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Master Thesis Report

Identification of green seed mutants in a *dog1-1* background

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CONTENT

Abstract

Seed dormancy is an important phase during seed maturation, and the plant hormone ABA is an important regulator in this phase. The transcription factor *ABA-INSENSITIVE* (*ABI*) *3* is found to control ABA-induced processes of seed maturation and germination, like seed dormancy and chlorophyll degradation. *Abi3* mutant seeds showed a reduction of dormancy and a low level of chlorophyll degradation. The *DELAY OF GERMINATION 1* (*DOG1*) gene is another key regulator of seed dormancy induction. The double mutant *abi3-1 dog1-1* produced green seeds, while both single mutants showed a brown seed colour. Thus, the *dog1-1* mutant acts as an enhancer of the weak *abi3-1* mutant. Therefore, we hypothesized that screening for green seed mutants in a *dog1-1* background could retrieve novel regulators of seed maturation. In an ethyl methanesulfonate (EMS) mutagenesis experiment, 40 plants with a green seed phenotype were found in the *dog1-1* background. After screening and identifying the stable green seed phenotype among these mutants, we continued with 8 mutant lines which were all new *abi3* alleles. In order to clean up the genetic background, the mutants were backcrossed to L*er*-0 and /or *dog1-1*. After three round of backcrossing, single *abi3* mutants would be isolated from the *dog1-1* background to check the green seed phenotype.

Keywords: green seed, seed dormancy, *dog1-1*, *ABI3*, Arabidopsis

Introduction

Seed maturation is an important phase during seed development. During this phase, storage compounds are accumulated, seed dormancy is induced, desiccation tolerance and seed longevity are acquired. Many factors are involved in this phase, for example, the *FUSCA3* (*FUS3*), along with the *LEAFY COTYLEDON 1* (*LEC1*) and *LEC2* gene could promote maternal abscisic acid (ABA) during the mid-maturation phase (Finkelstein, 2013). The ABA concentration is related to the induction of seed dormancy in this period (Karssen et al., 1983; Koornneef et al., 1989 and Finkelstein, 2013). Mutiple studies have shown in arabidopsis that seed dormancy and longevity could also be affected by environmental conditions, like light intensity and temperature (He et al., 2014; Finch-Savage and Leubner-Metzger, 2006; Reddy et al., 1985). Induction of seed dormancy at seed maturation phase is essential, if all seeds germinate at the same time, a generation might be lost because of a catastrophe. Therefore, it could let seeds wait for the most optimal conditions to germinate, and also allow more time for seed dispersal (Salazar et al., 2011).

Seed dormancy and germination could be regulated by endogenous ABA (Leung and Giraudat, 1998), and there are several ABA response mutants identified in arabidopsis. A mutant, which contains a lower level of endogenous ABA, is called *ABA-DEFICIENCY* (*aba*). The *aba* mutant has a reduced seed dormancy (Koornneef et al., 1982). For example, in *Arabidopsis thaliana*, the *aba* mutant lines with low level of endogenous *ABA* had symptoms of withering and lack of seed dormancy (Karssen et al., 1983). Other mutants with reduced responsiveness to ABA are *aba-insensitive* (*abi*) mutants, which are found to encode some proteins and regulators (Finkelstein, 2013). *ABI1* and *ABI2* encode the protein phosphatases and *ABI3*, *ABI4* and *ABI5* encode the transcriptional regulators. The *ABI3* transcription factor is assumed to control ABA-induced processes of seed maturation and germination. Besides seed dormancy, ABA could also affect the chlorophyll degradation (Nakajima et al., 2012). In *abi3* null alleles, the reduced chlorophyll degradation during seed maturation result in the typical green seed phenotype. When chlorophyll amounts in seeds reduce, germination rates of seeds are high (Jalink et al., 1998), therefore, the *abi3* mutant seeds showed a reduction of dormancy (Léon-Kloosterziel et al., 1996).

Another key regulator of dormancy induction is the *DELAY OF GERMINATION 1* (*DOG1*) gene (Bentsink et al., 2006). The *DOG1* gene was identified from two accessions: the laboratory strain Landsberg *erecta* (L*er*) with low dormancy and a high dormancy accession Cape Verde Islands (Cvi) (Alonso-Blanco et al., 2003). The expression of *DOG1* is mainly regulated by temperature and correlated with seasonal changes during seed maturation (Footitt et al., 2011). And the DOG1 protein acts as a timer for the release of seed dormancy (Nakabayashi et al., 2012). The research on the non-dormant mutant, *dog1-1*, showed that the mutant seeds could maintain 100% germination percentage during the dry storage after seed harvest, while the seeds of wild type L*er* needed around 50 days of dry storage to reach 100% germination percentage (Bentsink et al., 2006). The research from Bentsink et al. (2006) also showed that after 10 months of seed dry storage after seed harvest, germination percentage of *dog1-1* mutant decreased quickly, while L*er* seeds could maintain the 100% germination percentage even after 30 months of dry storage after

seed harvest. Therefore, it was suggested that *DOG1* could affect not only seed dormancy, but also seed longevity.

Earlier research also worked on the other functions of *DOG1* during seed maturation. The research showed *dog1-1* could regulate the green seed phenotype in a *abi3-1* background. The single mutant *abi3-1* and *dog1-1* both had brown seed phenotype as the wild type L*er*-0, and the single mutant of strong *abi3-5* allele showed a green seed phenotype. While the research showed that in the double mutants, *dog1-1 abi3-1* had green seed phenotype. Thus, the interaction between *ABI3* and *DOG1* has indicated that the *dog1-1* mutant acts as an enhancer of the weak *abi3-1* mutant (Dekkers et al., 2016). A similar result had shown by Clerkx et al. (2003) that seed storability was affected by the *GREEN-SEEDED* mutant (*grs*) in an *abi3-1* mutant background. The *GRS* gene also acted as an enhancer of *abi3-1* and the double mutant *grs abi3-1* gave the green seed phenotype, whereas the monogenic *grs* mutant was brown. Several regulators were also found to affect the green seed phenotype in the *abi3-1* mutant background. Koornneef et al. (1989) found that abnormal seed development, like green seed phenotype and desiccation intolerance, occurred in *aba1 abi3* double mutant, compared with wild type and monogenic mutants. Like the *dog1-1 abi3-1* double mutant, double mutants of *abi3-1 grs*, *aba1 abi3*, *abi3-1 abi3-7* and *abi3-1 abi3-4* all have green seed phenotype (Clerkx et al., 2003; Koornneef et al., 1989; Ooms et al., 1993). The mutant *dog1-1*, *grs*, *aba1*, strong *abi3* alleles, *abi3-7* and *abi3-4*, act as the enhancer in the *abi3-1* mutant background.

In the experiment made by Dekkers et al. (2016), the *dog1-1* acts as the enhancer in the *abi3-1* mutant background. Since *abi3-1* is a sensitized genetic background, it could identify genes acting as enhancers. The *abi3-1* mutant has low dormancy and an increased amount of chlorophyll during seed maturation (Léon-Kloosterziel et al., 1996). Therefore, we hypothesised that we might find mutations in the *dog1-1* background, resulting in the failure of chlorophyll degradation during seed maturation which could be identified by a green seed phenotype.

To find such mutants, 10000 arabidopsis seeds in the *dog1-1* background were treated with ethyl methanesulfonate (EMS) on 70 pools (Figure 1). After seeds set, seeds were harvested per pool. Each pool of seeds was checked for green seed phenotype by eye. Then green seeds were selected and sown again. Plants, which its seeds were green, were selected as the mutant lines. In total 351 plants were grown, and 40 plants with a green seed phenotype were found.

