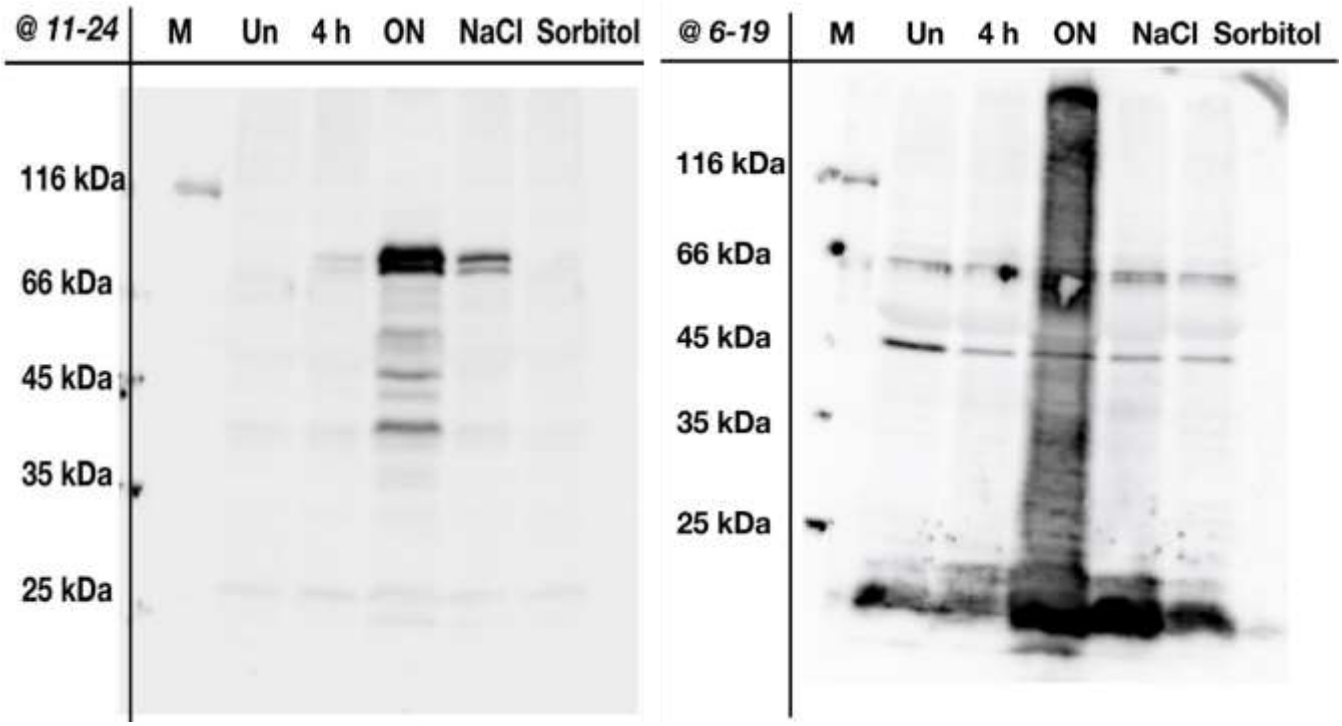


Figure 2.9. Immunoblotting of nitrocellulose membrane using the antibodies of CDeT11-24 (@11-24) and CDeT6-19 (@6-19).

Experiment 3: Promoter regulation studies

3.1. Detection of promoter activity *in situ* staining of the GUS reporter gene in transgenic plants

In this experiment, the ß-Glucuronidase (GUS) expression of five stress-related promoters isolated from *C. plantagineum* CDeT27-45/GUS, CDeT6-19/GUS, CDeT11-24/GUS, pcC13-62/GUS, and C2/GUS was investigated to analyze the impact of different stress parameters on their activity.

Two constructs were used for four chimeric genes whereas only one construct was used for pcC13-62/GUS. For CDeT16-19/GUS: CDeT6-19-1/GUS and CDeT6-19-2/GUS; for CDeT27-45/GUS: CDeT27-45-1/GUS and CDeT27-45-2/GUS; for CDeT11-24/GUS: CDeT11-24-16-4/GUS and CDeT11-24-16-11/GUS; and for C2/GUS: C2-10/GUS and C2-11/GUS.

The study of these promoters have been extended from previous studies conductinig analysis on seeds to transgenic seedlings to expound the impact of stress treatments and ABA-induction on gene expression in mature plant tissue soon after their germination. Similarly, former studies have shed the light on the accumulation patterns of transcripts in response to water stress after applying ABA exogenously. Nevertheless, factors other than changes in cellular ABA concentrations are involved in the activation of embryo development related genes and drought stress responses, implying that ABA-independent pathways may regulate the expression of drought-induced genes (Furini et al., 1996). Therefore, to investigate the previous hypothesis, individual seedlings from *A. thaliana* were not only treated with 100 µM ABA but they were also exposed to dehydration and dehydration-like conditions by applying 150 mM NaCl and 150 mM Sorbitol respectively. The strategy for the measurement of GUS accumulation involved measuring GUA accumulation after incubation and after dehydration. GUS-accumulation in the dehydration analysis was measured at three time frames: 0, 2, and 4 hours.

The promoters have been previously fused with GUS reporter gene and transformed into *A. thaliana* seedlings which were grown for six weeks in a growth chamber. After the successful treatment of seedlings, GUS activity was visualized by *in situ* staining. GUS staining takes advantage of a substrate called X-Gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide). X-Gluc is hydrolyzed by the GUS enzyme generating blue and insoluble dye (dichloro-dibromo-indigo) that can be easily detected under light microscope.

3.1.1. Analysis and comparison of promoter activity after two treatments: 24 hours after chemical treatment of seedlings of fresh leaves and 4 hours after dehyration

GUS activity was measured in developed plants. Fresh leaves taken from developed plants were exposed to NaCl, sorbitol, and ABA treatments. The fresh leaves were incubated and treated for 24 hours before staining them with GUS. To double check our findings using the orginal images showing the GUS-stained seedlings, refer to appenix 2 for the first stress streatment (figure 3.1) and refer to appendix 3 for the second stress treatment (figure 3.2).

High GUS activity was detected in the leaves of transgenic *A. thaliana* seedlings (Figure 3.1) transformed with the following constructs under untreated and treated conditions: CDeT6-19-1, CDeT6-19-2, CDeT27-45-1, C-2-10, and C-2-27 indicating that promoters were stably expressed in untreated leaves and leaves treated with NaCl, sorbitol, and ABA where as no GUS acitivty was identified in CDeT27-45-2. This indicates that CDeT6-19-1, CDeT6-19-2, CDeT27-45-1, C-2-10, and C-2-27 promoters that are expressed in fresh plants are mostly localized in leaves and expressed regularly without treatment and upon exposure to stress. Expression in response to NaCl indicates that the aforementioned promoters are induced due to salt accumulation in their leaves (error during the watering of the leaves). Also, induction as a result of sorbitol illustrates that water loss or osmotic stress induces the expression of these promoters.

Additionally, CDeT6-19-1 after 2 and 4 hours of dehydration in leaves and roots whereas CDeT6-19-2 was only expressed after 4 hours of dehydration. This suggests that CDeT6-19-1 promoter is expressed during dehydration and is important for the survival of leaves and roots. The fact that CDeT6-19-1 was expressed in hydrated tissue shows that the presence of a minimum sactivity of CDeT6-19-1 is important for the intial stress acclimation processes while the induction of expression of CDeT6-19-2 promoter indicates that it is more important in complementing the functions of other genes way after dehydration-induced process has been initiated.

C2-10 was only expressed in dehydrated plant tissues (figure 3.2) which contradicts our previous findings (figure 3.1) which state that C2-10 is expressed after stress treatment. This error may have been caused by mishandling of the tested samples. Similarly, C2-27 was not expressed in hydrated plant tissues (figure 3.2) eventhough they were expressed in fresh leaves (figure 3.1). However, C2-27 was responsive to stress treatment and it was expressed in leaves 2 and 4 hours into dehydration treatment.

On the other hand, it has been found that in leaves, leaf hairs, cotyledons, and roots, CDeT27-45-2-2 and pcC13-62 were silent in fully developed tissue after hydration with water and upon treatment with sorbitol, NaCl, and ABA. The presence of promoter in response to NaCl in roots indicates that salt accumualtion in roots is one of the primary inducers of pcC13-62 promoter.

Likewise, CDeT27-45-2-2 and pcC13-62 were not expressed in GUS-stained dehydrated plant tissues and no expression was detected in roots, leaves, leaf hairs, and cotyledons, even after 4 hours of dehydration (figure 3.2) signifying that CDeT27-45-2-2 may not after all be a stress-inducible gene. Similarly, CDeT27-45-2-1 was not detected in leaves, leaf hairs, and cotyledons, However, it was detected in roots with and without dehydration implying that it may be a constituitively expressed promoter that is localized to roots. It couldn't be concluded from our results if the gene is overexpressed afer dehydration and further quantification experiments should be performed.

Our results didn't show any expression of pcC13-62 promoters under any stress conditions whereas the findings of Giarola et al. (2018) show that pcC13-62 is expressed in the roots upon salt treatment and it is not expressed in hydrated plant tissue of A. thaliana seedlings, demonstrating that pcC13-62 is a stress-inducible promoter.

In fully developed plants, exogenous ABA had an effect on the induction of GUS of plants transformed with C2-27, CDeT11-24-16-11, CDeT27-45-1, and CDeT6-19-1 in root tissue (Figure 3.1).

Upon sorbitol treatment, CDeT26-19-1 is localized to the roots while CDeT6-19-2 is localized to leaf hairs. CDeT27-45-1 is loclaized to the cotyledons implicating it in several processes which may be responsible for stress-induced acclimation, due to water loss, in germinating seeds.

In summation, it has been found that not only the type of environmental stressor but also some limiting factors (e.g. the concentration of solutes, temperature changes, and water loss) play a major role in activity of promoter and determines its localization in plant tissue. ABA induced promoters suggest that regulatory pathways are important for determining the specificity of the promoter and its patterns of expression.

