Characterization of the role of *Arabidopsis thaliana* genes in desiccation tolerance during seed to seedling transition

MSc Minor Thesis Report

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Abstract

Dehydration stress, such as drought and desiccation tolerance (DT), affects the physiological and biochemical activities of seeds and seedlings through either suppressing or inducing stress responsive genes of plants. Responsiveness of genes related to DT can be mediated with increases in abscisic acid (ABA) level. Treatment of plants with exogenous ABA, therefore, induces responses of stress-associated genes. The objective of this study was to characterize Arabidopsis thaliana stress-associated genes for their role in desiccation tolerance in germinated Arabidopsis seeds. Four different mutant lines of Arabidopsis (*sod1, daa1, di19* and *vip1*) *were used, which stress-associated genes are knocked out. Seeds of these knock out mutants were selected at two seed germinated stages (RP-Radical Protrusion and RH-Root Hair), and analyzed for ability to acquire desiccation tolerance in mild-osmotic stress by apply Polyethylene Glycol (PEG). Levels of gene expression associated to the ABA pathway, which are associated to osmotic-stress response, were analyzed through RT-qPCR at RP and RH before and after PEG treatment. Eight key genes of the ABA response pathway were selected and checked for their expression level: two ABA receptors from the PYL/PYR/RCAR family (PYL7 and PYL9), five ABA biosynthesis genes (ABA1, NCED3, NCED 5, NCED 6 & NCED 9) and one ABA catabolic gene (CYP707A2). Quantitative gene expression data for RP and RH stages were obtained for PEG treated and untreated mutants and wild type. It showed an overall trend to increase the expression level after PEG treatment. Expression of genes of ABA biosynthesis and receptors increase in response to PEG treatment, while ABA catabolic gene decreases. We conclude that ABA genes are assumed to be involved in response to desiccation tolerance, although, three mutants: sod1, daa1, and di19 showed an increase of 20% in acquisition of desiccation tolerance compared to the wild type, genes involved in ABA pathway did not show difference related to phenotype.*

1. Introduction

The seed is an important dispersal unit in the plant life cycle with respect to its survival as a species. It can survive the period between seed maturation and seedling establishment. For successful survival, seeds should tolerate adverse environmental conditions such as drought, cold, salinity, low or high temperature as well as heavy metals (Bray *et al*., 2000; Krasensky and Jonak, 2012; Yang and Chen, 2013). Drought affects the physiological and biochemical activities of seeds through inducing stress-related gene expression (Yamaguchi-Shinozaki & Shinozaki, 2005; Shinozaki & Yamaguchi-Shinozaki, 2007). For this reason, traits such as desiccation tolerance (DT) and seed dormancy are very important.

According to the International Water Management Institute (IWMI) report, one-third of the world's population (~1 billion) will live in water deficit areas by the year 2025 (www.iwmi.org). This will result in severe economic impact on the agricultural sector. For this reason, it is vital to better understand stress tolerance mechanisms and develop new tools to engineer plants that are better adapted to water scarcity in these areas for better access to food and raw materials to support the agricultural sector.

There are two types of tolerance based on the critical water level. These are drought tolerance: the capacity to tolerate moderate dehydration, to moisture content below ~0.3g H_2O per gram dry weight (Hoekstra *et al*., 2001; Tripathi *et al*., 2014). And desiccation tolerance (DT) or anhydrobiosis: the tolerance of further dehydration, to water levels below 0.1g H_2O per gram of dry weight and successive re-hydration without permanent damage in periods of long dry periods (Hoekstra *et al.*, 2001; Maia *et al*., 2011; Dekkers *et al*., 2015).

Recently, varieties of molecular and genomic level research have been conducted in the model plant *Arabidopsis thaliana*. Stress-inducible genes, proteins and metabolites involved in desiccation tolerance are identified using microarray analysis (Shinozaki *et al.*, 2003; Todaka *et al*., 2012; Sarhadi *et al*., 2012; Maia *et al*., 2011). Most of the stress-inducible genes are found to be involved in the synthesis of osmolytes, dehydrins and enzymes that are involved in ABA biosynthesis (Bartels &

Sunkar, 2005). ABA biosynthetic genes and enzymes have been identified in *Arabidopsis thaliana*. For example, zeaxanthin epoxidase (ZEP) is one of these enzymes, which is encoded by the *ABA1* gene of *Arabidopsis thaliana* (Xiong *et al*. 2002; Xiong & Zhu 2003; Barerro *et al.,* 2006). It catalyzes a step of the ABA biosynthetic pathway through catalyzing epoxidation of zeaxanthin to antheraxanthin and all-*trans*-violaxanthin. All-*trans*-violaxanthin is then converted into the *cis*-neoxanthin and *cis* violaxanthin, which are cleaved by a 9-*cis* epoxy carotenoid dioxygenase (NCED) to form xanthoxin (Iuchi *et al*. 2000). Then xanthoxin is converted to abscisic aldehyde by the catalyzing ABA2 enzyme of *Arabidopsis thaliana.* The abscisic aldehyde is then oxidized to form ABA by the enzyme aldehyde oxidase 3 (AAO3). As a result, ABA is derived from the oxidative cleavage of a 9-*cis*-epoxy-carotenoid (Schwartz & Zeevaart, 2010).

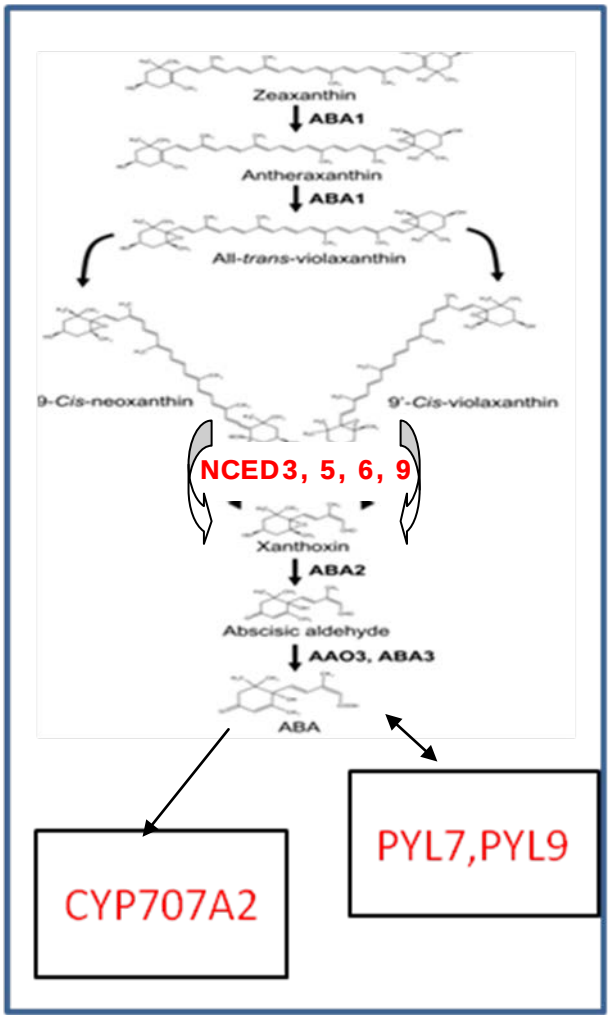


Figure 1 Biosynthesis of abscisic acid (ABA) from carotenoid precursors in higher plants. Adapted from Barerro *et al.,*(2006).

