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Map-Based Cloning and Molecular Characterization of the Seed Dormancy 10 Locus in Rice (Oryza sativa L.)

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MAP-BASED CLONING AND MOLECULAR CHARACTERIZATION OF THE *SEED DORMANCY 10* LOCUS IN RICE (*Oryza sativa* L.)

BY

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

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This dissertation is dedicated to my beloved parents Paisan and Wilai Pipatpongpinyo, my brother and sisters, my wife Maria Soledad Benitez Ponce, my daughter Maya Pipatpongpinyo, and my son Ananda Pipatpongpinyo for their love, endless support, encouragement, and sacrifices.

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ABBREVIATIONS

ABA	abscisic acid
aa	amino acid
APS	ammonium persulphate
BC	backcross
bp	base pair
C	Celsius
cm	centimeter
cM	centiMorgan
CTAB	cetyl trimethylammoniumbromide
Chr	chromosome
cDNA	complementary DNA
DAR	days of after ripening
DTF	day to flowering
DPA	day post anthesis
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
dsRNA	double-stand RNAs
EDTA	ethylenediaminetetraacetic acid
FT	Flowering time
FlcDNA	full-length cDNA
gDNA	genomic DNA
GA	gibberellic acid

g	gram
hpRNA	hairpin RNA
InD	insertion/deletion
IRS	inverted repeat sequence
IL	isogenic line
Kb	kilo-base
LL	leaf length
mM	milliMolar
Mb	mega-base
µg	micro-gram
µL	micro-liter
µM	micro-molar
mL	milli-liter
min	minute
M	molar
MD	morphological dormancy
MPD	morpho-physiological dormancy
NIL	near isogenic line
PL	panicle length
PYD	Physical dormancy
PD	physiological dormancy
PH	plant height
PCR	Polymerase chain reaction

PHS	pre-harvest sprouting
qRT-PCR	quantitative real time PCR
QTL	quantitative trait locus
RNA	ribonucleic acid
RM	rice microsatellite
RISC	RNA induced silencing complex
RNAi	RNA interference
s	second
SD	seed dormancy
SSR	simple sequence repeat
SNP	single nucleotide polymorphism
siRNA	small interfering RNA
SPP	spikelet per panicle
TF	transcription factor
TBE	tris-borate-EDTA
TE	tris-EDTA
TEMED	tetrametylethylenediamine
UV	ultraviolet

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ABSTRACT

MAP-BASED CLONING AND MOLECULAR CHARACTERIZATION OF THE *SEED DORMANCY 10* LOCUS IN RICE (*Oryza sativa* L.)

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Seed dormancy is a key adaptive trait of both ecological and agricultural importance. Although many quantitative trait loci (QTL) have been associated with seed dormancy in cereal crops or model plants, a majority of them remain unknown for molecular identities and functions. Cereal crops and wild/weedy relatives usually have weak and strong seed dormancy, respectively, such as Asian cultivated rice *vs*. weedy rice (*Oryza sativa* L.). Previous research identified a set of seed dormancy QTL, including *qSD10*, in single plant-derived BC_1F_2 and BC_1F_3 populations from the backcross between the weedy rice line SS18-2 and the cultivated rice line EM93-1. This dissertation aimed to clone *qSD10* and characterize the molecular functions of the QTL underlying gene(s). The first objective was to isolate *qSD10* as a Mendelian factor and narrow its size by fine mapping to identify candidate genes. A marker-assisted single-plant-selection technique was used to advance a BC_1F_3 plant for three generations to synchronize the genetic background of *qSD10*. A population of about 4000 BC_1F_6 plants segregating for the *qSD10*-containing region was used to identify recombinants between markers on a partial high-resolution map of 3 mega bases (Mb). The recombinants were selected for marker-assisted progeny testing to delimit *qSD10*. Finally, *qSD10* was narrowed to a genomic region of about 100 kilo bases (Kb). Physiological analysis based

on isogenic lines for the 100-kb region demonstrated that *qSD10* is involved in the development of primary dormancy by regulating dehydration and acquisition of desiccation tolerance during seed maturation. The progeny testing also identified a QTL for flowering time (*qFT10*) in the same 100-Kb region, with the *qSD10/qFT10* haplotype from EM93-1 enhancing seed dormancy and promoting flowering. There are 22 predicted genes in the 100-Kb region, including *Os10g32600* annotated as a Myb family transcription factor and previously reported as the *Early heading date 1* (*Ehd1*) gene.

The second objective of this dissertation was to clone the candidate gene *Os10g32600* and determine its role in seed dormancy and flowering. Both genomic DNAs (gDNAs) and full-length complementary DNAs (FlcDNAs) of *Os10g32600* were sequenced from EM93-1 and SS18-2. The sequence analyses revealed that both *Os10g32600* alleles consist of five exons and contain a regulatory domain and a helix-turn-helix DNA-binding domain, suggesting that it could be a Myb family transcription factor (TF) gene. There are seven point mutations that differentiate both alleles, one of which is located in the DNA binding domain. An RNA interference (RNAi) approach was used for functional analysis of the candidate gene. A cDNA sequence of 317 base pairs (bp) was used to design an inverted repeat sequence (IRS) to develop an *Os10g32600*-RNAi construct. The construct was used to transform the *japonica* cv. Nipponbare. Genetic analysis for a single copy of the *Os10g32600*-RNAi transgene in the T_2 and T_3 generations revealed that silencing *Os10g32600* delayed flowering and enhanced seed dormancy. These results revealed that *Os10g32600* is the underlying gene of *qFT10*, and has a pleiotropic effect on seed dormancy in the Nipponbare background. To determine the silencing effects in the EM93-1 background, a Nipponbare

Os10g32600-RNAi transgenic line was crossed with EM93-1 to develop BC_1F_1 and BC_2F_1 populations. Interestingly, silencing of *Os10g32600* delayed flowering in both BC_1F_1 and BC_2F_1 populations. However, silencing of *Os10g32600* enhanced the dormancy in the BC_1F_1 but reduced the dormancy in the BC_2F_1 population. These results suggest that *Os10g32600* could be the underlying gene for both *qSD10* and *qFT10*, but its effect on seed dormancy could be reversed by an unknown factor in the genetic background of Nipponbare. The other possibility could be that there is an unknown seed dormancy gene tightly linked to *Os10g32600* in the narrowed *qSD10* region.

In summary, this research isolated *qSD10* as a Mendelian factor and discovered that *qSD10* collocates with *qFT10* in weedy/wild rice. *qFT10* is underpinned by a Myb family transcription factor gene, which also has a pleiotropic effect on seed dormancy. The effect of this Myb gene on seed dormancy is strongly influenced by unknown factors in the genetic background. In addition, *qSD10* could be underlain by a gene tightly linked to *qFT10*. It is likely that the *qSD10/qFT10* haplotype was selected for early flowering in weedy/wild plants to adapt to hot, humid environments in tropical Asia. The narrowed *qSD10/qFT10* haplotype can be used to improve early flowering varieties for resistance to pre-harvest sprouting in rice.

Chapter 1 Introduction and Literature Review

Seed dormancy is an adaptive trait of both ecological and agricultural importance. This dissertation project aimed to elucidate genetic and molecular mechanisms of seed dormancy regulated by the quantitative trait locus (QTL) *qSD10* in rice (*Oryza sativa* L.), a model plant for grass species. This chapter begins with an introduction of seed dormancy and its related concepts, then review of some background information and recent reports about the research area, and ends with the rationale and objectives of this dissertation.

1.1 Introduction

1.1.1 Seed and disposal unit

A seed is a ripened ovule that is capable of germinating and developing into a new plant (Leubner 2017). The seed can be produced by sexual reproduction in general, or by apomixis in some cases. Seeds vary in structure, size, and composition with species.

Seed structures: A seed in flowering plants (Angiosperms) consists of three basic component tissues: embryo, endosperm, and testa. The embryo is a diploid tissue (2n) developed from a fertilized egg, which is the fusion of a haploid egg cell (n) with a haploid sperm (n), where n is a single set of chromosomes in a diploid (2n) species. A mature embryo may have one or two embryonic leaves, known as cotyledon. Based on the number of embryonic leaves, plants are divided into monocotyledonous and dicotyledonous species. The endosperm is a triploid (3n) tissue developed from a primary endosperm nucleus, which is the fusion of two haploid polar nuclei (2n) with the other sperm (n). A mature seed may have a well-developed endosperm or a degenerated endospermic tissue. Thus, seeds can be divided into endospermic seeds, such as those of

cereal crops, or non-endospermic seeds, such as those of many legume crops. The testa is developed from the inner cell layer of the ovule called integument, and is part of the maternal tissue (2n). The testa is also known as seed coat as it functions as a protective tissue (Fig1.1).

