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Genetic Component Effects of Two Loci on Seed Dormancy, Awn, Low-Temperature Germination, Plant Height, and Flowering Time in Rice (Oryza sativa L.)

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BY

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This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that conclusions reached by the candidate are necessarily the conclusions of the major department.

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I would like to dedicate this thesis to my family – my beloved wife Zehra Korkmaz for her endless love and support, my parents Mr. Nail Korkmaz and Mrs. Serpil Korkmaz, and my brothers Mr. Y. Emre Korkmaz and Y. Talha Korkmaz for their constant encouragement to accomplish the thesis work. Last but not least, this thesis is dedicated to my loving daughter Buglem Ikra for keeping my spirit up with all the innocence and she has accompanied me through every effort and thought of this thesis.

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ABBREVIATIONS

ABA	abscisic acid
aa	amino acid
APS	ammonium persulfate
BC	backcross
bp	base pair
C	Celsius
cm	centimeter
cM	centimorgan
CTAB	cetyltrimethyl-ammonium bromide
Chr	chromosome
CIM	composite interval mapping
DAR	days of after ripening
DNA	deoxyribonucleic acid
dNTP	deoxy nucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FT	flowering time
g	gram
GA	gibberellic acid
GP	germination percentage
GI	germination index
InD	insertion/deletion
IL	isogenic line
Kb	kilo-base
L	liter
LTG	low temperature germination
LR	likelihood ratio
M	molar

Mb	mega base
MD	morphological dormancy
MPD	morphological and physiological dormancy
mL	milli-liter
min	minute
NIL	near isogenic line
µg	micro gram
µL	micro liter
µM	micro molar
PCR	polymerase chain reaction
PD	physiological dormancy
PH	plant height
PY	physical dormancy
PHS	pre-harvest sprouting
QTL	quantitative trait locus
RM	rice microsatellite
SD	seed dormancy
SSR	simple sequence repeat
SNP	single nucleotide polymorphism
TBE	tris-boric-EDTA
TE	Tris-EDTA
TEMED	tetramethyl ethylene diamine
UV	ultraviolet

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ABSTRACT

GENETIC COMPONENT EFFECTS OF TWO LOCI ON SEED DORMANCY, AWN, LOW-TEMPERATURE GERMINATION, PLANT HEIGHT, AND FLOWERING TIME IN RICE (*Oryza sativa* L.)

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Seed dormancy (SD) is an adaptive trait of both ecological and agricultural importance. Cereal crops have been selected for reduced SD to promote germination, which also caused the pre-harvest sprouting (PHS) problem in crop production. The previous research identified a set of quantitative trait loci (QTL) for SD, including *qSD1-2* and *qSD8*, in the conspecific weedy and cultivated rice (*Oryza sativa*). *qSD1-2* carries a dormancy-enhancing allele from the cultivated rice and has a pleiotropic effect on plant height. *qSD8* carries a dormancy-enhancing allele from the weedy rice and tightly links to *qAL8* for awn length. The objectives of this research were: 1) to assemble the *qSD1-2* and *qSD8* alleles in the same genetic background as isogenic lines for fundamental research; and 2) to evaluate additive (*a*) and dominance (*d*) and epistatic (*i*) effects of these two loci on germination ability and its associated traits for use of the QTL alleles to breed cultivars resistant to PHS.

Single plants that are heterozygous for the *qSD1-2* and *qSD8* regions were selected from the BC_1F_1 to BC_1F_5 generations of a backcross (BC) to synchronize the genetic background. From the BC_1F_6 generation all the nine digenic genotypes for the

two loci were identified as isogenic lines (ILs). A total of 144 plants for the nine ILs were grown in a greenhouse, and evaluated for SD by a standard germination test (30°C) at 0 and 10 days of after-ripening (DAR), low-temperature (15°C) germination (LTG) ability, plant height, flowering time and percentage of awned seeds per panicle. QTL analysis confirmed the effects of *qSD1-2* on SD and plant height, and the associations of *qSD8* with the SD and awn traits. The analysis also identified new effects of the two QTL regions on LTG and flowering time, and the *qSD8* region on plant height. Multiple linear regression analysis revealed that the *qSD1-2* and *qSD8* loci influence the SD and flowering time traits by their main (*a* and/or *d*) effects, and the plant height and LTG traits by both main and epistatic effects. The regression analysis also revealed that *qSD1-2* had no effect, while *qSD8* alone had only a small effect (6.5%) on the awn trait.

This research provided new data to support the coevolution of SD with the adaptive traits awn, plant height, flowering time and LTG. The four homozygotes for the *qSD1-2* and *qSD8* loci were purified as a set of ILs. These lines are valuable for map-based cloning *qSD8* and for research molecular mechanisms regulating the SD development/release in the model system of rice. The dormancy-enhancing allele at *qSD1-2* is common in rice semidwarf cultivars. The estimated genetic component effects suggest that an addition of the dormancy-enhancing allele at *qSD8* to the genetic background of a semidwarf variety could reduce germination significantly (11% due to the additive effect). However, this addition may cause short-awned seeds, reduce LTG (8%), but have little influence on plant height and flowering time.

Chapter 1. Introduction and Literature Review

Seed dormancy has been associated with some other traits of ecological and agricultural importance, the latter including weed persistence, germination uniformity and resistance to pre-harvest sprouting. The thesis project aimed to identify the genetic basis underlying the phenotypic correlations in the conspecific weedy and cultivated rice. This chapter introduces some background information about the project, such as seed dormancy and related concepts, reviews genes or quantitative trait loci (QTL) associated with seed dormancy and defines the objectives and rationale of this research.

1.1 Introduction

1.1.1 Cultivated, wild and weedy rice

Rice is the most important food source for many countries in Asia, Africa, and Latin America. Rice supplies 35-60 % of the dietary calories for about 3 billion Asians. (Fageria, 2007). Rice is also a model system for grass species, including cereal crops, in fundamental research on plant adaptation and crop evolution.

The genus *Oryza* consists of 23 species, including the two cultivated species, *O. sativa* L. and *O. glaberrima* Steud (Chopra and Prakash, 2002). These 23 species are grouped into four distinct complex based on genomic composition: *O. sativa* (2n=24), *O. officinalis* (2n=24-48), *O. ridleyi* (2n=48), and *O. meyeriana* (2n=24) (Khush, 1997).The genome composition is determined by the meiotic pairing of the chromosome set. The two cultivated species belong to the *O. sativa* complex that share the AA genome with 2n=24 chromosomes.

Oryza sativa, also known as Asian cultivated rice, originated from the wild rice *O. rufipagon*, and is distributed throughout the tropics to temperate regions in the world. *O. glaberrima*, also known as Africa cultivated rice, originated from *O. barthii* and is endemic to West Africa. Both Asian and African cultivated rice share the "A" genome, as the chromosomes in the F_1 meiosis pair in normal. These two species are clearly distinguishable by morphologies. For example, *O. glaberrima* has fewer secondary panicle branches, shorter ligules, and thicker panicle axis than *O. sativa*, and *O. glaberrima* is completely annual (Oka, 1988).

Asia cultivated rice has differentiated into two main subspecies, *indica* and *japonica*, based on morphology, geographic distribution, and hybrid partial sterility. The *indica* and *japonica* subspecies each may originate from different population of the wild ancestors (Khush, 1997, Gao *et al.*, 2008). The *indica* subspecies has been widely grown in tropic areas, are usually tall in plant height, and has more tillers with long droopy light-green leaves. The *indica* rice has little tolerance to cold temperatures and responds in grain yield only to low applications of fertilizer. Seeds of *indica* cultivars are medium-long to long, and the starch amylose content is medium to high. When *japonica* and *indica* types are crossed with each other, the F_1 plants usually have a high degree of sterility.

The *japonica* subspecies could be domesticated from *O. rufipogon* in the middle area of Pearl River in southern China. The *japonica* varieties have more green and erect leaves and lower tillering capacity than the *indica* varieties. The *japonica* type of rice is usually resistant to lodging, and more nitrogen responsive in yield. The *japonica* type of

rice seed is shorter, wider and lower amylose content of the starch than *indica* types (Chandler, 1979).

There are many phenotypic differences between *O. sativa* and its wild relative(Morishima., 2002; Liang *et al*., 2006). Wild rice typically has long awns and seed shattering for dispersal, while the cultivated rice has short awns if it has and reduced shattering to increase the number of seeds to harvest. As a species, *Oryza perennis* has been used for perennial wild rice that found in Asia, Latin America, and Africa. There are some differences between *O. glaberrima* and *O. sativa*, like plant height, rounded ligule, glabrous lemma and palea, and panicle lacking secondary branches.

Weeds can be defined as "misguided plants" that grow where they should not be grown, thereby interfering with the objectives. Weeds cause a massive problem in crop production. Weedy rice is conspecific relatives of cultivated rice, including many forms intermediate between wild and cultivated rice. Weedy rice in the areas where wild rice was not present may originate from a de-domestication of rice cultivars. Weed rice has been notorious weeds in rice-growing areas worldwide.

Weedy rice is similar to cultivated rice in the genome size. The genome sequence is about 389 Mb. A total of 37.544 predicted protein coding genes were identified by the International Rice Genome Sequencing Project in 2005 (IRGSP and Sasaki, 2005). Thus, weedy rice has been a model system for genetic/genomic research on weed traits, including seed dormancy (Gu et al. 2003).

1.1.2 Seed, caryopsis, and dispersal unit

A seed can be defined as a "ripened ovule comprising of a miniature plant,a nutritive tissue (endosperm or perisperm), and in a protective tissue or seed coat, often accompanied by auxiliary structures, and capable under suitable conditions of independent development into a plant similar to the one that produced it (Webster, 1961)". However, seeds vary in structure and composition with species and may refer to as a fruit or a dispersal unit in literatures. The following section uses rice as an example to discuss differences among a seed, a fruit (caryopsis) and dispersal unit (Fig 1.1).

A true seed consists of three basic components, namely embryo, endosperm, and testa (seed coat). A dispersal unit refers to a mature seed or caryopsis that propagate together with additional maternal tissues or appendages, such as lemma, palea, or glumes. A dispersal unit may provide food reserves to sustain the growing seedling until establishes itself as an autotrophic organism (Bewley, 1997).

Seed development starts with the fusion of a male with a female gamete when both are completely mature. The fertilized egg cell develops into an embryo. A diploid (2n) embryo that typify the new or next generation evolve out of a fertilized egg, which is developed with the fusion of a haploid egg cell (n) and haploid sperm (n). A mature embryo consisting of precursor tissues for the leaves, stem, root and one or two cotyledons that are the seed leaves, residing to the embryonic axis. Based on the cotyledon number, plants are classified into monocotyledonous and dicotyledonous.

Endosperm is a triploid tissue (3n) developed from a primary endosperm nucleus. Endosperm development starts with the division of the triploid primary endosperm nucleus (Faure *et al.,* 2001). The endosperm continues after embryo development have

been completed. The endosperm stores starch, proteins, lipids and source of nutrients during germination and seedling development (Lopes, 1993).

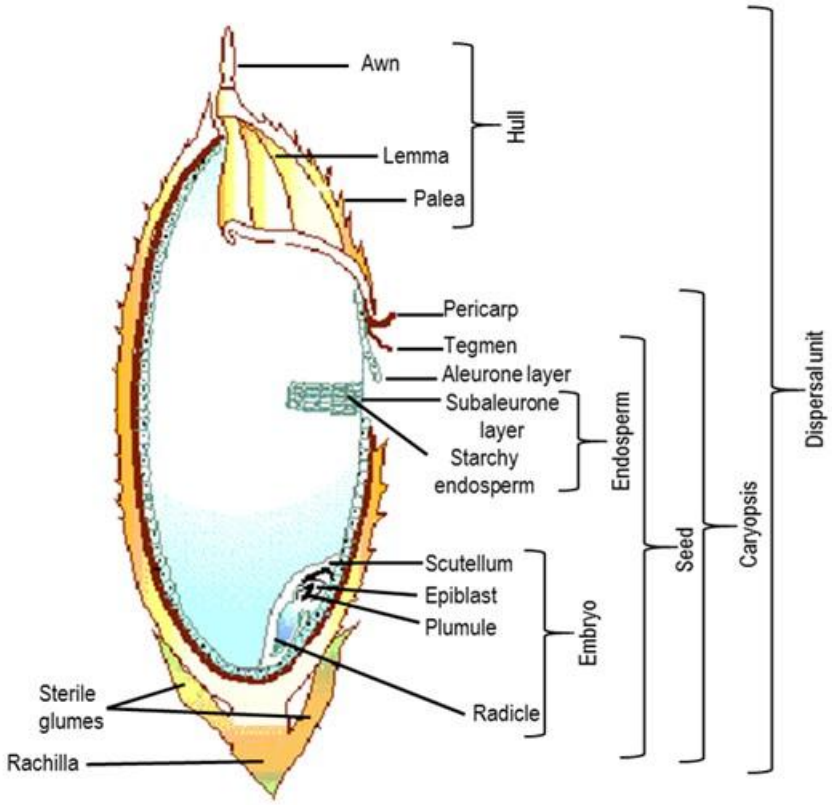

Figure 1.1 Morphology and structure of rice seed. The figure was modified from a diagram at http://jeaheerice.cafe24.com/e_03_01.html to show the relation of a seed with caryopsis and spikelet. The seed consists of the embryo, endosperm and testa (seed coat) tissues. The caryopsis (fruit) is a true seed enclosed within the pericarp tissue (fruit coat). The spikelet is a dispersal unit, which is a caryopsis enclosed by a hull (lemma and palea). Awn is the extended part of the lemma.

