Master Thesis Report

Screening *Seed Dormancy Modifier* Genes

Han Xia

Master thesis plant physiology

PPH-80436

Supervisor: Leonie Bentsink

Supervisor: Thu-Phuong Nguyen

Plant Physiology group

Wageningen Seed Lab

Introduction

Dormancy of seeds can be described as an inhibiting mechanism that prevents radical emergence[1]. Once dormancy released seeds become competent to germinate. Temporary failure to germinate during dormancy enables seeds to sense a favourable environmental condition for successful plant establishment[2], [3]. Seeds after harvesting accumulate a series of physical and biochemical changes before they are enabled to release dormancy and germinate. Such process is called after-ripening. Dry storage on room temperature is a common strategy for seeds after-ripening. DSDS50 is referred to as days of dry storage after harvesting required for seeds to reach 50 percent of germination[4]. It is used as an indicating parameter to represent seeds dormancy level. The higher DSDS50 value, the deeper dormancy level of the seeds is.

Dormancy is complex. Many loci are involved in physiological regulation of dormancy[5], [6]. A typical example is that dormancy is under regulation of intricate interaction and feedback of plant hormones[7], remarkably as abscisic acid (ABA) and gibberellins (GA)[8][9]. Previous research revealed that several Delay of Germination (*DOG*s) quantitative trait loci (QTL) have strong impact on seed dormancy[10], [11]. *DOG*6 was detected as the second strongest QTL amongst after *DOG*1[10]. Study on *NILDOG*6-sha, the Landsberg *eracta* (L*er*) background introgression line which consists of stronger *DOG6* loci from ecotype Shakdara (Sha), revealed its higher seed dormancy level than L*er* therefore confirmed the dormancy effect of *DOG*6[10]. In order to have a better understanding of *DOG*6 involved dormancy in Arabidopsis seeds, a forward genetics approach has been applied to exploit potential regulatory factors of DOG6.

Ethyl methanesulfonate (EMS) mutagenesis in dormant *NILDOG*6 background generated a few reduced seed dormancy mutants. Excluding mutants dependent on *DOG*1, ABA or GA regulation, 4 putative mutants are screened by their reduced seed dormancy phenotype, named seed dormancy modifier (*sdm*) 1 to 4. Apart from reduced-dormancy, *sdm*2 and

*sdm*4 also displays abnormal phenotype in seed mucilage. However, no evidence has shown direct coupling between this type of abnormal mucilage and reduced-dormancy. In other words, abnormal seed mucilage could be induced by mutations that are independent on dormancy related mutations.

Following the work described above, the subject of this thesis work was screening *seed dormancy modifier* genes from a potential candidate gene list. T-DNA insertion mutant lines of candidate genes were ordered and tested for their dormancy phenotype. Allelism test was performed by reciprocally crossing *sdm* mutant against corresponding T-DNA line and phenotyping F_1 seeds. Candidate genes that confirmed by allelism test are likely to be one of the same mutation of *sdm* and will serve as object for further analysis and research.

Materials and methods

Research approach

Forward genetics is applied as research approach for this work.

SHOREmap bulked segregant sequencing was applied to find mutation enriched loci in *sdm* genome. *Sdm* lines were backcross against their background lines *NILDOG*6-Sha to create mapping generation. Non-dormant or abnormal seed mucilage lines from F2 generation of backcross population were screened. By comparing genome of such mapping lines, most in common regions were considered most likely loci of *sdm* mutations. SHOREmap bulked segregant sequencing results indicated high mutation frequency regions in the genome. Potential candidate genes were selected from these regions for their corresponding *sdm*.

Sdm mutants are generated by EMS mutagenesis in *NILDOG*6 Background. *Sdm* mutant seeds behave differently from *NILDOG*6 seeds in germination processes, for example reduced dormancy level, seeds longevity and sensitivity to ABA. SHOREmap bulked segregant sequencing revealed genomic location information of *sdm*s. Putative candidate genes in such genomic location were selected. The assumption is that if F1 seeds of mutant plant of such candidate gene cross against corresponding *sdm* mutant plant display same trait of *sdm* mutant, the candidate gene was likely to contain point mutation induced by EMS assay in *sdm*.

So in this thesis study, T-DNA mutant of those candidate genes were ordered. Homozygosity of those T-DNA lines was tested by PCR using verification primers. Dormancy phenotype of T-DNA lines was tested by germination assay and calculated DSDS50. Homozygous t-DNA lines were grown and cross against their corresponding *sdm* mutant reciprocally in order to allelism but also maternal effect on seeds dormancy. F_1 seeds were tested for their dormancy trait by calculating DSDS50. Because of limited number of such crossing seeds in some lines, DSDS50 wasn't able to be calculated. Therefore the maximum germination percentage was recorded to represent dormancy level instead of DSDS50.