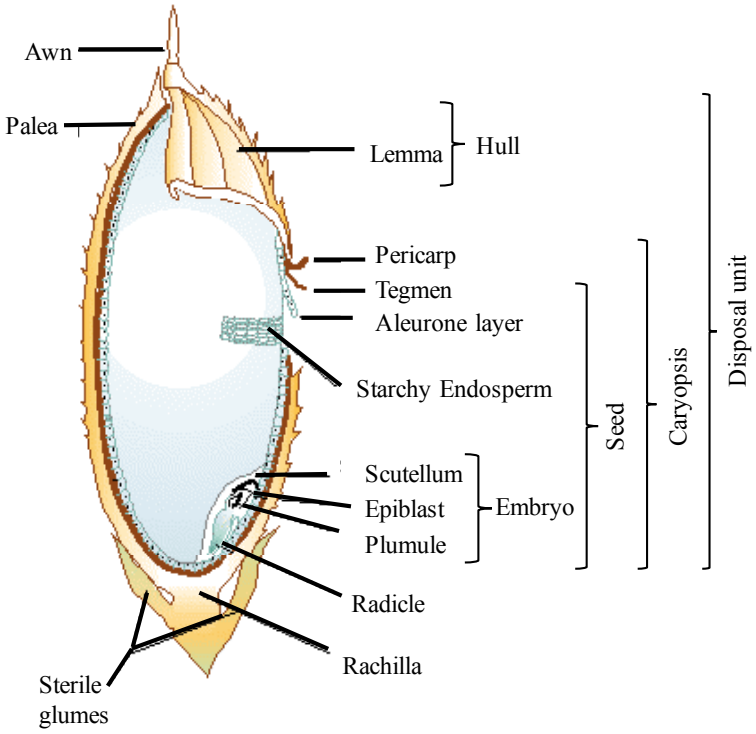

Figure 1.1 Structures of seed, caryopsis and spikelet in rice. This schematic was modified from a diagram at http://jeaheerice.cafe24.com/e_03_01.html by grouping individual parts into a seed, caryopsis or disposal unit.

A seed may refer to a dispersal unit. The structure of a dispersal unit also varies with species. A dispersal unit can be a caryopsis, which is a seed enclosed by the pericarp tissue or fruit coat, such as kernels of corn (*Zea mays*) or common wheat (*Triticum aestivum* L.). A dispersal unit can be a spikelet, which is a caryopsis enclosed by the lemma and palea tissues, such as the spikelet in rice and oats (Figure 1.1). In rice, a

spikelet may have an awn, which is an extended part of the lemma (Figure 1.1). This dissertation used spikelet (Fig1.1) to evaluate the degree of seed dormancy by standard germination testing.

1.1.2 Germination

Germination is the physiological process that initiates with water uptake by a dry seed and terminates with the protrusion of the embryonic axis, usually the radicle, from the covering tissues (Bewley 1997). This process can be divided into three phases based on changes in the water content of an imbibed seed. Phase I is characterized by a rapid increase in water uptake resulting in temporary membrane perturbation, which causes rapid leakage of metabolites into surrounding imbibition solution and the energy metabolism and respiratory activity in the quiescent seed reactive. Phase II (plateau phase) is characterized by the limitation of water uptake. Mitochondria undergo repair and replication, subsequently increasing oxidation reaction corresponding with the synthesis of new mRNA and protein. Phase III is characterized by resumption of water uptake and marked by the protrusion of the embryonic axes from the seed covering tissue (Bewley 1997). Seed dormancy is the most important internal factor that regulates germination. Dormant seeds may have some of the physiological and metabolic activities required for germination, but the radicle fails to elongate. Thus, a dormant seed stays in Phase II. In seed tests, a germinated seed is recognized when the radicle protrudes from the covering tissue at least 3 mm or longer in germination tests (Gu et al. 2003). Because seed dormancy delays germination or reduces the germination rate, the degree of seed dormancy can be measured by germination percentages.

1.1.3 Seed dormancy

Seed dormancy is defined as the temporary failure of a viable seed to complete germination under favorable conditions (Bewley 1997). Seed dormancy is a key adaptive trait of both ecological and agricultural importance. Seed dormancy is a critical checkpoint in plant development, since it regulates the timing and location of germination. For instance, dormant seeds in a seed bank may vary in germination time, which could result in the persistence of weeds when dormancy is delayed, particularly in a poorly managed crop field.

In agriculture, a moderate degree of seed dormancy is important. Domestication and breeding activities tended to reduce seed dormancy because of the selection for rapid uniform germination. An appropriate seed dormancy is required for cereal crops to resist pre-harvest sprouting (PHS), which is the germination on the plant before harvesting. On the other hand, strong seed dormancy would cause a problem in field management, such as non-uniform germination and weed persistence (Baskin and Baskin 2014; Bewley 1997; Finkelstein et al. 2008).

There are several classification systems for seed dormancy. First, based on seed component tissues inhibiting germination, seed dormancy can be classified into embryo dormancy, endosperm-imposed dormancy and maternal tissue-imposed dormancy. In some literature, the endosperm- and maternal tissue-imposed dormancy are grouped as "coat"-imposed dormancy (Gu et al. 2008). Embryo dormancy is the dormancy caused by factors within the embryonic tissue (Kermode 2005). Coat-imposed dormancy is the restriction of germination by the enclosing seed tissues such as the testa, pericarp, or endosperm (Bewley 1997).

Second, based on the timing of dormancy development or induction, seed dormancy can also be classified into primary and secondary dormancy (Finch-Savage and Leubner-Metzger 2006). Primary dormancy is acquired during seed development and is induced by the plant hormone abscisic acid (ABA) within the mother plant. Secondary dormancy is induced if the seed is not exposed to favorable germination conditions (e.g. light and/or nitrate) after primary dormancy is lost (Finch-Savage and Leubner-Metzger 2006). Thus, secondary dormancy is a response to adverse environmental conditions.

Third, based on seed morphological and physiological properties (Baskin and Baskin 2014; Baskin and Baskin 2004), seed dormancy can be divided into five classes (Baskin and Baskin 2014). 1) Physiological dormancy (PD). This is the most common form of seed dormancy found in gymnosperms and angiosperm. PD, caused by physiological status of the embryo, can be divided into three levels, deep, intermediate, and non-deep dormancy. Germination can occur in PD deep dormancy but requires a considerably long period of cold or warm dormancy breaking treatments or stratification. 2) Morphological dormancy (MD) can be found in seeds with underdeveloped embryos, however this can differentiate into cotyledons and hypocotyl-radicle. Dormancy breaking treatments are not required because MD's embryo is not physiological dormant, but needs time to germinate. 3) Morpho-physiological dormancy (MPD) is controlled by both morphological (underdeveloped embryo) and physiological factors. Long periods with dormancy breaking treatments are required before germination can occur. 4) Physical dormancy (PY) is caused by some mechanical characteristics which prevent water uptake, such as the water-impermeable layers of the palisade cells in the seed coat. Mechanical or chemical scarification is required to promote germination of PY by

weakening the embryo covering tissue. 5) Combination dormancy (PY+PD) is controlled by physical (water impermeable seed coats) and physiological (physiological embryo dormancy) components. The dormancy breaking treatment, such as dry storage (after-ripening) or cold stratification, is required to release PY+PD (Baskin and Baskin 2014; Finch-Savage and Leubner-Metzger 2006).

1.1.4 After-ripening

After-ripening refers to the physiological process in which the seed gradually releases the primary dormancy during a period of storage in a natural medium (e.g., soil) or in controlled conditions. Some species after-ripen fast in warm dry conditions, such as rice. Some other species require cold moist conditions to after-ripen or undergo stratification, such as *Arabidopsis* (Holdsworth et al. 2008). The length of the period of after-ripening varies depending on the species. Molecular mechanisms contributing to after-ripening are not yet known. Partial after-ripening in a controlled warm dry environment (e.g., lab room) for a given time can be used to evaluate genotypic variation in seed dormancy in a segregating population by standard germination testing (Gu et al. 2003).

1.1.5 Pre-harvest sprouting

Lack of seed dormancy is considered a major factor for pre-harvest sprouting (PHS) in wheat and other cereal crops. Pre-harvest sprouting is the germination in the plant, after seed maturation but before harvesting, also known as vivipary (Gao et al. 2008; Gubler et al. 2005; Li et al. 2004; Ogbonnaya et al. 2008; Zhang et al. 2014). Pre-harvest sprouting reduces the seed quality and limits seed end-use applications. These result in significant financial loss to farmers and food processors. An appropriate degree

of seed dormancy that allows uniform germination and prevents PHS can lead to maximum grain yield of crops. PHS is highly related to a low degree of seed dormancy (Bewley 1997; Li et al. 2004; Schramm et al. 2010). Therefore, knowledge about the genetic and molecular mechanisms of seed dormancy is fundamental for breeding varieties resistant to PHS.