Testa (also known as seed coat) is important for the seed because it is only protective tissue between the embryo and the external environment. Testa developed from the integument that is the inner cell layer of the ovule and one layer of the nucleus. Testa can be described to the presence of an outlier and inner cuticle, that often consists with fatty and waxy substances and different layers of thick-walled, protective cells.

A caryopsis is a true seed enclosed by the pericarp tissue. The pericarp tissue fuses with the testa as a protective tissue in grass species.

Dispersal unit can be a seed, a fruit, or a seed with appendages to facilitate the movement from the mother plant to a germination site, such as spike or spikelet in grass species. The dispersal unit may be named based on the species as caryopsis, (kernels of corn-*Zea mays)* that is a seed covered by pericarp, or fruit coat, or spikelet. Spikelet is the basic unit of a grass inflorescence; usually composed of two glumes and one or more florets on a rachilla (Orchidacearum, 1999).

1.1.2.1 Awn

The awn is an extension of the apex of the lemma. It is a needle-like structure to deter seed predation by birds and mammals (Furuta et al., 2015). In some situation, awn can provide movement into soil such as wild tetraploid wheat. On the other hand, long and sharp awns can obstruct manual harvesting in agriculture during the artificial selection of rice. Awns in some species such as barley can provide photosynthesis during grain filling. However, the awn in rice is not effectual because lacks chlorenchyma (Grundbacher, 1963). Some of the studies show that there is an adverse effect of removal awns of rice. Some researchers have previously proposed that there is a positive

correlation between awn length and dispersal unit burial, and they detected that this relationship increases the probability of seed survival in the soil. In addition, seed dormancy and awn are both adaptive trait for wild species. Seed dormancy was correlated with awn in weedy rice populations (Gu et al., 2005c; Mispan et al., 2013). Some researchers have previously proposed that there is a positive correlation between awn length and dispersal unit burial, and they detected that this relationship increases the probability of seed survival in the soil. In addition, seed dormancy and awn are both adaptive trait for wild species. Seed dormancy was correlated with awn in weedy rice populations (Gu et al., 2005c; Mispan et al., 2013).

1.1.2.2 Plant height

Plant height at maturation is the length of the main stem from the soil surface to the top of the panicle. Plant height is associated with lodging resistance in cereal crops. Traditional cultivars of rice have higher plant height compared to modern cultivars. The "green revolution" has been changed this scenario, which is characterized by semi-dwarf cultivars with 90-110 cm plant height (Fageria, 2007).

Another advantage of semi-dwarf varieties, except the lodging resistance, is high yield potential due to more tillers and responsive to increased fertilizer applications. However, extremely dwarf plant is a problem because grain yield increases with increasing plant height (Fageria, 2007). Environmental factors can affect plant height, but plant height is controlled largely by major genes, such as *semidwarf 1(sd1)* Thus, identification, selection or recombination with another trait is easy (Jennings *et al*., 1979).

Seed dormancy and plant height are adaptive traits for grass species. Domestication has reduced level of seed dormancy because of selection for rapid, uniform germination which also caused the pre-harvest sprouting problem in crop production (Basu *et al*., 2004). Many genes/ QTLs for plant height have been mapped in rice (Lin *et al*., 1998; Baskin *et al*., 2000; Alonso-Blanco *et al*., 2003; Gu *et al*., 2004b; Lee *et al*., 2012).

1.1.2.3 Flowering time

Flowering time is an adaptive trait that shows continuous variation in cereal crops (Cockram *et al*., 2007). The plants set their flowers at an optimum time for pollination, seed development, and dispersal. In addition, flowering time administrates the transition from vegetative to reproductive growth to complete the life cycle. Thus, it is crucial for local adaptability. Flowering time pre-determines the environmental conditions for the development of seed dormancy. Many of QTLs, that are associated with seed dormancy and heading date, detected in different populations in rice. For example, five heading date QTLs were detected on chromosomes 2, 3, 4, 6, and 7 in a BC_1F_5 populations derived from backcross population between *japonica* that is cultivar Nipponbare and *indica* cultivar Kasalath. Two of five QTLs on chromosome 3 and 7 were associated with both seed dormancy and heading date (Lin *et al*., 1998).

1.1.3 Seed dormancy (definition, importance, and classification)

Seed dormancy is defined as the temporary failure of an intact viable seed to germinate under favorable time(Bewley, 1997). It is an adaptive trait regulating the time and place of germination for wild species in natural ecosystems.

Seed dormancy is also of agricultural important. Cultivated plants show weak dormancy than wild type plants. When the degree of seed dormancy is high, it may cause a problem in field management like non-uniform germination or weed persistence such as unwanted crop plants or weed can germinate several years latter because there might be remaining un-germinated seed in soil bank (Bewley, 1997; Baskin and Baskin, 2004; Finkelstein *et al*., 2008). Most flowering plants need their seeds for the next generation. Delaying germination is the best way to protect the seeds from dry and cold winter mortality. However, a certain degree of dormancy in cereal crops is necessary to prevent preharvest sprouting (PHS), which is germination on the plants when moist conditions happen, or untimely rain occurs. Pre-harvest sprouting reduces seed quality and yield, resulting in the loss of money for farmers and food processors.

Seed dormancy can be classified in different systems such as primary and secondary dormancy. Primary dormancy is developed on the plant before maturation. There are two forms of primary dormancy: exogenous and endogenous dormancy. Generally, exogenous dormancy is related to the seed coat (physical properties). For instance, germination can be affected by embryo because the tissues covering the embryo may prevent water uptake or radicle emergence. Endogenous dormancy can happen by conditions within the embryo itself. Maturation and environmental conditions may affect the endogenous dormancy. Based on Finch-Savage and Leubner-Metzger (2006), primary dormancy occurs with the involvement of ABA during seed maturation on the mother plant. Secondary dormancy is a process to switch from non-dormant state to dormant state because of unfavorable germination conditions.

Another classification of seed dormancy has been created by Baskin *et al*. (1998;2004). Their classification system covers five classes of seed dormancy.

Physiological dormancy (PD) that is the most common type of dormancy. PD has three level of dormancy; non-deep, intermediate, and deep. Non-deep PD can be observed for short term and breaks with storage. In this level of dormancy, GA (gibberellic acid) promotes germination. Intermediate level of dormancy controls is reputed to be with seed coats and in tissues surrounding the embryo. When seeds keep in dry storage than can shorten the cold stratification period and GA promotes germination. PD- deep dormancy level is controlled by the embryo itself and GA does not promote germination in this level. To germinate seeds, some treatments or stratification can apply to break dormancy like a long period of cold or warm stratification.

Morphological dormancy (MD), the embryo is not entirely developed. The seeds, which have unachieved embryo does not have physiological dormancy level, do not need to dormancy-breaking treatment. However, it needs time to grow and radicle protrusion.

Morphophysiological dormancy (MPD) demonstrates some similarities with MD and PD. For instance, the seeds have an incomplete embryo and physiological components to dormancy. These seeds therefore require dormancy breaking treatment for germination.

Physical dormancy (PY) is known as water-impermeable seeds that remain dormant until to water uptake to seed. To germinate the seeds, some factor(s) should effect to testa or seed covering layer(s) to uptake water (Baskin *et al*., 2000). These

factors can be mechanical or chemical scarification, i.e. high temperature, freezing, drying, to weakening the embryo covering tissues.

Last class of the dormancy is that combination of physical and physiological dormancy (PD + PY). Combination dormancy illustrates both of water-impermeable seeds (hard impermeable testa) and physiological dormancy in the embryo. Before the germination, seeds will need to dormancy breaking treatment in dry storage, greenhouse conditions or cold stratification to release combination dormancy (Baskin *et al*., 2000; Baskin and Baskin, 2004; Finch-Savage *et al.,* 2006; Bentsink *et al.,* 2008).

The dormancy release is influenced by environmental and genetic factors. Some factors (after-ripening and stratification) affect dormancy release for many species. After-ripening (AR) is a period of warm dry storage after maturation. A recent definition of dormancy release by Finch-Savage and Leubner-Metzger (2006) is an increasing sensitivity of perception capacity of seeds to environmental conditions like water, gasses, temperature and light to promote germination. The molecular mechanisms of after-ripening are not clear enough, but we know that AR conditions vary with species. For example, *Arabidopsis* seeds need cold and light conditions to after-ripen. However, rice seeds need a warm dry environment (e.g., room or greenhouse conditions) to gain germinability. Briefly, the speed of AR and level of dormancy status can change, depending on environmental conditions during seed maturation, seed storage and germination conditions (Gu *et al.*, 2003; Holdsworth *et al.*, 2008). The most common method to break dormancy is dry after-ripening (Bewley, 1997), but the molecular mechanism of dry after ripening is not exactly clear. After-ripening to break dormancy is correlated with changes in ABA content during imbibition (Gubler *et al*., 2005). Several

studies show the application of GA can release dormancy in some species. Both of GA and light can release dormancy and increase germination rate in some species (Finch-Savage *et al.*, 2006; Graeber *et al*., 2012).

1.1.4 Germination

Germination is a physiological process starting with the uptake of water into the seed and ending with the protrusion of radicle from the surrounding structure. Based on the species; some of the seeds have ability to germinate for a few days after fertilization (Nonogaki *et al.*, 2010).

Seed germination is the most crucial stage in plant development because most of physical and metabolic events happen during germination process. This process can be grouped into three phases (Fig 1.2); water uptake, metabolic activations called as plateau phase and radicle protrusion(Ali *et al*., 2017). Phase I (water imbibition) starts with water uptake by dry seed's cell and go on until all of the matrices and the cells are full of water (Nonogaki *et al*., 2010). Increasing level of water in cells causes transitory membrane perturbation that promote leaking of solutes into surrounding imbibition solution resulting in reactive the quiescent seeds.

Phase II refers to limitation of water uptake and it is the most important phase because the main processes as physiological and biochemical happen in this phase such as biosynthesis, cell elongation, respiration, etc. Dormant seeds stay in this phase because some biological and physiological activities happen in this step.

Phase III starts with the protrusion of the embryonic axes from the testa or covering tissues and is characterized by increase in water content because of growth of new tissues (Nonogaki *et al.*, 2010).

1.1.4.1 Environmental influences on germination/dormancy

Moisture: Water is an essential requirement for germination. All seeds need enough moisture for enzyme activation, breakdown, and use of reserve storage material. In a dormant stage, seeds have a low level of moisture and metabolic activities are inactive. Dormant seeds may survive with a minimum level of metabolic activity that provides their long-term survival in the soil or dry storage. Favorable environment conditions or moisture varies with origin of species because many species have a different critical moisture content for germination such as moisture content in corn is 30%, wheat 40% and soybeans 50% (Shaban, 2013).

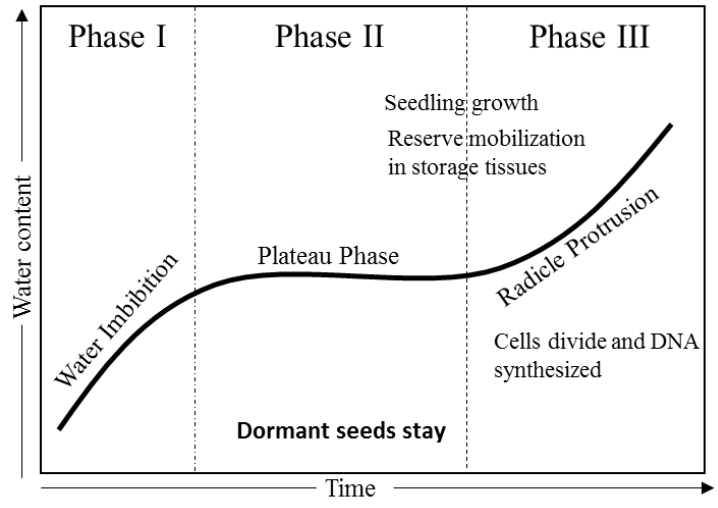

Figure 1.2 Phases of germination. The figure shows three phases divided based on the amount of water uptake. Most of metabolic activities ensue in phase I and II. Phase III is the seedling growth (Bewley, 1997).