By comparing dormancy phenotype of these reciprocal seeds and their parental seeds, gene contains mutation in *sdm* mutant will hopefully be found. However, candidate gene T-DNA lines are in Col-0 background whereas *sdm*s are in *NILDOG*6 background. Heterosis in such L*er* and Col-0 cross will complicate phenotyping seed dormancy.

Plant material

*NILDOG*6 is in L*er* background introgressed with stronger *DOG*6 QTL from Sha ecotype. Seed dormancy modifier (*sdm*) lines are reduced dormancy mutant of *NILDOG*6 induced by EMS mutagenesis. Four of these *sdm* lines (*sdm* 1 to 4) are studied here. T-DNA mutant lines of candidate genes have been ordered. M2 seeds of these T-DNA lines were provided by Phuong.Col-0 is used as wild type control to T-DNA lines.

Growing and harvesting condition

Seeds were sown on wet germination paper in plastic tray and put in light chamber for 5 days. Germinated seedlings were then transplant onto rockwool in climate chamber.

Plants were grown on rockwool plugs and watered with 1 g L21 Hyponex fertilizer (nitrogen:phosphorus:potassium,7:6:19) in a climate chamber at 20 °C day/ 18°C night with 16 h of light (35Wm^2) and at a relative humidity of 70%.

9 plants of each line were divided into three 3 plants block. In total 225 plants of these clusters were randomly distributed on the growing area. One plant of each line was randomly selected to do reciprocal cross, except for *NILDOG*6 and Col-0. Crossing seeds were harvested as siliques whereas seeds of rest plants were harvested as clusters. Seeds were harvested and were stored in paper bags at ambient condition.

Genotyping T-DNA lines

Homozygosity of T-DNA lines is tested by PCR using T-DNA verification primers. Seeds of original T-DNA lines provided by Phuong were grown and seeds of single plant were harvested and labelled. DNA was extracted from those seeds as template for verification PCR. PCR setup was either forward primer + reverse primer in pair with border primer + reverse primer or forward + boarder + reverse triple primer system. PCR programme was: 1. 95°C for 5 minutes, 2. 95°C for 30 seconds, 3. 55°C for 1 minute, 4. 72°C for 30 seconds. 30 cycles for step 2-4. 5. 72 °C for 10 minutes, 6. 4 °C incubation forever. PCR reaction system was 15 µL Firepol system based on lab protocol. PCR products were run on 1% agarose gel with 0.05% ethidium bromide. Gel images were taken from Biorad gel image system. Verification primer sequences was listed in appendix 1

Homozygote plants confirmed by genotyping were selected for each candidate genes. Seeds of those selected plant were sowed and grown 9 seedlings each. Seedlings were re-genotyped to reassure their homozygosity.

Reciprocal Crossing

Unopened flower buds were emasculated 5 weeks after transplantation onto rock wool. Artificial pollination was conducted 1 day afterwards. T-DNA lines were cross against their correspondent *sdm* lines reciprocally. Cross siliques that T-DNA lines serve as maternal plant were harvested approximate 20 days after pollination. Meanwhile cross siliques that *sdm* lines serve as maternal plant were harvested about 28 days after pollination because of longer maturation duration of its L*er* background.

Germination test

Approximately 30 seeds per sample, 6 samples were sown on two layers of germination paper pre-hydrated by 48 ml demi water in plastic tray. Trays were piled and wrapped with transparent plastic bag. Piles were in 22 °C continuous light incubator. The next day after sowing was treated as day 0. Pictures were taken daily over 7 days from day 0. Germination assays were performed 2, 5, 8, 11 days after harvesting for allelism test crossing lines meanwhile 4, 12, 14, 18 Days after harvesting for T-DNA lines, *sdm*, *NILDOG*6 and Col-0. Seed dormancy level was scored by DSDS50, which means days of dry storage after harvesting to reach 50% of germination. DSDS50 was calculated as the time point where 50% germination line cross to fitting curve of germination percentage curve.

Results

1 homozygosity of candidate gene T-DNA lines

21 T-DNA lines that consist of 15 candidate genes were verified as homozygote. These T-DNA lines were then grown to harvest seeds for germination test and reciprocal crossing against their corresponding *sdm*. There could be different T-DNA insertion in one gene result in different mutation for one gene. Table 1. is an overview of homozygosity verified candidate gene T-DNA lines

Table 1 sdm candidate gene T-DNA lines tested overview. Candidate genes were selected from a whole list. Labelled numbers are the correspondent working number. One candidate genes may have more than one T-DNA lines.