1.2 Literature review of current knowledge about seed dormancy

1.2.1 Development of primary seed dormancy

Seed dormancy is developed on the mother plant before seed maturation. Seed development can be divided into two phases: morphogenesis (embryogenesis and endosperm development) and maturation (filling, dehydration, physiological maturity). Embryogenesis initiates at the zygotic stage (the formation of the single cell zygote) and ends when reaching to the heart stage (the formation of the embryonic tissue). Next, the embryo is continuing to fill the embryo sac in the embryo growth phase. Cell division in the embryo is arrested once the embryo growth phase finishes. At this point, the seed maturation phase starts (Holdsworth et al. 2008). Seed maturation is a critical period of seed development and coincides with the accumulation of storage compounds, the acquisition of desiccation tolerance, the quiescence of metabolic activity, increased levels of the plant hormone abscisic acid (ABA) and the establishment of embryo dormancy (Holdsworth et al. 2008). It is well established that the transcription factors *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *FUSCA 3* (*FUS3*), *LEAFY COTYLEDON 1* (*LEC1*) and *LEC2* play a crucial role in the regulation of seed maturation and dormancy induction (Graeber et al. 2012; Holdsworth et al. 2008). Mutations in any of these transcription factors cause unusual seed maturation, resulting in reduced dormancy level (Graeber et

al. 2012; Holdsworth et al. 2008). Evidence demonstrated that mutations in *fus3*, *lec1*, and *lec2* cause a continuation of growth in immature embryos and mutation in *abi3* exhibit premature germination (Raz et al. 2001).

Dormancy is induced during seed maturation and increases until the seed is completely developed, then primary dormancy is established (Bewley 1997; Kucera et al. 2005). The induction of seed dormancy is controlled by several groups of regulators including plant hormones like ABA (Finch-Savage and Leubner-Metzger 2006), specific genes, and chromatin regulators (Graeber et al. 2012).

The ABA is considered as a positive regulator of both seed dormancy induction and maintenance (Finch-Savage and Leubner-Metzger 2006). The Arabidopsis *9-cis-epoxycarotenoid dioxygenase* (*AtNCED*), a key gene in a regulatory step during abscisic acid biosynthesis was used to study the importance of ABA in dormancy induction. Expression analysis of the members of the *AtNCED* gene revealed that *AtNCED9* was expressed in both embryo and endosperm during seed development and *AtNCED6* highly expressed at endosperm (Lefebvre et al. 2006). Further study on mutants showed the *Atnced6 -Atnced9* double mutant reduced dormancy phenotype (Lefebvre et al. 2006). These results indicate that ABA synthesized in both embryo and endosperm were required for seed dormancy induction (Lefebvre et al. 2006). Another gene that appears to be a key player of the seed dormancy induction process is *Delay Of Germination 1* (*DOG1*), the first seed dormancy specific gene identified in *Arabidopsis* (Bentsink et al. 2006). In rice, *qSD7-1* and *qSD12* appear to have important roles in inducing seed dormancy (Gu et al. 2011; Gu et al. 2010). The *qSD7-1* underlying gene induces

dormancy by promoting the accumulation of the dormancy-inducing hormone ABA in early developing seed (Gu et al. 2011)

It is widely understood that dormancy is maintained by renewed accumulation of ABA content during imbibition of dormant seed (Finch-Savage and Leubner-Metzger 2006; Finkelstein et al. 2008). To prove the role of ABA in dormancy maintenance, ABA content in dormant and non-dormant seed were investigated in *A. thaliana* ecotype Cape Verde Island (Cvi). The ABA content in dry dormant seed was high when compared to dry non-dormant seed. However, after imbibition, the ABA content of dormant seeds decreased at a slower rate compared to non-dormant seeds (Ali-Rachedi et al. 2004).

In plants, the organization of chromatin appears to also influence gene expression and affect seed dormancy (Graeber et al. 2012). Evidence for the involvement of chromatin modification in regulation of seed dormancy comes from the study of *KRYPTONITE (KYP)/SUVH4* in the control of *Arabidopsis* primary seed dormancy (Zheng et al. 2012). The *KYP/SUVH4* encode for histone methyltransferases to mediate histone H3 lysine 9 dimethylation. Further study of these genes demonstrated overexpression of *KYP/SUVH4* decreased dormancy (Zheng et al. 2012). On the other hand, kryptonite-2 (*kyp-2*) mutant enhanced dormancy, and promoted expression of seed dormancy related genes *DOG1* and *ABI3* (Zheng et al. 2012). Moreover, expression of *KYP/SUVH4* was up-regulated by gibberellic acid (GA) and down-regulated by ABA and epistatic to *DOG1* (Zheng et al. 2012). Those studies indicate that chromatin modification is a component of seed dormancy regulation.

Some evidence also shows that seed dormancy is initiated at early seed development stages and dormancy induction may involve GA. For instance, some author

proposed that *DOG1* promoted GA catabolism at low seed maturation temperatures during the induction of seed dormancy stage, resulting in strong dormancy (Kendall et al. 2011). Recent studies suggested that *DOG1* may use coat-imposed dormancy mechanisms to control seed dormancy (Graeber et al. 2014). Research in rice also found that the loss-of-function mutation of an allele of the GA synthesis gene located on chromosome 1 (*qSD1-2*) could promote dormancy (Ye et al. 2015). These pieces of evidence further indicate a role of GA in seed dormancy induction. However, it is widely accepted that the balance between the levels of ABA and GA hormones are a key factor for controlling induction and maintenance of dormancy and promoting germination (Graeber et al. 2012; Kucera et al. 2005).

1.2.2 Seed dormancy release and germination

Dormancy release and seed germination involve diverse mechanisms and regulators such as plant hormones (GA, ethylene, and brassinosteroids), and seed germination regulator gene (Finkelstein et al. 2008). Dormancy release and germination is determined by low ABA:GA ratios resulting from degradation of ABA and an increase of GA biosynthesis (Ali-Rachedi et al. 2004; Finch-Savage and Leubner-Metzger 2006; Kucera et al. 2005).

GA stimulates germination through many processes: 1) inducing hydrolytic enzyme that can weaken the seed protective tissue like the pericarp or testa; 2) inducing the seed storage mobilization and 3) expansion of the embryo (Finkelstein et al. 2008; Kucera et al. 2005).

Ethylene counteracts ABA effects on seed dormancy by interrupting the ABA signaling and promotes germination by promotion of radial cell expansion in the

embryonic hypocotyl, increased seed respiration, and increased water potential (Kucera et al. 2005). Another plant hormone, brassinosteroids, also promotes germination through enhancing the embryo growth potential, regulating elongation of shoots and photo-morphogenesis of seedling (Finkelstein et al. 2008; Kucera et al. 2005)

The seed germination regulator *MOTHER OF FT AND TFL1* (*MFT*) is a gene that encodes for a phosphatidylethanolamine-binding protein (Qin et al. 2010). The expression of *MFT* is observed in the radicle of germinating seeds and expression is mediated by *ABA-INSENSITIVE3* (*ABI3*) and *ABI5* transcription factor and *DELLA* protein, all part of the GA signaling pathway. Furthermore, *ABI5* was also directly repressed by *MFT*. This work suggests that *MFT* may regulate seed germination in response to ABA and GA through mediating negative feedback regulation of the ABA signaling pathway (Qin et al. 2010).

1.2.3 Genetic mechanisms of seed dormancy

Seed dormancy is a heritable quantitative trait as proved by many studies (Alonso-Blanco et al. 2003; Bentsink et al. 2010; Gu et al. 2003; Guo et al. 2004; Ye et al. 2010). In *Arabidopsis*, more than 20 QTLs associated with seed dormancy have been identified including the *Delay of Germination* (*DOG*) series (Alonso-Blanco et al. 2003; Van der Schaar et al. 1997). The *DOG1* to *DOG7* were identified on RILs derived from crosses between the weakly dormant Landsberg *erecta* (*Ler*) and strongly dormant Cape Verde Island (*Cvi*) accessions. *DOG1*, *DOG2*, *DOG3*, and *DOG6* appeared to have large phenotypic additive effects in *Ler* genetic backgrounds (Alonso-Blanco et al. 2003). Of those, *DOG1* accounted for up to about 19% of the phenotypic variances and have been widely accepted as a major seed dormancy gene.