Temperature: Temperature is another important environmental factor in soil for germination. It requires for embryo growth. Each species need different range of temperatures at which germination will occur. Imbibition can be possible in low temperature, but embryo growth can be affected by low temperature negatively. The optimum temperature for most of seeds is between 15°C and 30°C. The 30°C can be accepted as maximum temperature. There is a positive correlation between seed germination and temperature for most crop species (Nyachiro *et al*., 2002). On the other hand, some of crop species have high percentage of germination at lower temperature as recognized (Laude, 1956). For instance, seeds of Russian pigweed are reportedly can germinate in frozen soil or even on ice (Agriculture *Journal* 1935).

Oxygen: Air consists of about 20% oxygen, 0.03% carbon dioxide and about 80% nitrogen gas. Respiration goes up during seed germination and oxygen concentration of air effect germination. Imbibition occurs in the absence of oxygen but seed is prevented under anaerobic conditions (Copeland e*t al*., 1985; Simpson, 1990).On the other hand, seeds of some species can germinate better under oxygen concentration above that of air. Rice seeds can germinate underwater and the seed can be able to grow under anaerobic conditions.

Light: Light is required for seed germination after moisture, oxygen, and appropriate temperature. The mechanism of light in seed germination has some similarities with floral induction, root growth, radicle development, and pigment formation. The seed germination can be affected by both of light intensity, that can be varied based on species, and light quality that is color or wavelengths (Copeland *et al.*, 2001). In addition, light exposure is another factor for dormancy and germinability of

mature grain during grain development. Species can have different behaviors to light because of genetic or environmental factors. Lastly, primary and secondary dormancy can be influenced by light (Simpson, 1990).

1.2 Literature review of seed dormancy

1.2.1 Natural variation and inheritance of seed dormancy

Natural variation is the genetic diversity of a species in a trait due to allelic variants of genes (Alonso-Blanco *et al*., 2009). Seed dormancy is one of the most complex traits showing continuous variation in germination ability. Arabidopsis and rice have been used as model plants for research on the natural variation in seed dormancy. *Arabidopsis thaliana* as an annual plant has a range of difference in the degree of seed dormancy among natural accession collections (Bentsink *et al.*, 2010). This difference was associated with Quantitative Trait Loci (QTL) in Arabidopsis recombinant inbred lines (RILs) . Rice researchers have studied the inheritance of dormancy to improve the resistance of cultivars to preharvest sprouting. Some research focused on hull-imposed dormancy in cultivated rice. Heritability estimates for seed dormancy varied from 60% to 90% in weedy rice (Gu *et al*., 2003).

1.2.2 Physiological and molecular mechanism of seed dormancy

Plant hormones, such as abscisic acid (ABA), ethylene, gibberellins (GA), and brassinosteroids (BR), are involved in regulating the development, maintenance or release of seed dormancy or germination.

ABA is also known as abscisin II or dorman, with the molecular formula $C_{15}H_{20}O_4$. ABA is a positive regulator for seed dormancy induction and maintenance

(Gubler *et al*., 2005; Holdsworth *et al*., 2008). Generally, the level of ABA in seeds is low during early development and then it increases and peaks around mid-maturation. During late development, the ABA level in seed decreases precipitously, especially during the maturation drying phase (Bewley, 1997; Kermode, 2005). Although lack of ABA during seed development may cause the absence of primary dormancy in mature seeds, there is no certain relationship between ABA content and the degree of dormancy (Kermode, 2005). The environment conditions during seed development have an impact on ABA content. In addition, the ABA biosynthesis genes can affect seed ABA content. Thus, increasing ABA content in seeds can enhance seed dormancy or delay germination (Nambara *et al.*, 2003; Holdsworth *et al.*, 2008; Finkelstein *et al*., 2008). Increased endogenous ABA content may prevent germination late during development (Berry and Bewley, 1992). Breaking dormancy techniques (after-ripening, stratification, dark and smoke) are correlated with changes in ABA content during water uptake (Gubler *et al*., 2005). ABA biosynthetic genes that have been identified include *ABA1*, the *9-cis-epoxycarotenoid dioxygenase* (*NCEDs*), *ABA2/GIN1/SDR1* in *Arabidopsis* and several other species (reviewed by Nambara *et al.*, 2003). The *9-cis-epoxycarotenoid dioxygenase* (*NCEDs*) is a key gene that was used to understand the importance of ABA in dormancy induction. The four primary regulators, that are *FUS3, ABI3, LEC1 (LEAFY COTYLEDON 1)* and *LEC2*, control seed maturation and dormancy induction in *Arabidopsis* (Raz *et al*., 2001). *Delay of Germination 1* (*DOG1*) is the first seed dormancy gene that is another key player in *Arabidopsis*. There are two major seed dormancy genes that are *qSD7-1* and *qSD12* have important roles to induce seed dormancy in rice. The *qSD12* underlying gene promotes ABA accumulation in early

developing seeds to induce primary dormancy; the *qSD7-1* underlying gene induces dormancy by dormancy-inducing hormone ABA in early developing seed (Gu *et al*., 2010, 2011).

Gibberellins (GAs) are a large family of 136 tetracyclic diterpenoid acids and play an important role in plant development, like plant height, germination, flowering, dormancy, sex expression and plant stem elongation (Simpson, 1990; Lin *et al*., 1998; Finkelstein *et al*., 2008). When compared among seeds with different levels of dormancy and non-dormant seeds from *A.thaliana* ecotype Cvi, a set of GA-responsive genes are demonstrated with different expression levels (Cadman *et al*., 2006; Finch-Savage *et al*., 2006). GA promotes the embryo expansion and germination. In addition, seeds need GA to weaken the seed-covering layers (Finch-Savage and Leubner-Metzger, 2006). Based on Yamauchi *et al*., 2004, GA biosynthesis and response are activated during seed imbibition at low temperature. A low GA content in early developing seeds may cause more dormant seeds at maturation in rice (Hedden and Kamiya, 1997; Heng Ye, 2011). A loss-of-function mutation of the GA synthesis gene on chromosome 1 (*qSD1-2)* could promote dormancy (Ye *et al*., 2015).

1.2.3 Genetic mechanism of seed dormancy

Seed dormancy is controlled by many genes analyzed as QTL in many crops during the last two decades. Alonso-Blanco *et al*. (2003) identified seven QTLs, called *Delay of Germination* (*DOG*) from (*DOG 1*) to (*DOG 7*) from crossing between *Cvi- Cape verde Island-* (strong dormancy) and *Ler- Landsberg erecta* (weak dormancy). Germination test results illustrate that the DOG QTLs have a large additive effect in *Ler* genetic background (Alonso-Blanco *et al*., 2003).

More than thirty seed dormancy QTLs were detected on all chromosomes in rice (Table 1.1). Cultivated, weedy and wild rice were used to develop segregating populations to map seed dormancy QTLs. Five putative dormancy QTLs were mapped on chromosomes 3, 5, 7, and 8 using by backcross inbred lines (Lin *et al*., 1998). After these five seed dormancy QTLs, more than 20 seed dormancy QTLs were identified on all chromosomes except chromosome 4 and 10 (Cai *et al*, 2000). The rice genome was divided ten genomic regions as potentially containing genes that responsible for seed dormancy in cultivated and weed/wild rice. These 10 genomic regions, that are named as *qSD1-1, qSD1-2, qSD3, qSD4, qSD6, qSD7-1, qSD7-2, qSD8, qSD1*0, and qSD12, also include some not previously reported on chromosomes 4 and 10 (Gu *et al*., 2004b; Ye *et al*., 2010b).

In wheat, three seed dormancy QTLs reported on group 4 in a doubled haploid lines (Kato *et al*., 2001). *QPhs.ocs-4A* that is one of major QTLs in wheat detected the marker interval between *Xcdo 795* and *Xpsr115* on the long arm of chromosome 4A in wheat (*Triticum aestivum* L.). Another two seed dormancy QTLs on chromosome 4 detected on 4B and 4D were named *QPhs.ocs-4B* and *QPhs.ocs-4D*, respectively. All of these three seed dormancy QTLs have more than 80 % of the total phenotypic variance (Kato *et al*., 2001). Another two QTLs for seed dormancy detected on chromosome 3. *QPhs.ocs-3A.1* that is located on the short arm of the chromosome 3 and has 23-38 % of the phenotypic variance. The second QTL in the chromosome 3, *QPhs.ocs-3A.2*, is located on long arm of the chromosome. *QPhs.ocs-3A.2* had a minor effect on seed dormancy, and it was collocated with *taVp1* gene by around 50 cM (Osa *et al*., 2003). PHS is the common problem in the wheat and seed dormancy is associated with PHS.

TaSdr is a seed dormancy gene is in wheat and it is located on homologous group 2 chromosomes and it has tolerance to PHS. Based on cloning and characterization of *TaSdr* showed that it is an orthologue of *OsSdr4* (Zhang *et al*., 2014).

Seed dormancy is extremely important for barley because it influences malting yield and quality. A negative correlation between strong dormancy and malting yield or quality. Weak dormancy can cause pre-harvest sprouting in barley. Seed dormancy loci (SD1-SD4) were identified in a segregating population derived from Steptoe (dormant) × Morex (non-dormant). SD1 on chromosome 7 (5H) had the largest and consistent effect on seed dormancy and accounted for 55 % phenotypic variance (Han et al., 1999a). SD2 is promising to develop varieties with a moderate degree of seed dormancy in barley (Gao *et al*., 2003).

Discovering the genes underlying seed dormancy QTL is important for agriculture and ecology. Map-based cloning starts with narrowing down the QTL to a short DNA sequence, and continues with sequencing and functional analysis of the QTL underlying genes. Several seed dormancy QTLs have been map-based cloned and functionally confirmed in different species (Table 1.2). *Delay of Germination 1* (*DOG 1*) is the first seed dormancy QTL cloned in *Arabidopsis. DOG1* came from a small gene family of unknown molecular function from five members in *Arabidopsis* (Jowett *et al*., 2006). Some studies demonstrate that *DOG1* controls seed dormancy and flowering time in response to temperature in *Arabidopsis* and *DOG1* lead to temperature-dependent alterations in the seed GA metabolism (Graeber *et al*., 2014; Huo *et al*., 2016).

Table 1.1 List of QTL associated with seed dormancy in rice

Name	Chr.[a]	Marker[b]	R^2 (%)[c]	Donor[d]	Reference
NA	3	C1488	6	Kasalath	Lin et al. 1998
NA	5	R830	8	Kasalath	Lin et al. 1998
NA	7	R1440	11	Kasalath	Lin et al. 1998
NA	7	R1245	11	Kasalath	Lin et al. 1998
NA	8	C390	7	Nipponbare	Lin et al. 1998
qDOR-2	2	Amp1-RZ476	8-11	W1944	Cai & Morishima 2000
qDOR-3-1	3	G144-BCD454	13-17	W1944	Cai & Morishima 2000
qDOR-3-2	3	C12-Pgi1	8	W1944	Cai & Morishima 2000
qDOR-3-3	3	R1927-CDO122	14-15	W1944	Cai & Morishima 2000
qDOR-5-1	5	RZ296-BCD1072	7-8	W1944	Cai & Morishima 2000
qDOR-5-2	5	Bh2-R521	7	W1944	Cai & Morishima 2000
qDOR-6-1	6	Pgi2-Amp3	15	W1944	Cai & Morishima 2000
qDOR-6-2	6	R2171-RZ144	8-13	W1944	Cai & Morishima 2000
qDOR-8	8	RG181-Amp2	10-12	W1944	Cai & Morishima 2000
qDOR-9-1	9	Awn-Est12	8	W1944	Cai & Morishima 2000
qDOR-9-2	9	RZ792-C506	10	W1944	Cai & Morishima 2000
qDOR-11-1	11	G24-RZ141	8	W1944	Cai & Morishima 2000
qDOR-11-2	11	RZ141-APAGE2	8-22	W1944	Cai & Morishima 2000
qDOR-11-3	11	G257-CDO365	9	W1944	Cai & Morishima 2000

(continued)