*Sdm*1		*Sdm*2		*Sdm*3		*Sdm*4	
AT1G64583	46	AT1G65080	1	AT1G30470	48	AT5G06340	7
AT1G77500	47	AT1G65080	2	AT1G80940	42	AT5G06340	8
		AT1G68550	3			AT5G11580	18
		AT1G68550	4			AT5G11580	19
						AT5G11850	9
						AT5G13480	10
						AT5G15400	11
						AT5G15810	12
						AT5G15810	13
						AT5G15860	14
						AT5G15860	15
						AT5G15870	16
						AT5G15870	17

2 Dormancy phenotyping

DSDS50 was calculated as an indicator of seed dormancy. As plotted in figure 1, *sdm*1, *sdm*2, and *sdm*3 show significant lower DSDS50 than *NILDOG*6, which matches finding from Phuong's PhD thesis work. However comparing *sdm*4 and *NILDOG*6, there is no statistical significance with p-value 0.09 that is slightly beyond threshold p-value 0.05. However, DSDS50 of *NILDOG*6 displayed here is not accurate since the highest germination percentage of *NILDOG*6 is still below 50% at 18 DAH. Thus in real case DSDS50 of *NILDOG*6 is likely to be higher if further germination test after longer dry storage time could be performed. Consequently, *sdm* 4 in fact should also show significantly lower DSDS50 than *NILDOG*6.

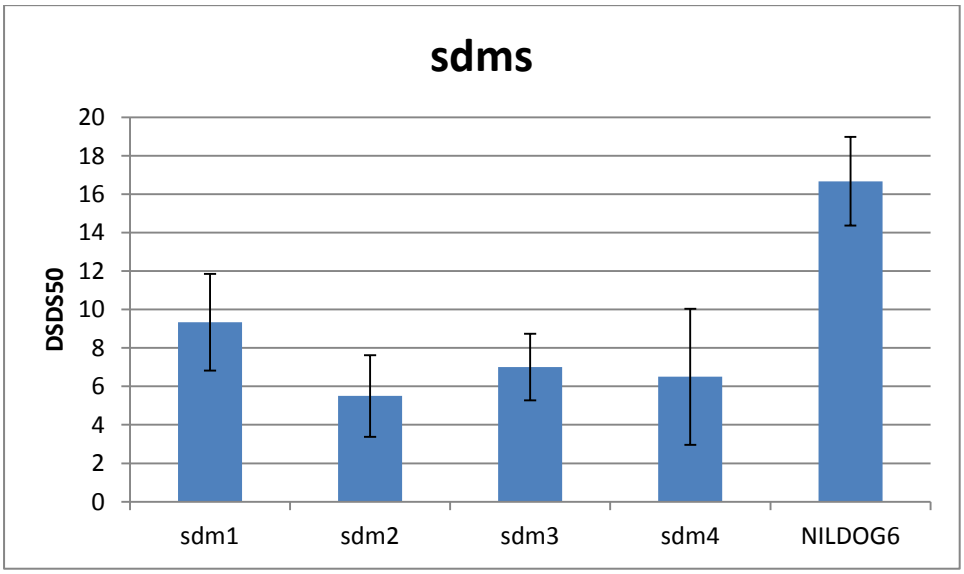

*Figure 1 Dormancy phenotype of 4 seed dormancy modifiers (*sdms*).*

None of 21 candidate T-DNA lines shows significant lower DSDS50 than Col-0. Label 46-6 refers to seeds from the 6th lines of candidate T-DNA lines 46. Line 1(Figure 3.), 48 (Figure 4) and 14(Figure5) doesn't have biological replicate. That is because heterozygote plants were found by genotyping during plant growth. Those heterozygous plants were discarded. Thus no error bar for the data of those three lines.

Two *sdm*1 candidates showed no difference in DSDS50 from Col-0(Figure 2). *Sdm*2 candidate line 3 and line 4 both have not significantly higher DSDS50 than Col-0(Figure 3). *Sdm*3 candidates line 42 have same level DSDS50 that of Col-0, whereas line 48 has a slightly higher DSDS50.(Figure4)