The main aim of the mutagenesis experiment is to identify novel regulators of chlorophyll degradation during seed maturation. The thesis work described here includes backcrossing of the mutants to L*er*-0 and/or *dog1-1* to clean up the genetic background as well as further characterization of some the identified mutants. Among the identified mutants are many novel *abi3* alleles however the *ABI3* sequence has not been analysed for all mutants yet. Therefore, for these mutants the *ABI3* cDNA will be sequenced and analysed first. If the green seed mutant is not a *abi3* mutant after the *ABI3* cDNA sequencing, DNA of the mutant will be sequenced and analysed to find the novel regulate gene.

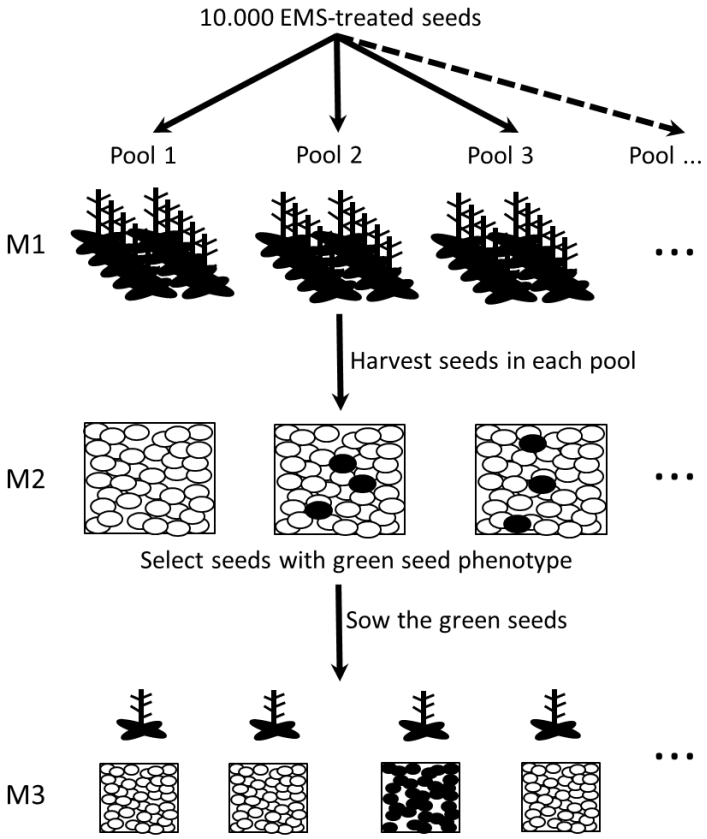

Figure 1. The methodology of mutagenesis experiment. Ten thousand seeds in the *dog1-1* background were treated with EMS on 70 pools, in nine trays (M1). After seeds set, seeds were harvested per pool. In M2, each pool of seeds was checked for green seed phenotype by eye. Then green seeds were selected and sown again. Plants, which its M3 seeds were green, were selected as the mutant lines.

Materials and Methods

Plant materials

Seeds of the *Arabidopsis thaliana* (L.) accessions Landsberg *erecta* (L*er*-0) were used in this experiment. L*er*-0, *dog1-1* and *abi3-1* were retrieved as described by Dekkers et al. (2016). Mutant lines in *dog1-1* background were produced by ethyl methane sulfonate (EMS) mutagenesis.

Plant growth conditions

Plants were grown on 4x4 cm Rockwool blocks in a growth chamber at 20°C /18°C (day/night) under a 16-hour photoperiod of artificial light (150 μmol·m^{-2}·s^{-1}) and 70% relative humidity. Plants were watered three times a week with a standard nutrient solution (He et al., 2014).

Mutant Backcrosses

In order to clean up mutant backgrounds, three rounds of backcross were planned to be done (Figure 2). In each backcross, plants of each mutant lines backcrossed with wild type L*er*-0 or *dog1-1*. All the F1 seeds were collected and sown to get F2 seeds. Genotyping the F2 plants or seeds to check the *dog1-1* mutation and continued with the homozygous plants, which showed green seed phenotype.

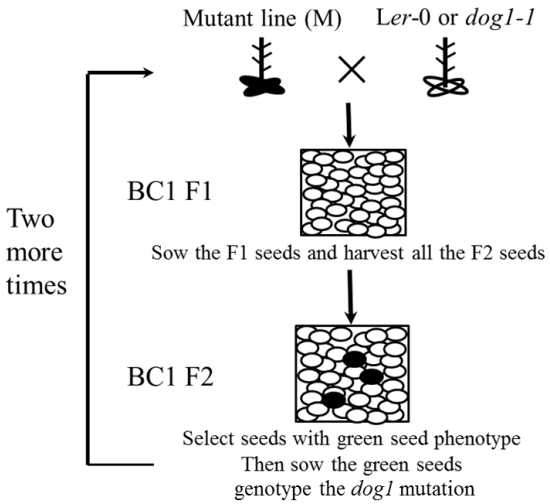

Figure 2. Mutants backcross processes. Single plant of each mutant lines were backcrossed with L*er*-0 or *dog1-1*. All the F1 seeds were harvested and sown to get F2 seeds. Green seeds were selected in F2. After around 6 weeks, genotyping was done to check *dog1-1* mutations in the plants with green seed phenotype. Repeated the backcrosses twice, in the end, get BC3 F2 seeds with green seed phenotype.

Green seed phenotyping

We used two ways to check the seed green seed phenotype. First, initial observation were made visually by using a stereo microscope. At the same time, weak mutants and strong mutants were identified according to the level of darkness of green. Except colour appearance, seed shape, dead seeds and pre-germinated seeds were also observed and counted as phenotypes of each mutant line.

To make quantitative measurements we measured chlorophyll fluorescence using the Pathoscreen machine (Phenovation Wageningen). The Pathoscreen is a machine that could obtain a measure of chlorophyll content, based on chlorophyll fluorescence. Wild type L*er*-0 was used as brown-seed control, while double mutant *dog1-1 abi3-1* was used as green-seed control. Photos of seed phenotypes both in colour and fluorescence were also taken by Pathoscreen.

Genotyping

DNA isolation

DNA was isolated from young leaves of 5-week-old plants. For each genotype, one young leaf was ground with a stainless steel ball in 200µl extraction buffer (2M NaCl, 200mM Tris-HCl pH 8.0, 70mM EDTA, 20mM $Na_2S_2O_5$), followed by an incubation step at 60^{o}C for 1 hour. Next, the sample was centrifuged for 5min at 2800g. 75µl of the supernatant was transferred to a new tube containing the mixture of 30µl 10 M NH_4Ac and 75µl isopropanol. After mixing, the sample was kept at room temperature for 15min. The mixture was centrifuged at max speed for 5 min to precipitate the DNA. The supernatant was removed and the pellet was washed by using 300µl 70% ethanol. After removal of the 70% ethanol, the pellet was dried in 60^{o}C oven for 5 min, followed by dissolving the DNA in 50-100µl MQ water, DNA was stored in -20^{o}C.

Genotyping

The *dog1-1* mutations were identified by the polymerase chain reaction (PCR) genotyping. The PCR mix and programme were summarized in Tables 1 and 2. The molecular marker used for the genotyping for *DOG1*-Cvi introgression in L*er*-0 was K15I22 (Bentsink et al., 2006).

After the PCR, gel electrophoresis was used to visualize and analyse the genotyping results. Loading buffer was added to the sample in the ratio of 1:5 (buffer: sample = 1: 5). 1% agarose gel was prepared with 4 g agarose and 20 µl ethidium bromide (EtBr) in 400 ml 1 x TAE buffer. Of each sample mix, 12 µl was loaded on the gel, using L*er*-0 and *dog1-1* as positive control and water as negative control, also 3 µl smart marker. Run the electrophoresis machine (PowerPac™ Basic Power Supply, BIO-RAD) around 80~100 volts.

Cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993) were used to detect *abi* mutations in this experiment. CPAS detect the genetic difference based on a distinct

endonuclease restriction sites. After the digestion, the difference could be detected on the gel according to the different DNA fragment length. When the single nucleotide polymorphism (SNP) of interest does not fit the recognition site of a restriction enzyme, a modified CAPS technique would be used, which is called derived cleaved amplified polymorphic sequences (dCAPS).

Table 1. Components[1] in a 15 µl polymerase chain reaction.

Taq DNA Polymerase (Firepol)	0.15 µl
10 mM dNTPs	0.3 µl
10X Buffer B	1.5 µl
$MgCl_2$	1.5 µl
10 µM Forward and Reverse Primers	0.4 µl
Nuclease-free H_2O	9.15 µl
DNA sample	2 µl

[1]All reaction components should be on ice and quickly transferring the reactions to a thermocycler machine.

Table 2. Temperature and time in PCR reaction.

Initial denaturation		95°C	5 min
	Denaturation	95°C	30 s
30 cycles	Annealing	59°C	30 s
	Extension[1]	72°C	75 s
Final extension		72°C	5 min
Hold		4°C	∞

[1]Extension times are generally 1 minute per kb.

Table 3. Temperature and time in a PCR reaction for restriction analysis.

Initial denaturation		95°C	5 min
	Denaturation	95°C	20 s
35 cycles	Annealing	57°C	30 s
	Extension[1]	72°C	30 s
Final extension		72°C	5 min
Hold		4°C	∞

[1]Extension times are generally 1 minute per kb.

Before the restriction analysis, PCR tubes were used to mix all the components (Table 1), and did the reaction in the PCR machine (Table 3). After the PCR, the PCR product was prepared for restriction analysis (Table 4). The restriction enzyme to detect SNP *abi3* mutant was generated on the website dCAPS Finder 2.0 (http://helix.wustl.edu/dcaps/). And the primer was generated on the website Primer3Plus (http://primer3plus.com/) according to the DNA sequence. The mixture was incubated for 1.5 hours at 37°C. Then, added 4 µl loading buffer to the mixture and loaded

12 µl on a 2~2.5% agarose gel. Gel electrophoresis was run by a machine (PowerPac™ Basic Power Supply, BIO-RAD) at 110 voltage.

Table 4. Components[1] in a 20 µl volume of restriction analysis.

Restriction endonuclease	0.2 µl
Restriction 10X buffer	2.0 µl
Nuclease-free water	12.8 µl
PCR product	5.0 µl

[1]All reaction components should be on ice and quickly transferring the reactions to a thermocycler machine.

Mutants sequencing

RNA isolation

For each genotype, two samples were taken to extract RNA, using the Hot-borate protocol (based on the procedures established by Wan and Wilkins, 1994). After the RNA isolation, three aspects were checked. First of all, the concentration of RNA was measured by the DropSense™ 16 'Touch & Go' reader. Second, the RNA integrity was tested by running the 150-200ng RNA per sample on the 2% agarose gel. The last thing, which was checked was potential contamination by DNA. The normal PCR was run to check whether there was any DNA in the RNA sample.

cDNA synthesis

The iScript™ cDNA synthesis kit (BIO-RAD) was used to synthesis cDNA. An amount of 700 ng RNA per sample and added water to 15 µl. Then a reaction, with 4 µl Buffer and 1 µl enzyme in the condition of no lid heating, was run in the following procedure, 5 min at 25°C, 20 min at 46°C and 5 min at 95°C. After synthesis, the cDNA was diluted with MQ water to 200 µl in total.

Amplifying *ABI3*

Using cDNA to run the PCR (the 72°C extension time was 2 minutes and 45 seconds) to amplify *ABI3*. The cDNA was used to do the Q5® high-fidelity PCR (Table 5 and 6). According to the *ABI3* primer sequences, annealing temperature in Q5® high-fidelity PCR was calculated in the NEB T_m Calculator (http://tmcalculator.neb.com/#!/).

DNA extraction from gel

After running the Q5® high-fidelity PCR product on the 0.8% agarose gel, excised the DNA fragment from the gel by using a clean scalpel. The protocol of DNA extraction from agarose gel was using the NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel (July 2014, Rev.03).

Sequencing and sequence analysis

Five tubes of each mutant sample were prepared in the way that each tube contains 5 µl of 50 ng/µl PCR product plus 5 µl of 5 pmole/µl primer. Then the samples were shipped to Macrogen Europe for sequencing.

The sequence results were analysed by CLC Workbench version 7.5.1 (Qiagen). In this program, we checked the mutation position of each mutant by making an alignment with the WT *ABI3* sequence.

Table 5. Components[1] in a 25 µl PCR using Q5® high-fidelity DNA polymerase.

5X Q5 Reaction Buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Forward Primer	1.25 µl
10 µM Reverse Primer	1.25 µl
Q5 High-Fidelity DNA Polymerase	0.25 µl
Nuclease-Free Water	9.75 µl
Template DNA	3 µl

[1]All reaction components should be on ice and quickly transferring the reactions to a thermocycler machine.

Table 6. Temperature and time in Q5® high-fidelity PCR.

Initial denaturation		98°C	30 s
	Denaturation	98°C	10 s
35 cycles	Annealing[1]	62°C	30 s
	Extension[2]	72°C	2 min 45 s
Final extension		72°C	2 min
Hold		4°C	∞

[1]ABI3 primer sequences:

ABI3 CDS FOR BD: ATGAAAAGCTTGCATGTGG

ABI3 CDS REV BD: TCATTTAACAGTTTGAGAAGTTGGT

[2]Extension times were generally 1 minute per kb. The ABI3 size was around 2.5 kb.

Results

Mutagenesis in *dog1-1* background

In order to find novel green seed regulators in *dog1-1* background, ten thousand *Arabidopsis thaliana* seeds in the *dog1-1* background were treated with ethyl methanesulfonate (EMS) and sown on 70 pools (Figure 1). After seeds set, seeds were harvested per pool. Each pool was checked for seeds with a green seed phenotype. Such green seeds were selected by eye and sown again. Plants, which produced green seeds, were selected as the mutant lines. In total 351 plants were grown, and 40 plants with a green seed phenotype were found.

The mutant *dog1-1* induced the green seed phenotype in weak *abi3-1* background (Dekkers et al., 2016), so first of all, the mutants in *dog1-1* background was checked whether they were *abi3* mutant. In these 40 green seed mutant plants, some of them have been identified and sequenced *ABI3* gene by amplifying the cDNA of *ABI3* (Table 7, No.1-11). The identified mutants were all *abi3* mutants. In these mutants, a base pair changed resulted in a stop codon, and their mutation positions located in different parts of *ABI3*, affecting different domains (Figure 3).

There were some mutants having the same mutation base pair, like M9-1 and M56-1, M11-1 and M16-3, M35-1 and M62-5, whereas they were grown in different pools independently. For these mutants, M56-1, M16-3 and M35-1 were chosen to continue.

Table 7. Overview of the identified *abi3* mutants.