(continued)

qDOR-11-4	11	CDO365-C6a	7	W1944	Cai & Morishima 2000
qDOR-11-5	11	R1465-RG1109	12	W1944	Cai & Morishima 2000
qSD1	1	RM220	7	SS18-2	Gu et al. 2004
qSD4	4	RM252	6-11	SS18-2	Gu et al. 2004
qSD7-1	7	RM5672	7-18	SS18-2	Gu et al. 2004
qSD12	12	RM270	48-54	SS18-2	Gu et al. 2004
qSD7-2	7	RM346	7	SS18-2	Gu et al. 2004
qSD8	8	RM135B	7	SS18-2	Gu et al. 2004
qSD1-1	1	RM220	8	SS18-2	Gu et al. 2006
qSDn-1	1	RM237-RM128	9-19	N22	Wan et al. 2006
qSDnj-3	3	RM231	6	Nnjing35	Wan et al. 2006
qSDn-5	5	RM480-RM413	6-16	N22	Wan et al. 2006
qSDn-7	7	RM234	4	N22	Wan et al. 2006
qSDn-11	11	RM21-RM229	7-12	N22	Wan et al. 2006
qSD1-2	1	RM3602	11	EM93-1	Ye et al. 2010
qSD3	3	RM520	9-11	SS18-2	Ye et al. 2010
qSD10	10	RM271	8-42	EM93-1	Ye et al. 2010
qSD1-1	1	RM23	12	cv.N22	Xie et al.2011
qSD1-2	1	RM488	13	cv.N22	Xie et al.2011
qSD2	2	RM525-RM240	8	cv.N22	Xie et al.2011

(continued)

(continued)

qSD3	3	OSR13-RM282	6	cv.N23	Xie et al.2011
qSD3	3	RM22-RM5819	2-7	PSRR-1	Subudhi et al. 2012
qSD7-3	7	RM5508-RM351	8	PSRR-1	Subudhi et al. 2012
qSD10	10	RM216-RM2504	4	Cypress	Subudhi et al. 2012
qSD6-1	6	RM314	6-15	LD	Zhang et al. 2017
qSD6-2	6	RM587	7	LD	Zhang et al. 2017
qSD6-3	6	RM528	8-18	LD	Zhang et al. 2017

[a] The chromosome location where QTL was detected

[b] The nearest or flanking markers on QTL

[c] Proportion of total variance explained by each QTL

[d] Donor parent of the QTL

The map-based cloning of *qSD1-2* identified a GA synthase enzyme gene (*OsGA20-ox2*) responsible for both seed dormancy and plant height (Ye *et al*., 2013, 2015). Specifically, the wild-type allele of *OsGA20-ox2* promotes stem elongation and germination (reduced primary dormancy), while the mutant allele, (*SD1*), reduces plant height but enhances the primary dormancy. *qSD1-2* locus associated with endosperm-imposed dormancy, regulating seed development and maturation programs in rice (Gu *et al*., 2015; Ye *et al*., 2015). *qSD7-1*/*qPC7* has pleiotropic effects for seed dormancy and pericarp color. *Os07g11020* was identified for the qualitative trait red pericarp color (*Rc*) (Gu *et al.*, 2011). The gene has pleiotropic effects control qualitative and quantitative traits with different physiological pathways. This gene has an increase in accumulation of the dormancy-inducing hormone.

Table 1.2 List of some seed dormancy QTL cloned from plant species

QTL	Molecular function	Physiological function	Species	Reference
DOG1	unknown protein		*Arabidopsis thaliana*	Bentsink *et al*. 2006
Sdr4	unknown protein		Rice	Sugimoto *et al*. 2010
qSD7-1	bHLH transcription factor	ABA & flavonoid synthesis	Rice	Gu *et al*. 2011
MFT	Phosphatidylethanolamine-binding protein		Wheat	Liu *et al*. 2013
RDO2	TFIIS transcription elongation factor	Transcription elongation	*A. thaliana*	Liu *et al*. 2011
qSD1-2	GA20-oxidase	Gibberellin synthesis	Rice	Ye *et al*. 2015
HUB1 (RDO4)	C3HC4 ring finger	Transcription elongation	*A. thaliana*	Liu *et al*. 2007

1.3 Rationale and objectives of this thesis project

1.3.1 Rationale

Although a majority of semi-dwarf cultivars of rice has the *qSD1-2* dormancy-enhancing allele, the effect of a single gene is not good enough to provide a sufficient level of primary dormancy for resistance to PHS. It is necessary to add one or more dormancy genes to the *qSD1-2* background to breed PHS-resistant semi-dwarf varieties.

Natural selection for seed dormancy may impact its interrelated traits such as plant height, flowering time, and awn. There is limited information about the selection for multiple trait. Seed dormancy is controlled by multiple genes. We need to know how they interact with each other to influence phenotypic variation.

Many QTLs for seed dormancy have been isolated as single Mendelian factors, including *qSD1-2* and *qSD8* in rice. The dormancy-enhancing allele of *qSD1-2* was derived from EM93-1. In the isogenic background, *qSD1-2* also has a pleiotropic effect on plant height, and *qSD8* was also associated with awn length. In addition, *qSD1-2* was cloned as the GA synthase enzyme gene *OsGA20-ox2* to control the hormone production in the developing seeds (Ye et al., 2013).

In addition to plant height and germination, the GA hormone is involved in regulating many other physiological processes, such as flowering time. There is no information about interaction between *qSD1-2* and *qSD8*, or the influence of GA on the awn elongation and the effect of *qSD8*.

The seed dormancy and awn association, or the *qSD8/qAL8* cluster, is underlain by two tightly linked genes. The effect of *qAL8* on awn length was enhanced when the

weedy form of the allele was present at *qAL4-1* (Gu *et al*., 2005). Seed dormancy were correlated with awn in weedy rice in four segregating populations (Gu *et al*., 2005; Mispan *et al*., 2013).

1.3.2 Objectives of the thesis project

Fine-mapping data from the previous research showed that the effects of *qSD8* and *qAL8* on germination capability or awn-length vary with genetic backgrounds. This suggested that *qSD1-2*, or *OsGA20-ox2*, could be involved in the epistatic interaction with *qSD8* and/or *qAL8*. Seed dormancy, plant height, flowering time, and awn length are all quantitative traits that are controlled by many genes and influenced by genetic backgrounds. For these two loci, the total genetic effect can be partitioned into additive (a), dominance (d) and epistatic (i) effects. Thus, the objectives of this research were to develop isogenic lines for all nine digenic genotypes of *qSD1-2* and *qSD8* and to evaluate all possible genetic component effects on seed dormancy, and the dormancy-related traits, including the previously reported plant height and awn, and the previously not reported traits flowering time and low-temperature germination.

Chapter 2. Genetic Dissection of Joint Effects for *qSD1-2* and *qSD8* on Seed Dormancy, Low Temperature Germination, Plant Height, Flowering Time, and Awn in Rice

2.1 Introduction

Seed dormancy as an adaptive trait is important for weedy species to survive in overtime. Cultivation has reduced seed dormancy because of selection for rapid and uniform germination for seedling establishment and field management. Lack of seed dormancy may cause the pre-harvest sprouting (PHS) problem in cereal crop. Pre-harvest sprouting is the seed germination on the plant after maturation but before harvesting under moist conditions. Seed dormancy controlled by multiple genes, which have been mapped as quantitative trait loci (QTLs) in barley, rice, wheat and some other crops (Reddy *et al*., 1985; Anderson *et al*., 1993; Han *et al*., 1999; Ye *et al*., 2010). One of the purposes to map the dormancy QTLs in cereal crops was to identify and use beneficial genes, or dormancy-enhancing alleles of the loci, to improve modern cultivars for resistance to PHS by marker-assisted selection.

Marker-assisted selection (MAS) for multiple QTL alleles in a breeding program requires knowledge about genetic component effects of the selected loci. For a single QTL, its gross genetic effect can be partitioned into additive and dominance for two or more QTL, the total genetic effect can be partitioned into additive, dominance, and epistasis (Collard *et al*., 2005).

Rice is a major crop and is also a model plant for genetic/genomic research on seed dormancy. Several seed dormancy QTLs have been identified for genetic component

effects on germination, such as the tri-genic system *qSD1-2, qSD7-1* and *qSD12* (Gu *et al.,* 2006) and the digenic system *qSD1-2* and *qSD7-2* (Ye *et al*., 2013). This research focused on the digenic system *qSD1-2* and *qSD8*.

The *qSD1-2* and *qSD8* alleles were introduced from the weedy rice lines SS18-2 into the genetic background of the cultivar rice line EM93-1 by recurrent backcross and marker-assisted selection for several generations. In the previous studies, *qSD1-2* was co-localized with *qPH1* for plant height, and *qSD8* was co-localized with *qAL8* for awn length. *qSD1-2/qPH1* was detected in the BC_1F_2 population and two BC_4F_2 (EM93-1//EM93-1/SS18-2) populations (Ye *et al*., 2010b), but not detected in the primary segregating (BC_1F_1)population (Gu *et al*., 2004a). The *qSD1-2/qPH1* cluster was cloned as the *semidwarf1* gene encoding a GA20-oxidase for the GA biosynthesis (Ye *et al*. 2015). The *qSD1-2/qPH1* allele from the weedy rice is functional, which reduces seed dormancy (or increases germination), but increases plant height. Whereas, the mutant allele from the semi-dwarf line EM93-1 enhances the primary dormancy and reduces plant height. *qSD1-2* was associated with the endosperm-imposed dormancy by regulating seed development and maturation programs in rice (Gu *et al*., 2015; Ye *et al*., 2015).

qSD8/qAL8 was identified as a cluster on chromosome 8 in several populations developed from different genotypes of weedy rice (Gu *et al*., 2004; Mispan *et al*., 2013a; Zhang *et al*., 2017). This cluster explained 27 % of the variance for seed dormancy and awn length range from 10% to 64 % of the variance. Most recently, the *qSD8/qAL8* cluster was identified as two closely linked loci (Pipatpongpinyo, 2018).

Semi-dwarf cultivars have been popular for the rice crop production since the Green Revolution in 1960s. The semi-dwarf cultivars contain the dormancy-enhancing allele at *SD1-2,* but many of cultivars are still susceptible to PHS. To improve the resistance of semi-dwarf varieties to PHS, it is necessary to introduce additional seed dormancy gene or genes to the *sd1* background. However, there is no information about interactional effects or epistasis between *qSD1-2* and *qSD8*. Therefore, the objectives of this research were; 1) to develop isogenic lines by introducing the *qSD1-2* and *qSD8* alleles into the same genetic background; 2) to confirm the effects of *qSD1-2* and *qSD8* on seed dormancy in an isogenic background; and 3) to estimate genetic component effects of the narrowed *qSD1-2* and *qSD8* regions on awned-seed percentage, flowering time, low-temperature germination and plant height, in addition to seed dormancy.

2.2 Material and Methods

2.2.1 Breeding scheme used to isolate both *qSD1-2* and *qSD8*

The *qSD8* seed dormancy QTL was identified in the BC_1F_1 (EM93-1//EM93-1/SS18-2) population. SS18-2 is the wild-like weedy line from Thailand and the donor parent of the dormancy enhancing alleles at *qSD8* (Gu *et al*., 2004). Whereas, the recurrent parent EM93-1 is an *indica* line of semi-dwarf cultivated rice and carries a dormancy-reducing allele at *qSD8*. *qSD1-2* was identified in single plant-derived BC_1F_2 and BC_1F_3 populations (Ye *et al*., 2010a). EM93-1 and SS18-2 carry the dormancy-enhancing and reducing alleles, respectively, at *qSD1-2*.

To develop a segregating population for both *qSD1-2* and *qSD8,* single plants that were heterozygous for the two loci were selected by MAS in each generation for three

more generations (BC_1F_6; Figure 2.1). A total of about 187 plants from the BC_1F_6 population were genotyped to select all nine genotypes for *qSD1-2* and *qSD8* loci as a digenic system for this research.

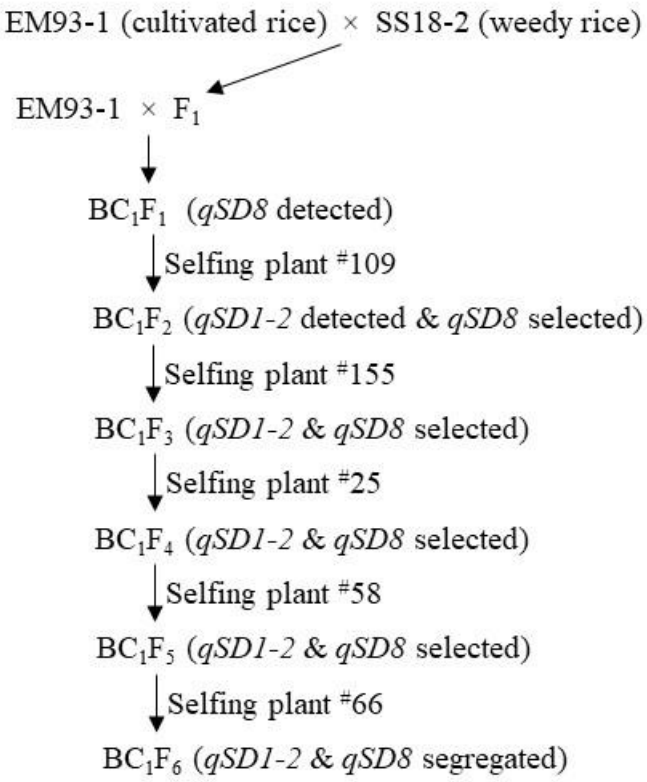

Figure 2.1 Breeding scheme used to develop a segregating population for *qSD1-2* and *qSD8*. Single plants from each of the BC_1F_1 (EM93-1//EM93-1/SS18-2) to BC_1F_5 populations were selected to develop the next populations by self-pollination and marker-assisted selection for *qSD1-2* and *qSD8*. Refer to Figure 2.2 for genotypes of plants #25 and #66.