DT is a complex trait that requires activities involved in abscisic acid (ABA) signaling. There are three core components that are involved in ABA signaling: PYR/PYL/RCAR receptor family consisting of pyrabactin resistance 1(PYR1)-like regulatory components of ABA receptors, the type 2C protein phosphatases (PP2Cs) and the sucrose-non-fermenting kinase 1-related protein kinase 2 (SnRK2s) family (Umezawa *et al.*, 2010; Komatsu *et al.*, 2013). PP2C phosphatases function as a negative regulator of ABA signaling components (Cutler *et al*., 2010) whereas the SnRK2s are positive regulator of ABA signaling (Umezawa *et al*., 2010). This indicates that signaling complex of these three protein families are important to regulate ABA response in plants. Under normal conditions, PP2C inactivates SnRK2 by dephosphorylating its multiple residues to repress the ABA signaling but when endogenous ABA is up regulated due to abiotic stress, the PYR/PYL/RCAR binds to ABA and interacts with PP2Cs to inhibit protein phosphatase activity. PYL proteins are therefore the most important to inhibit ABA-dependent PP2C phosphatase activity (Ma *et al*. 2008). The closed gate loop of PYLs is very important to seal the catalytic site of PP2Cs and expose the serine residue, an interface that interacts with group A PP2C upon ABA binding. Then, the ABA-bound receptor inhibits the PP2C phosphatase activity suggesting that PYLs are negative regulatory sub units of PPCs during ABA signaling (Ma *et al*., 2009). When the PP2C activity is inhibited, SnRK2 are released from PP2Cs-dependent regulation and turn to ABA signaling by phosphorylating the downstream basic leucine zipper *(*bZIP)-type transcription factors such as ABA response elements (ABRE)/ABRE binding factors (ABF) or membrane proteins (Umezawa *et al*., 2010).

In addition, five *ABA INSENSITIVE (ABI1 to ABI5)* loci that are involved in ABA downstream signaling are identified in Arabidopsis. They affect Arabidopsis germination and post-germination processes (Finkelstein, 2013). The *ABI1* and *ABI2* genes encode homologous type 2C protein Ser/Thr Phosphatases (PP2Cs). The remaining three *ABA Insensitive* genes, *ABI3, ABI4 and ABI5* encode transcription factors of the basic *B3*-domain, *APETALA2*-domain (*AP2*) and *basic leucine zipper*-(*bZIP*) domain families respectively (Lopez-Molina & Chua, 2000). They act as transcription factors found to be abundantly expressed in seeds and depending on the gene targeted; they act as either activators or repressors (Finkelstein, 2013). The

ABA Insensitive genes; *ABI3, ABI4 and ABI5,* regulate genes that are involved in many physiological processes such as lipid metabolism, postembryonic development, photosynthesis, response to ABA stimulus and chlorophyll metabolic processes (Wind *et al*., 2013). The ABA insensitive gene *ABI4* is essential for regulation of ABA and sugar signaling during seed germination and early seedling development (Huijser *et al*., 2000). It also activates expression of its own gene, *ABI5* and starch binding enzyme (*SBE2.2*) through binding directly to the promoter region of all three genes (Huijser *et al*., 2000). Dry seeds of A. thaliana *abi5* mutants showed lower levels of ABRE than the wild type suggesting that ABI5 has an important role in response to exogenous ABA during germination by protecting seeds from water loss during drought stress (Lopez-Molina *et al*., 2001).

Several studies have been conducted to explore the molecular basis of gain, loss and re-establishment of desiccation tolerance in germinated *abi* mutants of Arabidopsis (Maia *et al.*, 2011; Verdier *et al.*, 2013). Studies revealed that wild type Columbia (Col-0) seeds lose desiccation tolerance during germination and can re-acquire it during germination by application of a mild osmotic stress (Maia *et al.*, 2011). This has been proposed to be a good method to investigate the molecular basis of acquisition of desiccation tolerance. Previous studies have shown that over expression of stress-inducible genes that accumulate during the acquisition of desiccation tolerance are useful to improve it (Sun *et al.*, 2013). Objective of this study was to find out which genes were involved in desiccation tolerance in two germination stages (RP and RH) of Arabidopsis*.* Four different mutant lines of Arabidopsis were used, which they are knocked out. The *vip1* showed greatest result, where each gene related to ABA biosynthesis and receptors showed a decreased trend between treated and untreated stages. These results hypothesize that ABA biosynthesis and signaling are associated to its phenotype, which did not acquired DT at RH stage.

2. Materials and Methods

2.1. Genotyping the *Arabidopsis thaliana* mutant lines

DNA Extraction

DNA extraction was performed with unexpanded young leaves of the knockout mutants for selection of homozygous T-DNA lines. The tissue material was grinded into non-sterile polypropylene 1.2 ml tubes (Collection Micro tubes (racked, 8 x 12) having one metal bead inside and 300µl extraction buffer (Table 1) Then the samples were shaken for 1 minute at 30Hz, and incubated in an oven at 60^0C for 1 hour. Afterwards, samples were centrifuged at 3920 rpm for 15 minutes and 75µl of the upper watery phase of each sample (supernatant) was pipetted into new microplates. Then 75µl isopropanol and 30µl 10mM NH_4AC were added to each sample in order to precipitate the nucleic acids. Samples were kept for 15 minutes at room temperature. Then the samples were centrifuged at 3920 rpm for 15 minutes. The supernatant was poured off and 50µl 70% ethanol was added; centrifuged at 3920 rpm for 5 minutes to wash the pellet and ethanol was poured off. The pellets were dried at room temperature for 30 minutes and re-suspend in 50µl MQ water. Quality of the DNA was checked using a Nanodrop®.

Table 1 Extraction buffer preparation

Extraction buffer stock
0.35 M Sorbitol
0.1 M Tris-HCl pH 8.0
5 mM EDTA pH 8.0

PCR Assays

In order to investigate the presence or absence of the T-DNA insertion, PCR analysis with the border primers SAIL_534_B01 (for the mutant AT1G33970) & SALK for the other nine mutant lines (Table 2) and primer pairs of the respective mutants were performed. Gel electrophoresis was used to check the DNA samples and the corresponding wild type control for homozygosity. Therefore, four knockout mutant lines of *Arabidopsis thaliana* that were previously identified as been homozygous were used for further analysis.

Table 2 Knockout mutants used for selection of homozygous T-DNA lines

Locus	Description	T-DNA line
AT1G33970	P-loop containing nucleoside triphosphate hydrolases superfamily protein; FUNCTIONS IN: GTP binding; INVOLVED IN: response to bacterium	SAIL_534_B01
AT4G01450	Other names: UMAMIT30, USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 30; nodulin MtN21-like transporter family protein	SALK_146977
AT1G04300	TRAF-like superfamily protein; FUNCTIONS IN: molecular function unknown; INVOLVED IN: biological process unknown;	SALK_026088
AT2G04240	Other names: XERICO; Encodes a small protein with an N-terminal trans-membrane domain and a RING-H2 zinc finger motif located at the C-terminus. Gene expression is induced by salt and osmotic stress. Transcrips levels are induced by DELLA	SALK_075188C
AT3G14880	FUNCTIONS IN: molecular function unknown; INVOLVED IN: response to karrikin; BEST Arabidopsis thaliana protein match is: transcription factor-related (TAIR:AT4G18650.1); Has 30201 Blast hits to 17322 proteins in 780 species: Archae - 12; Bacteria - 1396; Metazoa - 17338; Fungi - 3422; Plants - 5037; Viruses	SALK_056670
AT4G25800	Calmodulin-binding protein; FUNCTIONS IN: calmodulin binding; INVOLVED IN: biological process unknown	SALK_040280C
AT5G0340	Other names: POLYUBIQUITIN 3, UBQ3; Encodes ubiquitin that is attached to proteins destined for degradation. UBQ3 is most homologous with UBQ4, and is expressed in higher levels in vegetative tissue but lower levels in flowers than UBQ4. UBQ3 encodes different number	SALK_148831C
AT1G53780	peptidyl-prolyl-cis-trans isomerases;hydrolases;nucleosidetriphosphatases;ATPbinding;nucleotide binding; ATPases; FUNCTIONS IN: in 6 functions; INVOLVED IN: protein folding, protein catabolic process	SALK_106176C
AT2G470	Zinc finger C-x8-C-x5-C-x3-H type family protein; FUNCTIONS IN: zinc ion binding, nucleic acid binding; INVOLVED IN: biological process unknown	SALK_054523C
AT4G1610	Other names: ATOEP16-2, ATOEP16-S Homologous to pea OEP16 and barley pPORA (OEP16), a member of Arabidopsis OEP16 family. Two OEP16 genes are closely related to each other and are conserved in all land plants, OEP16-2, also named OEP16-S	SALK_025109
AT5G04750	F1F0-ATPase inhibitor protein, putative; FUNCTIONS IN: ATPase inhibitor activity	SALK_023158C

Source: http://www.arabidopsis.org

2.2. Phenotyping the *Arabidopsis thaliana* mutant lines

Seeds of the wild type accession, Columbia (Col-0) and four knockout mutant lines of *Arabidopsis thaliana* (Table 3) have been used for this study.