CDeT6-19-1

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	++	-	++	-
NaCl	++	-	-	-
Sorbitol	++	-	-	++
ABA	++	-	-	++
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT6-19-2

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	++	-	-	-
NaCl	++	++	-	-
Sorbitol	++	++	-	-
ABA	++	-	++	++
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT27-45-1

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	+	-	-	-
NaCl	+	-	-	+
Sorbitol	+	-	+	+
ABA	+	-	-	+
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT27-45-2

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	-	-	-	-
NaCl	-	-	-	-
Sorbitol	-	-	-	-
ABA	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT11-24 16-4

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	+	-	-	-
NaCl	+	-	-	+
Sorbitol	+	-	-	-
ABA	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT11-24 16-11

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	-	-	-	-
NaCl	-	-	-	-
Sorbitol	-	-	-	-
ABA	+	-	-	+
Positive control	++	++	++	++
Negative control	-	-	-	-

C2-10

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	+	-	-	-
NaCl	+	-	-	-
Sorbitol	+	-	-	-
ABA	+	-	-	+
Positive control	++	++	++	++
Negative control	-	-	-	-

C2-27

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	+	-	-	-
NaCl	+	-	-	-
Sorbitol	+	-	-	-
ABA	+	-	-	+
Positive control	++	++	++	++
Negative control	-	-	-	-

pcC13-62

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
Water	-	-	-	-
NaCl	-	-	-	-
Sorbitol	-	-	-	-
ABA	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

Figure 3.1. Activity of pcC13-62/GUS promoter and GUS chimeric promoter contructs CDeT6-19-1/GUS, CDeT6-19-2/GUS, CDeT27-45-1/GUS, CDeT27-45-2/GUS, CDeT11-24-16-4/GUS, CDeT11-24-16-11/GUS, C2-10/GUS, and C2-11/GUS without treatment (water), with NaCl treatment

(NaCl), with sorbitol treatment (Sorbitol), and with ABA-induction (ABA). The results show GUS accumulation after incubation. Two controls: positive control and negative control were used for each construct. One (+) sign was used to identify positive accumulation of GUS in untreated and treated seedlings where two (+) signs were used to identify the presence of GUS in positive control samples.

CDeT6-19-1

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	+	-	-	-
2 hours	+	-	-	+
4 hours	+	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT6-19-2

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	+	-	-	-
2 hours	-	-	-	-
4 hours	+	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT27-45-1

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	-	-	-	+
2 hours	-	-	-	+
4 hours	-	-	-	+
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT27-45-2

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	-	-	-	-
2 hours	-	-	-	-
4 hours	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT11-24 16-4

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	+	-	-	-
2 hours	+	-	-	-
4 hours	+	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

CDeT11-24 16-11

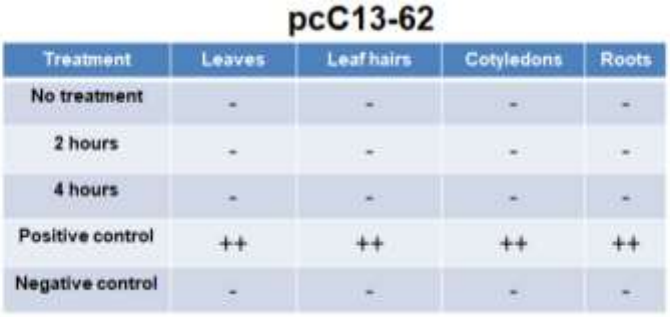
Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	-	-	-	-
2 hours	+	-	-	-
4 hours	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

C2-10

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	+	-	-	-
2 hours	-	-	-	-
4 hours	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

C2-27

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	-	-	-	-
2 hours	+	-	-	-
4 hours	+	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-



pcC13-62

Treatment	Leaves	Leaf hairs	Cotyledons	Roots
No treatment	-	-	-	-
2 hours	-	-	-	-
4 hours	-	-	-	-
Positive control	++	++	++	++
Negative control	-	-	-	-

Figure 3.2. Activity of pcC13-62/GUS promoter and GUS chimeric promoter contructs CDeT6-19-1/GUS, CDeT6-19-2/GUS, CDeT27-45-1/GUS, CDeT27-45-2/GUS, CDeT11-24-16-4/GUS, CDeT11-24-16-11/GUS, C2-10/GUS, and C2-11/GUS without dehydration (no treatment) and after 2 and 4 hours of dehydration (2 hours and 4 hours respectively. Two controls: positive control and negative control were used for each construct. One (+) sign was used to identify positive accumulation of GUS in untreated and treated seedlings where two (+) signs were used to identify the presence of GUS in positive control samples.

3.2. Promoter analyses using transient expression via particle gun bombardment

CDeT11-24 promoter activity was also investigated in transiently transformed *C. plantagineum*. The transformation process involved the integration of a constitutively expressed promoter (35SCaMV) fused to GFP reporter gene as control and LEA-like CDeT11-24 promoter fused to GUS reporter gene. Unlike stable transformation, transient integration process only displays short-term expression of the promoter. CDeT11-24 promoter was coated onto gold nanoparticles and delivered onto second and third whorl *C. Plantagenium* leaves, which have been previously hand-picked and placed on petri-dishes, using particle gun bombardment.

3.2.1. Promoter analyses using transient expression via particle gun bombardment

The long oval structures are endodermal cells whereas the small structures are stomatal cells (figure 3.3). In a typical expiremental setup with successful results it would be expected to obtain a relatively equal expression of GFP and GUS as well as GUS spots should be present in ABA treated leaves due to the fact that genes encoding LEA Proteins (11-24) are ABA-inducible. Also, GUS should be detected in Sorbitol-treated leaves due to the fact that genes encoding LEA Proteins (11-24) are osmotic stress inducible.

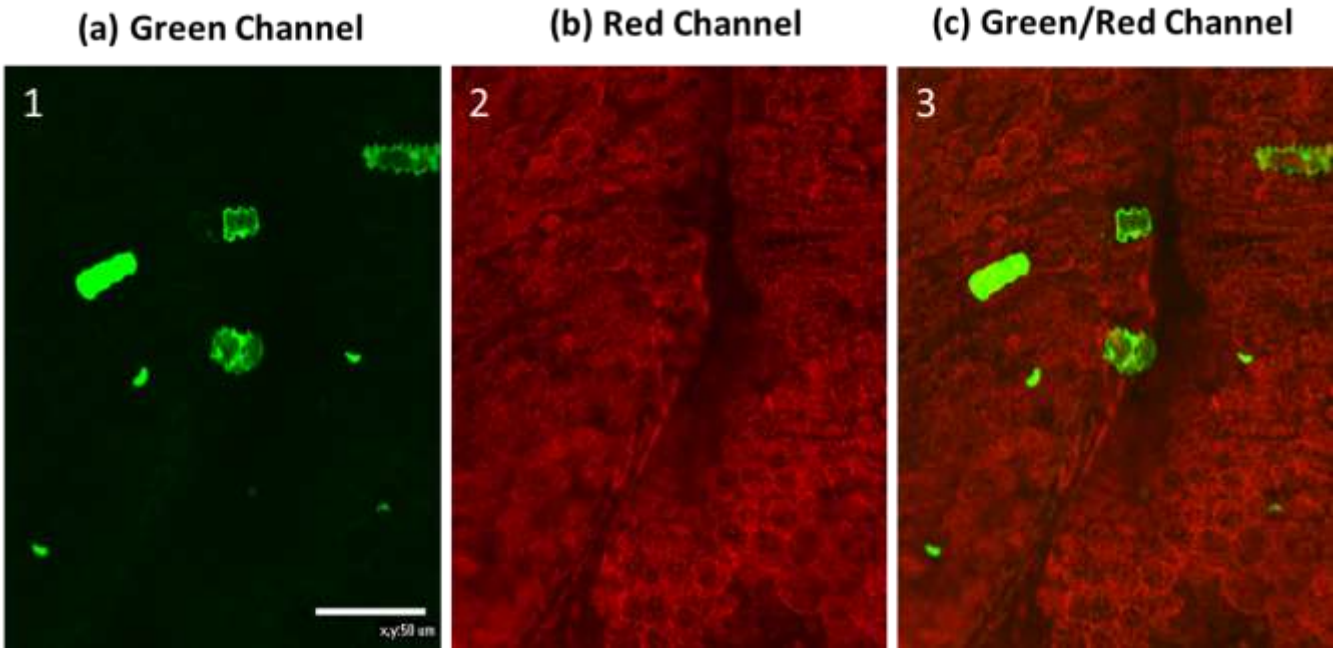


Figure 3.3. Confocal Microscopy images of a C. plantagineum leaf showing epidermal leaf cells and stomata transformed with 35ScaMV/GFP construct. The bright green signal is GFP and the red signal is autofluorescence of the chloroplasts. (a) shows green channel, (b) shows red channel, and (c) shows green/red channel overlay.

However, relative GUS expression in figure 3.4 (a) is relatively low in both Sorbitol- and ABA- treated leaves with almost 10% relative GUS expression. This contradicts with the findings of van den Dries et al. (2011) (figure 3.4; b) which signifies that upon mannitol (an isomer of sorbitol) and ABA treatment, an increase in GUS expression from 10% in water to 81% in Sorbitol treated transgenic leaves and 68% in ABA treated transgenic leaves was detected, corroborating the previous hypothesis which states that CDeT11-24 is an osmotic and ABA inducible promoter.

In summation, the tested CDeT11-24 promoter fused to the GUS reporter gene was not highly expressed in the transformed cells treated with sorbitol and ABA indicating that the promoter was not activated due to either osmotic stress or ABA exposure. An important hurdle to particle gun bombardemnt is that the gold particles must be treated with car and caution otherwise mispipetting will diminsh the amount of available particles for coating, thereby affecting the subsequent transformation-related steps.