In rice, there are more than 30 seed dormancy QTLs (Table 1.1) mapped into all of 12 chromosomes (Cai and Morishima 2000; Gu et al. 2006; Gu et al. 2005b; Lin et al. 1998; Subudhi et al. 2012; Wan et al. 2006; Xie et al. 2011; Ye et al. 2010; Zhang et al. 2017). Seed dormancy in rice is controlled by a great number of genes, affecting different levels of dormancy. For example, Cai and Morishima (2000) conducted QTL mapping and identified more than 20 seed dormancy QTLs dispersed on all chromosomes except for chromosomes 4 and 10. Lin et al. (1998) used backcross inbred lines and mapped five putative dormancy QTLs to chromosomes 3, 5, 7 (two loci), and 8 (Lin et al. 1998). In fact, some of rice chromosomes appear to harbor more than two independent dormancy loci such as chromosomes 1, 3, 6, 7, 9, and 11 (Cai and Morishima 2000; Lin et al. 1998; Mispan et al. 2013; Ye et al. 2010; Zhang et al. 2017). Ten genomic regions across the rice genome have also been identified as potentially containing genes responsible for seed dormancy in wild/weedy and cultivated rice, including some not previously reported on chromosomes 4 and 10 (Gu et al., 2004b, Ye et al., 2010) and named as: *qSD1-1, qSD1-2, qSD3, qSD4, qSD6, qSD7-1, qSD7-2, qSD8, qSD10, and qSD12*. Out of those, the seed dormancy QTL locus on chromosome 1(*qSD1-2*) and 10 (*qSD10*) was identified as a new QTL associated with seed dormancy in BC_1F_2 population and accounted up to 11% and 42% of the phenotypic variances, respectively (Ye et al. 2010). The QTL *qSD10* was only detected after other major QTL alleles comprised of *qSD1-1*, *qSD4*, *qSD7-1*, *qSD7-2*, *qSD8* and *qSD12* were removed from the genetic background (Ye et al. 2010), which indicated that *qSD10* effect on seed dormancy was masked by other seed dormancy QTLs; hence remaining undetected in previous reports in rice (Cai and Morishima 2000; Gu et al. 2003; Lin et al. 1998). The QTL *qSD12* on chromosome 12, accounted for 49-

54% of phenotypic variances in BC_4F_2 (Gu et al. 2005a). This research indicates that the weedy/wild rice display divergence in the degree of seed dormancy, which can be an excellent resource to study the natural variation of seed dormancy.

There are several seed dormancy QTLs identified in wheat (Kato et al. 2001; Osa et al. 2003; Torada et al. 2005). One of the major QTLs named *QPhs.ocs-4A* was detected on the long arm of wheat chromosome 4A. Two other wheat QTLs for seed dormancy were mapped to the short and long arms of chromosome 3A and were named *QPhs.ocs3A.1* and *QPhs.ocs3A.2,* respectively. *QPhs.ocs3A.1* had a major effect on seed dormancy and explained up to 38% of the phenotypic variance. The *QPhs.ocs3A.2* had a minor effect and collocated with *TaVp1* gene that is an orthologue to *Vp1* gene of maize. The *Vp1* gene encode for transcription factors associated with dormancy (Osa et al. 2003). By comparative mapping, the *QPhs.ocs-4* and *TaVp1* genes are homologous to the *SD4* gene in barley (Kato et al. 2001) and *Vp1* gene in maize (Osa et al. 2003), respectively.

In barley, seed dormancy locus *SD1* was detected on the centromere region of chromosome 7 (5H) by QTL analysis in a Steptoe x Morex mapping population. *SD1*is considered as a major QTL in barley due to its large and consistent effect on dormancy (Han et al. 1999). Another seed dormancy QTL named *SD2* was mapped near the chromosome 7 (5H) L telomere (Gao et al. 2003). Seeing that *SD2* had a moderate seed dormancy effect, it is a promising QTL to develop moderate levels of seed dormancy in barley (Gao et al. 2003). All the above research suggests that natural variation in seed dormancy exists in model plants, wild, weeds or crops.

Table 1.1 List of seed dormancy QTL reported for 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
qDOR-11-4	11	CDO365-C6a	7	W1944	Cai & Morishima 2000
qDOR-11-5	11	R1465-RG1109	12	W1944	Cai & Morishima 2000
qDOR-11-6	11	RG1109-RZ536	13-16	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

(To be continued)

(Table 1 continued)

Name	Chr[a]	Marker[b]	R^2 (%)[c]	Donor[d]	Reference
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	Nanjing35	Wan et al. 2006
qSDn-5	5	RM480-RM412	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 at al. 2010
qSD3	3	RM520	9-11	SS18-2	Ye at al. 2010
qSD10	10	RM271	8-42	EM93-1	Ye at al. 2010
qSD-1-1	1	RM23	12	cv. N22	Xie et al. 2011
qSD1-2	1	RM488	13	cv. N22	Xie et al. 2011
qSD-2	2	RM525-RM240	8	cv. N22	Xie et al. 2011
qSD-3	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] Chromosome where a QTL was located.

[b] The nearest or flanking markers.

[c] Proportion of the total variance explained by the QTL.

[d] Donor parent of the dormancy-enhancing allele.

1.2.4 Genes underlying seed dormancy QTL

Seed dormancy has been associated with multiple quantitative trait loci (QTL) in several crops and model plants. These QTL varied in their effect on germination and are presumably underlain by genes differentiated during evolution. Cloning and characterization of the dormancy QTL underlying genes could provide in-depth insights into developmental mechanisms of seed dormancy, seed dormancy evolution, and provide candidate genes to manipulate crop varieties for germination capability.

Map-based cloning (a.k.a. positional cloning) is the process of identifying a QTL underlying gene (based on its map position) by narrowing down the QTL to a short DNA sequence, followed by molecular characterization of the sequence for structure, function, and allelic variation of the cloned gene. Some underlying seed dormancy genes have been cloned and functional studies have been performed in *Arabidopsis*, weedy rice, wheat, barley and lettuce (Table 1.2) (Argyris et al. 2011; Bentsink et al. 2006; Graeber et al. 2010; Gu et al. 2011; Liu et al. 2011; Sugimoto et al. 2010; Ye et al. 2015). In 2003, *Delay of Germination 1* (*DOG1*) gene was identified as a locus affecting seed dormancy in *Arabidopsis* (Alonso-Blanco et al. 2003). Three years later, by using a Map-Based cloning approach Bentsink et al (2006) proposed *DOG1* as the first seed dormancy gene that have been cloned. *DOG1* encoded protein with unknown molecular function. The putative orthologous of Arabidosis *DOG1* (*AtDOG1*) gene was identified in Brassicaceae relatives such as *Lepidium sativum* (*LesaDOG1*) and *Brassica rapa* (*BrDOG1*) by cross-species approaches (Graeber et al. 2010). Further analysis at the promotor region found that both *AtDOG1* and *BrDOG1* contain a RY repeat required for *ABI3/VP1*-mediated

gene expression (Graeber et al. 2010; Graeber et al. 2012), which suggested that *DOG1* may regulate germination by a highly-conserved dormancy factor (Graeber et al. 2012).

The molecular mechanism through which some of the dormancy-related genes regulate seed dormancy have been described for a subset of genes. Approaches used to clone and investigate seed dormancy related genes include map-based cloning in different crops, including rice. For example, in rice the *qSD1-2* underlying gene was cloned and pleiotropic effects were observed on seed dormancy and plant height (Ye et al. 2015). The *qSD1-2* locus was mapped into the long arm of chromosome 1 of rice genome (Ye et al. 2010). The *qSD1-2* associated with endosperm-imposed dormancy and was also responsible for variation of plant height (Gu et al. 2015; Ye et al. 2013; Ye et al. 2015). Further *SD1-2* sequence analysis revealed that *SD1-2* gene encoded for *OsGA20ox2* gibberellin synthase which is also known as "the rice green revolution gene" or "*semi-dwarf1* (*Sd1*) gene" (Ye et al. 2015). The *SD1-2* displays loss-of-function mutation, due to deletion of about 382 base pair (bp) in EM93-1, which enhanced seed dormancy and reduced plant height. Additional studies found that the mutant allele of the *sd1-2/ph1* gene reduced seed GA content at the early stage, resulting in decelerate endosperm cell differentiation, delayed abscisic acid accumulation and postponed dehydration (Ye et al. 2015). Therefore, Ye H., et al (2015) proposed that *qSD1-2* may regulate the seed dormancy development through desiccation mechanisms induced by GA particularly within the triploid (3n) tissue (endosperm).