2.2.2 Plant cultivation and seed harvesting

To develop a segregating population, seeds from plant #66 in BC_1F_5 were after-ripened and germinated at 30°C for 5 days. Seedlings were transferred to 200-cell Plug

Trays, with one plant per cell and cultivated with the rice nutrition solution for rice. Seedlings at 3-leaf stage were transplanted into pots (12 cm × 12 cm × 15 cm^2), with one plant per pot. The pots were filled with clay soil and greenhouse medium (Sunshine Mix #1; SUNGRO Horticulture Ltd., Canada) in a 3:1(clay soil/medium) ratio. The plants were grown in a greenhouse with temperatures set at 29°/21°C (day/night). Plants were tagged for flowering time when the first panicle in a plant engendered from the leaf sheath. Seeds were harvested at 40 days after flowering. The harvested seeds were cleaned by removal of immature seeds. The mature seeds were air-dried in greenhouse temperature for 3 days, and then stored at -20°C freezer to maintain the status of primary dormancy.

2.2.3 Phenotypic assessment for traits

2.2.3.1 Seed dormancy

Seed dormancy was measured by germination percentage under controlled conditions (Gu *et al*., 2003). Seeds from each of the BC_1F_6 plants were divided into two sets to after-ripen (stored at the room temperature 24.0±0.6 °C) for 0 and 10 days, respectively. Germination tests were replicated three times with about 50 seeds per replication at 0 and 10 days after ripening (DAR). Seeds were placed in 9-cm petri dishes that were lined with a Fisherbrand P5 grade filter paper and soaked with 8 ml water. The prepared petri dishes were placed in an incubator at 30°C and 100 % relative humidity in the dark for 7 days. Germinated seeds were defined as the protrusion of the radicle from the hull by at least 3mm and counted daily from day 2 to day 7 visually. Germination percentage (GP) was calculated as:

$$GP = \Sigma n_i / N \ (\%) \qquad \text{Equation 2.1}$$

where, Σn_i is the number of germinated at day 1 to day 7, and N is the total number of seeds in a sample. Mean GP of three replicates for each plant was used to estimate the degree of seed dormancy at given DAR.

Germination data were also used to calculate germination index (Reddy et al., 1985b). Germination index (GI) was calculated as:

$$GI = (7\times n_1+6\times n_2+5\times n_3+4\times n_4+3\times n_5+2\times n_6+1\times n_7)10\times N \qquad \text{Equation 2.2}$$

where, n_i (i=1 to 7) is the number of seeds germinated after day 1 to day 7, and N is the total number of seeds in a sample.

2.2.3.2 Low temperature germination

Seed samples were stored at the room (warm & dry) condition for more than two months to release dormancy completely. Three replications of seed samples from each of the plants were germinated at 15°C (Sasaki, 1983), and the other conditions were same as the germination test for seed dormancy. Germination parameters were calculated using the above-described methods.

2.2.3.3 Plant height, flowering time, and percentage of awned seed per panicle

The flowering time was recorded by emergence of the first panicle from the leaf sheath. The period (d) from germination to flowering was used for data analysis.

Plant height was measured as the length of the main stem from the soil surface to the top of a panicle of the main stem at harvest.

The awn trait was quantified by percentage of awned seeds on per panicle. Seeds were accounted for 100 seeds as awn and without awn visually.

2.2.4 DNA extraction

Plant DNA was extracted the leaf tissue using the CTAB (Cetyl trimethyl-ammonium bromide) method. Leaf fragments from each seedling were placed in a 1.5 ml microcentrifuge tube and fresh leaves were ground into powder in liquid nitrogen. The tubes were incubated at 60°C water bath in CTAB extraction buffer (2% Cetyl trimethyl-ammonium bromide, 100 mM pH 8.0 Tris-HCI, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol) for 30 min. shaking tubes every 10 min. Equal volumes of chloroform was added and mix well 5 min and leave tubes at room temperature for 10 min. The tubes were in centrifuge at 4 °C for 15min at 13000 rpm. The upper aqueous layer was transferred into a new tube and mixed with 0.7 volume of isopropanol gently for 10min. and incubated 4 °C for 15 min. at 13000 rpm. The supernatant was discarded very carefully, and the DNA pellets washed with ice cold 70% ethanol for two times. The DNA pellets were air-dry to remove the rest of ethanol and then dissolved in 100µ TE buffer (10 mM Tris pH 8, 1 mM EDTA) according to the amount of tissue. The samples were stored at 4 °C for overnight or in a 60°C water bath to elute the DNA. The DNA concentrations were quantified with a Thermo Scientific NanaDrop^{TM}1000 Spectrophotometer.

2.2.5 Marker development and polymerase chain reaction (PCR)

Marker development: Rice microsatellite (RM) or simple sequence repeat (SSR)) markers were used to genotype individual plants The sequence of SSR markers were

obtained from the Gramene database (www.garamene.org) and synthesized in Integrated DNA Technologies, Inc. (IDT) (www.idtdna.com).

Polymerase Chain Reaction (PCR): A program was set up in a total volume of 15 or 20µl containing 50 ng of DNA-template, 3 µl of 5× Green Go Taq® reaction buffer (Promega, Madison, WI), 0.2 unit of Taq polymerase, 200 µM of dNTP (Fisher BioReagents™ Nucleotides) and 20 µM of each primer. The PCR cycles start with denaturation at 95°C for 5 min., 40 cycles of denature at 94°C for 20 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and 72°C for 10 min. followed by 4°C forever.

Gel electrophoresis and imaging: The PCR products were separated by electrophoresis on non-denature gel, which consist of 6 % acrylamide, and 0.1 % APS, 0.01 % TEMED in 0.5 ×TBE solution. The electrophoresis was run in 0.5 ×TBE buffer at 300 volts for 2-3 hours. The gel was stained with ethidium bromide. The gel glass plates were imaged under UV light by using the AlphaEaseFC™ (Alpha Innotech) gel imaging system. Genotypes for a marker were coded as 1,2, and 3 for to EM93-1 like homozygote, heterozygous, and SS18-2 like homozygote, respectively.

2.2.6 Construction of linkage map

Marker genotyping data from the F_2-like segregating populations of 144 plants were used to develop partial linkage maps for the *qSD1-2* and *qSD8* regions. The computer software MAPMAKER/EXP (version 3.0b) (Lander et al., 1987) was used for the map construction. Genetic distance in centiMorgans (cM) was developed by Kosambi's mapping function (Kosambi, 1943). The markers were grouped at the

minimum LOD (log-likelihood) threshold of 3.0 and the maximum genetic distance of 40 cM.

2.2.7 QTL mapping

The Windows QTL Cartographer V2.5_009 composite interval mapping (CIM) was used to confirm the *qSD1-2* and *qSD8*. The interval mapping program was set up at 1 cM walking speed and 1000 random permutations at the significant level of 0.05. The composite interval mapping program was used to show QTL peak position, likelihood ratio, additive (α) and dominance (d) effects, and the proportion of the variance explained be a QTL (R^2). EM93-1-like homozygous, heterozygous, SS18-2-like homozygous genotypes for a marker were coded as 1,2, and 3, respectively to determine the *a* and *d* effects for plant height (PH), flowering time (FT), germination percentage (GP), low-temperature germination percentage (LTG), and percentage of awned seeds per panicle (AWN).

2.2.8 Linear regression analysis for genetic component effects of the two loci

Gene additive (*a*), dominance (*d*), and epistatic (*i*) effects of the *qSD1-2* and *qSD8* loci, which were represented by nearest markers, were estimated using the linear regression model:

$$y_{ijk} = \mu + a_1 x_i + d_1 z_i + a_8 x_j + d_8 z_j + i_{a1a8} w_{ij} + i_{a1d8} w_{ij} + i_{d1a8} w_{ij} + i_{d1d8} w_{ij} + \varepsilon_{ijk} \quad \text{Equation 2.3}$$

where, y_{ijk} is the phenotypic value of the plant k (k=1 to N, the number of plants evaluated for germination, plant height, flowering time, awn length); μ is the mean of the model; x_i and x_j are variables for additive effects of *qSD1-2* (a_1) and *qSD8* (a_8), respectively, which are coded as -1 for the EM93-1-like homozygote, 0 for heterozygote,

or 1 for the SS18-2-like homozygote; z_i and z_j are variables for dominance effects of *qSD1-2* (d_1) and *qSD8* (d_8), respectively, which are coded as 0.5 for the heterozygote or -0.5 for the homozygotes; w_{ij}s are variables for epistatic interactions between the additive (i_{a1a8}), or dominance (i_{d1d8}) effects, or between the additive and dominance effects (i_{a1d8} & i_{d1a8}); ε_{ijk} is the random error and effect that cannot be explained by the genetic effect. The regression analysis was performed by the SAS procedure REG with a stepwise selection set at a significant level of 5 % (Gu *et al*., 2006)

2.3 Results

2.3.1 Genotypes of initial plants

Marker genotyping data revealed that the BC_1F_3 plant #25 (Fig. 2.1A) retains segments on chromosomes 1, 3, 4, 5, 8, and 10 from SS18-2 (Fig. 2.2A). The plant #25 was not selected to develop a segregating population for *qSD1-2* and *qSD8*, because it is also heterozygous for the other four chromosomal segments, including one containing *qSD3.* Segregation for the additional four heterozygous regions may interference with the effect estimation for the *qSD1-2* and *qSD8* loci.

After two generations of self-self-pollination and marker-assisted selection, the BC_1F_5 plant #66 became heterozygous only for the *qSD1-2* and *qSD8* regions (Fig. 2.2B). Thus, seeds from this plant were used to identify all 9 genotypes for *qSD1-2* and *qSD8*.

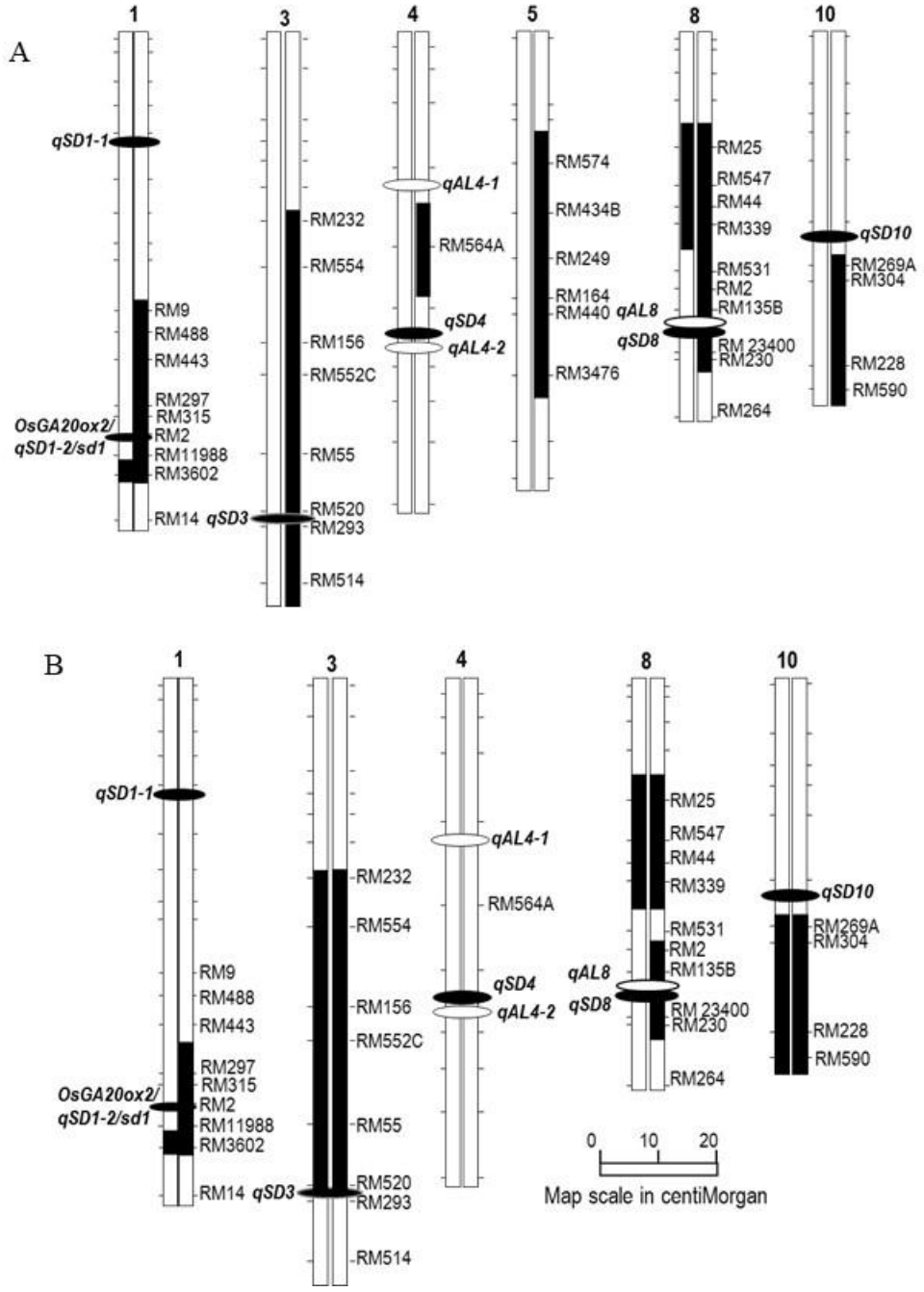

Figure 2.2 Graphic representation of genotypes for the BC_1F_3 plant #25 (**A**) and BC_1F_5 plant #66 (**B**). Only the chromosomes that contain segments from the donor parent SS18-2 (dark) in the background of the recipient parent EM93-1 (open) are shown. Rice microsatellite (RM) markers were used to genotype the plants. Ovals indicate positions of QTLs for seed dormancy (*qSD*) or awn length (*qAL*).