Table 3 Knock-out mutants used for evaluation of desiccation tolerance

Gene	Function	Other Names	Description
AT1G12520	Copper chaperone	ATCCS, CCS, Copper chaperone for SOD1	Copper-zinc superoxide dismutase copper chaperone (delivers copper to the Cu-Zn superoxide dismutase). Up-regulated in response to copper and senescence.
AT1G64110	Coding for ATPase	DUO1-Activated ATPase1(DAA1)	Target promoter of the male germline-specific transcription factor DUO1
AT1G56280	Drought induced gene family	ATDI19,Drought-Induced 19 (DI19)	Encodes a gene whose transcript level in root and leaves increases to progressive drought stress. The increase in transcript level is independent from abscisic acid level. It appears to be a member of plant-specific gene family.
AT3G01310	Phosphate metabolism	Arabidopsis Homolog of Yeast VIP1 1 (ATVIP1)	Encodes a myo-inositol hexakisphosphate kinase.

Source: http://www.arabidopsis.org

First, seeds of the above mentioned four knockout mutant lines of *Arabidopsis thaliana* and the accession Columbia (Col-0) were put in 9 cm Petri dishes on two layers of blue filter paper (Anchor paper Co.) moistened with 10 ml demi-water. Then, seeds were put in a dark room to be stratified at 4°C for 72 hours to break dormancy. After stratification, the seeds were transferred to an incubator with constant white light at 22°C and allowed to germinate. After approximately 28 hours (RP) and 32 hours (RH) (Figure 2), the germinated seeds were selected at the specific stages by hand using a stereomicroscope. Three hundred seeds of each biological replicate for each stage were selected and 150 seeds were stored in -80°C and 150 seeds were used for treated with polyethylene glycol (PEG 8000 with a concentration of -2.5MPa).

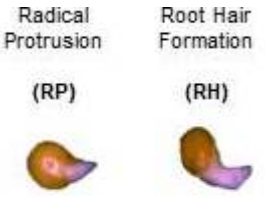


Figure 2 Seed-to-seedling developmental stages of Arabidopsis seeds

PEG Treatment

Many studies have been conducted on the attainment of desiccation tolerance during seed development and on its loss upon germination. In order to re-establish DT in the seeds of the four tested mutants (*sod1*, *daa1, di19 and vip1),* a PEG treatment was performed as described by Maia *et al*., (2011) with slight modifications. A total number of 150 germinated seeds each for the RP and the RH stage for each line and replica were selected and incubated for three days in a PEG8000 solution with an osmotic potential of -2.5MPa. After incubation, seeds were washed with distilled water to remove the remaining PEG and kept at -80^{o}C.

RNA Extraction

RNA extraction was performed using 150 germinated seeds of RP and RH stages after PEG incubation and 150 non-treated germinated seeds at the same developmental stages. Seeds were put into 2ml tubes and stored at -80^{o}C prior to grinding. Total RNA was extracted according to the hot borate protocol (Wan and Wilkins, 1994). The RNA pellets were dissolved in 20µl DEPC MQ water and the RNA quality was checked by agarose gel electrophoresis. The absorbance (A260, 280 and 230) was measured using a Nanodrop® to check the quantity and quality of RNA.

cDNA Synthesis

cDNA was synthesized using the iScript™ cDNA synthesis kit (Bio-Rad) protocol. First, 5µl of master mix which contained 4µl 5x iScript reaction buffer and 1µl iScript reverse transcriptase was added to each tube. Then, 1µg of each RNA sample and MQ water was added to a final reaction volume of 20µl. The reaction mix was incubated at 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and subsequently kept at 4°C. Finally, one tenth of the cDNA reaction volume was diluted 10x with sterile MQ water followed by RT-qPCR using gene-specific primers.

Real-time quantitative PCR (RT-qPCR) Analysis

Real-time quantitative PCR (RT-qPCR) was performed on CFX96 connect ™ Real-Time PCR Detection System (Bio-Rad). Amplification was done in a 10µl reaction mixture containing, 5µl SYBR Green, 1µl of 10µM primers, 2.5µl cDNA and 1.5 µl MQ water. Primer pairs were designed using the CLC Main Workbench software. The RT-qPCR program run with the first step at 95°C for 3 minutes, followed by 40 cycles at 95°C for 10 seconds and 60°C for 30 seconds followed by a melt curve (55°C to 95°C; increasing 0.5°C for every 5 seconds . Data were analyzed using CFX Data Analysis Manager Software and qbase+®. Primer efficiencies were measured with LinRegPCR software and most of the primers showed efficiencies higher than 90%. Melting curves were analyzed and a single peak was observed for all primers, confirming the specificity of the primers. Four reference genes, described by Dekkers *et al.* (2012), were used (At1g16970, At2g43770, At3g12210, and At4g02080) and each of them was checked for their expression stability in geNorm® software. Normalization of reference genes were done according Dekkers *et al.* (2012). Finally, calibrated normalized relative quantity (CNRQ) values were exported from qbase+® and statistically analyzed.

3. Results

3.1. Assessment of DT re-establishment in germinated *Arabidopsis thaliana* mutant lines

Four knock out mutants and the wild type Col-0 were selected according to their response in acquisition of DT (Figure 3) to check if ABA pathway genes are associated to DT in germinated seeds after PEG treatment at RP and RH stages. Mutants were at1g12520 gene that codes for copper chaperone (*sod1*), at1g64110 codes for ATPase (*daa1*), at1g56280 codes for drought induced gene family member (*di19*) and at3g01310 codes for a phosphoglycerate mutase-like gene (*vip1*). The superoxide dismutase (SOD) enzyme catalyzes the dismutation of superoxide radicals and involved in defense against reactive oxygen species (Beyer *et al.,* 1991). The *SOD1* gene is shown up regulated in response to copper limit great than 15.7 mg/kg dry weight and senescence because of abiotic stresses like desiccation (Abdei-Ghany *et al.,* 2005). Due to this reason, the *SOD1* gene is supposed to perform down regulated in the *sod1* mutant during desiccation tolerance. The DAA1 gene is also known as DUO1-Activated ATPase1(DAA1) which is a target of the male germ line-specific R2R3 MYB transcription factor DUO1 and induced by cold, drought and ABA (Borg *et al*., 2011; Ali *et al*., 2013). The drought induced gene family member *DI19* is a dehydration-induced, ABA-independent gene that has a role in DT (Milla *et al*., 2006). Phosphoglycerate mutase-like gene, *VIP1* encodes a myo-inositol hexakisphosphates (InsPs) and has a role in phosphate metabolism by controlling the anti-oxidant tocopherol, stress responses and hormonal signaling through providing a chemical code for plants to respond to these signals (Barker & Berggren, 2013; Munnik & Vermeer, 2010). The *SOD1, DAA1, DI19* and *VIP1* genes are suggested to be involved in DT process. Expression of two ABA receptors from the PYL/PYR/RCAR family (PYL7 and PYL9), five ABA biosynthesis genes (ABA1, NCED3, NCED 5, NCED 6 & NCED 9) and one ABA catabolic gene (*CYP707A2*) in response to DT acquisition at RP and RH, therefore, were investigated in the knock-out mutants.