Seed dormancy 4 (*Sdr4*) was previously mapped into rice chromosome 7 and cloned by map-based cloning. Sugimoto et al. (2010) suggested *Sdr4* may act as an intermediate regulator of dormancy during seed maturation because the expression of

Sdr4 was positively regulated by *OsVP1* – a global seed maturation regulator (Sugimoto et al., 2010). The novel protein encoded by *Sdr4* shows no similarity to proteins with known function.

Seed dormancy is a major factor associated with PHS in common wheat (*Triticum aestivum L.*). Wheat seed dormancy gene, *TaSdr*, located on homoeologous group 2 chromosomes and associated with tolerance to pre‑harvest sprouting. Cloning and characterization of this gene showed that *TaSdr* is an orthologues of *OsSdr4* gene by exhibiting 77.6 % of sequence similarity (Zhang et al. 2014).

1.2.5 Associations of seed dormancy with the other adaptive traits

Adaptive traits associated with seed dormancy have been identified in different plant species. In some instances, they have been identified in the same chromosome region as genes associated with seed dormancy. In rice, many reports have shown the association of dormancy QTLs with adaptive and agronomic traits, such as red grain color, black hull color, shattering, awn length, plant height, and flowering time (Gu et al. 2005c; Lin et al. 1998; Mispan et al. 2013; Takeuchi et al. 2003; Ye et al. 2013). For instance, seed dormancy QTL on chromosome 4 (*qSD4*) was confirmed to have co-evolved with other weedy/wild trait like black hull color. Seed dormancy QTL chromosome 8 (*qSD8*) appears to collocate with seed shattering, awn length, and percentage of awn (Gu et al. 2011; Gu et al. 2005c; Mispan et al. 2013) and seed dormancy/plant height QTL clusters were identified at chromosome 1 (*qSD1-2/qPH1*) and chromosome 7 (*qSD7-2/qPH7*) (Ye et al. 2013).

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

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

[a] QTL name

[b] Predicted molecular function of cloned QTL

[c] Predicted physiological function of cloned QTL

Some of the QTL clusters are controlled by tightly linked genes such as seed dormancy/heading date cluster controlled by *Sdr1* and *Hd8* genes (Takeuchi et al. 2003). Other dormancy QTL clusters arise from pleiotropic effect of one single gene. For instance, the association of seed dormancy and pericarp color was linked to *qSD7-1/qPC7* QTL cluster. The *qSD7-1/qPC7* cluster was delimited to the *Os07g11020* or *Rc* locus, encoding for a basic helix-loop-helix transcription factor that regulates abscisic acid and flavonoid synthesis (Gu et al. 2011). Another QTL cluster in rice genome controlled by pleiotropic effect was detected at the *qSD1-2/qPH1*cluster which enhanced seed dormancy and reduced stem elongation (Ye et al. 2013). The *qSD1-2/qPH1* cluster was narrowed to a 20-kb genomic region which encompassed the semidwarf1 (*sd1*) locus for gibberellin synthesis (Ye et al. 2013; Ye et al. 2015).

1.2.6 Seed dormancy and flowering time

Seed dormancy and flowering time are two important adaptive traits. Seed dormancy controls the transition from seed to seedling (germination). Flowering time orchestrates the transition from vegetative to reproductive development to complete the plant's life cycle. Although seed dormancy and flowering time have been investigated in plant species like *Arabidopsis* (Chiang et al. 2009; Huo et al. 2016; Taylor et al. 2017), and rice (Doi et al. 2004; Lin et al. 1998; Takeuchi et al. 2003), little is known how these two traits interact with each other to regulate plant adaptation to diverse ecosystems.

Flowering time predetermines the environmental conditions for the development of seed dormancy. In rice, given that the period of the reproductive stage (panicle initiation to flowering) is relatively constant among cultivars, typically about 35 days, the variation in flowering time is determined during the vegetative stage (seed germination to

panicle initiation) (Gu et al. 2004a; Zhou et al. 2001). Different studies have shown a significant association between seed dormancy and flowering time. For instance, Lin et al. (1998) detected QTLs associated with seed dormancy and heading date in rice BC_1F_5 line derived from a backcross between *japonica* cultivar (Nipponbare) and *indica* cultivar (Kasalath). Five QTLs associated with heading date were detected on chromosomes 2, 3, 4, 6 and 7. Of these the seed dormancy/heading date QTL clusters were detected on chromosomes 3 and 7 (Lin et al. 1998). However, this only detected a significant negative correlation of seed dormancy/heading date QTL clusters on chromosome 3. Several years later, Yano et al., (2001) detected 14 additional QTLs associated with heading date (*Hd1* to *Hd14*) in progeny derived from a cross between Nipponbare (*japonica*) and Kasalath (*indica*) rice. The *Hd1-Hd14* were detected in almost every rice chromosomes, except chromosomes 1, 5, 9, and 11. As previous result, the QTL of *heading date 8* (*Hd8*) was also mapped on to chromosome 3 and tightly linked to QTL of *seed dormancy 1* (*sdr1) (Takeuchi et al. 2003).* Therefore, the tightly linked *Hd8* and *sdr1* would explain the significant correlation between seed dormancy and heading date on chromosome 3 previously identified by Lin et al. (1998).

A key regulator of flowering time, *Early heading date 1* (*Ehd1)* promotes flowering time under short day condition. *Ehd1* function as floral inducer, independent from short day promotion pathway mediated by rice *Heading date1* (*Hd1*) or *Arabidopsis CONSTANS* (*CO*). *Ehd1* gene encodes a B-type response regulator, and its putative orthologue has been identified in *Sorghum* (Murphy et al. 2011). Interestingly, the orthologue of *EHd1* has not been identified in *Arabidopsis* or in temperate cereals such as wheat and barley (Doi et al. 2004; Greenup et al. 2009). *Ehd1* promote flowering by

activating downstream genes such as FT-like genes and MADS-box genes, which are involved in the floral transition (Doi et al. 2004). On the other hand, *Ehd1* was regulated by *OsMADS1*, *OsMADS14 (Doi et al. 2004),* and *OsMADS51* gene, localized upstream (Kim et al. 2007). Kim et al. (2007) proposed that *OsMADS51* transmits the flowering signal from *OsGI* (*GIGANTEA*) to *Ehd1* under the short day promotion pathway. However, some research suggested that *Ehd1* may promote flowering regardless of photoperiod when mediated by *Rice Indeterminate 1* (*Osld1*) (Park et al. 2008). *OsLFL1*(*Late Flowering 1*) has also been reported to interact with *EHd1* to control flowering time. *OsLFL1* appear to suppress the expression of *EHD1* gene, causing the late flowering phenotype (Peng et al. 2007). Moreover, in the promoter region, *EHd1* carries 3 copies of RY *cis*-elements (CATGCATG), which are recognized as a binding site by B3 DNA binding domain of *OsLFL1* (Peng et al. 2007). Many researchers have been studying the effect of *EHd1* gene on flowering time. However, evidence about the effect of *EHd1* on seed dormancy has never been reported.

In *Arabidopsis thaliana*, recent studies discovered that some major flowering time genes regulate seed germination. The *FLOWERING LOCUS C* (*FLC*) was found to mediate germination through genes in flowering pathway such as *FT, SOC1,* and *AP1*. *FLC* repressed *FT* and *SOC1* resulting in low expression of *AP1*. During seed development, low level of *AP1* influences the abscisic acid catabolic pathway (via *CYP707A2*) and gibberellins biosynthetic pathway (via *GA20ox1*) through unknown pathways in germinating seeds (Chiang et al. 2009). Moreover, another study demonstrated a linkage between seed dormancy and flowering time phenotype and

suggested that the major seed dormancy gene *DOG1* can also regulate seed flowering time (Huo et al. 2016).

1.3 Rationale and objectives of this research

qSD10 was one of the seed dormancy QTL identified in advanced generations of populations from a backcross (BC) using the weedy rice line SS18-2 as the donor parent and the cultivar rice line EM93-1 as the recurrent parent. *qSD10* was mapped to a genomic segment of about 5 Mega base pairs (Mbp) and accounted for up to 42% of the phenotypic variance in germination percentage (Ye et al. 2010). Different from many of the seed dormancy QTL identified in the cross, *qSD10* has the dormancy-enhancing allele from EM93-1. Furthermore, recent research in the Seed Molecular Biology Laboratory at SDSU showed that *qSD10* is also associated with flowering time. Interestingly, *Ehd1* was also located in the 5-Mb region but was never reported for an effect on seed dormancy or germination. Seed dormancy develops after floral initiation in the life cycle. It is unknown if the association rises from a tight linkage or from pleiotropic effects of a single gene. Despite intensive study about a relationship between seed dormancy and flowering time little is known about connecting pathway of this relationship to control life history. The answer to these questions is important not only for understanding the evolutionary mechanism of these two adaptive traits but also for crop breeding to manipulate germination ability and flowering time of new cultivars.