2.3.2 Confirmation of *qSD1-2* and *qSD8*

Phenotypic variations were observed for seed dormancy, low-temperature germination (LTG), plant height, flowering time, and awn traits in BC_1F_6 population, as shown by genotypic means in Table 2.1.

Table 2.1 Summary of genotypic means for traits evaluated in the BC_1F_6 population.

Genotype[a]	N[b]	PH[c]	DTF[d]	Awn[e]	GP1[f]	GP2[g]	LTG[h]
AABB	7	59.9±7.9	84.0±1.5	0.0±0.0	42.8±17.1	88.1±7.4	66.0±24.0
AABb	8	57.8±2.1	84.8±1.8	3.4±4.7	26.1±17.0	72.7±19.1	56.5±11.5
AAbb	10	60.8±3.9	87±3.4	12.2±13.8	29.6±11.9	78.4±13.7	65.1±19.3
AaBB	23	77.3±6.7	83.4±3	0.1±0.7	50.7±16.9	88.2±8.1	76.9±14.8
AaBb	36	84.3±8.7	82.0±2.5	2.5±5.8	36.7±11.9	81.9±9.7	72.1±19.1
AaBB	16	83.2±7.8	83.9±2.2	15.2±16.2	40.8±14.2	86.9±9.1	61.0±16.2
aaBB	7	99.3±9.7	82.7±3.0	0.0±0.0	55.2±7.1	87.6±4.2	72.1±19.1
aaBb	24	105.5±6.1	81.6±2.7	5.7±11.2	42.6±9.6	83.4±8.4	79.1±11.3
aabb	13	97.5±3.3	83.3±2.5	10.7±9.9	44.7±14.1	84.8±5.9	76.1±13.5

[a]Digenic genotypes for *qSD1-2* (A/a) and *qSD8* (B/b), with the upper-/lower-case letters indicate the alleles from the parents SS18-2 and EM93-1, respectively; [b]N, number of plants; [c]PH, plant height in cm; [d]DTF, days to flowering; [e]Awn, percentage of awned seeds; [f]GP0, germination percentage at 0 DAR; [g]GP10, germination percentage at 10 DAR; and, [h]LTG, low-temperature germination percentage.

QTL analysis confirmed effects of *qSD1-2* and *qSD8* on germination at 0 and 10 DAR (Figure 2.3). The *qSD1-2* region was also detected effects on LTG, plant height, and flowering time (Figure 2.3A), with the allele from SS18-2 reducing seed dormancy, promoting stem elongation and LTG, and delaying flowering. Similarly, the *qSD8* region

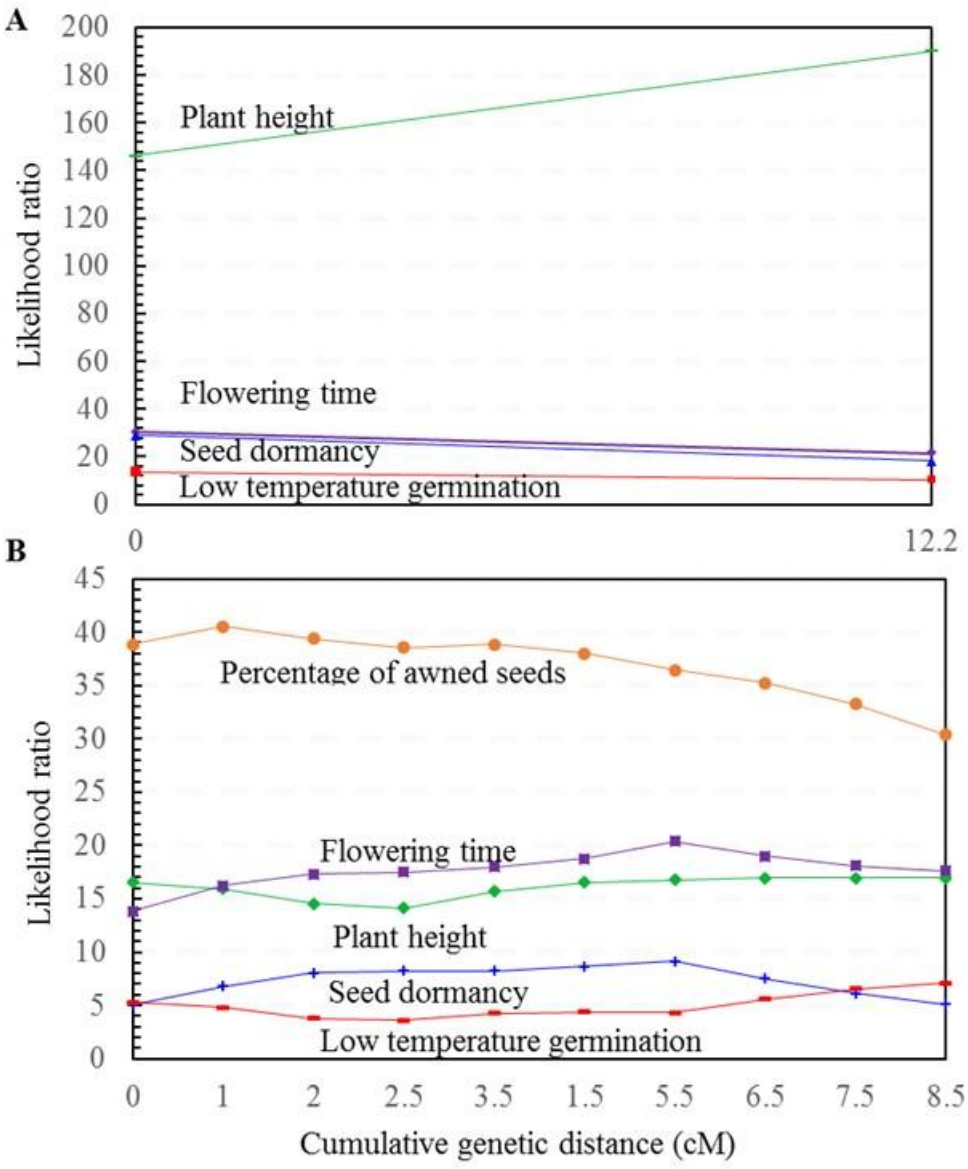

Figure 2.3 Likelihood distributions for seed dormancy and interrelated traits on chromosomal segments containing *qSD1-2* (**A**) and *qSD8* (**B**). The data were exported from the composite interval mapping program.

was also detected for effects on the LTG, plant height, flowering time, and awn traits (Figure 2.3B), with the allele from SS18-2 enhancing seed dormancy, increasing percentage of awned seeds, plant height, and LTG, and also delaying flowering time a little.

2.3.3 Genetic component effects of *qSD1-2* and *qSD8* on individual traits

2.3.3.1 Seed dormancy

2.3.3.1.1 Phenotypic variation patterns

Similar patterns of variation for germination percentage (GP) and index (GI) were observed in the BC_1F_6 population (Figure 2.4). Frequency distributions for GP and GI were approximately normal at 0 DAR, falling in the range from 7% to 95% for GP or from 3% to 70% for GI (Figure 2.4A). The distributions at 10 DAR slightly skewed to the high percentage end, and the parameter GI increased the normality a little (Figure 2.4B).

2.3.3.1.2 Genetic component effects

Both *qSD1-2* and *qSD8* had significant additive effects on germination at 0 DAR (Table 2.2). For the additive component, the alleles from the parents EM93-1 and SS18-2 at *qSD1-2* and *qSD8* reduced germination or enhanced seed dormancy, respectively. In addition to the additive effect, *qSD8* also had significant dominance effect on germination at 0 DAR. Different from 0 DAR, *qSD1-2* had an additive effect and *qSD8* had a dominance effect on reducing germination at 10 DAR.

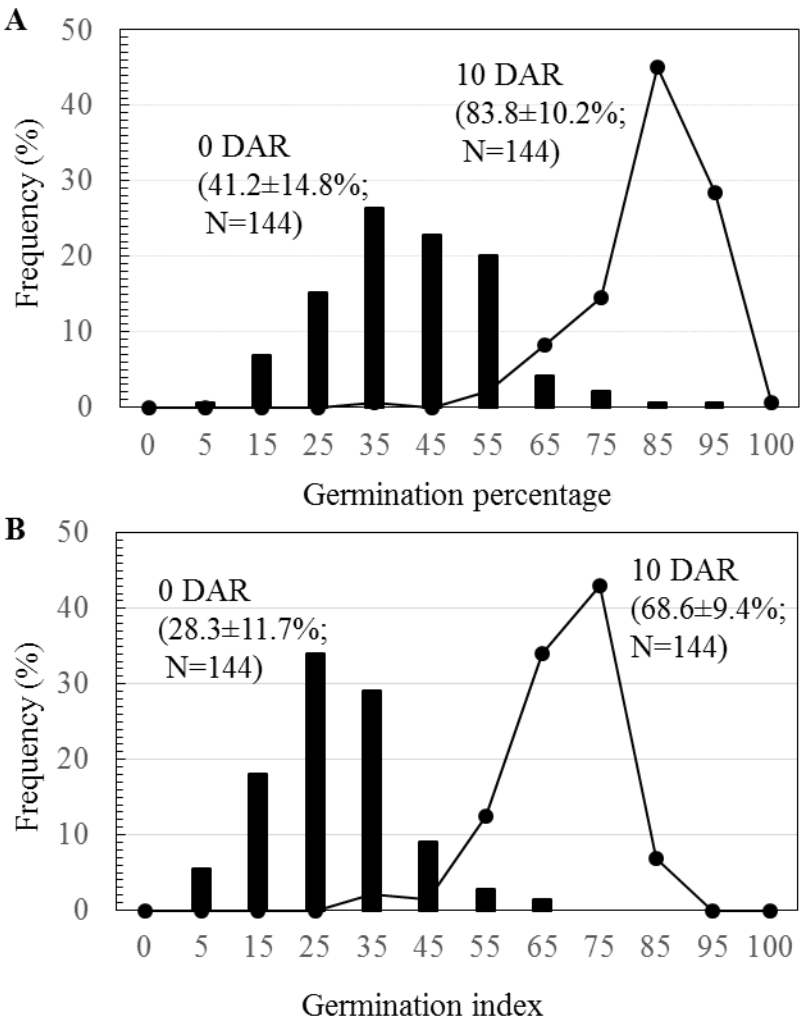

Figure 2.4 Frequency distributions for germination percentages (**A**) and index (**B**) at 0 and 10 days of after-ripening (DAR) in the BC_1F_6 population. Mean, standard deviation and sample size are shown on the figure.

Table 2.2 Genetic component effects of *qSD1-2* and *qSD8* on germination at 0 and 10 DAR.

Parameter	Germination at 0 DAR		Germination at 10 DAR	
	Effect (%)	Probability	Effect (%)	Probability
	(Germination percentage at the 7th day)			
μ	40.1	<.0001	83.3	<.0001
a_1	7.1	<.0001	2.7	0.0307
a_8	-5.7	0.0003		
d_8	-8.5	0.0002	-5.0	0.003
	(Germination index)			
μ	27.3	<.0001	67.9	<.0001
a_1	6.6	<.0001	3.9	0.0005
d_1			3.6	0.0166
a_8	-4.5	0.0002		
d_8			-4.8	0.0015

Only the significant parameters in Equation 2.3 were listed. μ is the model mean; a_1 and a_8 are additive effects for *qSD1-2* and *qSD8*, respectively; and d1 and d8 are dominance effects for *qSD1-2* and *qSD8*, respectively.