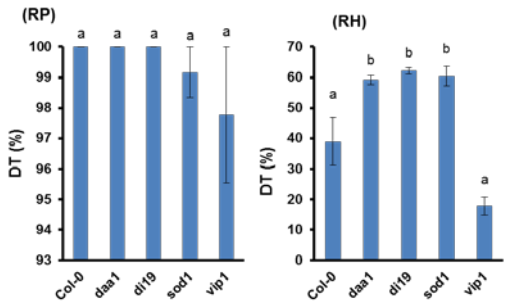
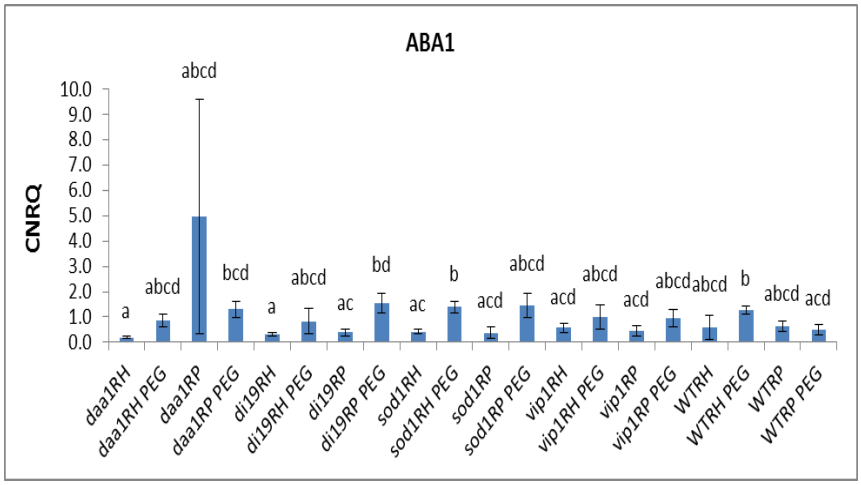


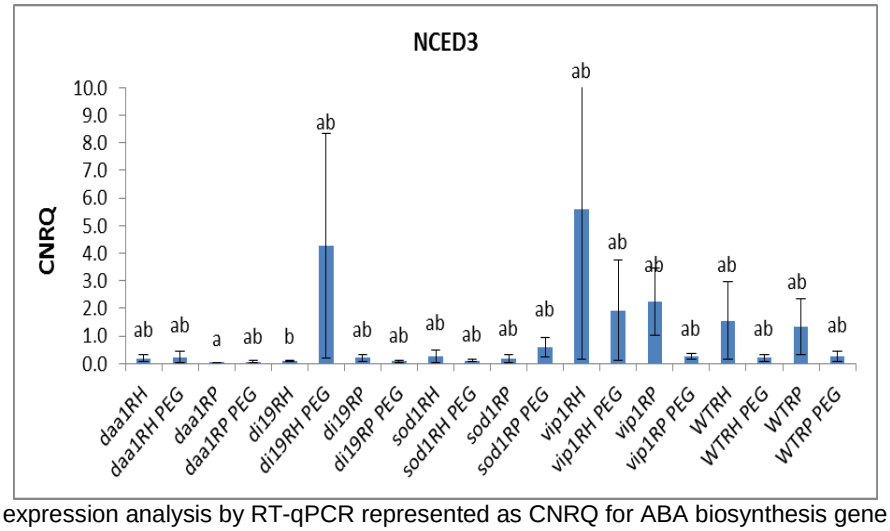
Figure 3 Response of mutant lines in acquisition of DT

3.1.1. ABA biosynthesis genes

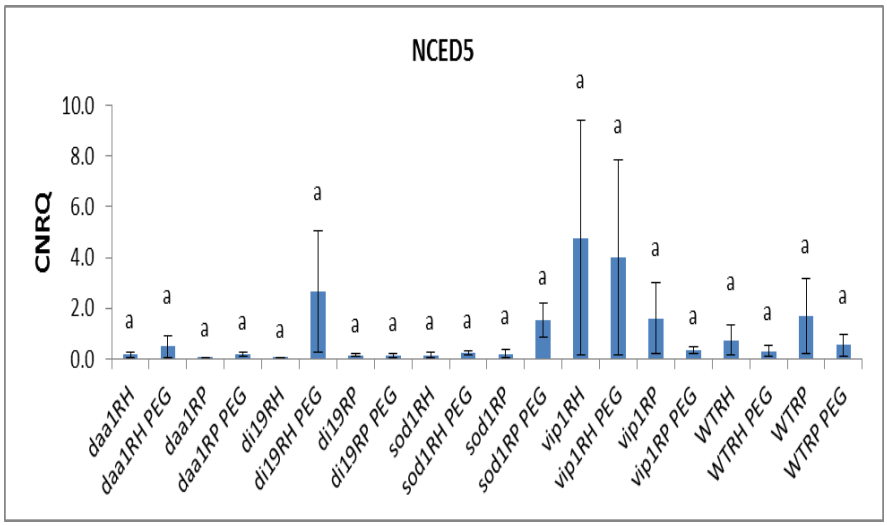
No ABA biosynthesis genes showed significant changes in their expression levels after PEG treatment when compared to untreated seeds in both stages of development (Figure 4). The expression of NCED3 and NCED5 did not increase significantly with PEG treatment. Generally, there was a trend to increase in expression level after PEG treatment in contrast to the untreated seeds. These might suggest that, ABA content increases in response to PEG treatment.



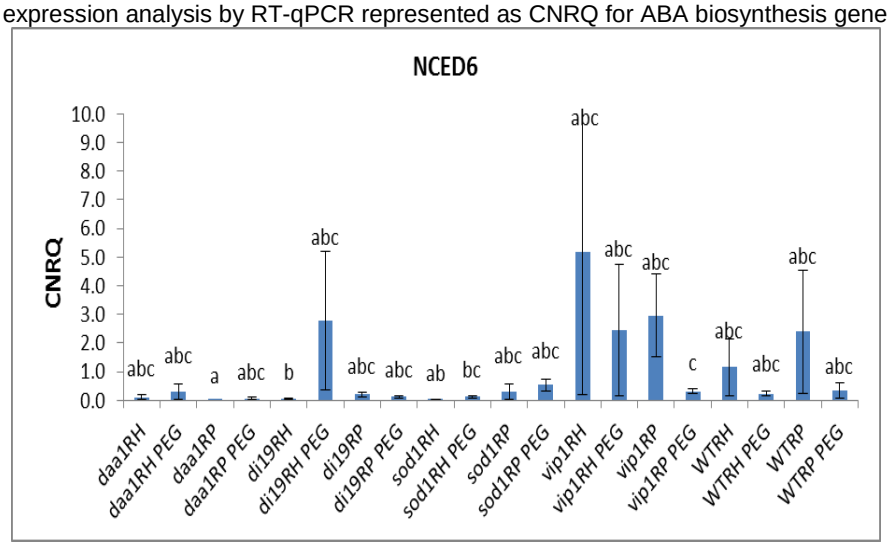
a) Gene expression analysis by RT-qPCR represented as CNRQ for ABA biosynthesis gene ABA1