Therefore, the objectives of this dissertation project were to: 1) isolate *qSD10* as a Mendelian factor and narrow it with a high-resolution map to identify its candidate genes; and 2) to clone the candidate gene *Os10g32600* and determine its functions for seed

dormancy and flowering in different genetic backgrounds using an RNA interference (RNAi) approach.

It is expected that this research will provide new knowledge on the induction, maintenance or release of seed dormancy, as well as on co-evolutionary mechanism between seed dormancy and flowering time. Novel QTL alleles or candidate genes for crop breeding could be used to improve new varieties for the resistance to pre-harvest sprouting (PHS) and early maturation. The co-evolutionary mechanism between seed dormancy and flowering time may have some implication to weed evolution in agro-ecosystems.

Chapter 2 Fine Mapping *qSD10*, a Quantitative Trait Locus for Seed Dormancy in Rice

2.1 Introduction

Seed dormancy has been associated with some other adaptive traits, such as red grain color in wheat, spikelet morphology in oats, and some wild or crop-mimic characteristics in weedy rice. As expected, most QTL for seed dormancy were collocated or linked with QTL for the interrelated traits, including seed shattering, black hull color, red pericarp color, awn, plant height, and flowering time in weedy rice (Gu et al. 2011; Mispan et al. 2013). Such a phenotypic association could be caused by pleiotropic effects of a single gene or a tight linkage between genes for the associated traits (Gu et al. 2011; Ye et al. 2013). For instance, the gene underlying the QTL cluster *qSD1-2/qPH1* has pleiotropic effects on seed dormancy and plant height (Ye et al. 2015). Also, the association between seed dormancy and heading date was controlled by a close linkage between *Sdr1* and *Hd8* (Takeuchi et al. 2003). Thus, fine mapping is necessary to clone a QTL underling gene and to determine if a QTL has pleiotropic effects on different traits.

Fine mapping is a genetic approach used to delimit the position of a gene or cytogenetic feature on a high-resolution genetic and/or physical map. This approach is particularly useful to delimit quantitative trait locus (QTL) to a short genomic region, identify its candidate gene(s) and dissect it from closely linked genes for other traits. QTL for many adaptive traits, including seed dormancy, tend to collocate or cluster together on the same genomic region in plants. This has been shown, for instance, for corn [*Zea mays* (Capelle et al. 2010)], rice [*Oryza sativa* L. (Cai and Morishima 2002; Gu

et al. 2005c)], sunflower [*Helianthus annuus L.* (Gandhi et al. 2005)], and wheat [*Triticum aestivum L*. (Huang et al. 2006)].

In rice, the QTL *qSD10,* a QTL associated with seed dormancy, is located on a genomic region of about 10 centiMorgan (cM) on chromosome 10 (Ye et al. 2010). *qSD10* was detected in the advanced backcross populations BC_1F_2 (#109) and BC_1F_3 (#109-33). In the BC_1F_3 (#109-33) population of 48 plants, *qSD10* was the only QTL segregating for seed dormancy, which accounted for 42% of the variance in germination percentage (Ye et al. 2010). In addition, *qSD10* was detected in the populations where the dormancy-enhancing alleles of the *qSD1*, *qSD4*, *qSD7-1*, *qSD7-2*, *qSD8* and *qSD12* loci were removed (Ye et al. 2010), indicating that the effect of *qSD10* may vary with genetic backgrounds in rice.

The backcross populations used to detect *qSD10* was made from EM93-1, a weakly dormant genotype of cultivated rice, and SS18-2, a strongly dormant genotype of weedy rice, with EM93-1 being the recurrent parent. A total of 10 QTL for seed dormancy were detected from the BC_1F_1 to BC_1F_3 generations. Of the 10 loci, eight have the dormancy-enhancing alleles derived from SS18-2, and the remaining two (*qSD1-2* and *qSD10*) have the dormancy-enhancing allele derived from EM93-1 (Ye et al. 2010). Map-based cloning demonstrated that *qSD1-2* is identical to *sd1* (Ye et al. 2015). It is known that the dormancy-enhancing allele at *qSD1-2* was retained in the cultivated rice because of the artificial selection for the mutant gene *semidwarf1* (*sd1*) (Ye et al. 2015). However, little is known about the mechanism for which the dormancy-enhancing allele at *qSD10* was retained in the cultivated rice.

The objectives of this study were: 1) to map *qSD10* on a high-resolution map to delimit the seed dormancy QTL; 2) to identify additional traits associated with the delimited *qSD10* region, such as flowering time, in an isogenic background; and 3) to determine physiological mechanisms for the development of seed dormancy regulated by *qSD10*.

2.2 Material and Methods

2.2.1 Plant material and breeding scheme

Advanced backcross (BC) populations: Previous research detected *qSD10* in a BC_1F_2 population and confirmed *qSD10* in a BC_1F_2 plant (#109-33) -derived BC_1F_3 population, which were derived from a BC_1F_1 (EM93-1//EM93-1/SS18-2) population. SS18-2 is a line of weedy rice with strong seed dormancy, while EM93-1 is a line of cultivated rice with weak seed dormancy (Gu et al. 2003). For the BC_1F_3 plant #109-33 it was confirmed with marker genotypes that all other seed dormancy QTL-containing regions were synchronized by the EM931 genome, except for *qSD10* (Ye H., et al. 2010). In this research, a BC_1F_3 plant, which was heterozygous for a *qSD10*-containing region, was advanced to a BC_1F_7 population by self-pollinating from single plants to select for recombinants (Fig. 2.1).

Recombinants and progeny lines: To delimit *qSD10*, five recombinants were selected from the BC_1F_7 to develop the BC_1F_8 progeny lines. Two recombinants from the BC_1F_8 were selected to develop the BC_1F_9 progeny lines. One recombinant from the BC_1F_9 was selected to develop the BC_1F_{10} progeny line and one recombinant from the BC_1F_{10} was selected to develop the BC_1F_{11} line (Fig. 2.1). From these lines, two plants, which were homozygous for the dormancy-enhancing and reducing alleles at *qSD10* from

EM93-1 (IL_{SD10}^{E}) and SS18-2 (IL_{SD10}^{S}), respectively, were selected from the BC_1F_9 (R40-05-03) line as a pair of isogenic lines.

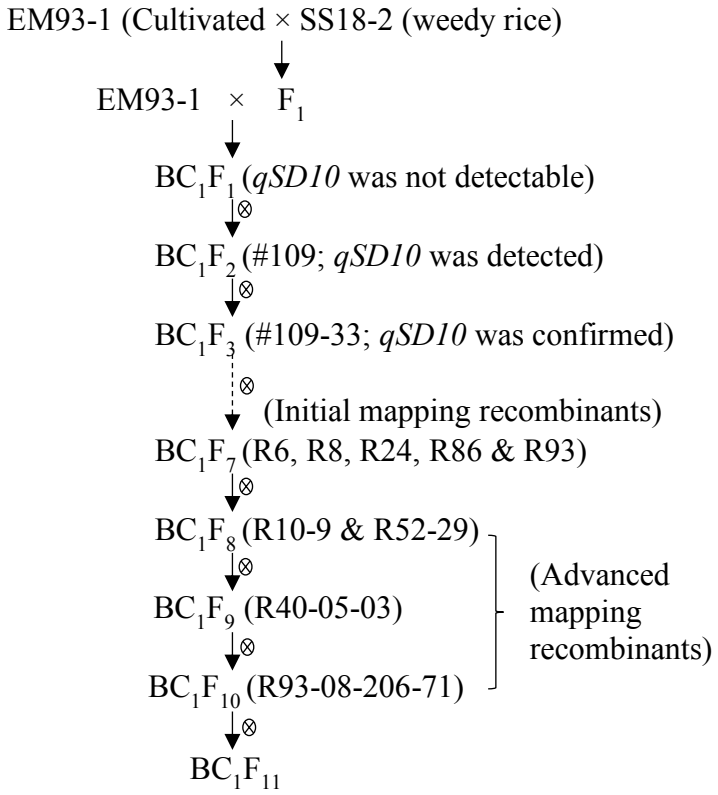

Figure 2.1 Breeding scheme used to select recombinant to delimit *qSD10*. *qSD10* was detected in the single plant (BC_1F_1 plant #109)-derived BC_1F_2 population, where chromosomes or chromosomal segments containing the seed dormancy loci *qSD1-1*, *4*, *7-1*, *7-2*, and *12* were substituted by the genome of the cultivated rice line EM93-1 (Ye et al. 2010). The initial mapping recombinants were selected from single plant-derived BC_1F_7 population where the *qSD1-2* and *8* loci were substituted by the EM93-1 genome.