No.	Mutant	cDNA (L*er*-0 sequence)[1]	Protein[2]	Domains affected
1	M60-2	SNP 481 **C**AA --> **T**AA	aa161 Q --> *	Before B1
2	M18-2	SNP 610 **C**AA --> **T**AA	aa204 Q --> *	Before B1
3	M9-1	SNP 874 **C**AG --> **T**AG	aa292 Q --> *	In B1
4	M56-1	SNP 874 **C**AG --> **T**AG	aa292 Q --> *	In B1
5	M58-1	SNP 900 TG**G** --> TG**A**	aa300 W --> *	In B1
6	M11-1	SNP 904 **C**AA --> **T**AA	aa302 Q --> *	In B1
7	M16-3	SNP 904 **C**AA --> **T**AA	aa302 Q --> *	In B1
8	M1-1	SNP 1333 **C**AG --> **T**AG	aa445 Q --> *	In B2
9	M35-1	del 1773-1776 (AGAA is missing)	from aa592 7 erroneous aa followed by a *	In B3
10	M62-5	del 1773-1776 (AGAA is missing)	from aa592 7 erroneous aa followed by a *	In B3
11	M2-1	SNP 1857 TG**G** --> TG**A**	aa619 W --> *	In B3
12	M66-1			
13	M14-1			
14	M67-1			
15	M68-1			
16	M69-1			

[1]Indicated the location of single nucleotide polymorphism (SNP) in the cDNA of *ABI3* in L*er*-0 sequence.
[2]Amino acids (aa) location in *ABI3* protein. (Q: Glutamine; W: Tryptophan; *: stop codon)

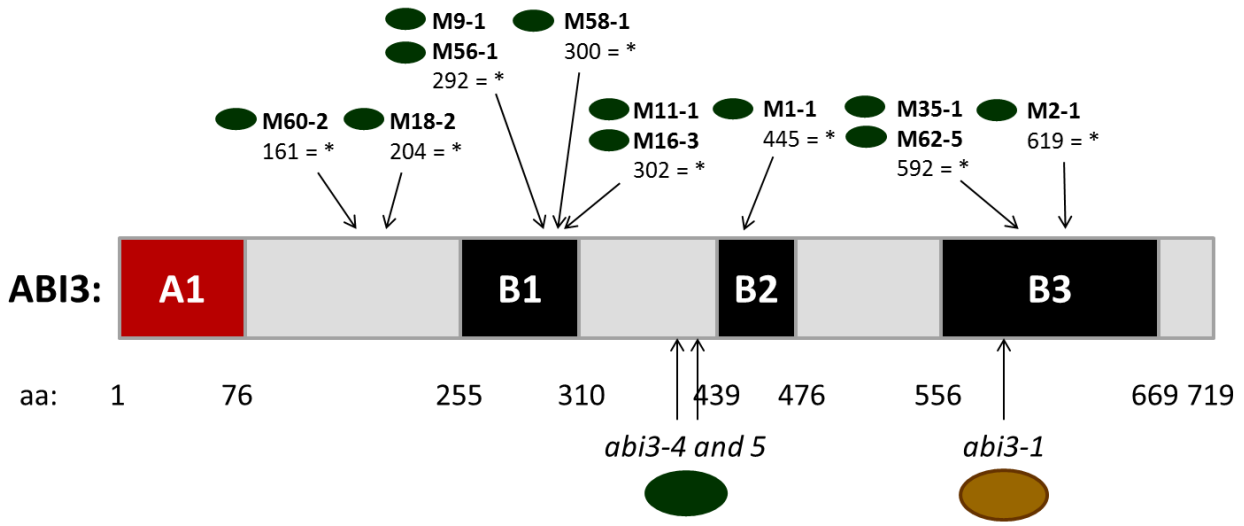

Figure 3. Overview of mutation positions of identified green seed mutants on ABI3 protein. The location of each mutant showed a base pair changed resulted in an amino acid changing to a stop codon (*), comparing with strong alleles *abi3-4* (SNP 1654 C --> T) and *abi3-5* with green seed phenotype and weak allele *abi3-1* (SNP 2143 G --> A) with brown seed phenotype. The number of each mutant meant the amino acids (aa) location in *ABI3* protein. Protein ABI3 contains 719 amino acids (aa) in total, and followed by a stop codon, TGA. There are three basic domains in ABI3 protein, B1, B2 and B3, also the A1 domain. The A1 is a domain in the acidic N-terminal of the protein (Giraudat et al., 1992); B1 could have interactions with ABI5; B2 and B3 are DNA-binding domains (Nakamura et al., 2001; Suzuki et al., 1997 and Brady et al., 2003).

This thesis work is part of this mutagenesis experiment which consisted of two parts: one is analysing and sequencing the remaining mutants, M14-1, M66-1, M67-1, M68-1 and M69-1 (Table 7, No.12-16) and the other one is backcrossing the green seed mutants.

Part 1. The remaining mutants analysis

Phenotyping results of remaining green seed mutants

Of the sixteen mutants, five have not been characterized (Table 7, No.12-16). Pathoscreen was used to make phenotype comparisons between the remaining mutants by visual appearance and chlorophyll fluorescence measurements (Figure 4, 5 and 6). As wild type L*er*-0 and *dog1-1* were used as brown-seed controls, and double mutant *dog1-1 abi3-1* was used as green-seed control, to which both the natural colour phenotypes and fluorescence images of the other mutants were compared. The values of mutant chlorophyll fluorescence were measured to show which mutant had significant different phenotypes from the mutant *dog1-1* (Appendix 1). The results of each mutant would be described one by one.

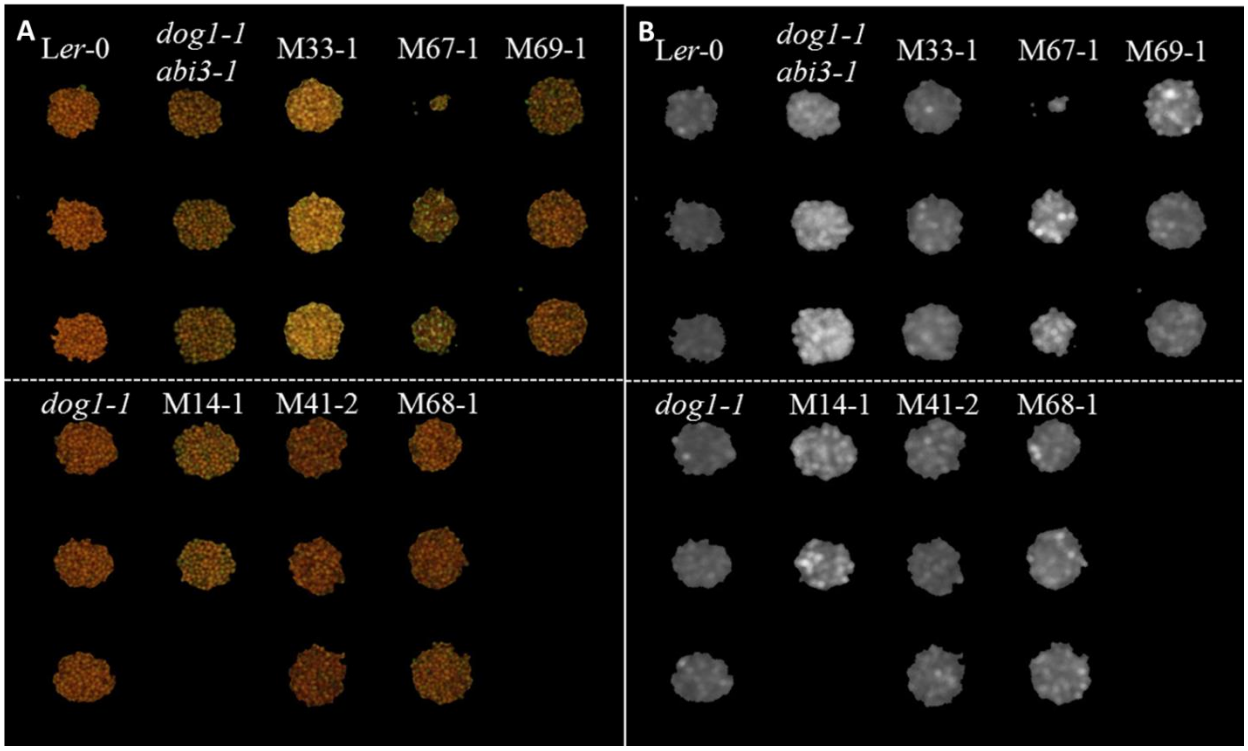

Figure 4. Seed phenotypes of different mutants and wild type L*er*-0 taken by Pathoscreen. A: seed natural colour; B: fluorescence image. For each genotype, three pools were used as replicates, but M14-1didn't have enough seeds for the third pool. Seeds in each pool were put randomly.