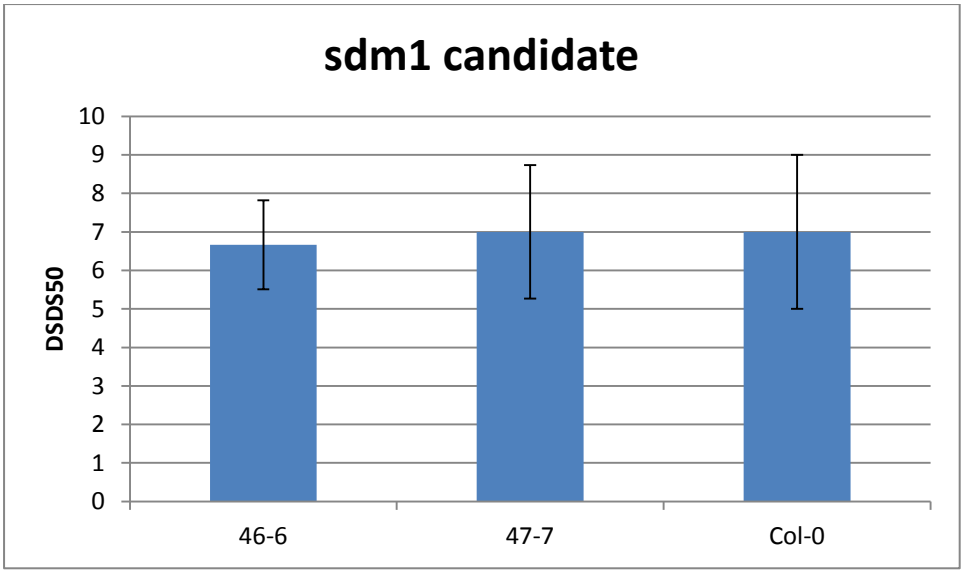

Figure 2 dormancy of sdm*1 candidate gene T-DNA lines presented as DSDS50. 46-6 refers to as line 46 in Table 1 and the 6th line within line 46 population.*

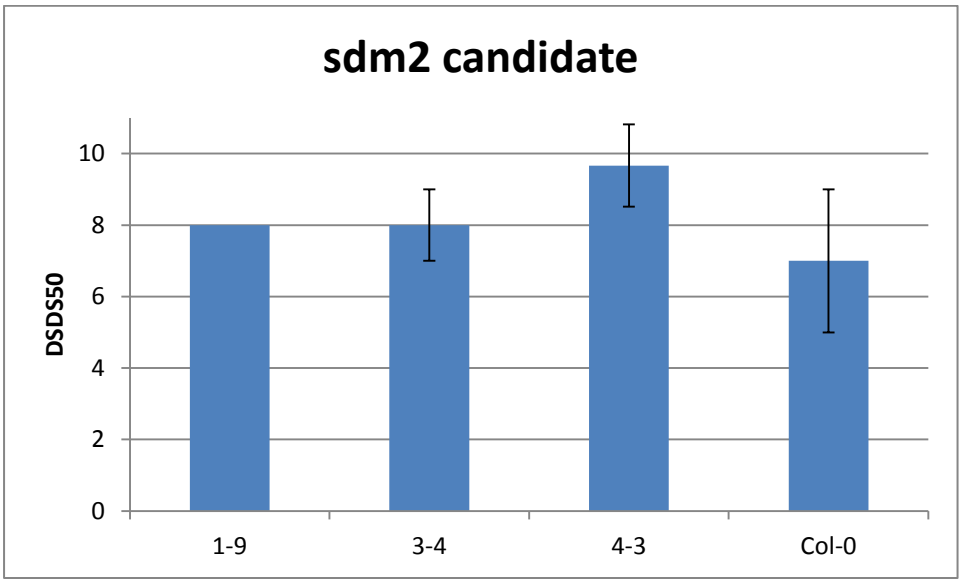

Figure 3 dormancy of sdm*2 candidate gene T-DNA lines presented as DSDS50.*

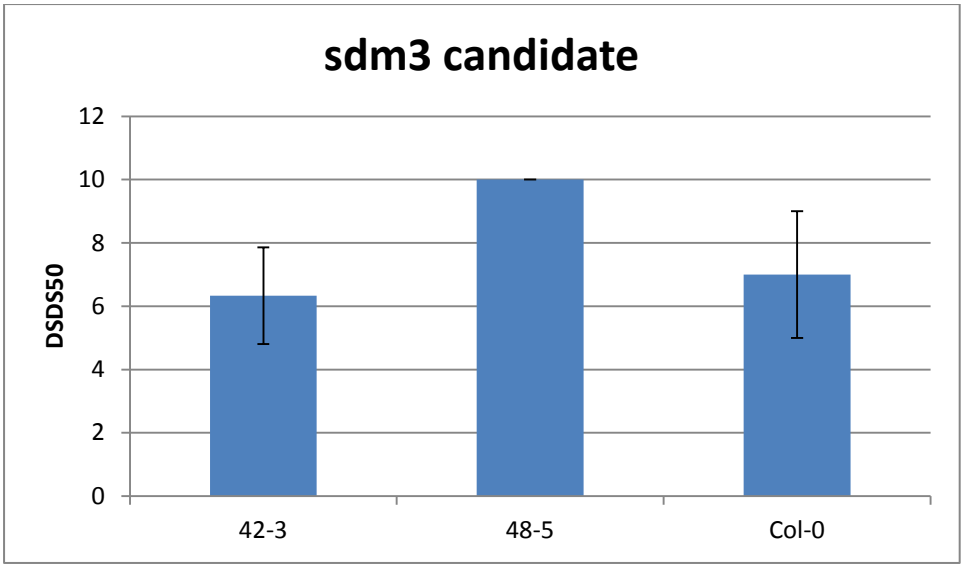

Figure 4 dormancy of sdm*3 candidate gene T-DNA lines presented as DSDS50*

*Sdm*4 candidate lines vary in DSDS50 (Figure 5) yet no obvious reduced dormant line7 could be found. Line 7 has the lowest DSDS50 5.2 days, however, it is still not significantly differs from Col-0. When plants still in growth, we observed that early dropped seeds of line 7 germinate quickly after attached rockwool, forming a layer of seedling compared to Col-0 and other T-DNA lines.