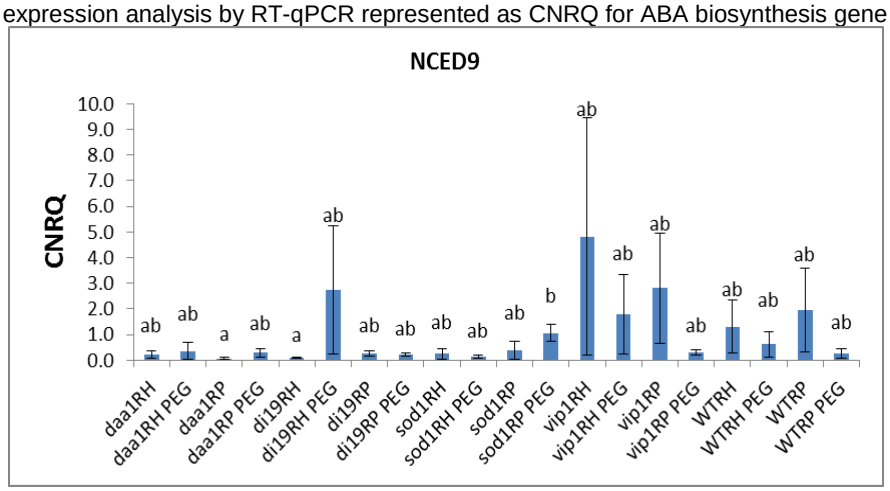
b) Gene expression analysis by RT-qPCR represented as CNRQ for ABA biosynthesis gene NCED3



c) Gene expression analysis by RT-qPCR represented as CNRQ for ABA biosynthesis gene NCED5



d) Gene expression analysis by RT-qPCR represented as CNRQ for ABA biosynthesis gene NCED6



e) Gene expression analysis by RT-qPCR represented as CNRQ for ABA biosynthesis gene NCED9

Figure 4 Gene expression analysis by RT-qPCR represented as CNRQ for ABA biosynthesis genes ABA1, NCED3, NCED5, NCED6, and NCED9. Gene expression is shown for untreated seeds and seeds treated for 3 days in PEG (-2.5 MPa). Bars represent the mean values ± SE of three independent biological replicates. Small letters represent statistical differences ($p \leq 0.05$) between different developmental stages and different treatments. Bars with the same letter(s) are not significantly different at $p < 0.05$.

3.1.2. ABA catabolic gene

The ABA catabolic gene, CYP707A2 does not show significant changes in its expression level after PEG treatment when compared to untreated seeds in both stages of development (Figure 5). However, there was a tendency to decrease in expression level after PEG treatment in contrast to the untreated seeds. This might suggest that, the CYP707A2 gene degrades the ABA content in PEG treated seeds and as a result, PEG-treated seeds might not have accumulated ABA compared to untreated seeds.

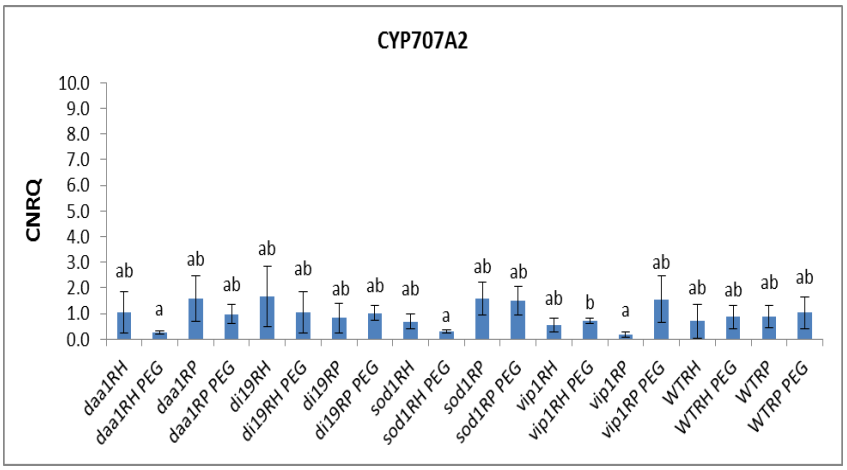


Figure 5 Gene expression analysis by RT-qPCR represented as CNRQ for ABA catabolic gene CYP707A2. Gene expression is shown for untreated seeds and seeds treated for 3 days in PEG (-2.5 MPa). Bars represent the mean values ± SE of three independent biological replicates. Small letters represent statistical differences ($p \leq 0.05$) between different developmental stages and different treatments. Bars with the same letter(s) are not significantly different at $p < 0.05$

3.1.3. ABA receptors from the PYL/PYR/RCAR family

The expression levels of *PYL7* and *PYL9* did not show significant changes after PEG treatment when compared to untreated seeds in both stages of development (Figure 6).. Generally, there was a trend to increase in expression level after PEG treatment in contrast to the untreated seeds. This might suggest that these genes are involved in DT regulation in response to osmotic stress and increased ABA sensitivity in germinating seeds.

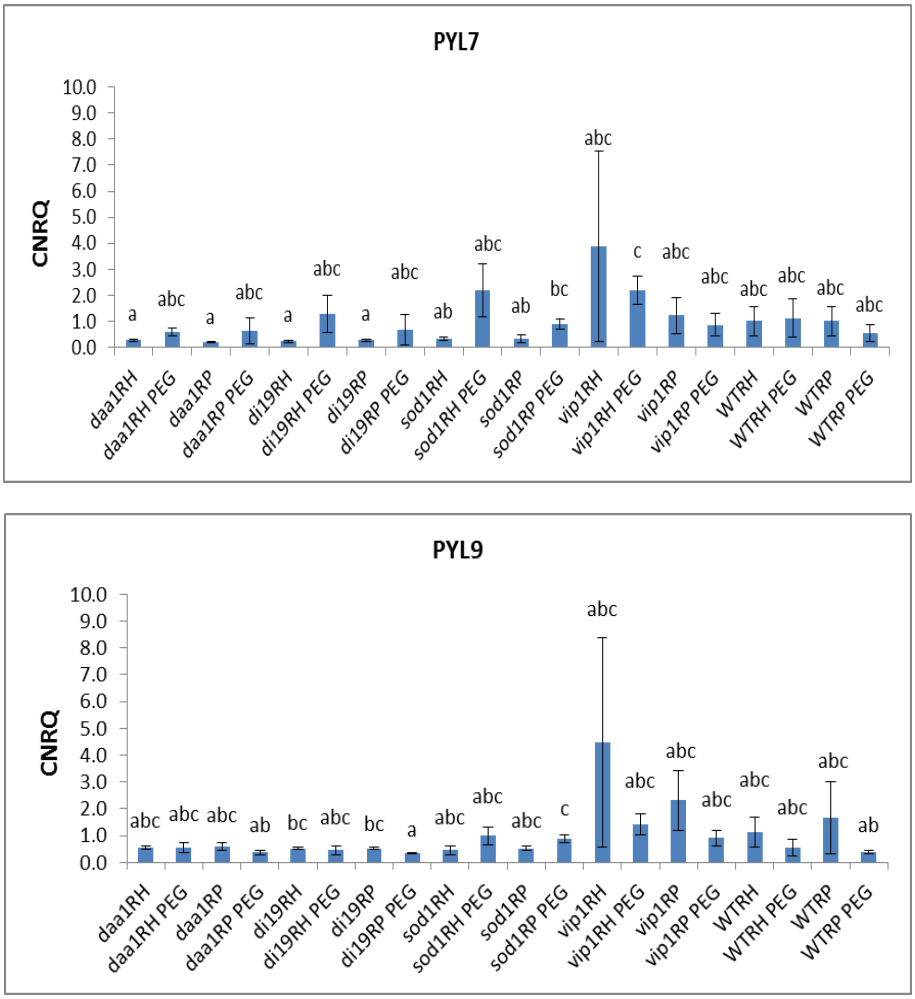


Figure 6 Gene expression analysis by RT-qPCR represented as CNRQ for ABA receptors from the PYL/PYR/RCAR family. Gene expression is shown for untreated seeds and seeds treated for 3 days in PEG (-2.5 MPa). Bars represent the mean values ± SE of three independent biological replicates. Small letters represent statistical differences ($p \leq 0.05$) between different developmental stages and different treatments. Bars with the same letter(s) are not significantly different at $p < 0.05$.