The seeds of mutant M33-1 showed bright yellow compared with other mutants and wild type (Figure 4A). When the seeds of M33-1 were observed by stereo microscope individually, the bright yellow phenotype was found to have transparent seed coat, which was a typical phenotype of *transparent testa* (*tt*) mutant (Shirley et al., 1995). In the fluorescence image, M33-1 had the same phenotype with L*er*-0 and *dog1-1*, and its chlorophyll fluorescence value did not have significant difference with *dog1-1* (Figure 4B and 6). Therefore the mutant M33-1 was cancelled.

An interesting phenotype was found in M67-1. The seeds of M67-1 had both green and brown seeds in natural colour image and the fluorescence image showed some bright spots (Figure 4). After the first round of backcross, some interesting phenotypes were shown, which contained greenish seeds, abnormal shapes of seeds, pre-germinated seeds and empty or dead seeds (Figure 5). These abnormal seeds were selected and sown, but these phenotypes disappeared in the next generation. The chlorophyll fluorescence value also showed M67-1 did not have significant higher chlorophyll fluorescence compared with *dog1-1* (Figure 6). Therefore, we would not continue with this mutant line.

The mutant M69-1 had the same phenotypes with M67-1 in natural colour, fluorescence image and value (Figure 4 and 6). Seeds (BC1 F2) from backcross M69-1 x L*er*-0 showed the same phenotypes with M67-1 as well, so M69-1 might be mixed with M67-1. Since it did not have stable green seed phenotype, M69-1 would not be continued.

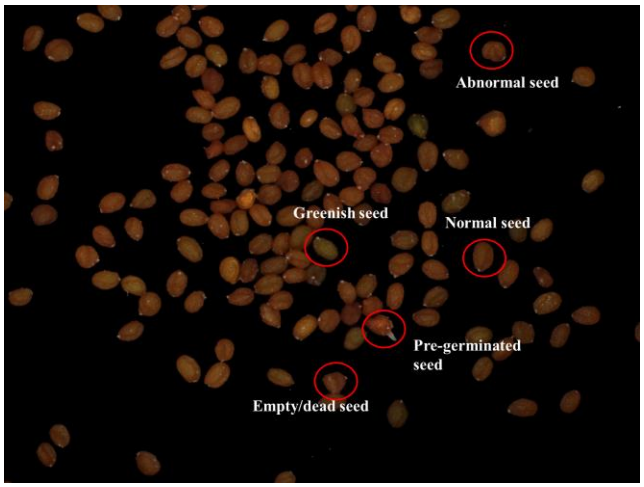

Figure 5. Seed phenotypes of M67-1 after the first round of backcross taken by Pathoscreen. It included greenish seeds, abnormal seeds, empty/dead seeds, pre-germinated seeds and normal seeds.

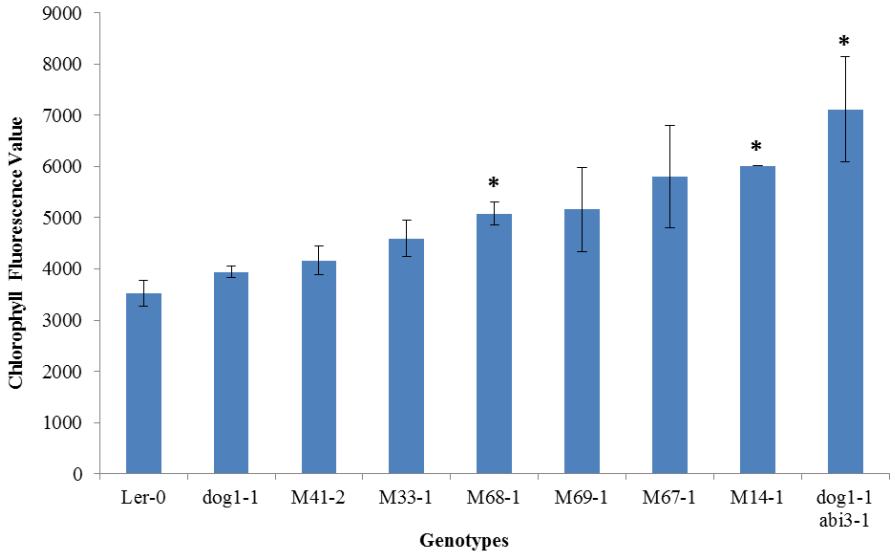

Figure 6. A bar chart of chlorophyll fluorescence values of different genotypes measured by Pathoscreen (Appendix 1). The error bars indicated the population standard deviation. Genotypes were ordered by chlorophyll fluorescence value, which from lowest to highest. The asterisks indicated the mutants had significant high chlorophyll fluorescence compared with both L*er*-0 and *dog1-1*, and these mutants had the same chlorophyll fluorescence level with *dog1-1 abi3-1*.

The mutants M14-1 had similar phenotype with *dog1-1 abi3-1*, which had greenish seeds (Figure 4A). Meanwhile, the M14-1 also had bright fluorescence image compared with other genotypes and double mutant *dog1-1 abi3-1* (Figure 4B). In the bar chart of chlorophyll fluorescence, M14-1 showed significant difference, compared with both L*er*-0 and *dog1-1* (Figure 6). These results showed M14-1 was a green seed mutant and would continue to sequence.

Another mutant M41-2 had brown seed phenotype and same chlorophyll fluorescence image with L*er*-0 and *dog1-1* (Figure 4). Its chlorophyll fluorescence value was also in the same level of wild type (Figure 6). Therefore the mutant line would not be continued.

The last mutant, M68-1, had the same natural colour and fluorescence image with L*er*-0 and *dog1-1* (Figure 4). However, in the bar chart of chlorophyll fluorescence value, M68-1 was significant different with dog1-1. The reason might be some immature seeds inside. Since M68-1 had clear brown seed phenotype, this mutant line was also cancelled.

In the end, the mutants, M33-1, M41-2, M67-1, M68-1 and M69-1, were cancelled since they did not have stable green seed phenotype. The mutant M14-1 could be continued to sequence and analysis because it had clear green seed phenotype. Another mutant, M66-1, had been checked the green seed phenotype before I involved in this experiment, but it have not be sequenced yet. Therefore, the cDNA of *ABI3* in M14-1 and M66-1 would be sequenced to check if they were *abi3* mutants.

The green seed mutants M14-1 and M66-1 are novel *abi3* mutant alleles affecting the B3 domain

The *dog1-1* mutant is an enhancer of the green seed phenotype in the weak *abi3-1* background (Dekkers et al., 2016), and because of this we expected to retrieve *abi3* mutants in this mutant screen as well. Therefore, first of all, we amplified and sequenced the *ABI3* cDNA of the mutants in *dog1-1* background was checked whether it was *abi3* mutant or not. Here we have sequenced and analysed the cDNA of *ABI3* of the mutants M14-1 and M66-1. In order to confirm the sequencing result, the dCAPS was used to detect *abi3* mutation positions in these two mutants. The *ABI3* primers were designed and restriction enzymes were selected respectively for each mutant.