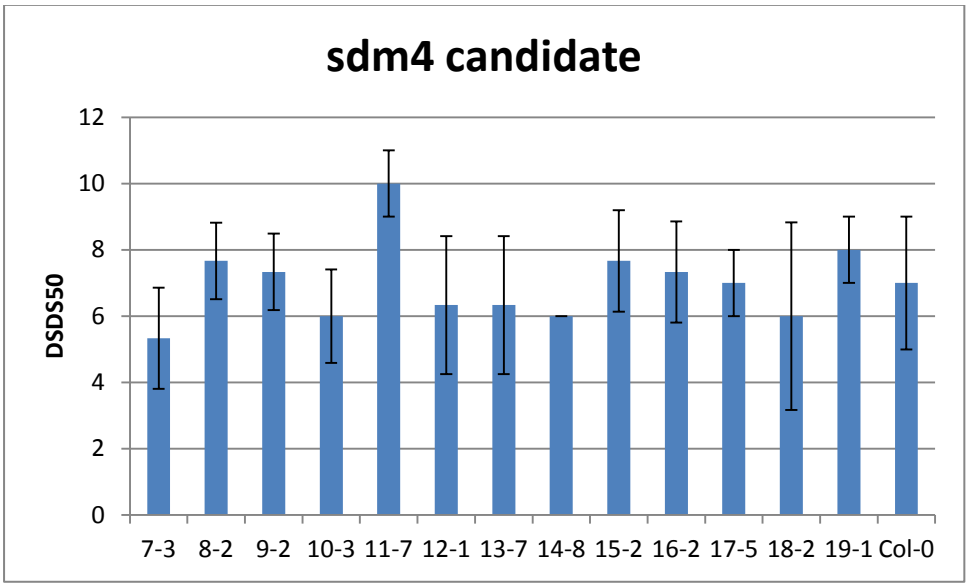

Figure 5 dormancy of sdm4 candidate gene T-DNA lines presented as DSDS50.

3 Allelism test

DSDS50 can hardly be calculated for crossing lines due to limited amount of seeds harvested. Instead of DSDS50, germination percentage can be treated as a compromise to give some impression of how dormant these seeds are.

Crossing of *sdm*1 and *sdm*3 as maternal plant lines shows higher germination percentage than their counterpart on 8 days after harvesting (Figure 6 & Figure 8). While, for *sdm*2 and *sdm*4 crossing lines, there is no similar pattern. *Sdm*2 x 3 and *sdm*2 x 4 lines siliques failed to develop seeds so that no these two data. Moreover for *sdm*2, 5 out of 5 *sdm*2x1 seeds germinated resulted in 100 percent germination (Figure 7).

Line 11 shows high germination at 100% on 5 days after exception that only 2 viable seeds were available for germination test and both of them germinated (Figure 9). Lack of seeds here in the test makes it hard to draw conclusion.

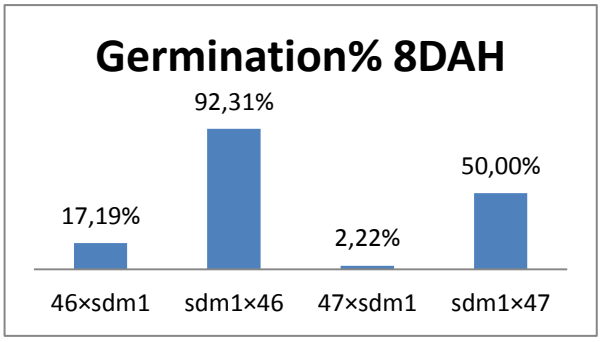

Figure 6germination percentage of sdm*1 and candidate T-DNA line reciprocal crossing seeds..*

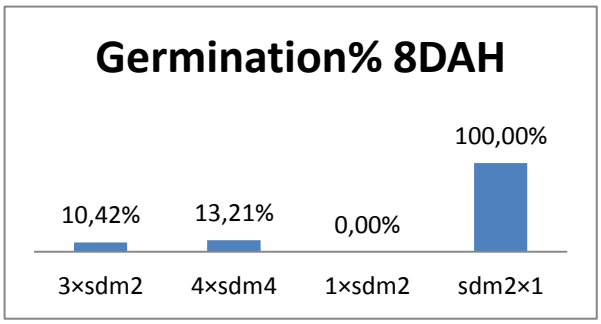

Figure 7 germination percentages of sdm*2 and candidate T-DNA line reciprocal crossing seeds*