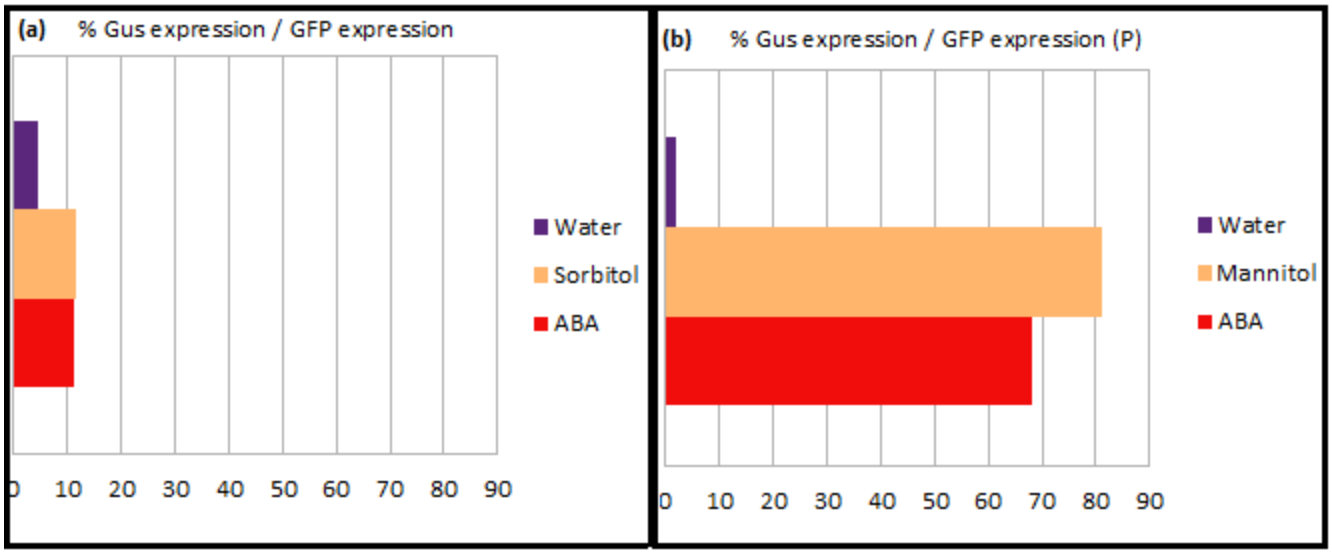


Figure 3.4. Relative expression of GUS with respect to GFP. (a) Relative expression in expirment 3.2 in water, sorbitol, and ABA-treated transgenic *C. plantagenium* leaves. (b) Relative expression of transgenic C. plantagineum leaves in response to water, sorbitol, and ABA-treatments; exported from van den Dries et al. (2011).

3.3. Promoter analyses using transient expression via agro-infection

The activity of CDeT11-24 promoter was investigated in *C. plantagineum* transiently transformed using agro-infection. Similar to the previous transformation, agro-infection utilized the integration of a constitutively expressed promoter (35SCaMV) fused to GFP reporter gene as control and LEA-like CDeT11-24 promoter fused to Gus reporter gene. After preparing *Agrobacterium tumerfaciens* electro-competent cells, the constructs were transferred into the plasmids of *A. tumerfaciens* using electroporation method (figure 3.5). Afterwards, colony PCR was performed to identify positive clones which have the constructs successfully inserted into their plasmids (figure 3.6). Clone number 1 was chosen for plant infection. Clone 1 of *A. Tumerfaciens* was used to infect *C. Plantagineum* leaves. Three stress treatments were conducted: 0.5 M sorbitol, 100 µM ABA, and water as control. After 48 hours of incubation, GUS assay was performed by staining the transformed leaves with X-Gluc and subsequently destained. Leaves were inspected under the binocular light microscope (figure 3.7).

Since CDeT11-24 is ABA and sorbitol inducible, it would be expected to see GUS expression after ABA and sorbitol treatments.

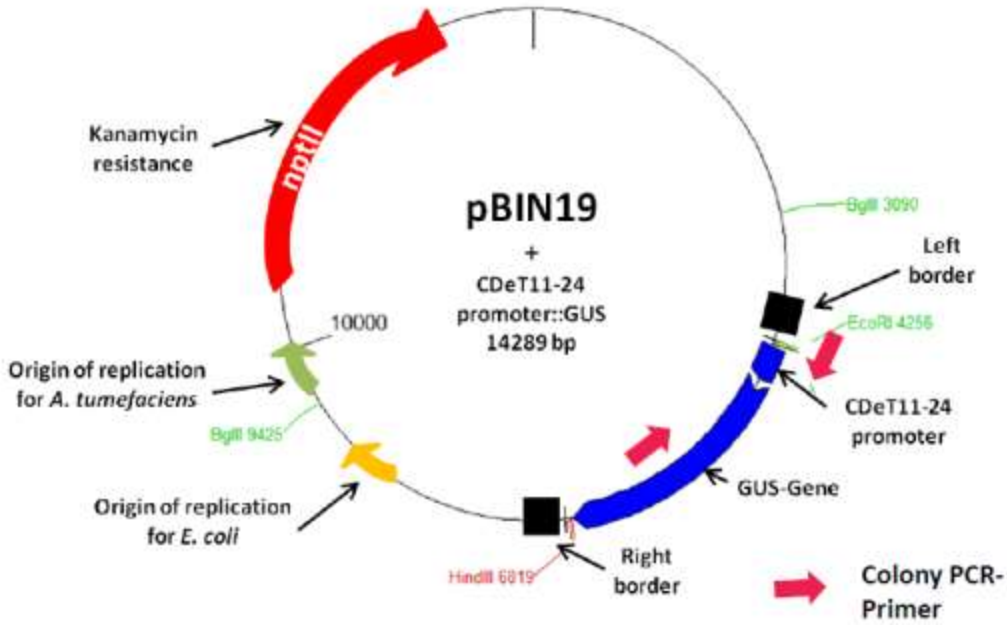


Figure 3.5. pBin19 A. tumerfaciens plasmid with the fused LEA-like CDeT11-24/GUS construct.

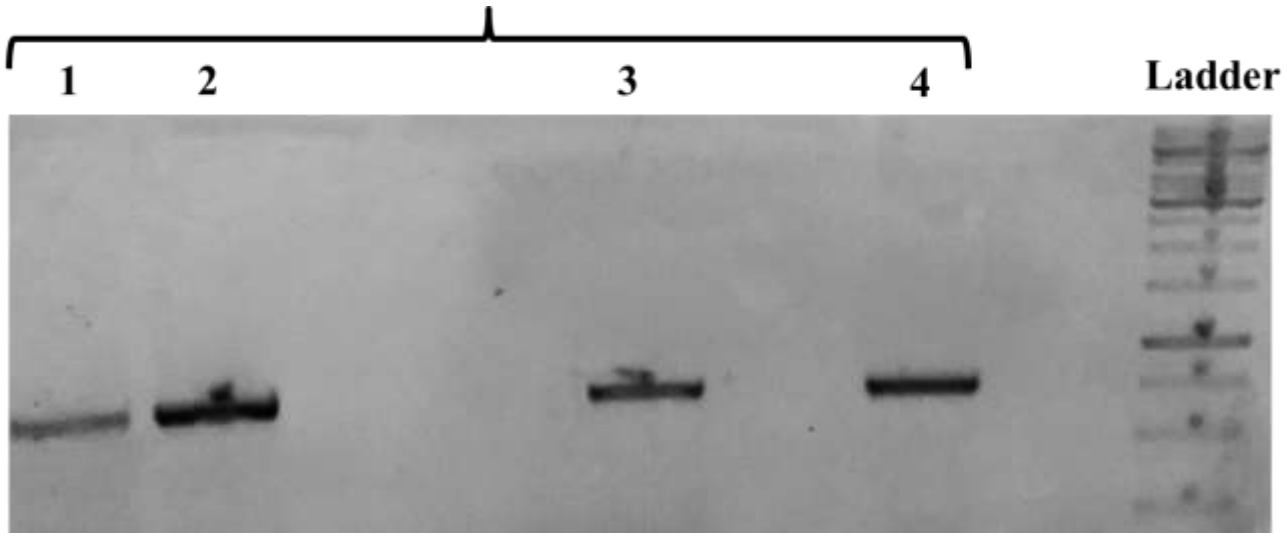


Figure 3.6. Screening *A. tumerfaciens* colonies for positive clones. 1, 2, 3, and 4 represent positive clones. Clone number 1 was used to infect *C. plantagineum* leaves.

Low amounts of GUS were detected in water treated samples. As compared to control, ABA (figure 3.7; e-h) and sorbitol (figure 3.7; i-l) showed strong induction of CDeT11-24. These findings are in accord with the experiments carried out in previous sections. By comparing the results in figure 3.7 to those in figure 2.7, the same expression patterns after ABA and sorbitol treatments could be inferred.

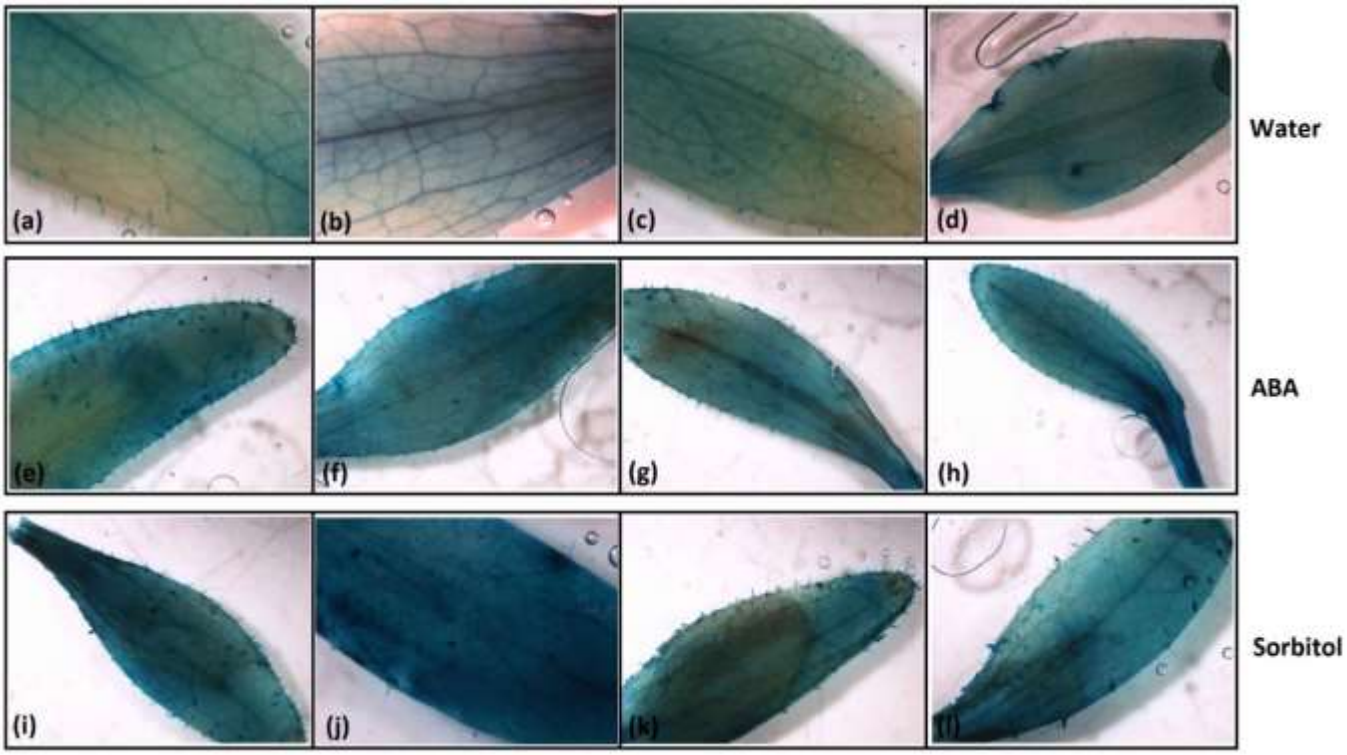


Figure 3.7. GUS staining of transgenic C. plantagineum leaves carrying transiently expressing the CDeT11-24/GUS construct. Images (a) to (d) show control leaves irrigated with water; water was used as control. Images (e) to (h) show ABA-treated leaves. Images (i) to (l) show sorbitol-treated leaves.