Sequence analysis of *ABI3* gene in the green seed mutant M14-1

The sequencing result of M14-1 showed that M14-1 was an *abi3* mutant, which affected B3 domain of *ABI3* protein (Figure 7). A base pair changed in intron 3 of *ABI3* gene (SNP 1872, the purine guanine (G) to the purine adenine (A)). This was a mutation in a splicing site, which resulted in the retention of intron 3 in *ABI3* transcript. Immediately after the mutation, a stop codon was encoded which resulted in a shorter *ABI3* protein of 625aa in M14-1 compared with WT (719aa).

Based on the sequence analysis, *ABI3* primers for M14-1 were designed:

Forward: **GCGGGGCGGGGGCACGGGGGG**TTTGGAACATGCGCT**C**CAG
Reversed: AAAACGTACCGGTGTTCTCG

The underlined base pair in red was a mismatched base pair. A modified region, GCGGGGCGGGGGCACGGGGGG, was added to the forward primer since there was a mismatched base pair in the forward primer. The region extended the 20-bp of the product, so the digested sequence could be visualized on gel clearly.

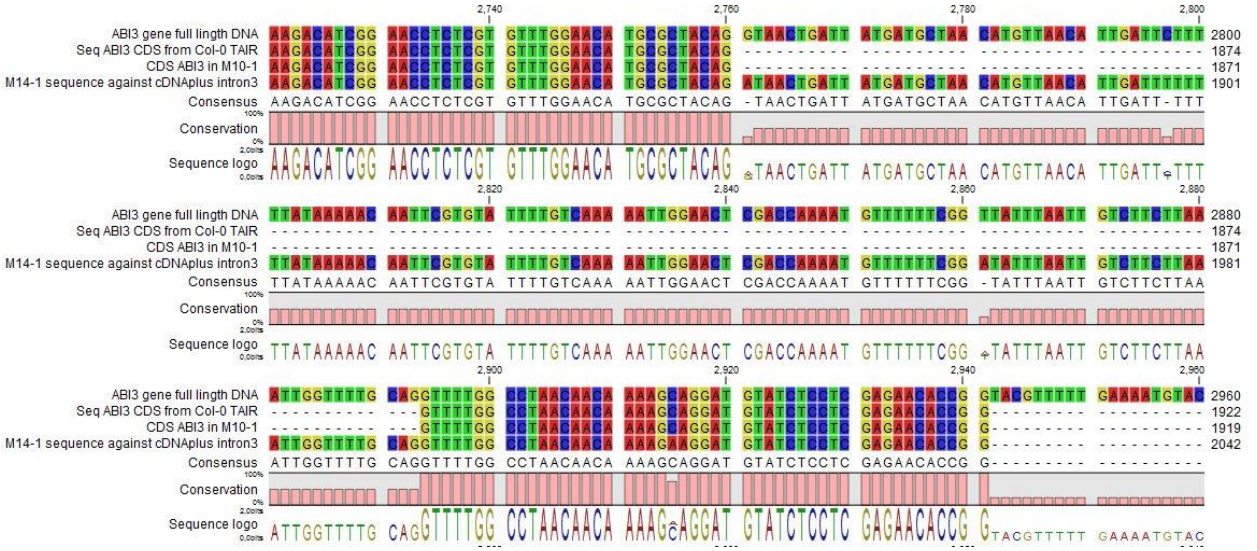

Figure 7. The alignment image of M14-1 cDNA of *ABI3* sequence result. A base pair changed in SNP 1872 resulted in the retention of intron 3 in M14-1 cDNA of *ABI3*. The M14-1 of intron 3 retention sequence was compared with *ABI3* gene full length DNA, *ABI3* cDNA sequence of Col-0 and *ABI3* cDNA sequence in M10-1. The mutant M10-1 is wild type in *ABI3* gene, so it was used as the *ABI3* cDNA of L*er*-0. The numbers at the right side and at the top of each alignment line showed the SNP positions in *ABI3*.

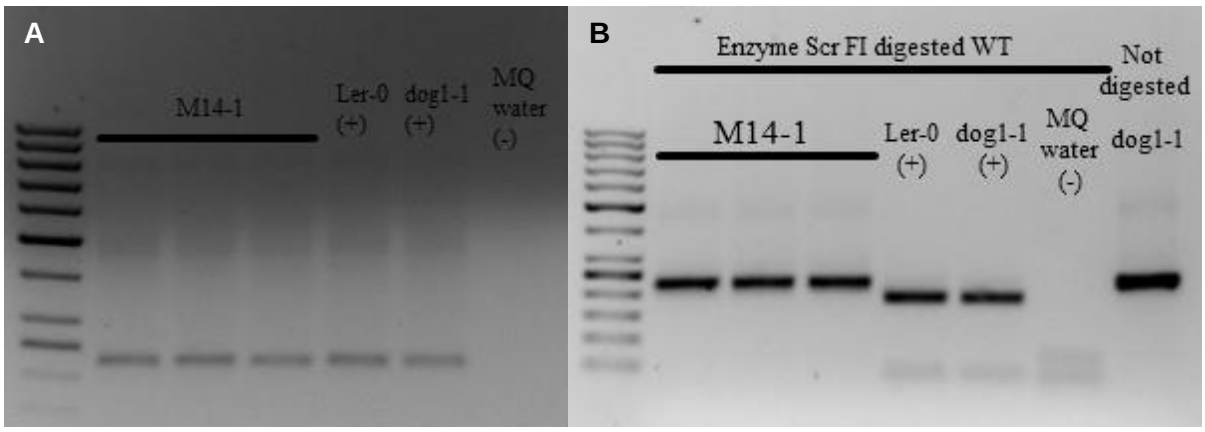

Figure 8. Gel results of restriction analysis of M14-1. The DNA of M14-1 was isolated from 3 different plants, which were used as replicates. L*er*-0 and *dog1-1* were used as positive controls and water was used as negative control. A: The *ABI3* primers were tested in M14-1, compared with positive and negative controls. B: Restriction analysis by using the ScrFI restriction enzyme, which digest wild type *ABI3* sequence (in L*er*-0 and *dog1-1*). The band on the right hand site of not digested *dog1-1* indicated the M14-1 samples were not digested as expected.

The primers were tested in M14-1, positive controls (L*er*-0 and *dog1-1*) and negative control (water). The gel result showed that *ABI3* gene of all the lines were amplified by primers and all the bands were in the same height (Figure 8A). For the M14-1, the restriction enzyme, ScrFI, was selected to cut the wild type sequence (Figure 8B). The result showed that the positive control L*er*-0 and *dog1-1*, which were both wild type for ABI3, were digested by the enzyme. The mutants were the same with *dog1-1* that was not digested. These results confirmed the mutation position of M14-1 in *ABI3*.

Sequence analysis of *ABI3* gene in the green seed mutant M66-1

When we analysed the sequence result of M66-1, first, we compared in the cDNA level (Figure 9). *ABI3* cDNA of M66-1, L*er*-0 and Col-0 were compared, and the result showed a base pair in SNP1919 was missing, which is the first base pair in exon 5. EMS mainly induces base pair changes resulting in C/G to T/A (99%) conversions (Kim et al., 2006), and it is less likely to cause base pair deletions. Therefore, we compared the sequence on the DNA level with *ABI3* transcript. In intron 4, the last two base pairs are AG, which is a splicing site. Then we made an assumption that the last base pair in intron 4 changed from G to A, resulting in the splicing site changed and 1-bp of the recognition site shifting to right, so that 1-bp was missing in exon 5.