4. Discussion

4.1. Genotyping the *Arabidopsis thaliana* mutant lines

It is possible to detect the presence of a T-DNA insertion of a given gene in Arabidopsis knock-out mutants by PCR using one T-DNA specific primer and gene specific primers to the respective knock-out mutant. As a result, if the T-DNA is landed in the gene of interest, a PCR product is formed through the amplification process. The chance of a T-DNA insert to be found within a gene is determined by genome size, gene size and number of T-DNA inserts in the population (krysan *et al*., 1999). Since Arabidopsis is reproduced by self-fertilization, heterozygosity is rarely a problem. However, all of the tested mutant lines were heterozygous for the T-DNA insertions. This might be due to disruption of gene function, homozygous lethal mutations or additional segregation of the T-DNA insertion in to different genes. For example, the 5 to 25kb long T-DNA insert can cause disruption of gene function because of very little intergenic material and small introns in Arabidopsis plants (krysan *et al*., 1999). Due to this disruption of gene function, homozygous lethal mutations can happen resulting in vitality of only heterozygous plants (krysan *et al*., 1999). Also endogenous interference or silencing of the T-DNA insert might be a cause.

4.2. Gene expression analysis of the ABA pathway

ABA is known to be involved in regulating plant physiological processes such as seed dormancy. Seed dormancy can be influenced by altered ABA biosynthesis even though ABA deficient and ABA insensitive (ABI) mutants can produce desiccation tolerant (DT) seeds during maturation (Cutler *et al.*, 2010). However, DT was shown to be lost during germination, on which the *Arabidopsis thaliana* seeds become sensitive to desiccation (Maia *et al.*, 2011). Then, DT can be re-established when mild osmotic stress (like for example PEG) is applied to germinated seed up to radicle protrusion (RP). When seeds are at root hair (RH) stage the ability to re-acquire DT drops dramatically (Maia *et al.*, 2011). The mechanisms involved in the seed or seedling to perceive this osmotic signal is not clear yet. Therefore, knowing the key genes involved in ABA biosynthesis, signaling and degradation is very important.

4.2.1. ABA biosynthesis genes

The level of ABA biosynthesis and ABA sensitivity determines the response of plants to ABA. It is proposed that increased ABA level triggers ABA mediated stress response in desiccation sensitive Arabidopsis seeds through the application of mild osmotic stress (PEG) (Maia *et al*, 2014). However, our data revealed that no ABA biosynthesis genes showed significant changes in their expression levels at both stages of development even though there was a trend to increase in expression levels after PEG treatment. This might suggest that ABA content increases in response to the PEG treatment. The present result was in agreement with Maia *et al* 2014, who showed that all ABA biosynthesis genes showed a tendency to increase in their expression levels upon PEG treatment. In addition, DT can be rescued by PEG treatment regardless of increased ABA levels; since all NCEDs in this experiment showed a tendency to increase their level of expression in PEG treated Arabidopsis seeds. According to a report by Westwood *et al.*, (2013), plants expressing cucumber mosaic virus factors showed increased drought tolerance without over accumulation of ABA. This suggests that adjustment of ABA signalling and/or perception can be sufficient to induce a proper stress response besides enhancing ABA levels since increased ABA level is not always necessary to elicit DT (Maia *et al* 2014). One of the reasons why ABA biosynthesis genes are not up regulated in response to PEG treatment might be due to the presence of the ABA catabolic genes such as the CYP707A family (Okamoto *et al*., 2006); inhibitors of ABA biosynthesis (Debeaujon & Koornneef, 2000) and/or ABA conjugation products (Lim *et al*., 2005). It might also be due to the balance between ABA biosynthesis and ABA degradation. Furthermore, it might also be due to ABA translocation from one sub-organelle to the other. For example, NCEDs are found in the plastid but they differ in their binding to the thylakoid membrane. NCED2, NCED3 and NCED6 are localized in both stroma and thylakoid, whereas NCED9 is localized only in the stroma and NCED5 is bound by the thylakoid membrane. This difference in localization might affect the release of xanthoxin to the cytoplasm and some enzymatic activities during ABA biosynthesis.

4.2.2. ABA catabolic gene

ABA levels can also be regulated through catabolism in addition to ABA biosynthesis. ABA catabolism has two pathways, namely hydroxylation of ABA by the P-450 type monoxygenases to give unstable 8′-OH-ABA and esterification/conjugation of ABA to ABA-GE (Lim *et al*., 2005). The ABA-8′-hydroxylases are encoded by the CYP707A family having different patterns of expression in both time and space but all induced by both dehydration and subsequent rehydration. Because of spatial and temporal differences in expression patterns, each member of this family has different developmental and/or physiological role(s) (Okamoto *et al*., 2006). For example, CYP707A1 and CYP707A3 have a role for ABA catabolism in the middle of seed development and CYP707A2 during imbibition and at the end of seed development (Okamoto *et al*., 2006). It has been shown that, CYP707A mutants in *Arabidopsis* accumulate a higher amount of ABA than lines over expressing ABA biosynthetic enzymes (Umezawa *et al*. 2006). In addition, overexpression of CYP707A effectively reduces endogenous ABA (Okamoto *et al*. 2006, Umezawa *et al*. 2006). As a result, CYP707As has a role in controlling the level of ABA biosynthesis. However, our data show that the ABA catabolic gene, CYP707A2 does not show significant changes in its expression level after PEG treatment when compared to untreated seeds in both stages of development. Nevertheless, there were lines with a tendency to increase in expression level after PEG treatment. For example, the At1g56280 gene knocked out mutant line that code for drought induced gene family (*di19*) and the At3g01310 gene knocked out mutant line that codes for a phosphoglycerate mutase-like gene (*vip1*) showed increase in level of expression up on PEG treatment. The reason for down regulation of CYP707A2 in response to PEG treatment might be due to the fact that the CYP707A2 Abscisic Acid 8′-Hydroxylase degrades the ABA content in PEG treated seeds. As a result, PEG-treated seeds might not accumulate active ABA when compared to untreated seeds. It might also be due to continuous balance between ABA biosynthesis and ABA catabolism or these products that were not detected in the PEG treated seeds due to some catabolic inhibitors that targets CYP707A. Whereas the *di19* mutant that has dehydration-induced and ABA-independent DT mechanism but not through ABA catabolism since it was ABA-independent. In addition, the *vip1* mutant that encodes InsPs has a role in phosphate metabolism by controlling the anti-oxidant tocopherol, stress responses and

hormonal signaling through providing a chemical code for plants to respond to these signals including signals of ABA catabolism. Due to these reasons, both mutants were shown up-regulated up on PEG treatment through detecting signals of ABA catabolism.