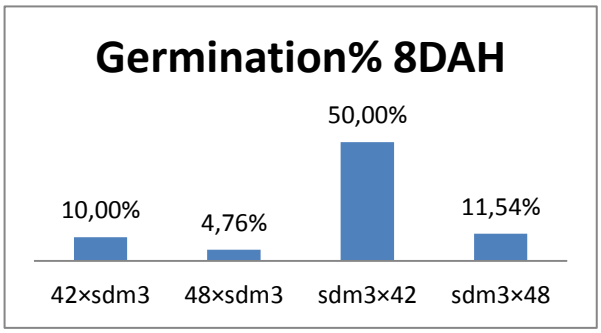

Figure 8 germination percentages of sdm*3 and candidate T-DNA line reciprocal crossing seeds*

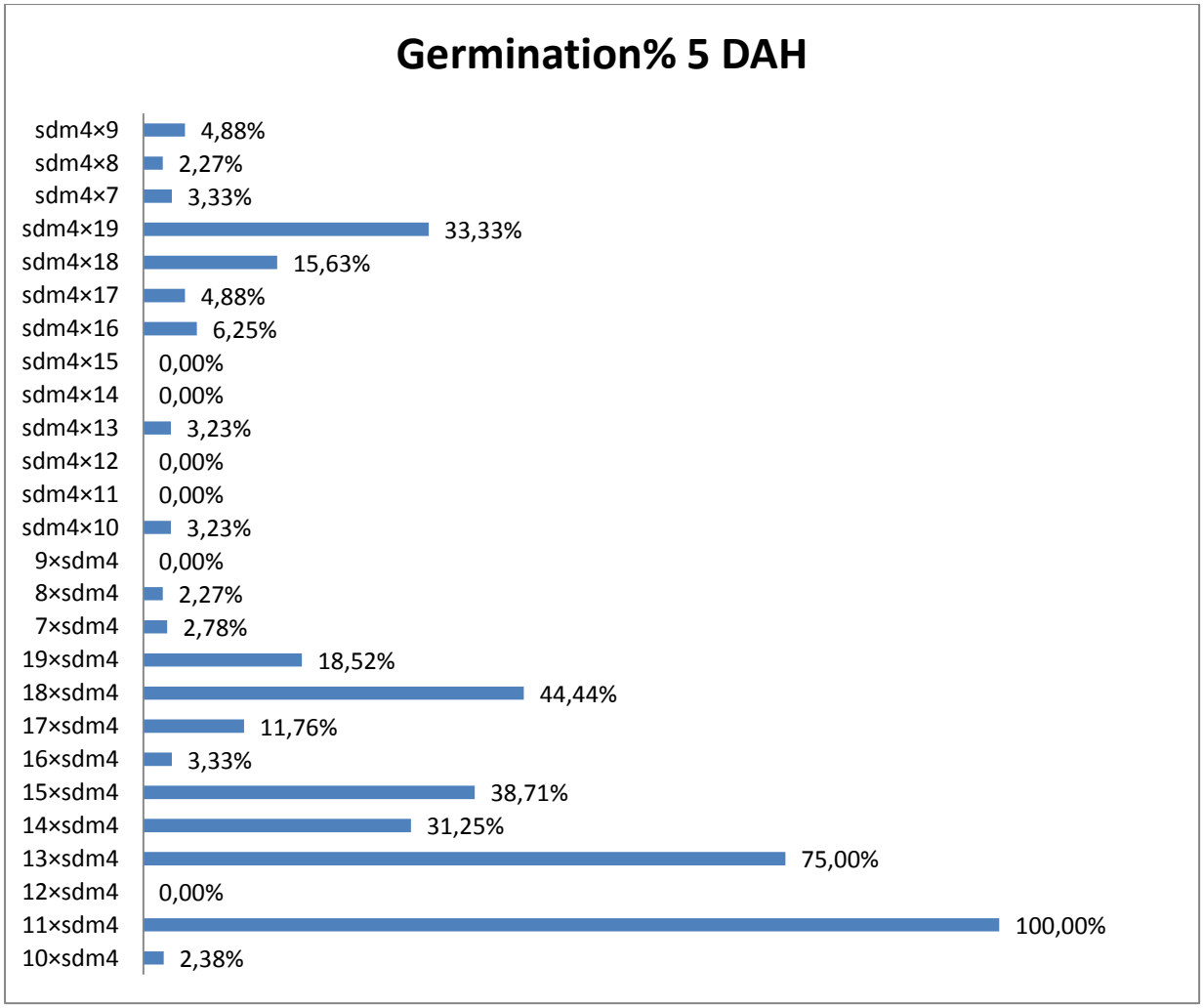

Figure 9 germination percentage of sdm4 and candidate T-DNA line reciprocal crossing seeds

Beside limited number of crossing seeds for germination test, no proper control for such between background L*er* × Col-0 crossing in the experiment makes there germination data invalid to determine actual dormancy phenotype. In the experiment design, two controls have been assigned to be *NILDOG*6 and Col-0. However, it is not clear whether the crossing seeds resemble *NILDOG*6 more or Col-0 in dormancy phenotype. It will be more logical to use *NILDOG*6 × Col-0 seeds as control for those reciprocal crossing seeds. This control should have been included in the experiment.