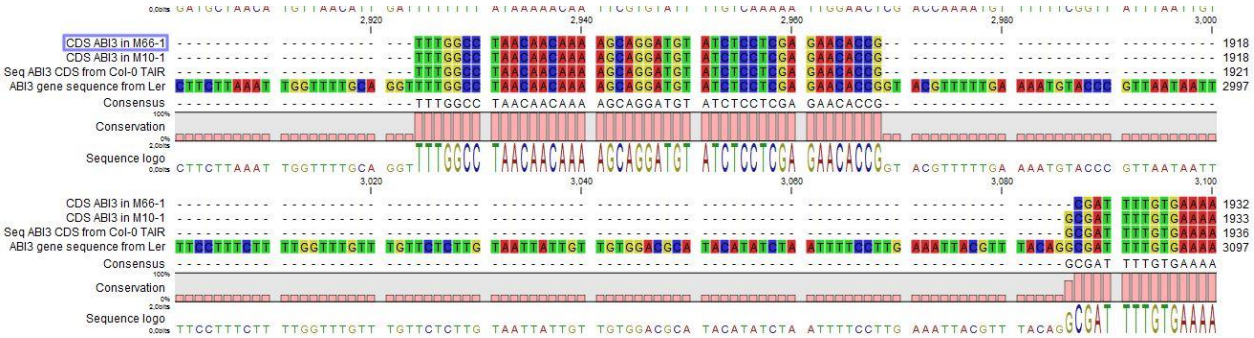

Figure 9. The alignment image of M66-1 cDNA of *ABI3* sequence result. A base pair changed in intron 4 resulted in a base pair missing in SNP 1919. Sequence result of M66-1 cDNA of *ABI3* was compared with *ABI3* gene full length DNA, *ABI3* cDNA of Col-0 and *ABI3* cDNA in M10-1. The mutant M10-1 is wild type in *ABI3* gene, so it was used as the *ABI3* cDNA of L*er*-0. The numbers at the right side and at the top of each alignment line showed the SNP positions in *ABI3*. A (adenine), T (thymine), C (cytosine) and G (guanine) are four kinds of base pairs in DNA.

As at this time we have not analysed this DNA sequence of *ABI3*, we aimed to confirm this assumption using a genetic marker. Based on the sequence analysis, *ABI3* primers for M66-1 were designed:

Forward: CAAAAATTGGAACTCGACCAA
Reversed: **GCGGGGCGGGGGCACGGGGGG**CCCATTGGTTTTCACAAAATCGA

Like M14-1, in M66-1, a modified region, GCGGGGCGGGGGCACGGGGGG, was added to the reversed primer where a mismatched base pair located in. The underlined base pair in red was a mismatched base pair. The region extended the 20-bp of the product, so the digested sequence could be visualized on gel clearly.

An *ABI3* primer was designed for the M66-1, then it was tested in M66-1 and positive controls, L*er*-0 and *dog1-1* which both contain a wild type *ABI3* gene (Figure 10A). The gel image showed the dCAPS primer pair amplified a fragment on the expected height in all samples. The fragments were digested with the restriction enzyme ClaI, which should cut the mutant sequence (Figure 10B). The result indicated that the mutants were digested by the enzyme, while the positive controls L*er*-0 and *dog1-1*, which were both wild type in *ABI3*, were not. This restriction analysis results confirmed the assumption of the underlying mutation in M66-1.

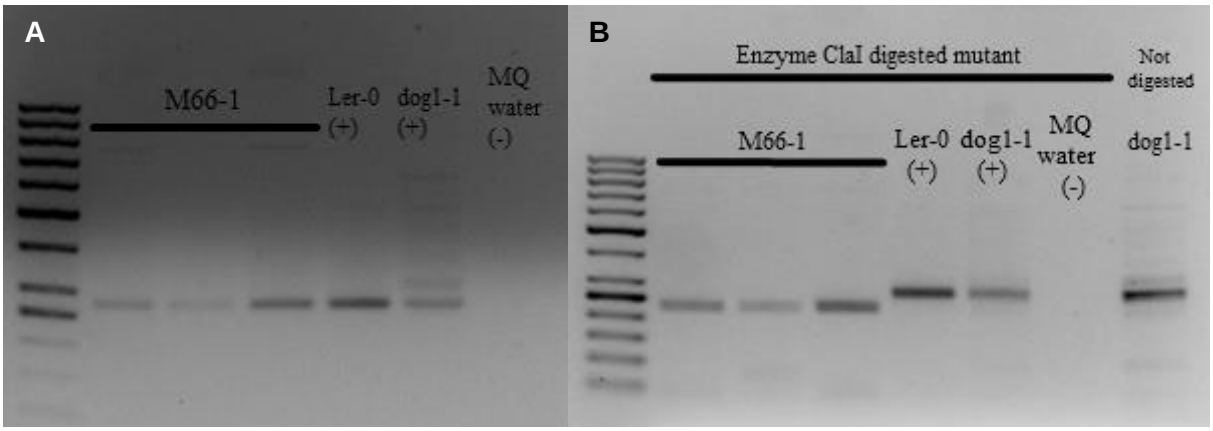

Figure 10. Gel results of restriction analysis of M66-1. The DNA of M66-1 was isolated from 3 different plants, which were used as replicates. L*er*-0 and *dog1-1* were used as positive controls and water was used as negative control. A: The *ABI3* primers were tested in M66-1, compared with positive and negative controls. B: Restriction analysis using the ClaI restriction enzyme which digests the mutant *ABI3* sequence in M66-1. The band of not digested *dog1-1* indicated the WT *ABI3* sequence were not digested.

Part 2. Mutants backcrossing

In order to clean up the genetic background, all the green seed mutant lines were backcrossed to L*er*-0 and/or *dog1-1* (Figure 2). After phenotyping the remaining green seed mutants, some of these mutants were cancelled because of the unstable green seed phenotype. For now, in total, 8 mutant lines were backcrossed (Table 8). At least three pollinations were done for each genotype, and all F1 seeds were harvested and sown in order to get enough seeds for next generation. The mutant M1-1 was backcrossed with *dog1-1* in the BC1 (Table 8), because when it was backcrossed with L*er-0*, the siliques didn't develop. For each genotype, twenty F2 green seeds were selected randomly by eye and sown in 10 wool-rock blocks (two seeds in each block).

After six weeks of sowing F2 seeds, each plant was genotyped to check *dog1* mutation(Table 8). In the end, for the mutants had been genotyped, M60-2 only had heterozygous in *dog1-1* and other mutants all had homozygous plants in *dog1-1*. In the next generation, M60-2 would be genotyped again to get homozygous *dog1-1* plants from segregation.

After genotyping the *dog1* mutation, the backcross processes would be repeated twice (Figure 2). In total, three rounds of backcrosses will be done. Due to the time limitation, the backcrosses were in the second round for now.

Table 8. Backcrosses (BC) of mutants in *dog1-1* background overview.

Mutant	**Crossed with**		**Current stage**	**Selection of double mutants**[1]
	BC1	BC2		
M60-2	L*er*-0	L*er*-0	BC2 F1	heterozygous
M56-1	L*er*-0	L*er*-0	BC2 F2	homozygous
M16-3	L*er*-0	L*er*-0	BC2 F2	homozygous
M1-1	*dog1-1*	L*er*-0	BC2 F2	homozygous
M35-1	L*er*-0	L*er*-0	BC2 F1	homozygous
M2-1	L*er*-0		BC1 F2	
M14-1	L*er*-0	L*er*-0	BC2 F1	homozygous
M66-1	L*er*-0	L*er*-0	BC2 F1	homozygous

[1]Mutants were genotyped to check *dog1* mutation in BC1 F2.