Experiment 4: Screening of an *Arabidopsis thaliana* T-DNA insertion population in search for mutations in specific genes

Insertional mutagenesis has proven to be an essential tool in understanding the underlying functions of newly discvoered genes. The method utilizes a sequence called insertion, which is a segment of foreign DNA that will be inserted into the genome of the desired plant thereby generating various mutations (e.g., knock-out, knock-down, or overexpression). The effect of a mutation on a mutagen can vary from loss-of-function to overexpression. The insertion can be a semirandom translocation of a highly active transposable element (Parinov et al., 1999) or the result of a highly efficient transformation of *A. thaliana* with *Agrobacterium tumerfaciens* harboring a Ti plasmid (Tumer-inducing plasmid) (Parinov et al., 1999; Krysan et al., 1999) termed T-DNA tagging. The natural process of infection and transformation of wounded plants termed T-DNA tagging is utilized by researchers to transform plants by modifying the T-DNA segment of the Ti plasmid. The T-DNA region can then be transferred to the host plant and incorporated stably into its genome therefore altering the normal functions of the genes.

One of the prominent features of insertional mutageneis is that DNA elements are inserted at random within chromosomes. As opposed to transposons, using T-DNA as the insertional mutagen doesn't result in the transposition of the insertion subsequent to integration within the plant's genome and T-Dna insertions can remain emically and physically stable through multiple generations (Krysan et al., 1999; (Radhamony et al., 2005). A second advantage of using T-DNA manifests due to the fact that the insertion doesn't only disrupt the gene but it also act as a marker for the identification of the mutation. Since the introns of *A. thaliana* are too small, an insertion on the order of of 5 to 25 kb disrupts the function of a gene (Radhamony et al., 2005). Thirdly, by using T-DNA insertions, it is possible to maintain mutations that are homozygous lethal in a population in the form of heterozygous plants (Radhamony et al., 2005).

On the other hand, one troublesome disadvantage of T-DNA isnertions is that the structure of these insertions are much more complex involving multiple copies of T-DNA. In unusual cases, deletions, rearrangements, and/or duplications of genomic regions may be accompanied T-DNA insertions (Tax and Veron, 2001; , Nacry et al., 1998). resulting in the identification of false positives during the screening process unless very specific precautions are taken (Stepanova and Alonso, n.d.).

Nonetheless, using transposons may prove beneficial in special situations in which multiple members of a gene family are arranged in tandem along a chromosome, then, the ability of a transposon to hop to neighbouring genes may provide a convenient method for creating mutations within all of the members

of the targeted gene family (Krysan et al., 1999). Yet, if the plant under investigation has active transposons, several experimental approaches must be followed to ensure the elimination of potential false positives due to somatic transpositions (Wisman et al., 1998).

Three objectives were accomplished. First, three mutant lines have been identified from a large population (39,700 plant line) of *A. Thaliana*. Moreover, the location of the insertion site of T-DNA (in exon or intron) was acknowledged and the nature of the insertion was classified into concatemeric or non-concatemeric.

4.1. PCR Strategy

Since there is no efficient homologous recombination system in *A. thaliana* which could specifically and systematically generate knockouts for all *A. thaliana* genes, the generation of large collections of insertions at random (Parinov and Sundaresan, 2000) by using a large population of T-DNA–transformed lines, may give us a reasonably good chance of finding a transgenic plant carrying a T-DNA insert within any gene of interest.

To screen the collections of generated mutant lines for a knock-out in a specific gene, two different strategies are commonly employed: a gene-specific PCR-based approach (Winkler et al., 1998) and systematic sequencing of insertion sites. However, only the gene-specific PCR approach was followed in this experiment. Since the sequence of the inserted element is known to us Polymerase Chain Reaction (PCR) can be used to recover the gene with the desired insertion (Radhamony et al., 2005) thereby isolating the plants which carry the T-DNA mutation of interest (McKinney et al., 1995; Krysan et al., 1996). It should be noted that both gene-specific primers and T-DNA primers were used to identify the mutants; more about the primers will be discussed in the ensuing sections.

Once the type of mutagenesis has been chosen and the gene-specific PCR approach has been developed (i.e. ordering the required gene-specific and T-DNA primers), the next critical decision is to decide wether the plants should be grown in pools or individually. In this experiment the bi-dimensional strategy was followed twice to facilitate running the PCR in fewer samples. Therefore, 39,700 transgenic lines has been arranged into pools of 100 plants (P-100). Then every 4 pools of 100 (P-100) were combined to form 1 pool of 400 (P-400). Then 20 pools of 4000 (P-4000) were formed by combining 10 pools of 400 (P-400). Since the bidimensional strategy is being followed (i.e. 10 of the P-4000 pools are letter pools and 10 of the P-4000 pools are number pools), in the end we had 10 P-4000 letter pools termed (A, B, C, D, E, F,

G, H, I , and J) and 10 P-4000 number pools numbered (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10) representing 39,700 P-400 transgenic lines twice; once in the letter pool and once in the number pool (see appendix 1).

Four primer combinations have been chosen for this experiment indicating that only 20 PCR reactions are required for each primer combination (80 PCR reactions in total). When the samples are run on agarose gels with ethidium bromide (first PCR round), two bands in the letter pools and two bands in the number pools are expected to be found for every identified mutant line. The first PCR round narrows down our search for mutant lines from 39,700 plants to 400.

After identifying the mutant lines, they are run using their respective primer combinations in a second PCR reaction. A positive control has been run next to the mutant lines samples. Thus, 6 individual PCR reactions (2 for positive control and 4 for the P-100 lines) are prepared. The second PCR round narrows down our search for mutant lines from 400 to just 100.

Furthermore, subsequent to identifying the mutant lines from P-100, the seeds of the identified mutant lines are sterilized then spread on MSAR Agar plates to germinatre and grow for 3 weeks. Next, DNA was extracted from the leaves of every P-100 muant line. Prior to running the PCR reactions, the primer combinations which gave positive results in previous PCR rounds were chosen, and a new bi-dimensional strategy was devised. Each of the P-100 plants was represented twice, one time in a number pool and one time in a number pool. Afterwards, the PCR reactions were run on agaorse gels and the mutant lines were identified. The third PCR round narrows down our search for mutant lines from 100 to just 3.

4.2. Results and Discussion

4.2.1. Primer Combinations

For performing the PCR reactions, two primers are needed, one gene primer and one T-DNA primer. In the end, four primers producing four bands must be used to identify one mutant on the gel. For performing the first PCR reactions, four possible primer combinations, shown in figure 4.1, were compiled.

Primer Name	Forward and Reverse Primers	
P1	5´gene primer	Left Border T-DNA Primer
P2	5´gene primer	Right Border T-DNA Primer
P3	3´gene primer	Left Border T-DNA Primer
P4	3´gene primer	Right Border T-DNA Primer

Figure 4.1. The four primer combinations (P1, P2, P3, and P4) used to perform first round of PCR.

4.2.2. First PCR Round

In figure 4.2, the letters and number pools of the first PCR round for ALDH 3H1 genes is shown. The results indicate that the bands are strong and no background has been seen interferring with the results.

The molecular-weight size marker was used to identify the approximate size of a DNA in base pairs (bp). The wild type (WT) bands were strong and their sizes corresponded to those found in the database (3000 bp). Nevertheless, two ghost bands were detected in number pool 6 and letter pool G-P3. Only one mutant was identified on the gel (Mutant G4). Yet, only one band was detected in the number pool 4 (P4) and one band was detected in the letter pool G (P4).

These results shows only one mutant G4 (figure 4.2) and misses the second mutant B8. Also only two bands instead of four were detected in G4. This may be caused by using the wrong primers or mispipetting the master mix or any of its components. Due to the detection of only one band, no conclusions can be made from figure 4.2 regarding the size of the gene of G4.