Discussion& conclusion

Results of germination test are not enough to reveal any of tested candidate genes to be *sdm* mutation. Firstly, germination time span is relatively short due to time limitation. The last germination test was performed on 18 days after harvest, which is too early to end the test in that some lines didn't reach 90 percent germination at last as described by He et al[4]. Especially for *NILDOG*6, its germination percentage didn't even reach 50% to calculate a valid DSDS50. Data from more data points in a longer time span after harvest will contribute to a more accurate DSDS50 to evaluate dormancy phenotype. Secondly, replicate of each candidate gene T-DNA lines is three, therefore small sample size result in lower reliability for statistics. Thirdly, *sdm*s are in *NILDOG*6-sha background that consists of strong *DOG*6 loci whereas *DOG*6 loci in Col-0 seem not have such strong dormancy effect. Hence, it is doubt-worthy whether there will be obvious reduced dormancy phenotype for those T-DNA lines in Col-0 given the insertion mutation is the same mutation in *sdm*s. It will provide clearer evidences if T-DNA lines in other ecotype background rather than Col-0 with stronger *DOG6* loci were available.

It is also possible that other genes in the candidate list which haven't been tested yet to be the genes mutated in *sdm*s.

Shortage in crossing seeds resulted in not enough data points to fit germination percentage curve in order to calculate DSDS50. No clear indication was found from allelism test dormancy phenotype from the limited germination percentage data. It is not eligible to interpret these data. It is also a mistake that the control for allelism test, which were Col-0 and *NILDOG*6, is not proper and somehow vague provided that there were pretty good DSDS50 data. Either *NILDOG*6 or Col-0 cannot serve as proper control independently for such L*er*× Col-0 crossings. Heterosis of L*er* × Col-0 crossing may affect dormancy phenotype from each other. *NILDOG*6 ×Col-0 crossing seeds can serve as a suitable control since it includes heterosis effect of L*er* ×Col-0 in comparison to those reciprocal crossing lines in allelism test.

Reduced number of seeds was developed in crossing siliques. That could be either the crossing induced fertilization malfunction or technical failure during emasculation or manual pollination Allelism test results do not count that much when no potential candidate gene could be found from first germination test either.

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Appendix 1

Verification primer for T-DNA lines genotyping.

Gene		Primer L	Primer R
AT1G64583	46	CATAGGCAAGCGAAGAACAAC	TCTACTTAATTAAGCGGCCCC
AT1G77500	47	CTTGTCCACTCTCTTTGGTGG	TGAAATTCTTCGCAATTCACC
AT1G65080	1	GCAGTCCTGCTTTGTTTTACG	TCTTCAGATTATGGACGCATG
AT1G65080	2	TTGGCTATGCTATTCCACAGG	CCGGAAATTCTAACCTTGGAG
AT1G68550	3	GCTTCTCCTGCTGTTCCTAGG	TCAGAATCTAACGCCCAACTG
AT1G68550	4	TCAGAATCTAACGCCCAACTG	TGGTGTTAGGCAAAGGAAATG
AT1G30470	48	CTCAATGACCTCAGCTGCTTC	AAGACTTTTGCTCTTGTTGCG
AT1G80940	42	ACACTCGAAATTCGTGGTCTG	CTTTCGTTTTGATCTGATCCG
AT5G06340	7	TTCGGTGAGCATGTACTTTCC	CAAAAGGCCATTGAGCTATTG
AT5G06340	8	AACCAATCACCGATTGTTCTG	TGTAACTTGTTCGCCATTTCC
AT5G11580	18	AGTTCTTCCGACGAGAAGAGC	TGGTCAAATCAGCAACACAAG
AT5G11580	19	GAGTGCTGATGATCGGTGAAG	ATGGCAAGAGACATCATGGAG
AT5G11850	9	GTTACTCGTCCCCCAAATTTC	TCAAACTTTGCATCAGCTGTG
AT5G13480	10	TAATGTTAATGGCGATGCCTC	AAGAATTCAACTGGGGAAAGC
AT5G15400	11	GAACTCGTCTGGTATTTCCCC	GAGCTTGCCATGACTTTGAAC
AT5G15810	12	GGAAAACCATTACTTTCACATGG	CCAAAGAGGAAGAACCTTTGC
AT5G15810	13	CAAGCAGCTCATTGCAATTC	CCAAAGAGGAAGAACCTTTGC
AT5G15860	14	GAGAGCATCTCCTGGACAGTG	ATCAACATCACCTGTGGTCTG
AT5G15860	15	TGGGTCAAATAAATTTGGTGG	CTAGGATCTACCTGATGGGGC
AT5G15870	16	TGCACATGACTGATTCTTCCC	GCTTAATGGAAGTTTCGGACC
AT5G15870	17	AGACATTTGTTGAGACGGTGG	GTCCGGCCATATCTCTTCTTC