2.3.3.2 Low temperature germination

2.3.3.2.1 Phenotypic variation pattern

The largest variation in LTG among the 9 genotypes occurred at the 5th day after imbibition (Figure 2.5A), when the frequency distribution skewed to the high percentage end (Figure 2.5B). Frequency distribution for GI was approximately normal falling in from 29.7 % to 42.9 % (Figure 2.5B).

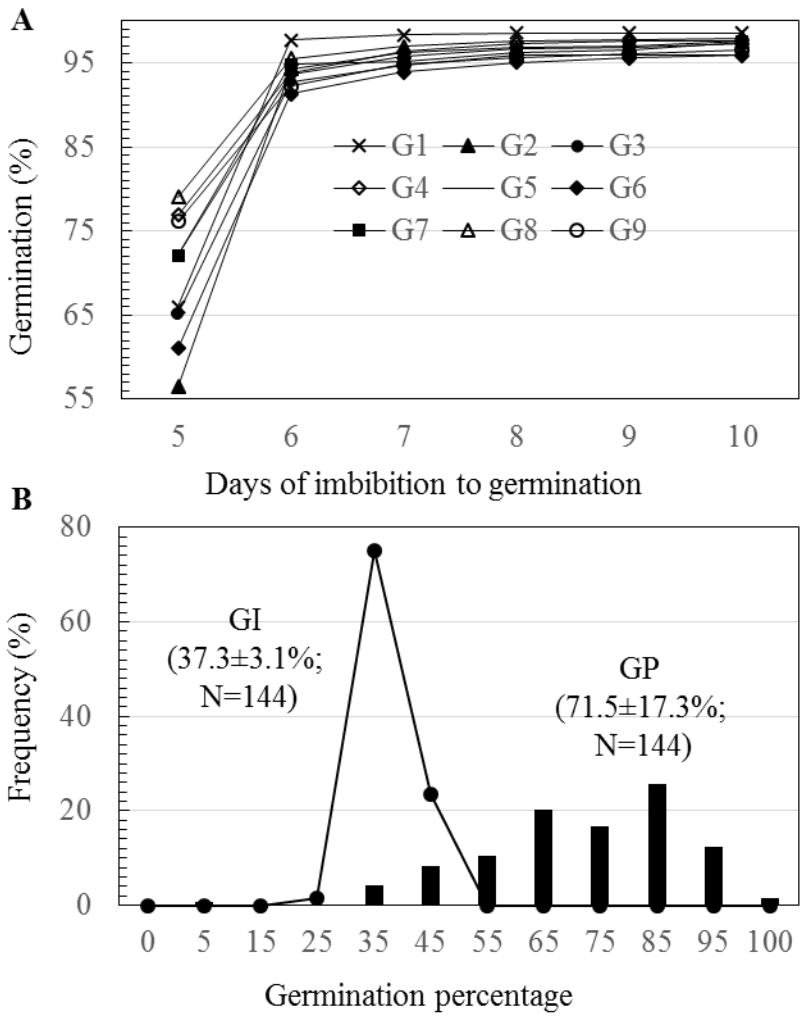

Figure 2.5 Germination percentage (**A**), germination index and frequency distribution (**B**) of low-temperature germination in the BC_1F_6 population. Mean, standard deviation and sample size are shown on the figure.

2.3.3.2.2 Genetic component effects

Both *qSD1-2* and *qSD8* had additive, but not dominance, effects on germination under the low temperature (15°C) condition (Table 2.3). For the additive component, the allele from EM93-1 at qSD1-2 and the allele from SS18-2 at *qSD8* reduced germination. In addition, the epistatic interaction (id1a8) between *qSD1-2*'s dominance effect (d_1) and

qSD8's additive effect (a_8) was also significant, based on germination percentage (Table 2.3).

Table 2.3 Genetic component effects of *qSD1-2* and *qSD8* on low-temperature germination

Parameter	Effect (%)	Std error	F value	Probability
		(Germination percentage at the 7th day)		
μ	70.3	1.3	2543.8	<.0001
a_1	7.0	1.9	12.6	0.0005
a_8	-3.9	1.8	4.5	0.0355
i_{d1a8}	-9.0	3.7	5.6	0.0186
		(Germination index)		
μ	37.2	0.2	21133.7	<.0001
a_1	1.0	0.3	8.1	0.005
a_8	-0.9	0.3	7.7	0.006

Only the significant parameters in Equation 2.3 were listed. μ is the model mean; a_1 and a_8 are additive effects for *qSD1-2* and *qSD8*, respectively; and i_{d1d8} is the epistatic effect between *qSD1-2*'s dominance effect and *qSD8*'s additive effect.

The $d_1 \times a_8$ epistasis was characterized mainly by the genotypic difference of *qSD1-2* varied with the genotypes of *qSD8*, with the difference being largest when *qSD8* was heterozygous (Figure 2.6). In addition, the *qSD1-2* heterozygote decreased in LTG with the increase in the number of the dormancy-enhancing allele at *qSD8*.

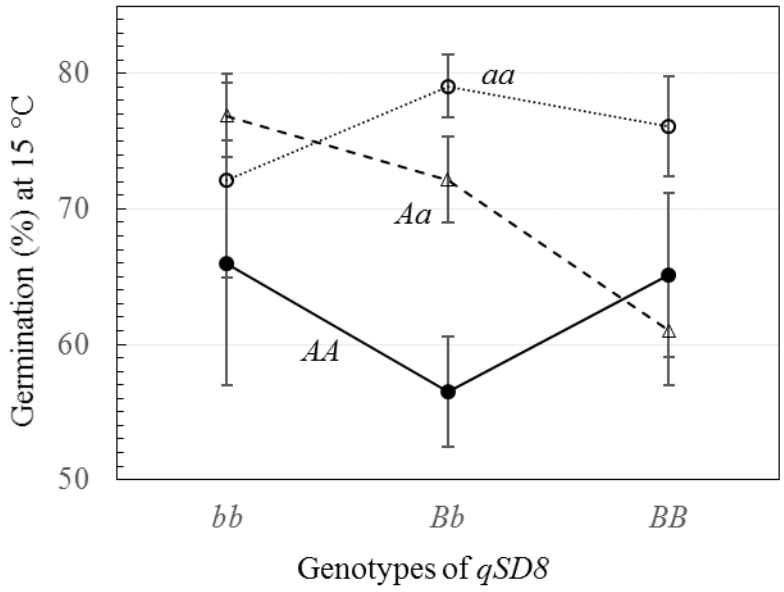

Figure 2.6 Digenic epistasis between *qSD1-2* and *qSD8* for low-temperature germination in the BC_1F_6 population. Italic upper/lower cases of *A/a* and *B/b* denote seed dormancy enhancing/decreasing alleles at *qSD1-2* and *qSD8*, respectively.

2.3.3.3 Plant height

2.3.3.3.1 Phenotypic variation pattern

The BC1F6 population varied in plant height from 53 to 118 cm, with the mean being 85 cm (Figure 2.7). The frequency distribution appeared to be binominal, suggesting that there is a gene in the *qSD8* region modifying the phenotypic variation.

2.3.3.3.2 Genetic component effects

qSD1-2 had a major additive effect (21 cm) on plant height, with the allele from SS18-2 promoting stem elongation (Table 2.4). *qSD8* had a small, but significant, dominance effect (3 cm) on plant height. In addition, two types of epistasis between the additive and dominance effects of these two loci were also significant (Table 2.4).

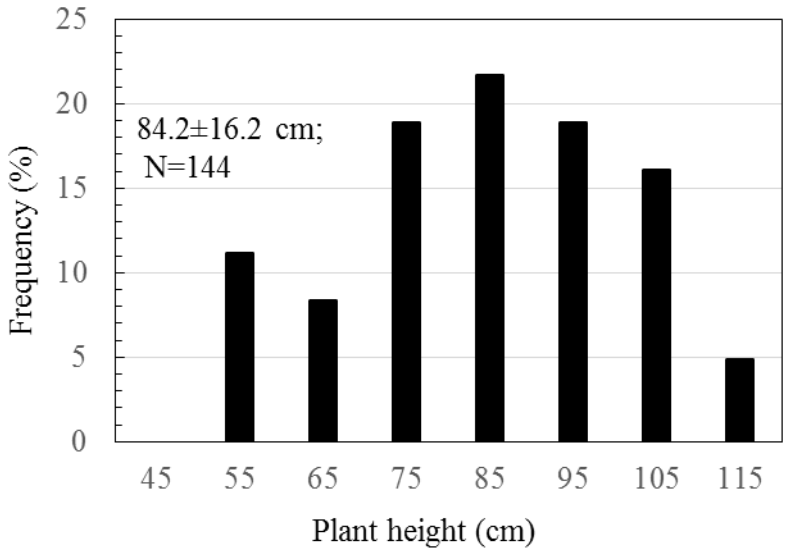

Figure 2.7 Frequency distribution of plant height in the BC_1F_6 population. Mean, standard deviation and sample size are shown on the figure.

Table 2.4 Genetic component effects of *qSD1-2* and *qSD8* on plant height

Parameter	Effect (cm)	Std error	F value	Probability
μ	81.5	0.6	17611.3	<.0001
a_1	21.0	0.8	571.1	<.0001
d_8	3.3	1.2	7.5	0.007
i_{a1d8}	4.1	1.7	5.5	0.0196
i_{d1a8}	3.3	1.6	3.9	0.0488

Note: Only the significant parameters in Equation 2.3 were listed. μ is the model mean; a_1 is the additive effect of *qSD1-2*; d_8 is the dominance effect of *qSD8*; and i_{a1d8} and i_{d1a8} are the epistatic effects between the dominance and additive effects of the two loci.

The two epistatic interactions were characterized mainly by the genotypic difference of *qSD1-2* varied with the genotypes of *qSD8*, with the difference being largest when *qSD8* was heterozygous (Figure 2.8).

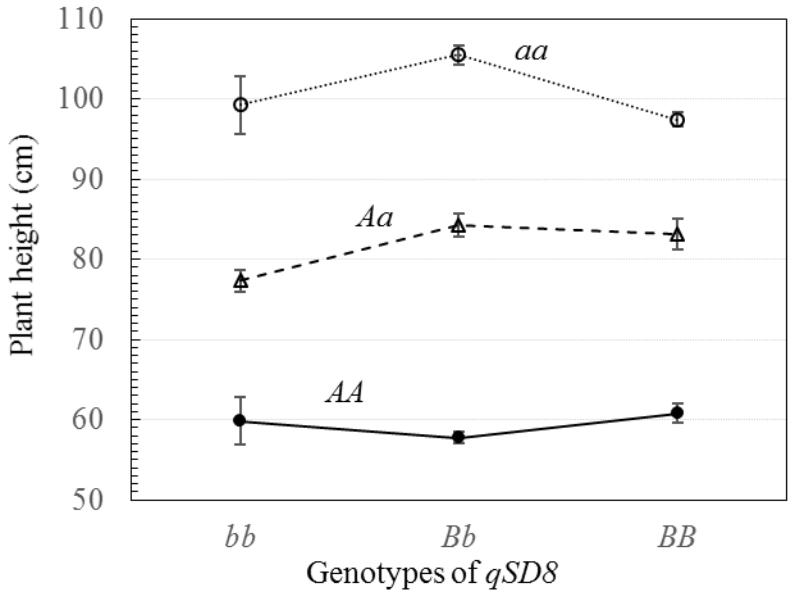

Figure 2.8 Digenic epistasis between *qSD1-2* and *qSD8* for plant height in the BC_1F_6 population. Italic upper/lower cases of *A/a* and *B/b* denote seed dormancy enhancing/decreasing alleles at *qSD1-2* and *qSD8*

2.3.3.4 Flowering time

2.3.3.4.1 Phenotypic variation pattern

The BC_1F_6 population varied in days to flowering from 75 to 92 (Figure 2.9). The frequency distribution was normal with the mean being 83 d. Both *qSD1-2* and *qSD8* were not reported for an effect on flowering time. However, the QTL mapping (Figure 2.3) and the distribution pattern suggest that these two loci may have pleiotropic or linkage effects on flowering time.

2.3.3.4.2 Genetic component effects

The linear regression analysis revealed that *qSD1-2* had both additive and dominance effects, with the allele from SS18-2 delaying flowering (Table 2.5). Different

from *qSD1-2*, *qSD8* had only a dominance effect, with the allele from SS18-2 also delaying flowering (Table 2.5). There is no any interaction between *qSD1-2* and *qSD8* in influencing flowering time.