2.2.2 Plant cultivation and seed harvesting

To develop progeny lines, after-ripened seeds from selected recombinants were germinated at 30°C for 7 days. The seedlings were moved to 200-cell plug trays, with one seedling per cell, and cultured with the rice nutrition solution (Yoshida et al. 1976). After

2 to 3 weeks, the seedlings were transplanted into pots (12 cm× 12 cm × 15 cm dimensions) with one plant per pot. The pot was filled with a mixture of clay soil and greenhouse medium (Sun Gro Horticulture) in a 3:1 ratio. The day/night temperatures were set at 29°/21°C and the relative humidity was 60.8±11.1% in the greenhouse. Flowering time was recorded as the date when the first panicle of a plant exerted from the leaf sheath. Seeds were harvested at 40 days after flowering, air dried in the greenhouse for 3 days, and stored at a -20°C freezer to maintain the status of primary dormancy.

2.2.3 Phenotypic characterization

Seed dormancy: The degree of seed dormancy was measured by germination percentage of partially after-ripened seeds. After-ripening treatment was conducted by storing seed samples at room temperature (25°C) for 7 or 14 days. The time period (d) of after-ripening for a progeny line or a segregating population was determined based on results from a preliminary test, where the mean germination percentage was 40-60 %. Germination testing was conducted using a standard protocol (Gu et al. 2003). A sample of about 50 well-developed seeds was placed in a 9-cm Petri dish, which was lined with a filter paper and soaked with 8 mL water. The Petri dishes with soaked seeds were placed in an incubator for 7 days under conditions of 30°C, 100% relative humidity in dark. Germinated seeds were determined based on the radicle protrusion from the hull of at least 3 mm. Germination percentages of three replicates from each of the plants in a progeny line were averaged to represent the degree of seed dormancy at the days of after-ripening (DAR).

Flowering time: Flowering time was determined by days from seedling to flowering date or day to flowering (DTF).

Plant/leaf morphology: Plant morphology, such as plant height (PH), leaf length (LL), and panicle length (PL) were recorded. PH was performed by measuring the length of the main stem from the base to the top of the leaf sheath of a matured plant, LL was estimated as the mean of the length of the first and second top leaves, and PL was calculated as the average of three normally developed panicles.

2.2.4 Marker development and genotyping

DNA extraction: DNA samples were extracted leaf tissue using the CTAB (Hexadecyl trimethyl-ammonium bromide) method. Young leaves from a plant were cryogenically ground to powder in liquid nitrogen. The ground tissue was placed in a 1.5 ml microcentrifuge tube and incubated with the CTAB extraction buffer (2% Hexadecyl trimethyl-ammonium bromide, 100 mM pH8.0 Tris-HCl, 1.4 M NaCl, 20 mM EDTA, and 0.2% β-Mercaptoethanol) in a water bath at 60°C for 30 min. Following the incubation period, an equal volume of chloroform was added, gently mixed for 5 min, and centrifuged at 13000rpm for 20 min. at 4°C. The supernatant was transferred into a new tube. To precipitate DNA, 0.7 volumes of ice cold isopropanol was added, mixed gently, and incubated at -20°C for 10 min. The sample was centrifuged at 13000 rpm. for 10 min. at 4°C. Supernatant was decanted without disturbing the DNA pellet and subsequently washed twice with ice cold 70% ethanol. DNA pellets were air-dried to remove the residual ethanol and then re-suspended in TE buffer (10 mM Tris pH 8, and 1 mM EDTA) in a water bath at 60°C to completely elute the DNA.

Marker development: Simple sequence repeat (SSR) and insertion/deletion (InD) markers were used to develop a high-resolution map for the *qSD10* region. The primer information on known SSRs were obtained from Gramene database (www.gramene.org).

New InD primers were developed by examining the reference genome sequence of japonica rice (*O. sativa spp* cv. Nipponbare). The new developed InD markers were tested for polymorphism between two parental lines (EM93-1 and SS18-2). Eleven polymorphic InD markers (Appendix 1) were selected for genotyping the recombinants and their progeny lines.

Polymerase Chain Reaction (PCR): The PCR reaction for SSR and InD markers was performed in 20 µL containing 50 ng DNA-template, 4 µL of 5× Green GoTaq® reaction buffer (Promega, Madison,WI), 200 µM of dNTP, 20 µM of forward and reverse primers, and 0.2 unit of Taq polymerase. The PCR cycle consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, and 72°C for 10 min.

Electrophoresis and imaging: The PCR products were analyzed for size on a 6% non-denaturing polyacrylamide gel, containing 6% acrylamide, 0.1% APS, 0.01% TEMED, and 0.5× TBE buffer and separated with ~300 Volt for ~3 hours. The electrophoresis gel was imaged under UV light and recorded using the AlphaEaseFC™ (Alpha Innotech) gel imaging system.

2.2.5 Data analysis and genetic effect estimation

To determine whether a selected recombinant contains the *qSD10* underlying gene(s), marker-trait correlation analysis was performed for genotypic and phenotypic data from the progeny line. A significant correlation indicates that the recombinant was heterozygous for the seed dormancy locus. If the correlation was significant, data from the progeny line were used to estimate gene additive and dominance effect by the linear regression model:

$$Y_{ij} = \mu + ax + dz + \varepsilon_{ij} \quad \text{Equation 2.1}$$

where, Y_{ij} is the trait value for the j^{th} plant of the i^{th} marker genotype; μ is the model mean; x is the dummy variable for the additive component and was coded as -1, 0, and 1 for i = 1 (EM93-1-like homozygote), 2 (heterozygote), and 3 (SS18-2-like homozygote), respectively; z is the dummy variable for the dominance component and was coded as 0.5 for $i = 2$, or -0.5 for $i = 1$ or 3; a and d are the regression coefficients and estimates of the additive and dominance effects, respectively; and εij is the error term of the model . Correlation and regression analyses were estimated using SAS program (Base 2011).

2.2.6 Annotation of predicted genes in a narrowed *qSD10*-containing region

qSD10 was narrowed to a genomic region of ~100 kb based on the reference genome sequence from Gramene database. The Gramene and MSU (Kawahara et al. 2013) databases were used to predict the number of genes encompassed by narrowed *qSD10* region and to annotate each of the predicted genes for their molecular function, protein product, and amino acid length.

2.2.7 Seed moisture content and germination testing

The development time of primary dormancy coincides with the seed maturation programs such as dehydration and acquisition of desiccation tolerance. The two isogenic lines IL_{SD10}^{E} and IL_{SD10}^{S} were used to determine the dormancy development mechanism regulated by *qSD10*. Three biological replicates of the IL_{SD10}^{E} and IL_{SD10}^{S} plants, 21 plants per replicate, were grown in a greenhouse. Spikelets on the main tillers were marked for anthesis dates. About 200 seeds were sampled from each replicate at 20, 25, 30, 35, and 40 days post anthesis (DPA) and divided into two groups to measure seed weight and germination percentage, respectively. To measure the moisture content, the

first group of seeds was weighed immediately after harvest to obtain fresh weight (FW), and weighed again after 72 hours of drying at 105°C to obtain the dry weight (DW). The moisture content as a percentage by fresh weight was calculated as:

$$\text{Seed moisture content (\%)} = (FW–DW)/FW) \times 100 \qquad \text{Equation 2.2}$$

The other group of seeds was after-ripened (air dried) for 7 days and germinated at 30°C and 100% relative humidity in the dark.

2.3 Results

2.3.1 Initial mapping identified association of *qFT10* with flowering time

A positive correlation (r = 0.52) between germination percentage and flowering time was observed in the initial mapping population (Fig. 2.2). The positive correlation indicates that early flowering plants tended to have stronger seed dormancy than the late-flowering plants.

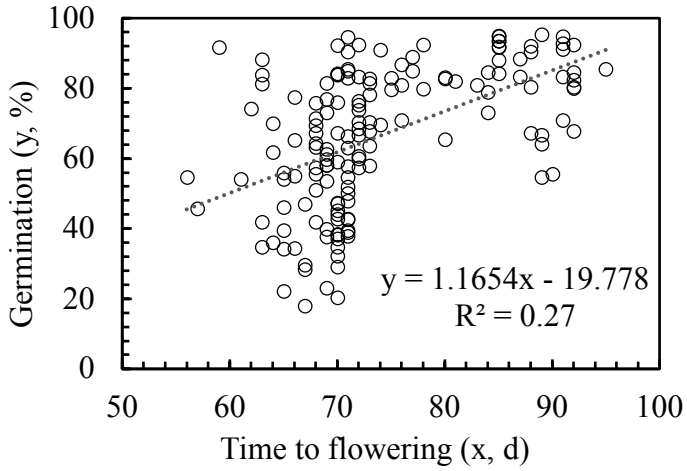

Figure 2.2 Scatter plot of seed dormancy and flowering time in the initial mapping population. R^2 is a goodness-of-fit measure for linear regression model.