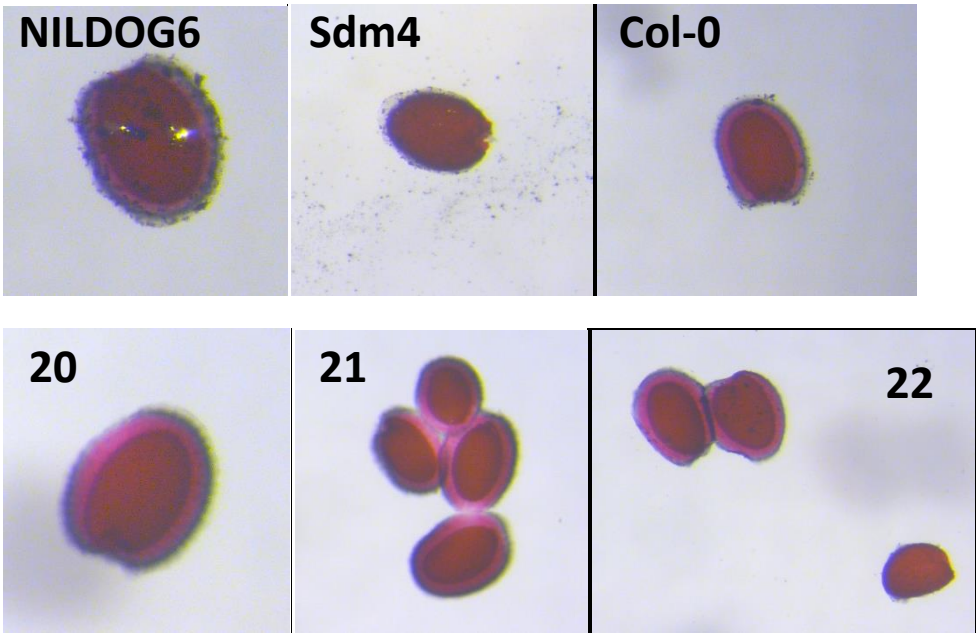
Appendix 2

Sdm4 mucilage phenotyping

Apart from reduced dormancy phenotype, *sdm*4 has abnormal seed mucilage phenotype as well. SHOREmap bulk segregant sequencing also isolated the high snp frequency loci for *sdm*4 mucilage gene. In this thesis we also tried to phenotype *sdm*4 candidate gene T-DNA lines for mucilage.

Mucilage staining

Seeds were imbibed with 1 ml MilliQ water in 12 well cultivation plate for 1 hour. Then water was removed as much as possible by careful pipetting and 1 ml 0.01% ruthenium red was added to the well staining for 1 hour. Then stain solution was removed following washing with milliQ water. Then the seeds were observed and pictured under stereo



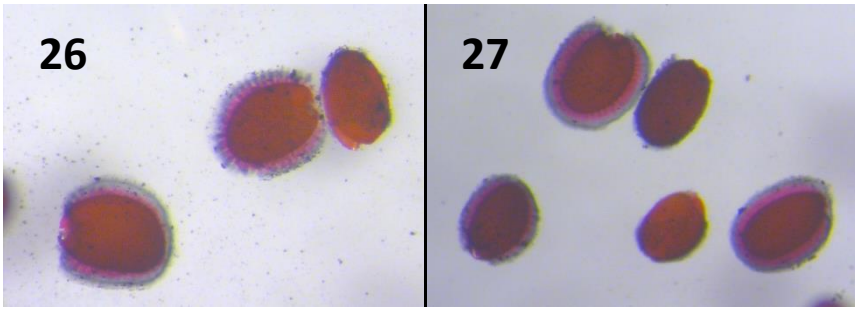


Figure 10. *phenotype of sdm4 mucilage mutant candidate gene T-DNA lines. Line 20 contains mutation in AT1G60940, line 21,22 in AT1G62310, line26,27 in AT1G63300. Dark dots in the picture are insoluble debris of staining but not fungi debris.*

microscope (Stemi SV 11 Zeiss binocular).

*Sdm*4 phenotype is lacking of the radiant skirt. No *sdm*4 mucilage phenotype found in line 20 or line 21. Both wild type and *sdm*4 phenotype were observed in line 22, 26 and 27, where a small proportion of total seeds display *sdm*4 phenotype. We cannot conclude whether these candidate genes were *sdm*4 mucilage mutant gene.

One explanation could be this abnormal seed mucilage phenotype is under coupling regulation of the smd4 mucilage gene and the strong DOG6 loci. As consequence, DOG6 in Col-0 background is not strong enough to induce this coupling regulation.