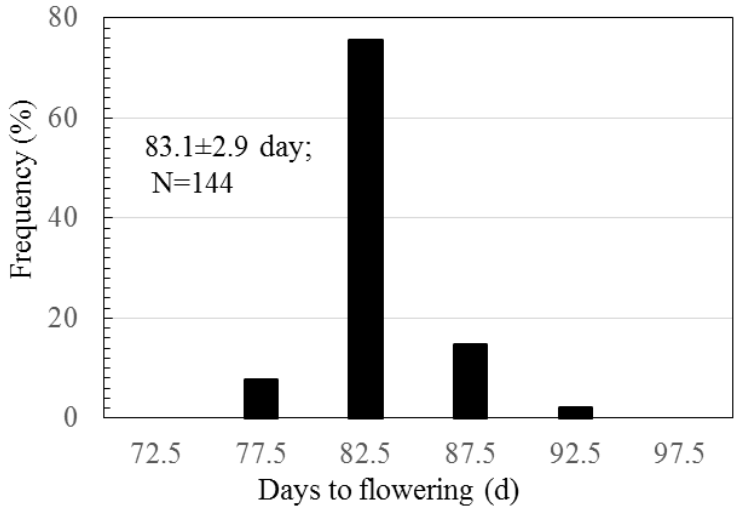

Figure 2.9 Frequency distribution of days to flowering in the BC_1F_6 population. Mean, standard deviation and sample size are shown on the figure.

Table 2.5 Genetic component effects of *qSD1-2* and *qSD8* on flowering time

Parameter	Effect (d)	Std error	F value	Probability
μ	83.3	0.22	137301	<.0001
a_1	-1.4	0.33	18.0	<.0001
d_1	-0.9	0.45	4.1	0.0447
d_8	-1.5	0.44	11.8	0.0008

Only the significant parameters in Equation 2.3 were listed. μ is the model mean; a_1 and d_1 are the additive and dominance effects of *qSD1-2*, respectively; d_8 is the dominance effect of *qSD8.*

2.3.3.5 Percentage of awned seeds per panicle

2.3.3.5.1 Phenotypic variation pattern

The BC_1F_6 population consisted of ~70% awnless and ~30% awned plants (Figure 2.10). The awned plants also varied in the percentage of awned seeds per panicle from 0 % to 60.2% (Figure 2.10).

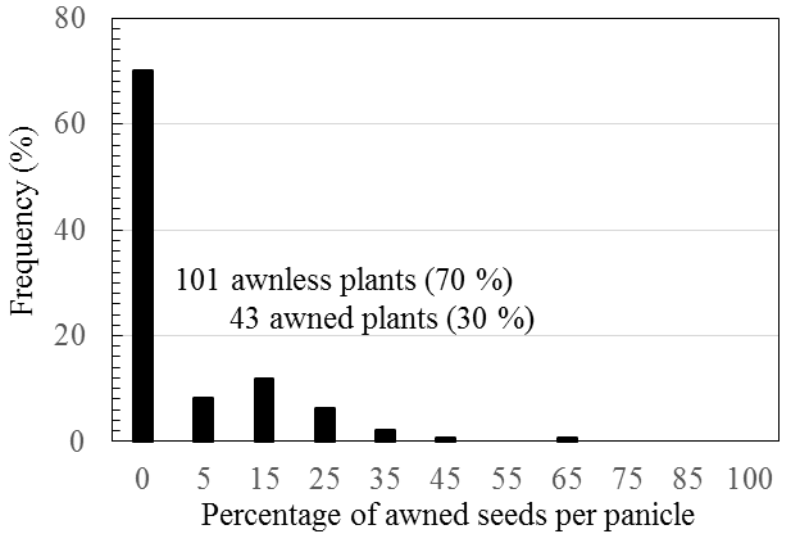

Figure 2.10 Frequency distribution of awned-seed percentage in the BC_1F_6 population.

2.3.3.5.2 Genetic component effects

The linear regression analysis detected only an additive effect (a_8) for *qSD8/qAL8*, with the allele from SS18-2 increasing the presence of awn (Table 2.6).

Table 2.6 Genetic component effects of *qSD1-2* and *qSD8* on awned-seeds percentage

Parameter	Effect (%)	Std error	F value	Probability
μ	5.1	0.7	46.5	<.0001
a_8	6.4	1.0	38.0	<.0001

Only the significant parameters in Equation 2.3 were listed. μ is the model mean; a_8 is the dominance effect of *qSD8*

2.3.4 Correlations among the traits tested

Phenotypic correlations were significant between some of the traits evaluated for the BC1F6 population (Table 2.7). The strongest correlation occurred between flowering time and plant height as negatively. There is a positive correlation between plant height and low-temperature germination, while low-temperature germination and percentage of awned seeds per panicle were negatively correlated. In addition, positive correlation was observed between flowering time and germination percentage at 0 days of after-ripening.

Table 2.7 Summary of correlation coefficients (r) between traits in the BC_1F_6 population

Trait	PH	DTF	Awn	GP0	GP10	LTG
PH		**-0.5274**	0.034	0.1263	0.065	**0.2375**
DTF	**<.0001**		0.1073	**0.1939**	0.0796	-0.1175
Awn	0.687	0.2006		-0.1132	-0.1429	**-0.1904**
GP0	0.1328	**0.0199**	0.1768		**0.5851**	0.0918
GP10	0.4406	0.3428	0.0876	**<.0001**		0.1241
LTG	**0.0043**	0.1607	**0.0223**	0.274	0.1384	

Note: PH, plant height in cm; DTF, days to flowering; Awn, percentage of awned seeds per panicle; GP0, germination percentage at 0 DAR; GP10, germination percentage at 10 DAR; and LTG, low-temperature germination percentage. Listed below and above the diagonal line are probability (P) levels and r values, respectively. Values significant at $P<0.05$ are shown in bold.

2.4 Conclusion and Discussion

2.4.1 Summary

This research developed a digenic system segregating only for the *qSD1-2* and *qSD8* regions. The previous research identified a set of dormancy QTLs *qSD1-2, qSD4, qSD6, qSD7-1*, *qSD7-2*, and *qSD8* segments in BC_1F_1 population. The parental lines EM93-1 and SS18-2 were used to develop an F_2-like backcross population and single plants were heterozygous were selected by using marker-assisted selection for each segregating populations from BC_1F_1 to BC_1F_6. The donor parent SS18-2 is the wild-like rice line with seed dormancy enhancing alleles, *indica* type of rice from Thailand, while the recurrent parent EM93-1 is cultivated rice line with seed dormancy reducing alleles (Gu *et al*., 2003).

The six heterozygous chromosomes 1, 3. 4, 5, 8, and 10 containing regions were detected plant $^{\#}25$ in BC_1F_3 population. After two generations, plant $^{\#}66$ that is heterozygous for only *qSD1-2* and *qSD8* in BC_1F_5 was selected to develop BC_1F_6 segregating population. 144 plants were selected by using marker-assisted selection to distinguish nine genotypes. The synchronized genetic background improved estimation genic effects for seed dormancy, low-temperature germination, plant height, flowering time and percentage of awned seeds per panicle. Effects of *qSD1-2* and *qSD8* on germination at 0 and 10 DAR were confirmed by QTL analysis. The *qSD1-2* region has effects for low-temperature germination, plant height, and flowering time, with reducing allele for seed dormancy from SS18-2. The *qSD8* region has also significant effects on low-temperature germination, plant height, flowering time, and percentage of awned per panicle, with seed dormancy enhancing allele from SS18-2, increasing plant height, low-

temperature germination, and percentage of awned seeds and also it is delaying flowering time for a few days.

Both *qSD1-2* and *qSD8* had significant additive effects and *qSD8* had significant dominance effect at 0 DAR. The *qSD1-2* had additive and dominance effects at 10 DAR, while the *qSD8* had only dominance effect at 10 DAR. There is no any detected interaction between *qSD1-2* and *qSD8* at 0 and 10 DAR.

Both of two loci had additive effects on germination under the low temperature conditions. The interaction was detected between *qSD1-2*'s dominance effect (d_1) and *qSD8*'s additive effect (a_8) based on germination percentage. Largest difference can be seen when *qSD8* was heterozygous with *qSD1-2*.

The genetic effect of two clusters of QTLs have main effect and epistasis. *qSD1-2* had a major additive effect that reduce plant height about 21 cm with 78 % (R^2) of the phenotypic variance. *qSD8* had a small significant dominance effect (3 cm) on plant height. *qPH1* or *qSD1-2* had a greater main effect than *qSD8*. *qSD1-2* varied with the genotypes of *qSD8* and largest difference can be seen when *qSD8* was heterozygous.

Based on the linear regression analysis (Equation 2.3), *qSD1-2* had additive and dominance effects that the allele from SS18-2 delaying flowering. At same time, *qSD8* had dominance effect that also delaying flowering time due to allele from SS18-2.

The linear regression analysis based on the model (Equation 2.3) detected only additive (6.4 %) for *qSD8/qAL8*. SS18-2 allele increases the percentage of awned seeds per panicle.

Homozygotes were selected from the BC_1F_6 population as isogenic lines for these two loci for future research.

2.4.2 Discussion

This research evaluated genetic component effects of *qSD1-2* and *qSD8* on five traits in the same genetic background. These two loci mainly were additive and had no epistatic effect on germination.

Based on the experimental results, addition of the dormancy-enhancing allele at *qSD8* to the genetic background of a semi-dwarf cultivar could enhance the degree of dormancy, or the resistance to PHS. It is expected based on the additive effect of -4.5% at 0 DAR (Table 2.2), that the addition of the *qSD8* dormancy could reduce germination at harvest by 9%. It is also estimated that the addition of a genomic segment encompassing *qSD8* would have a little effect on low-temperature germination, plant height, and flowering time.

Seed dormancy and plant height co-evolved and they have interaction on chromosome segments encompassing *qSD1-2/qPH1* and *qSD8/qAL8*. *qSD1-2* was cloned as the GA synthase enzyme gene *OsGA20ox2* to control the hormone production in the developing and germinating seeds. *qSD1-2* is semi-dwarf gene that is also known as green revolution gene. Almost all cultivars are homozygous for *qSD1-2* that is mutant gene. This gene also enhanced seed dormancy or enhanced the pre-harvest sprouting (Ye *et. al.* 2010).

Seed dormancy and awn are two traits that have adaptive significant for wild species. However, long awns are not favorable during harvest and storage; hence, this

trait was artificially selected during domestication. Even so long awns are retained in some cereal crops, such as wheat and barley, because long awns contribute significantly to photosynthesis and yield (Abebe *et al*., 2010). Long awns are important for seed dispersal in wild rice (O.*rufipogon*), but longs awns are absent in cultivated rice (O. *sativa*). The genetic mechanism involved in loss-of-awn in cultivated rice remains unknown. Seed dormancy and awn correlation was associated with a few QTL cluster, including *qSD8/qAL8.* The effect of *qAL8* on awn-length was enhanced when the weedy form of the allele was present at *qAL4-1*(Gu et al., 2005). Seed dormancy were correlated with awn in weedy rice in four segregating populations(Gu et al., 2005; Mispan et al., 2013b).

qSD8/qAL8 accounted for 27% and 64% of the variances for SD and AL, respectively. Where SS18-derived allele reduced germination (~11.21%) and increased awn length (9.1 mm). The seed dormancy and awn association, or the *qSD8/qAL8* cluster, is underlain by two tightly linked genes (unpublished data). *qSD8* was genetically dissected from *qAL8.* To understand the relationship between seed dormancy- plant height and regulatory mechanism, cloning the candidate gene of *qSD8/qAL8* is necessary.

It is concluded that the dormancy-enhancing allele at *qSD8* is a candidate gene for breeding varieties resistant to PHS in the rice crop.

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Appendixes

Appendix 1. DNA extraction

To prepare 1L 0.5M EDTA (pH8.0) stock solution, 186.1 g EDTA and 20g NaOH were dissolved in 800 ml ddH_2O. Adjust the pH to 8.0 with 1M NaOH. Sterilize by autoclaving and store at room temperature.

Appendix 2. Gel electrophoresis

TBE buffer was dissolved 54g of Tris-base. 27.5g of boric acid, and 20ml of 0.5M pH 8.0 EDTA in 1L ddH_2O

TE buffer was diluted 1ml 1M pH8.0 Tris-HCI and 2ml 1M pH8.0 EDTA in 1L ddH_2O. Sterilize by autoclaving and store at room temperature.

To prepare 1M Tris-HCI stock solution, dissolve 121g Tris-base in 800 ml ddH_2O.Adjust pH to the desired value by adding concentrated HCI. Adjust the volume to 1L with ddH_2O, sterilize by autoclaving and store at room temperature.