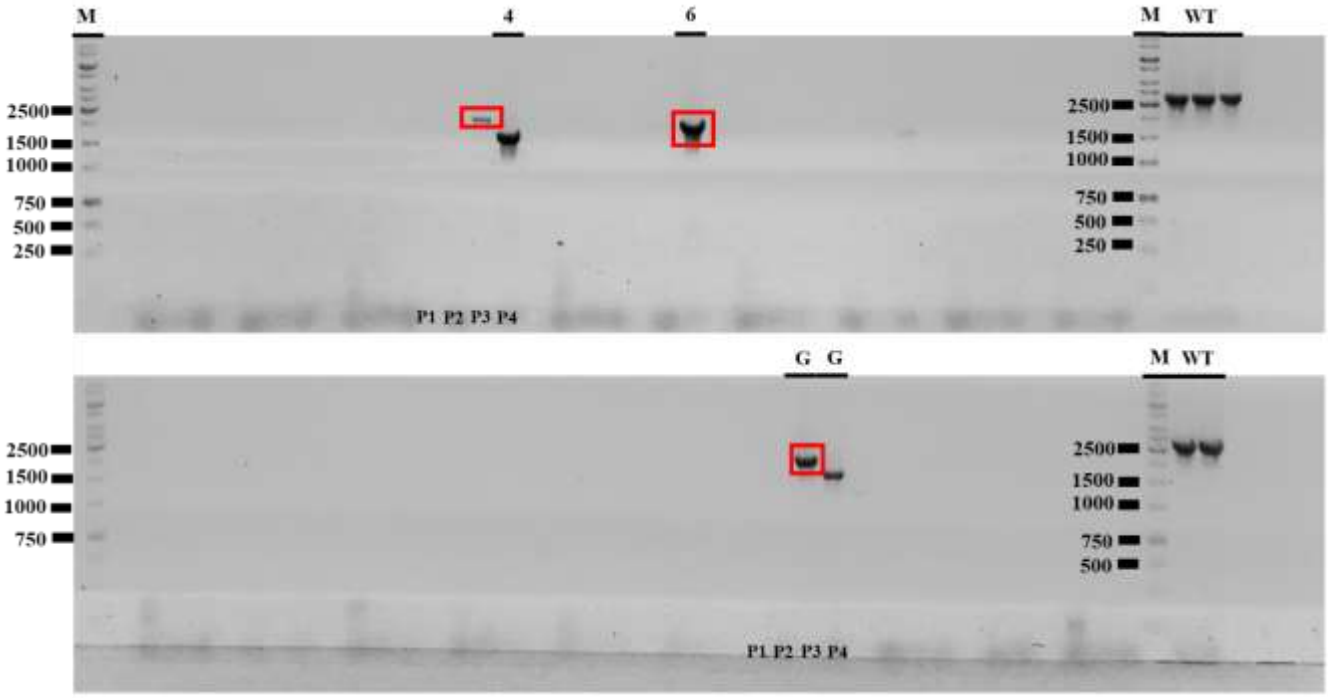


Figure 4.2. The agarose gel picture of ALDH3H1 gene for P-4000 pools. The red boxes show the ghost bands. The molecular-weight size marker shows the size of DNA fragments in bp. The lower line labelling shows the primer combinations (P1, P2, P3, and P4).

Former year's results (figure 4.3) also showed two ghost bands in the number pool 4-P3 and the letter pool G-P3. Since the same ghost bands were detected in this year's results (figure 4.2) these ghost bands may be the result of unwanted insertion in an unwanted location that has been maintained throughout the years. For primer combination P1 and P4, the mutant G4 was identified. Two strong bands were detected in letter pool G and number pool 4 (figure4.3). Whereas for primer combination P1 and P3, two bands were detected in letter pool B and only one band was detected in number pool 8-P1. Concerning the size of the bands, the size of ALDH3H1 in mutant G4 and B8 can be calculated by adding up the sizes of the two bands, however, the marker can't be clearly seen in figure 4.3 and the calculation of the sizes could be done after performing the second PCR round.

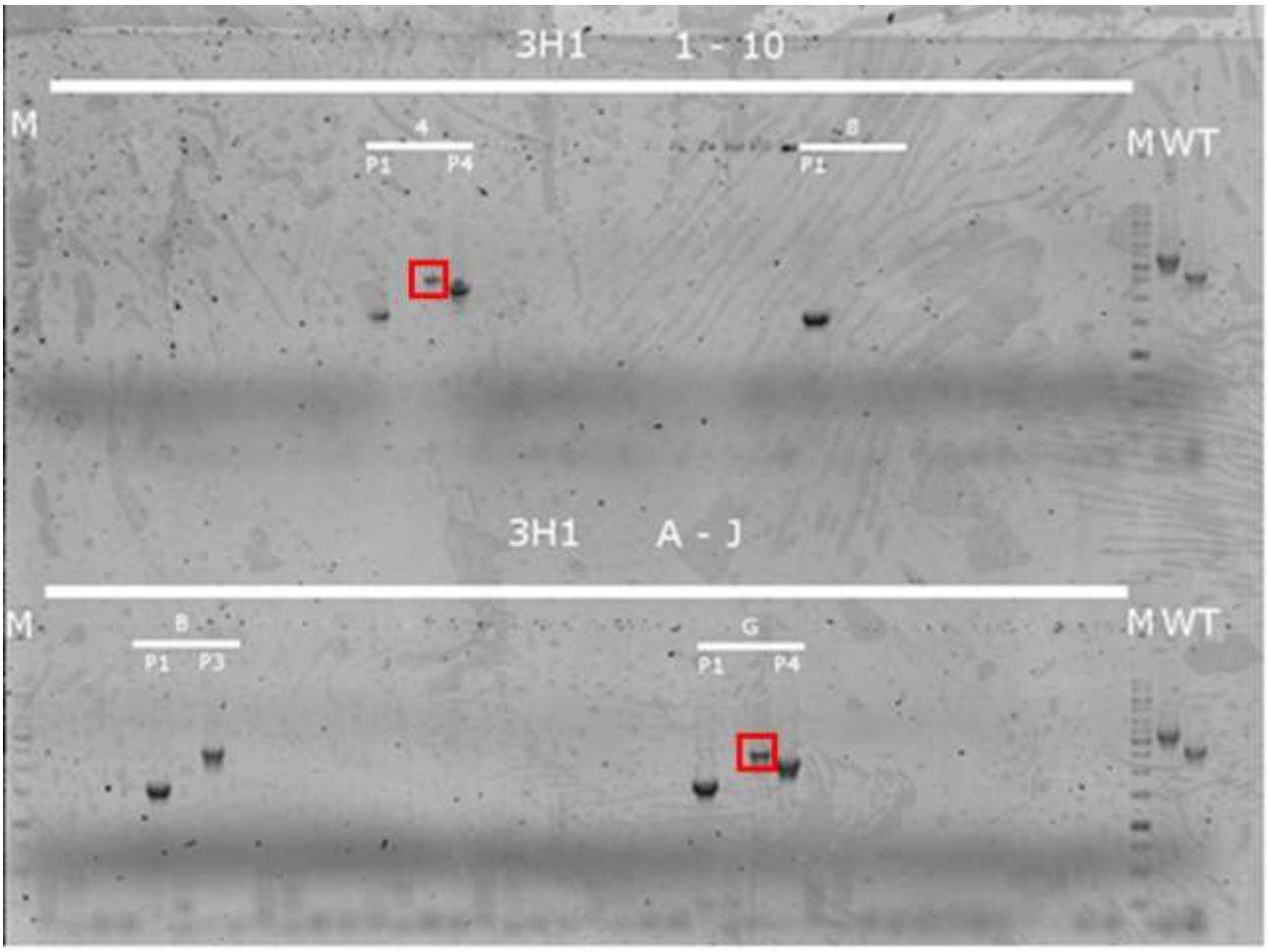


Figure 4.3. The agarose gel picture of former year's results for ALDH3H1 gene for P-4000 pools. The red boxes show the ghost bands. The molecular-weight size marker shows the size of DNA fragments in bp.The lower line labelling shows the primer combinations (P1, P2, P3, and P4). Two primer combinations (P1-P4) and (P1-P3) could be successfully detected.

In figure 4.4, the letters and number pools of the first PCR round for ALDH3I1 genes is shown. The results indicate that the bands are strong and no background has been seen interferring with the results. Two bands were detected in number pool 6 and one in letter pool I. However, ghost bands were detected in letter pools F, G, and J.

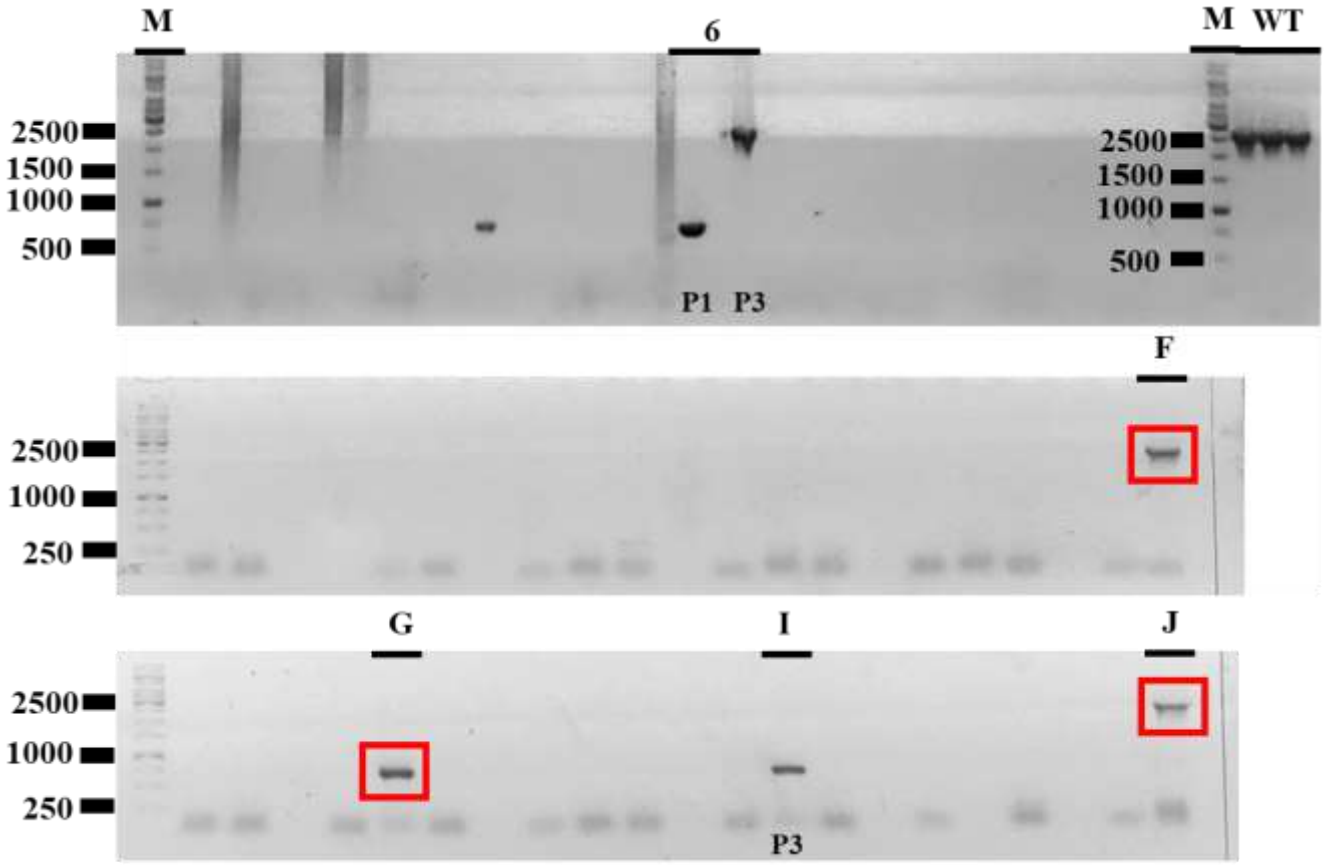


Figure 4.4. The agarose gel picture of former year's results for ALDH3I1 gene. The red boxes show the ghost bands. The molecular-weight size marker shows the size of DNA fragments in bp.The lower line labelling shows the primer combinations (P1, P2, P3, and P4). The primer combination (P1-P3) was successfully detected.