4.2.3. ABA receptors from the PYL/PYR/RCAR family

Three core components that are involved in ABA signal transduction pathways have been identified after intensive study on ABA (Miyakawa *et al*., 2013). These are PP2Cs, PYR/PYL/RCARs and SnRK2s (Umezawa *et al.*, 2010; Komatsu *et al.*, 2013). The PYR/PYL/RCAR receptor consists of pyrabactin resistance 1(PYR1)-like regulatory components of ABA receptors that inhibits protein phosphatase activity through inactivating PP2Cs (Ma *et al*., 2009). The PP2C phosphatases function as a negative regulator of ABA signaling components through inactivating SnRK2s (Cutler *et al*., 2010) whereas the SnRK2s are positive regulator of ABA signaling (Umezawa *et al*., 2010). Under normal conditions, PP2C inactivates SnRK2 by dephosphorylating its multiple residues but when endogenous ABA is up regulated due to abiotic stress such as desiccation and drought, the PYR/PYL/RCAR binds to ABA and interacts with PP2C to inhibit protein phosphatase activity. According to Gonzalez-Guzman *et al*., (2012), the PYR/PYL/RCAR protein family except PYL13 is capable of activating ABA-responsive gene expression through binding to the PYL/RCAR receptors that confirms these ABA receptors other than PYL13 have a role to regulate ABA signaling in response to PEG treatment in germinated seeds. However, our data revealed that no ABA receptors from the PYL/PYR/RCAR family showed significant changes in their expression levels at both stages of development even though there was a fashion to increase in expression levels after PEG treatment. The present result was in contrast with Maia *et al*., 2014, who found increased expression of PYL7 and PYL9 in response to PEG treatment in both RP and RH stages. The reason for non-significant changes in expression levels after PEG treatment might be due to conformational changes during ABA binding to the PYR/PYL/RCARs or different receptor proteins are involved in the ABA signaling pathway since some PYR/PYL proteins such as PYL9 and PYL5 have preference on binding to different ABA arrangement (Santiago *et al*., 2009). Therefore, analysis that

is more careful during RNA extraction and cDNA synthesis has to be repeated to confirm the hypothesis.

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Supplementary Figures

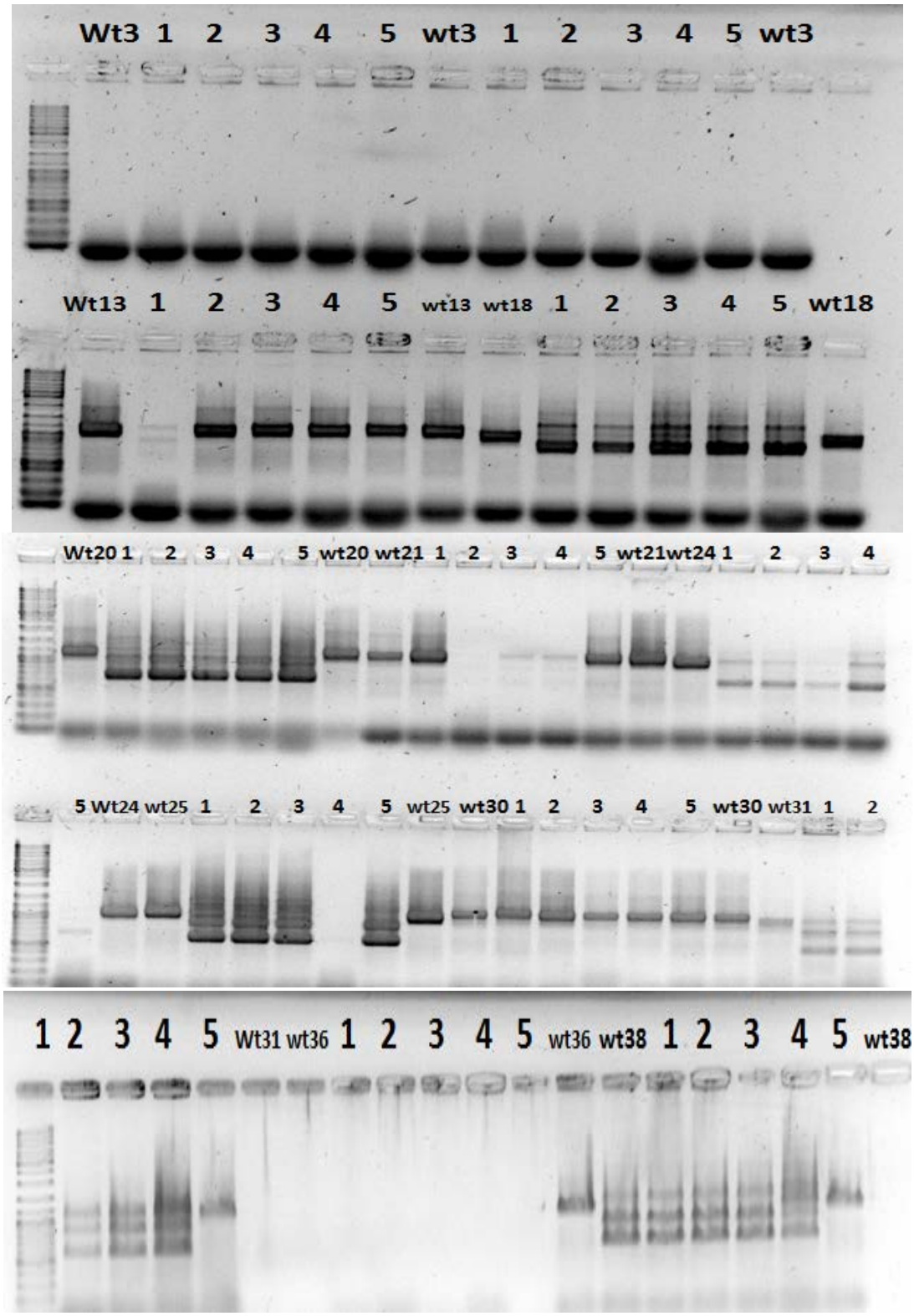
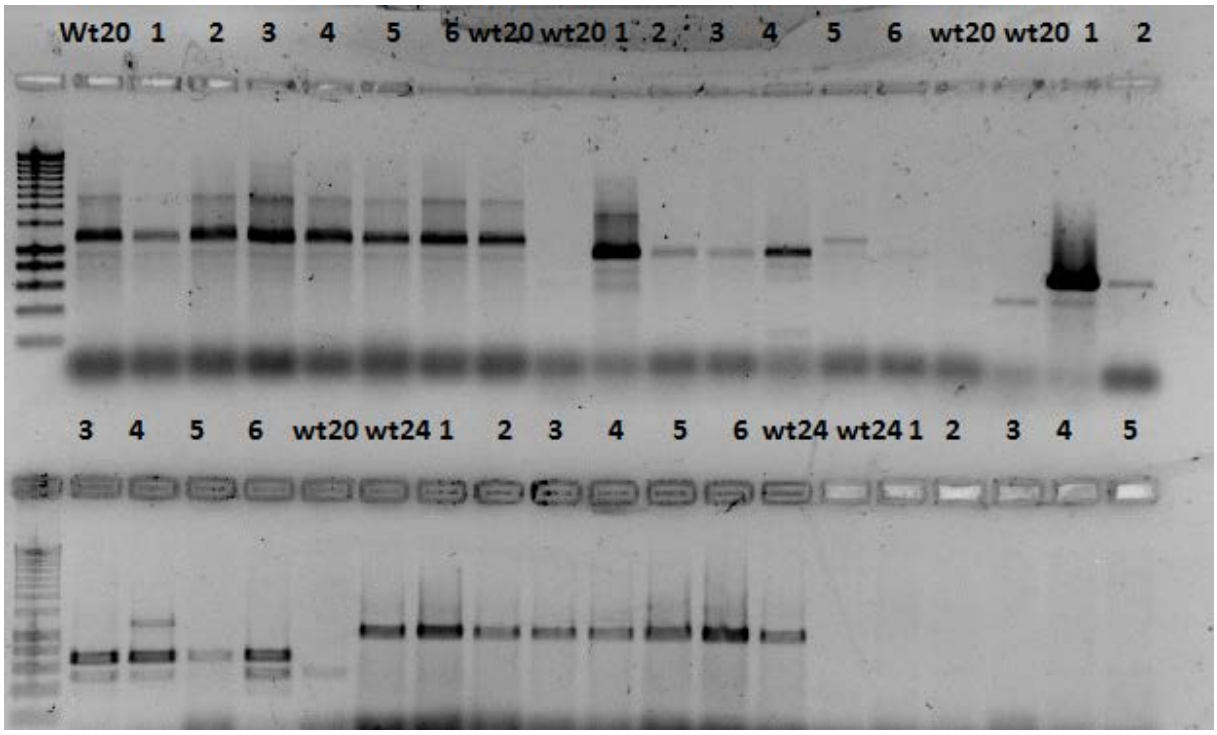