Discussion

Mutant identification

The aim of this experiment is to find the green seed mutants in *dog1-1* background to identify novel regulators of seed maturation. However, so far, all the identified mutants in this mutagenesis experiment are *abi3* mutants. The ABI3 protein plays important roles on the processes during seed maturation (Clerkx et al., 2003; Parcy et al., 1994; Ooms et al., 1993), and *ABI3* is a central regulator in ABA signaling (Zhang et al., 2005; Stone et al., 2006; Finkelstein et al., 2002). However, we cannot conclude that *ABI3* is the only regulator on the green seed mutant in a *dog1-1* background. The reason we didn't find novel regulators might be related to the way we selected the mutant lines in the mutagenesis experiment (Figure 1). In M2, the green seeds were selected under the stereo microscope by eye, in this situation, only seeds with severe green seed phenotype could be found. Therefore, the possible solution to find green seeds regulated by additional factors is selecting green seed mutant lines by measuring chlorophyll fluorescence. The Pathoscreen could take fluorescence image and measure chlorophyll fluorescence at the same time (Figure 4-6), which is easy to do the following analysis. Therefore, we could select the mutant lines with higher chlorophyll fluorescence measurement, even if their green seed phenotype is weak.

The mutation position of identified mutants located in the different domains in ABI3 protein (Figure 3). The sequence results of identified mutants showed a base pair (bp) changed causing a stop codon in *ABI3* sequence (Table 7), which reduce the length of ABI3 protein. For the mutants before or in B2 domain, when they are isolated from the *dog1-1* background, large part of ABI3 protein will not be functional. They might have similar severe seed phenotypes with strong alleles *abi3-4* and *abi3-5*, for example seeds will fail to degrade chlorophyll during maturation and keep seeds green (Giraudat et al., 1992). For the mutants located in B3 domain, like *abi3-1*, the B3 DNA-binding domain could be partly activated to enable the transcription activity of ABI3 (Ezcurra et al., 2000).

For the mutants I analysed, M14-1 and M66-1, dCAPS markers were designed to confirm the mutation position (Figure 7-10). In the following research, the CAPS markers could also be used to select the single *abi3* mutant without *dog1-1* background. For other mutants, which were identified before, CAPS markers could also be developed to confirm the mutation positions and select the single mutants isolated from the *dog1-1* background.

Green seed phenotype regulation

Green seed phenotype is caused by a lack of chlorophyll degradation during the last stages of seed development. Severe alleles in *abi3* mutants (like *abi3-4* and *abi3-5*) show non-dormant and green seed phenotypes (Nambara et al., 1995). Earlier research on the mutants in *abi3-1*

background showed that double mutant *dog1-1 abi3-1* had green seed phenotype, while single mutants *dog1-1* and *abi3-1* had brown seed phenotype. So *dog1-1* mutant is an enhancer of the weak *abi3-1* allele (Dekkers et al., 2016). In the mutagenesis experiment in the *dog1-1* background might retrieve additional regulators that regulate chlorophyll degradation and/ or seed maturation.

The double mutant *dog1-1 abi3-1* showed green seed phenotype, and in this experiment *abi3* mutants in *dog1-1* background also showed green seed phenotype. Both experiments indicate *DOG1* and *ABI3* interact with each other. The genetic relationship between *DOG1* and *ABI3* on how these two genes regulate chlorophyll degradation is not clear yet. There are two hypothesis, the first is one of these two genes might upstream of the other, and second is they work parallel (Figure 11). For the first one, if *ABI3* is downstream regulated by *DOG1* (Figure 11A), the mutants in *dog1-1* background could not actively express *ABI3*. On the other hand, *dog1-1* could be in a quiescent state for the *abi3-1* mutant, *DOG1* gene cannot be expressed actively. Secondly, when *DOG1* and *ABI3* work parallel (Figure 11B), *dog1-1* and *abi3-mutant* can be expressed independently. These two hypothesis need further research to confirm.

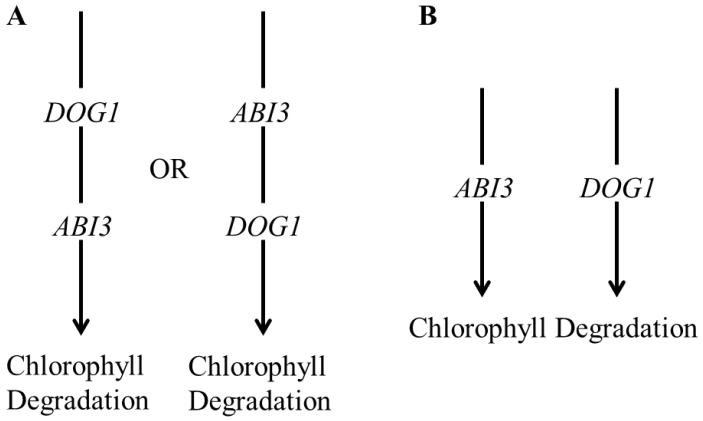

Figure 11. Two hypothesis how *DOG1* and *ABI3* interact with each other and how they regulate the chlorophyll degradation. A: *DOG1* and *ABI3* work in one line, one gene might upstream of the other. B: *DOG1* and *ABI3* work parallel, they regulate the chlorophyll degradation together but independently.

All the green seed mutants found in this experiment are *abi3* mutants, which confirms *ABI3* that is the main regulator in chlorophyll degradation. However, how the interactions between *ABI3* and *DOG1* causing a green seed phenotype is still not clear. As we known, *ABI3* is a central regulator in ABA signalling (Zhang et al., 2005; Ooms et al., 1993) and *DOG1* is a dominant factor by influencing ABA signalling in seed dormancy (Footitt et al., 2011). Thus, the changes in ABA level might influence chlorophyll degradation directly, then resulting in the green seed phenotype, but this still need to be investigated. Meanwhile, both ABA and *DOG1* play important roles in seed dormancy (Stone et al., 2006; Nakabayashi et al., 2012), therefore *DOG1* co-operate

with ABA levels in seed to regulated the seed dormancy. However, how this interaction works and is this interaction related to *ABI3* are the unknown questions which need further work.

Further research

The further research will continue the three rounds of backcrosses to clean up the genetic background of these green seed mutants in *dog1-1* background (Figure 2 and Table 8). Both homozygous double mutants (*abi3-/dog1-1*) and single mutants (*abi3-/DOG1*) would be selected and checked green seed phenotypes.

Using Arabidopsis to work on the green seed phenotype could be aware of the effects on seed quality causing by the failure of chlorophyll degradation. Therefore, producing high quality seeds without green seeds could be an improvement in the application of agriculture.

Acknowledge

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Appendix 1. Data and statistical analysis of chlorophyll fluorescence measurement

Table 9. The chlorophyll fluorescence (Chl.Fl.) of each genotype measured by Pathoscreen.

Pool No.[1]	Genotypes	Chl.Fl.	Average Chl.Fl.	σ[2]	*P*-value[3]
1		3876			
6	L*er*-0	3313	3528	249	0.101
11		3394			
2		5871			
7	*dog1-1 abi3-1*	7073	7111	1028	0.012*
12		8388			
3		4133			
8	M33-1	4674	4598	353	0.065
13		4987			
4		4442			
9	M67-1	6822	5805	1002	0.059
14		6150			
5		6326			
10	M69-1	4534	5165	822	0.105
15		4634			
16		3780			
21	*dog1-1*	4048	3938	114	1.000
26		3985			
17	M14-1	6014	6016	2	0.000*
22		6018			
18		4464			
23	M41-2	3786	4163	282	0.354
28		4240			
19		4815			
24	M68-1	5349	5083	218	0.003*
29		5086			

[1]The number in a 5x6 (row x column) pool was counted from left to right first, then from top to bottom.

[2]The σ meant population standard deviation. Formula: $\boldsymbol{\sigma = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(x_i - \mu)^2}}$

[3]A t-test was conducted to check the difference between the mutant lines and *dog1-1*. If the difference was significant, *P*-value would be less than 0.05. The asterisks (*) showed these mutant lines had significant higher chlorophyll fluorescence compared with *dog1-1*.