The results of former years shows that the bands for insertions in ALDH3I1 gene were strong and no background interference was detected (figure 4.5). Also, no ghost bands were recognized. One mutant was identified (I6) and four bands were detected; two bands in the letter pool (I) and two bands in the number pool (6). By this stage, three mutants have been idetifed with each one of them present once in the letter pool and once in the number pool (mutants lines 4G, B8, and I6). Appendix 1 can be used to determine the P-400 pools which contain the mutant lines by crossing the numbers and letter, that is, for the mutant line 4G four P-100 pools numbered 64, 164, 264, and 364 were taken for the second round of PCR. The same is applied for B8 (the four P-100 pools are 18, 118, 218, and 318) as well as for I6 (the four P-100 pools are 86, 186, 286, and 386). The corresponding P-400 pools will be used along with their primer combinations in the second PCR to narrow down the number of plants from 400 to 100 for each mutant line.

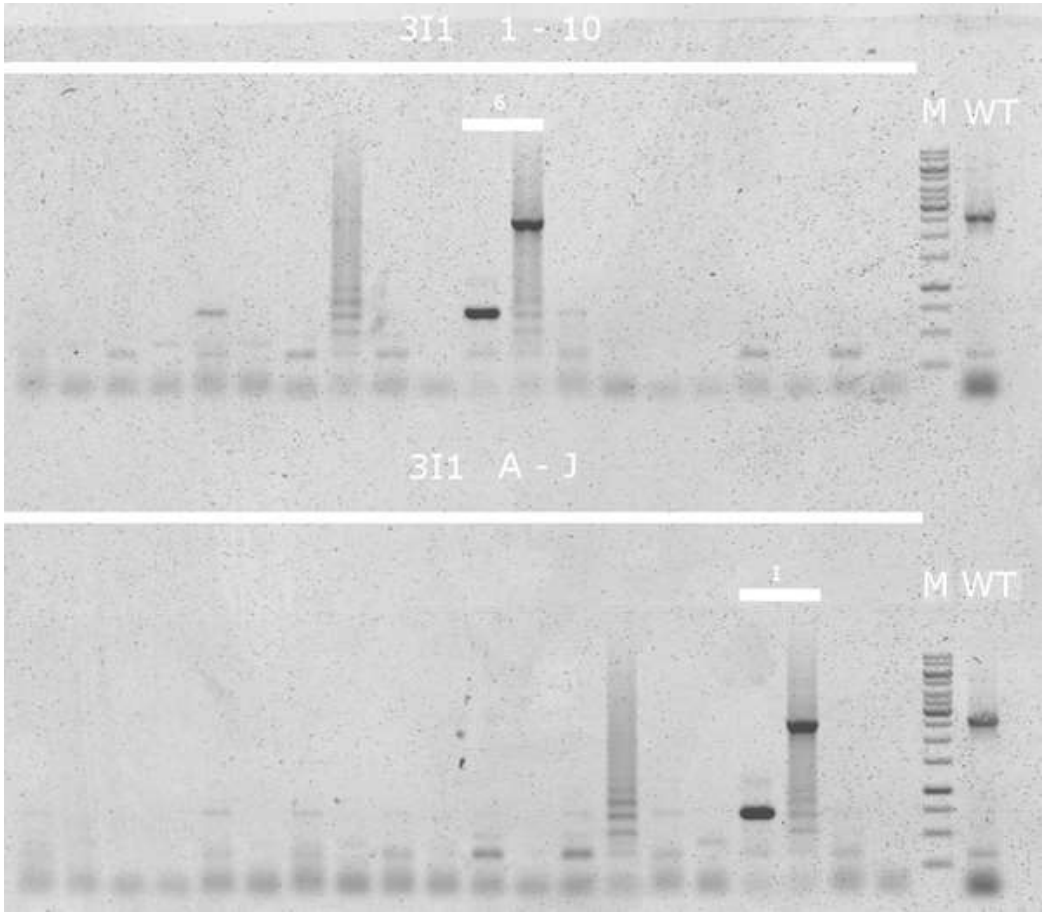


Figure 4.5. The agarose gel picture of former year's results for ALDH3I1 gene for P-4000 pools. The molecular-weight size marker shows the size of DNA fragments in bp. The primer combination of the letter and number pools was P1 and P3.

4.2.3. Second PCR

The prevously identified P-400 pools were run on agarose gel to identify the mutant lines in the P-100 pools. The positive control in red for G4, B8, and I6 were consistent with the previous run in the second PCR and showed bands of the same sizes. Three pools with the mutant lines were identified: P-100 pool number 164 for G4, P-100 pool number 218 for B8, and P-100 pool number 386 for I6 (see figure 4.6).

P-100 pools number 218 and 164 represent plant lines with possible insertions affecting the ALDH3H1 gene whereas the P-100 pool number 386 represents plant lines with possible insertions affecting the ALDH3I1 gene. After finding the P-100 pools, further steps must be taken before performing the third and final PCR round.

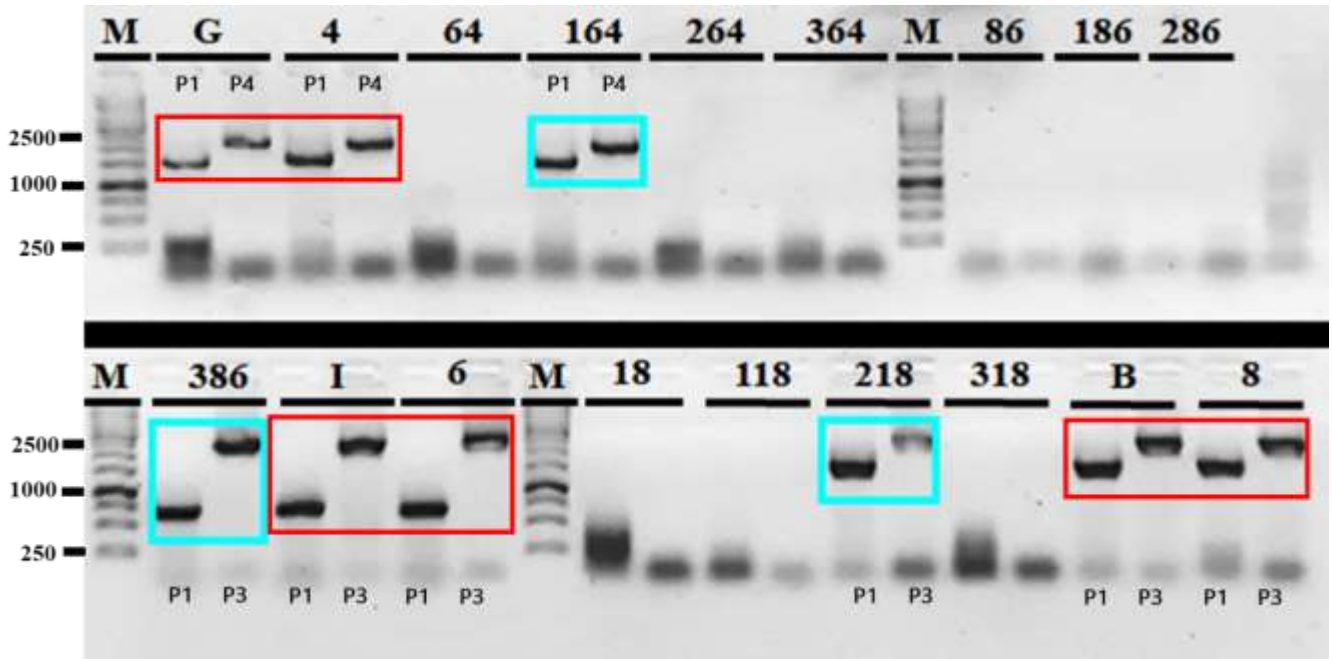


Figure 4.6. The agarose gel picture of the second PCR for P-400 pools of ALDH3H1 and ALDH3I1 genes. The molecular-weight size marker shows the size of DNA fragments in bp. The primer combination P1 and P3 was used for I6 and B8 while primer combination P1 and P4 was used for G4. The red boxes show the positive control for letter andnumber pools representing the P-400 pools. The light blue boxes show the P-100 pools which contain the desired mutant line.

4.2.4. Third PCR

Subsequent to identifying the three P-100 pool (164, 218, and 386) which contains the mutant line, the seeds from these pools were sterilized and dried, then sown on MSAR AGAR plates such that only 1 line is represented by an individual plate. The petri-dishes were transferred to the growth chamber and the seeds were left to germinate for 2 weeks. Plates were inspected afer one week to check for any contaminations and only one plate which was heavily conaminated was discarded. The lab supervisor confirmed that the discarded plate didn't contain any of the three mutant lines. Finally, the genomic DNA from the leaves of the grown seedlings was isolated using the protocol offered by Rogers and Bendich (1985) and the three mutant lines were identified.

Nonetheless, a new bidimensional strategy have been developed for the third PCR. Each individual plant was present in two pool, once in the letter pool and once in the number pool, that is, every P-100 pool which had 100 independent plant lines were divided into ten groups each containing ten individual plant lines (3 leaves from each line were used to perform the PCR reactions). Every group of ten was represented once in the letter pool and once in the number pool. In the end 60 PCR reactions must be performed (30 for letter pools and 30 for number pools) and the same primer combinations used in the first and second PCRs are ought to be used.

The gel run for the third PCR is shown in figure 4.7. In the first P-100 letter andnumber pools, no mutant lines were identifed, however, it was expected from previous years that for P-100 number 218, bands in lanes 5 and H should be identified. Nevertheless, bands were successfully identified in P-100 pools number 164 (lanes 8 and E) and 386 (lanes 2 and G).

Consequently, appendix number 2 is used to cross the letter and number coordiantes to identify the mutant lines. The muant *A. thaliana* lines for H5, E8, and G2 are 21825, 16398, and 38612 respectively (see appendix 2).