Figure 1: Genotyping mutant lines using forward, reverse and border primers in a pool of five plants per sample

Key: 1,2,3,4,5 are samples of the mutants in a pool of five each sample and wt13= wild type for line 13; wt20= wild type for line 20; wt25= wild type for line 25; wt31= wild type for line 31; wt36= wild type for line 36, wt38=wild type for line 38.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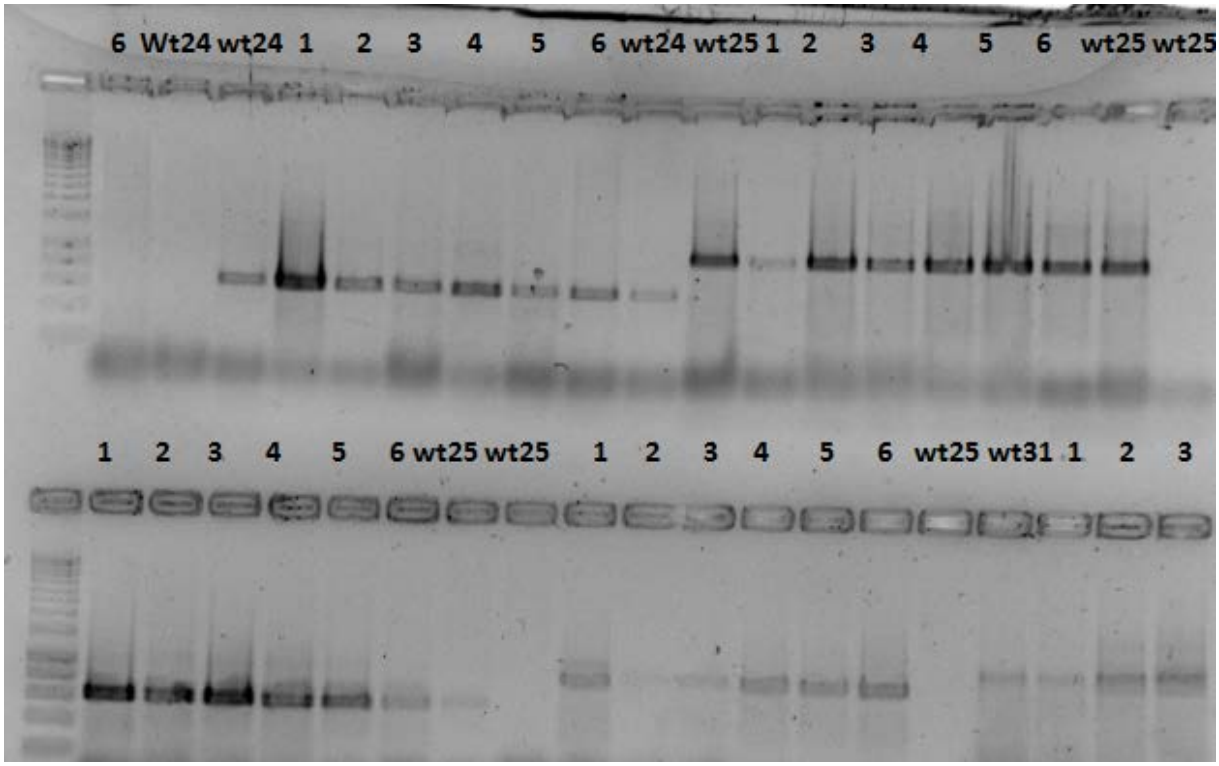
3 4 5 6 wt20 wt24 1 2 3 4 5 6 wt24 wt24 1 2 3 4 5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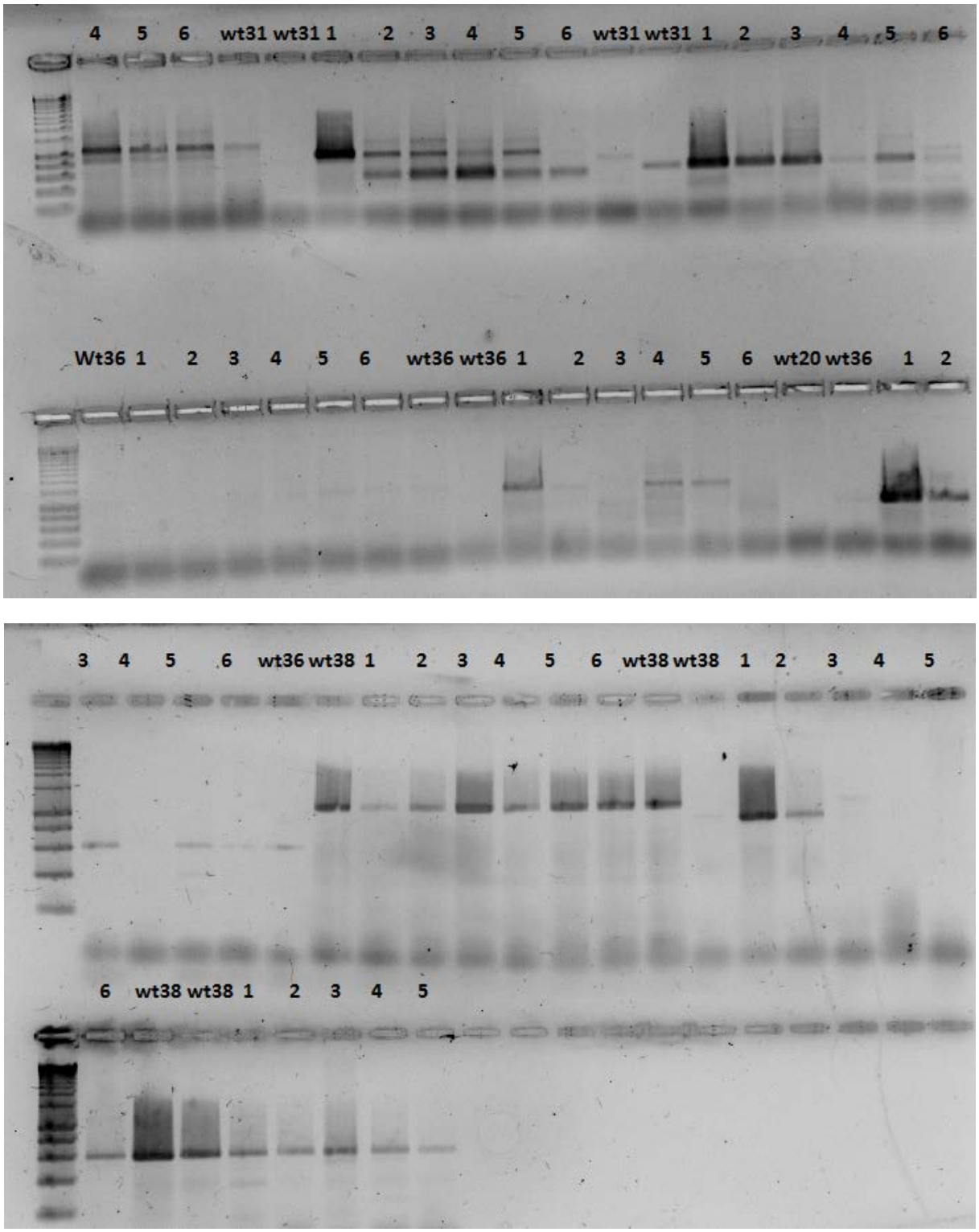

Figure 2: Gel electrophoresis image using the forward and reverse (F+R), the forward and the border (F+B) and the reverse and border (R+B) primers respectively.

Key: 1, 2,3,4,5 are samples of the mutants and wt20= wild type for line 20; wt25= wild type for line 25; wt31= wild type for line 31; wt36= wild type for line 36, wt38=wild type for line 38.