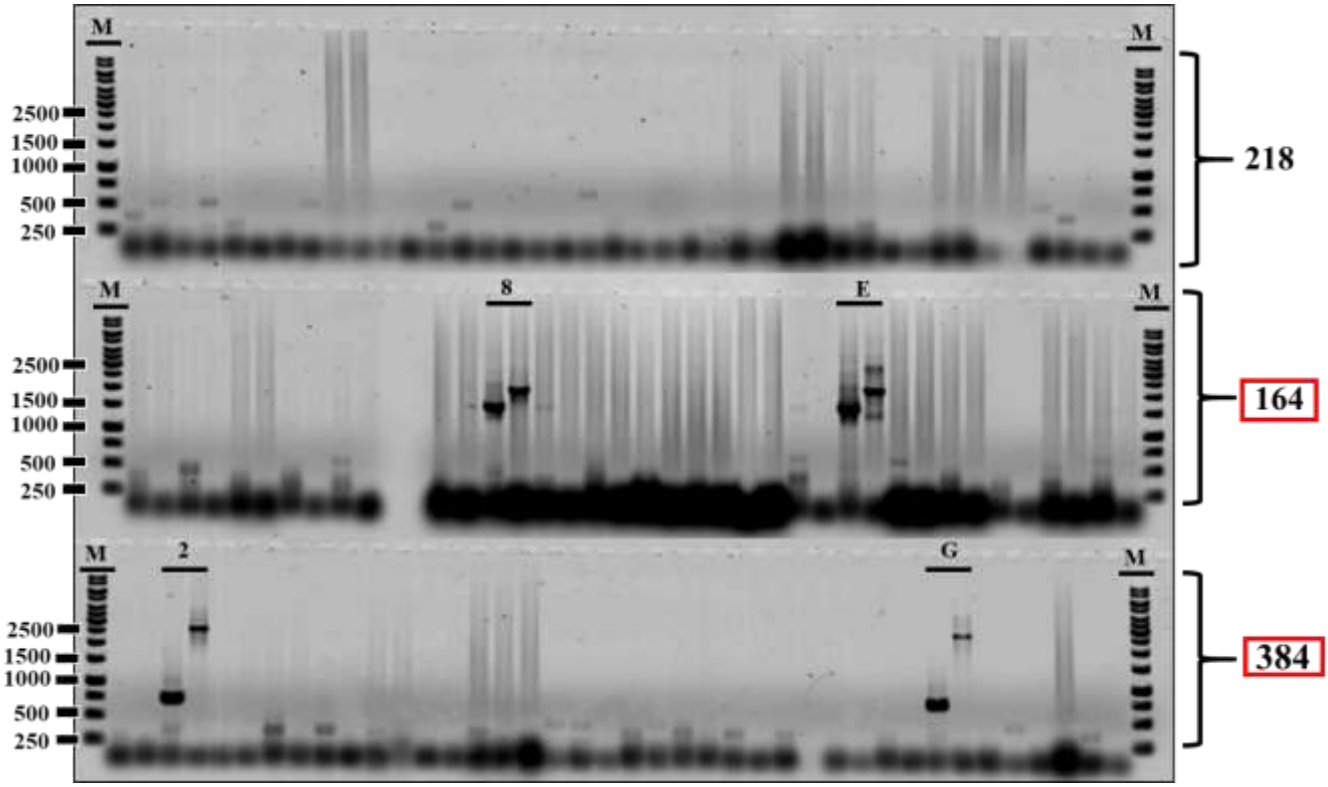


Figure 4.7. The agarose gel picture of former year's results. Third PCR for P-100 pools of ALDH3H1 and ALDH3I1 genes. The molecular-weight size marker shows the size of DNA fragments in bp. The primer combination P1 and P3 was used for I6 and B8 while primer combination P1 and P4 was used for G4. The red boxes show the mutant lines which have been positiviely identified.

4.2.5. Analysis of the identified insertions in ALDH3H1 and ALDH3I1 genes

4.2.5.1. Position of the three insertions

Before locating the position of the insertions, it is imperative to estimate the sizes of the bands for every mutant line that has been detected. The size of the gene in mutant lines 21825, 16398, and 38612 representing 218, 164 and 386 respectivey was estimated using the results of the second shown in figure 4.6. The ALDH3H1 gene in mutant lines 21825 and 16398 had a total size of 3500 bp which is closely similar to the expected size of 3359 bp. Concerneing the gne ALDH3I1 in mutant line 38612, the total size was determined (2625 bp) which is 58 bp less than the expected size (2683 bp) (see figure 4.8).

	Gene		
	ALDH3H1		ALDH3I1
Mutant Line	21825 (P-218)	16398 (P-164)	38612 (P-386)
Lower Band (bp)	1400	1500	625
Upper Band (bp)	2100	2000	2000
Total Size (bp)	3500	3500	2625
Expected Size (bp)	3359	3359	2683

Figure 4.8. The sizes of the lower band, upper band, and total size of gene for mutant lines 21825, 16398, and 38612. P-218, P-164, and P-386 represent the P-100 pools. The sizes of PCR fragments are in base pairs (bp).

After performing the PCR, the PCR products are usualy sen for sequencing to determine the exact location of the insertion in the mutant gene. Howeve, due to the lack of time, no PCR products were sent for sequencing. Therefore, we were provided with an estimation of the size of mutant gene (around 500 bp or less). To provide a theoratical estimation for the position of the insertion, 500 is divided by two yielding two hypothetical fragments of 250 bp in length. Then, 250 bp was subtracted from the size of the lower band of all three mutant lines yielding 1150 for mutant line 21825, 1250 for mutant line 16398, and 490 bp for mutant line 38612 (see figure 4.9). It should be noted that the two insertions were identified in the second intron of ALDH3H1 and ALDH3I1 genes. The third insertion musst be the in the second intron, however, our results indicated that the insertion in 38612 is in the first exon. Therefore, we made an assumption that the length of the integrated mutant gene was less than 500.

4.2.5.2. Determining the nature of the insertion

The presence of one left border and one right border in inserted T-DNA indicate that the T-DNA insertion is non-concatemeric, whereas the presence of two left border regions in inserted T-DNA indicate that the T-DNA insertion is concatemeric. The latter can be detected by using only the left border primer in both primer combinations to amplify the T-DNA region. Both 38612 of ALDH3I1 and 21825 of ALDH3H1 are concatemeric while 16398 of ALDH3H1 is non-concatemeric (see figure 4.9).

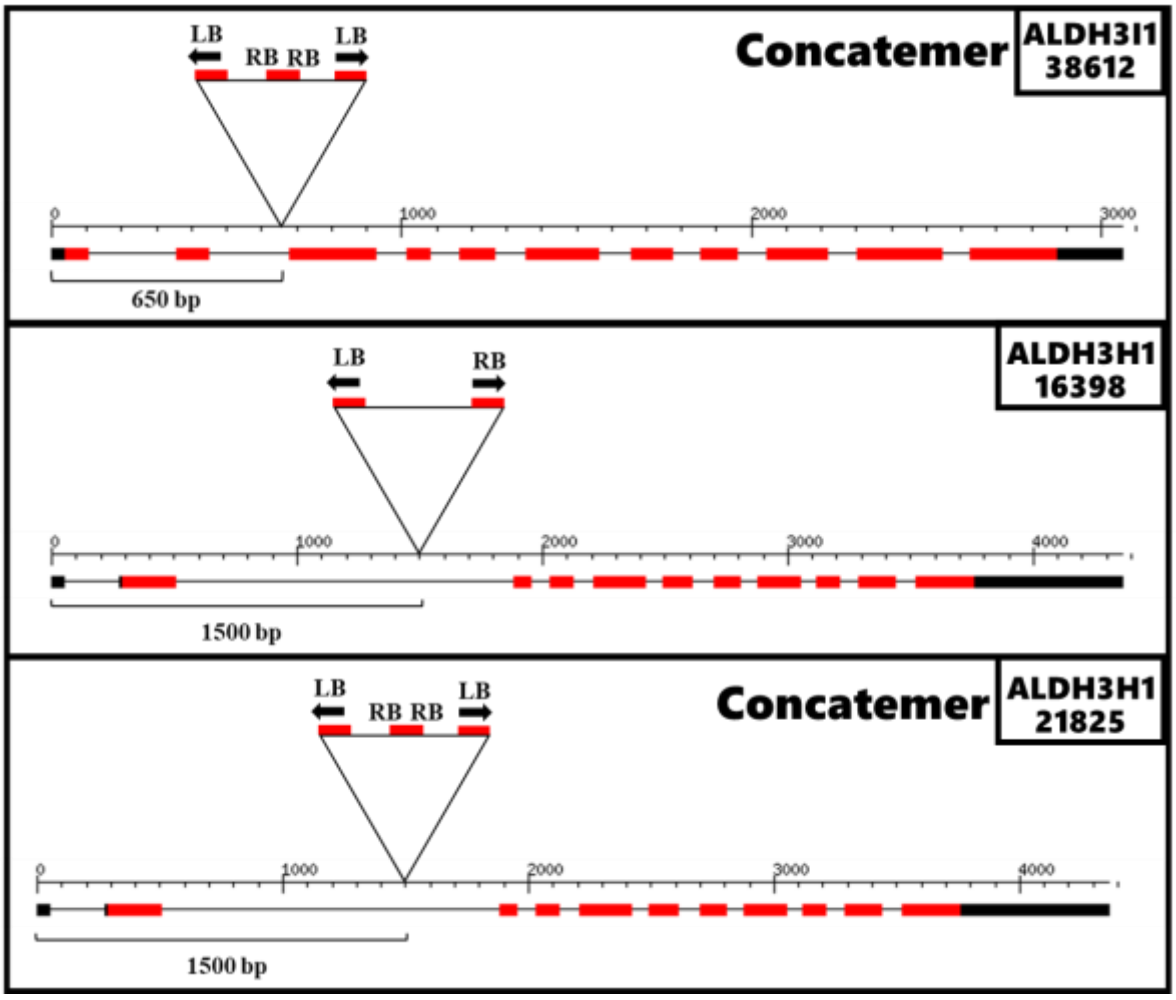


Figure 4.9. The position of the insertions for mutant lines 21825 and 16398 in ALDH3H1 and 38612 in ALDH3I1. The T-DNA primers specific to the PCR reactions are shown above the triangles. LB means left border and RB means right border.

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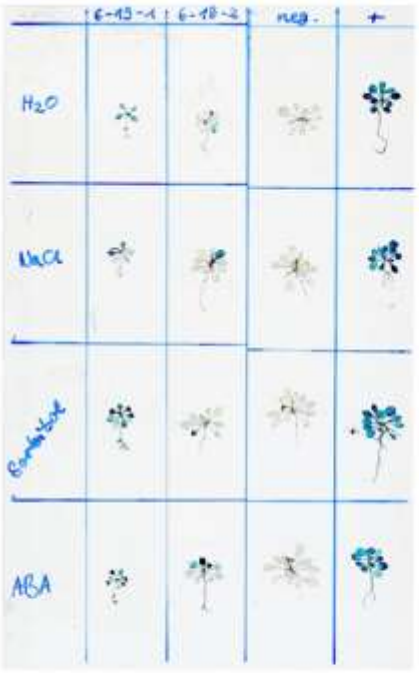
Supplementary Data

Appendix 1. Relative ion leakage dataset for after 3 days and 7 days of NaCl treatment.

	Relative ion leakage (%)			
[NaCl]	*Arabidopsis thaliana*		*Eutrema salsugineum*	
	After 3 days	After 7 days	After 3 days	After 7 days
0 mM	7	12	14	18
100 mM	19	66	23	35
200 mM	27	68	16	29
300 mM	31	79	10	88
600 mM	58	99	23	77

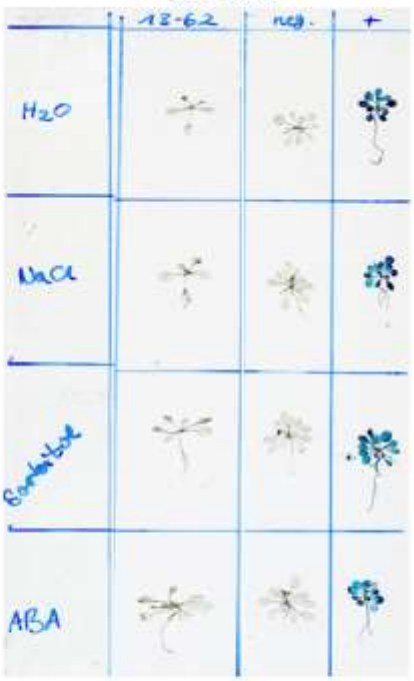
Appendix 2. Activity of pcC13-62/GUS promoter and GUS chimeric promoter contructs CDeT6-19-1/GUS, CDeT6-19-2/GUS, CDeT27-45-1/GUS, CDeT27-45-2/GUS, CDeT11-24-16-4/GUS, CDeT11-24-16-11/GUS, C2-10/GUS, and C2-11/GUS without treatment (water), with NaCl treatment (NaCl), with sorbitol treatment (Sorbitol), and with ABA-induction (ABA). The results show GUS accumulation after incubation. Two controls: positive control (+) and negative control (neg) were used for each construct.

CDeT6-19-1 and CDeT6-19-2

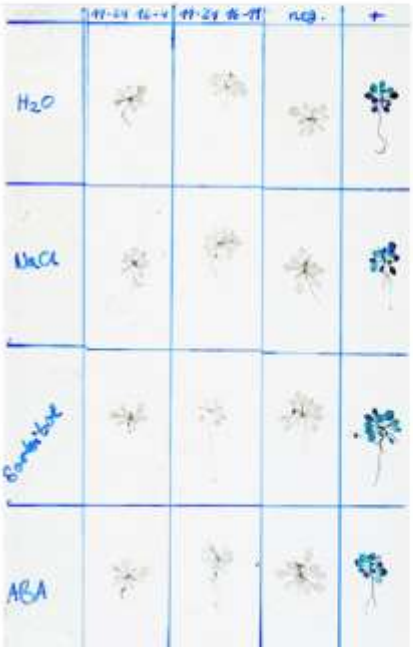


CDeT27-45-1 and CDeT27-45-2

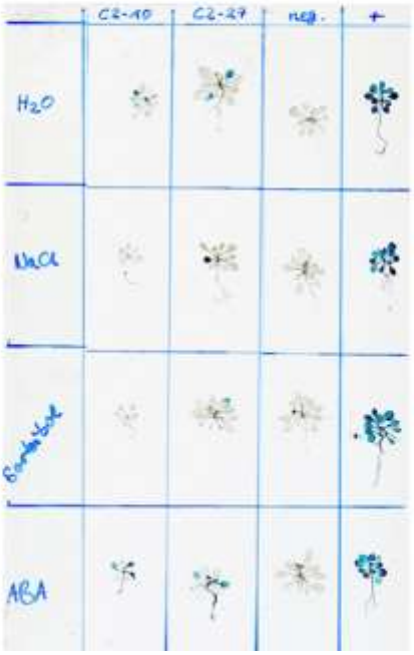
pcC13-62



CDeT11-24-16-4 and CDeT11-24-16-11



C2-10 and C2-27



Appendix 3. Activity of pcC13-62/GUS promoter and GUS chimeric promoter contructs CDeT6-19-1/GUS, CDeT6-19-2/GUS, CDeT27-45-1/GUS, CDeT27-45-2/GUS, CDeT11-24-16-4/GUS, CDeT11-24-16-11/GUS, C2-10/GUS, and C2-11/GUS without dehydration (no treatment) and after 2 and 4 hours of dehydration (2 hours and 4 hours respectively). Two controls: positive control and negative control were used for each construct.

Appendix 4. The Bi-dimensional strategy for the 20 pools of 4000 (P-4000) which were divided into 10 letter pools of 400 plants each and 10 letter pools of 400 plants each.

	1	2	3	4	5	6	7	8	9	10
A	1	2	3	4	5	6	7	8	9	10
	101	102	103	104	105	106	107	108	109	110
	201	202	203	204	205	206	207	208	209	210
	301	302	303	304	305	306	307	308	309	310
B	11	12	13	14	15	16	17	18	19	20
	111	112	113	114	115	116	117	118	119	120
	211	212		214	215	216	217	218	219	220
	311	312	313	314	315	316	317	318	319	320
C	21	22	23	24	25	26	27	28	29	30
	121	122	123	124	125	126	127	128	129	130
	221	222	223	224	225	226	227	228	229	230
	321	322	323	324	325	326	327	328	329	330
D	31	32	33	34	35	36	37	38	39	40
	131	132	133	134	135	136	137	138	139	140
	231	232	233	234	235	236	237	238	239	240
	331	332	333	334	335	336	337	338	339	340
E	41	42	43	44	45	46	47	48	49	50
	141	142	143	144	145	146	147	148	149	150
	241	242	243	244	245	246	247	248	249	250
	341	342	343	344	345	346	347	348	349	350
F	51	52	53	54	55	56	57	58	59	60
	151	152	153	154	155	156	157	158	159	160
	251	252	253	254	255	256	257	258	259	260
	351	352	353	354	355	356	357	358	359	360
G	61	62	63	64	65	66	67	68	69	70
	161	162	163	164	165	166	167	168	169	170
	261	262	263	264	265	266	267	268	269	270
	361	362	363	364	365	366	367	368	369	370
H	71	72	73	74	75	76	77	78	79	80
	171	172	173	174	175	176	177	178	179	180
	271	272	273	274	275	276	277	278	279	280
	371	372	373	374	375	376	377	378	379	380
I	81	82	83	84	85	86	87	88	89	90
	181	182	183	184	185	186	187	188	189	190
	281	282	283	284	285	286	287	288	289	290
	381	382	383	384	385	386	387	388	389	390
J	91	92	93	94	95	96	97	98	99	100
	191	192	193	194	195	196	197	198	199	200
	291	292	293	294	295	296	297	298	299	300
	391	392	393	394	395	396	397	398		

Appendix 5. The Bi-dimensional strategy for the three P-100 pools which contain the mutant lines. Each pool had 100 plants which were divided into 10 letter pools of 400 plants each and 10 letter pools of 400 plants each.

ALDH3H1 - Pool 164:

Pools	1	2	3	4	5	6	7	8	9	10
A	16351	16352	16353	16354	16355	16356	16357	16358	16359	16360
B	16361	16362	16363	16364	16365	16366	16367	16368	16369	16370
C	16371	16372	16373	16374	16375	16376	16377	16378	16379	16380
D	16381	16382	16383	16384	16385	16386	16387	16388	16389	16390
E	16391	16392	16393	16394	16395	16396	16397	16398	16399	16400
F	16401	16402	16403	16404	16405	16406	16407	16408	16409	16410
G	16411	16412	16413	16414	16415	16416	16417	16418	16419	16420
H	16421	16422	16423	16424	16425	16426	16427	16428	16429	16430
I	16431	16432	16433	16434	16435	16436	16437	16438	16439	16440
J	16441	16442	16443	16444	16445	16446	16447	16448	16449	16450

ALDH3H1 - Pool 218:

Pools	1	2	3	4	5	6	7	8	9	10
A	21751	21752	21753	21754	21755	21756	21757	21758	21759	21760
B	21761	21762	21763	21764	21765	21766	21767	21768	21769	21770
C	21771	21772	21773	21774	21775	21776	21777	21778	21779	21780
D	21781	21782	21783	21784	21785	21786	21787	21788	21789	21790
E	21791	21792	21793	21794	21795	21796	21797	21798	21799	21800
F	21801	21802	21803	21804	21805	21806	21807	21808	21809	21810
G	21811	21812	21813	21814	21815	21816	21817	21818	21819	21820
H	21821	21822	21823	21824	21825	21826	21827	21828	21829	21830
I	21831	21832	21833	21834	21835	21836	21837	21838	21839	21840
J	21841	21842	21843	21844	21845	21846	21847	21848	21849	21850

ALDH3I1 - Pool 386:

Pools	1	2	3	4	5	6	7	8	9	10
A	38551	38552	38553	38554	38555	38556	38557	38558	38559	38560
B	38561	38562	38563	38564	38565	38566	38567	38568	38569	38570
C	38571	38572	38573	38574	38575	38576	38577	38578	38579	38580
D	38581	38582	38583	38584	38585	38586	38587	38588	38589	38590
E	38591	38592	38593	38594	38595	38596	38597	38598	38599	38600
F	38601	38602	38603	38604	38605	38606	38607	38608	38609	38610
G	38611	38612	38613	38614	38615	38616	38617	38618	38619	38620
H	38621	38622	38623	38624	38625	38626	38627	38628	38629	38630
I	38631	38632	38633	38634	38635	38636	38637	38638	38639	38640
J	38641	38642	38643	38644	38645	38646	38647	38648	38649	38650