About 4000 plants were genotyped to select recombinants for a *qSD10*-containing region of <3 Mb. The five recombinants, R6, R8, R24, R86, and R93, selected from the BC_1F_7 line for initial mapping (Fig. 2.1), were generated from crossovers on a segment of about 2 Mb between RM25483 and RM25574, based on 10 polymorphic markers (Fig. 2.3). The marker- trait (germination percentage) correlation was significant (r_g = 0.45 - 0.68) in the progeny lines of R86, R93, and R24, which are all heterozygous for a genomic region between RM25532 and RM5620, but not in the progeny lines of R6 and R8, which are homozygous (fixed) for the RM25532 - RM5620 region (Fig. 2.3). Results from the five progeny lines revealed that *qSD10* locates in the narrowed region of <400 kb between RM25521 and RM5620. The results also verified that seed dormancy enhancing allele was derived from cultivar parent EM93-1 (Table 2.1), which is consistent with the previous report by Ye et al. (2010). Moreover, the narrowed region was also significantly correlated with days to flowering (r_f = 0.91-0.96), where flowering enhancing allele was derived from EM93-1 (Table 2.1). Therefore, five recombinants can be divided into two groups based on the phenotypic variation of seed dormancy and flowering time in the progeny lines. Group 1 consists of lines R24, R86, and R93, which showed large variation in seed dormancy and flowering time, and these two traits correlated with each other (Fig 2.2). These results indicate that there is a QTL for flowering time, designed *qFT10*, in the *qSD10*-containing region. Group 2 consists of lines R6 and R8, which showed little variation in seed dormancy and flowering time (Fig. 2.3 and Table 2.1).

The initial mapping with these five recombinants confirmed *qSD10*, and also further narrowed the QTL and discovered that the narrowed genome region associates

with both seed dormancy and flowering time, which was not detected in the previous research (Ye et al. 2010).

Phenotypic variation for germination (%) and DTF was observed among individual lines derived from R24, R86, and R93 lines. Genic effects of *qSD10* were varied in different population. For instance, progeny test of R86-derived lines showed strong additive effects (20%), and accounted for large proportion (46%) of the phenotypic variance. On the other hand, R93-derived lines had small additive effects (8%) and strong dominance (15%), and accounted for small proportion (28%) of the phenotypic variance. Similarly, R24-derived lines demonstrated additive (11%) and dominance (11%), and explained small proportion (20%) of the phenotypic variances (Table 2.1). These results indicated that seed dormancy is greatly influenced by environmental factors. The flowering time of progeny lines (R24, R86, and R93), *qFT10* had strong additive effect (9-14 days) and strong dominance (7 days) effect, and accounted for a large proportion (83% to 96%) of the phenotypic variances (Table 2.1).

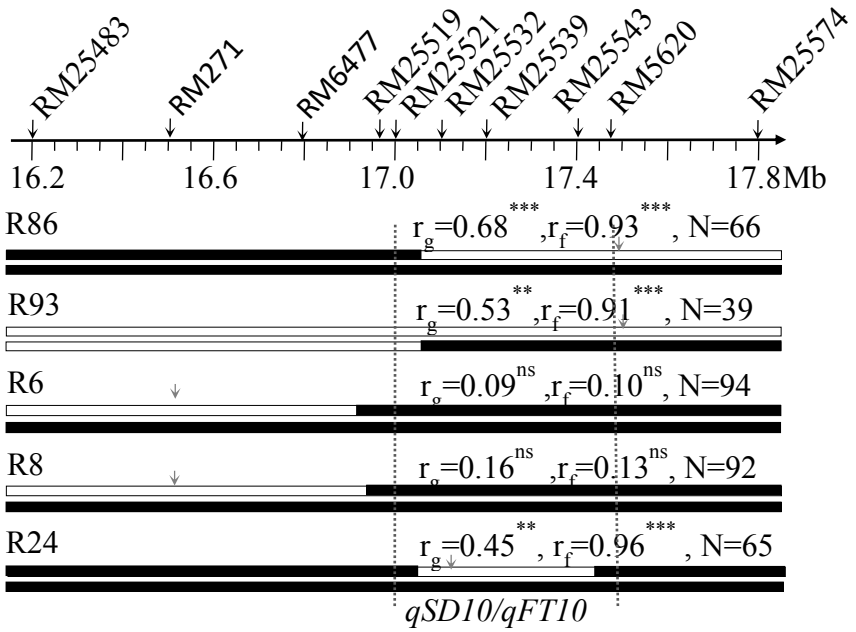

Figure 2.3 Initial map of *qSD10*. Physical map (top), and selected recombinant and progeny lines of *qSD10*. Arrows indicates position of marker on reference genome (Nipponbare). The genotype of recombinants (R#) were represented by chromosomal segments from the parental lines SS18-2 (dark bar) and/or EM93-1 (empty bar). Each recombinant was used to evaluate for seed dormancy, and flowering time by progeny testing. The pointed grey arrow indicate marker used to genotype the progeny line derived from recombinant line. Data shown are correlation coefficients (r) between marker (pointed grey arrow) genotypes and germination percentages (r_g) and, day to flowering (r_f) in the progeny line of N plants. The correlation significance at $P<0.05$, $P<0.01$, $P<0.001$, or non significant are show as *, **, *** or ns. The vertical dotted line show the narrowed *qSD10* region

Table 2.1 Summary of genic effects of *qSD10* on germination and flowering time in progeny line in initial mapping populations.

Recombinant (marker)	Progeny line									
	Geno[a]	n	Germination (%)[b]				Days to Flowering			
			Mean ± SD	*a*	*d*	R^2	Mean ± SD	*a*	*d*	R^2
R86	EE	22	43.6 ± 15.5				66.6 ± 2.8			
(rm5620)	ES	32	61.6 ± 17.3	20.1^{***}	3.5^{ns}	0.46	72.4 ± 2.5	8.6^{***}	-5.4^{ns}	0.87
	SS	12	84.6 ± 8.9				84.8 ± 2.1			
R93	EE	7	76.4 ± 11.4				63.7 ± 2.9			
(rm5620)	ES	19	68.5 ± 18.2	7.6^{*}	-15.2^{**}	0.28	69.4 ± 1.5	10.1^{***}	-6.7^{**}	0.83
	SS	13	87.5 ± 5.6				82.8 ± 5.2			
R6	EE	24	63.7 ± 17.8	Fixed for			73.0 ± 5.9	Fixed for		
(rm271)	ES	45	65.6 ± 17.2	SS18-2-like allele			72.0 ± 7.7	SS18-2-like allele		
	SS	25	68.1 ± 18.0				73.6 ± 5.0			
R8	EE	15	82.8 ± 10.6	Fixed for			77.9 ± 5.2	Fixed for		
(rm271)	ES	48	79.2 ± 11.6	SS18-2-like allele			79.3 ± 5.7	SS18-2-like allele		
	SS	26	82.6 ± 10.0				79.8 ± 3.8			
R24	EE	6	56.6 ± 12.7				71.8 ± 4.9			
(rm25532)	ES	25	63.2 ± 19.0	10.8^{**}	-10.8^{*}	0.2	80.1 ± 2.4	13.8^{***}	-7.5^{**}	0.93
	SS	17	77.8 ± 13.2				108.8 ± 1.8			

[a] Genotype (Geno.) for alleles of the marker locus from the parents EM93-1 (E) and SS18-2 (S);

[b] Gene additive (*a*) and dominance (*d*) effects. A positive *a* or *d* value indicates the allele from SS18-2 increased germination or delayed flowering time. R^2 indicates the proportion of the variance explained by the marker. The superscripts denote the significance at the probability level of 0.05 (*), 0.01 (**), or 0.001 (***), or not significant (ns).

2.3.2 Advanced mapping delimited *qSD10/qFT10* to a genomic region of 100 kb

To further narrow down the *qSD10/qFT10* cluster, four recombinants between RM25527 and RM5620 were selected for progeny testing. R10-9 and R52-29 were selected from the BC_1F_7 lines, R40-05-03 was selected from the BC_1F_8 line, and R93-08-206-71 was selected from the BC_1F_9 line. The marker - trait correlation was significant for germination percentage (r_g = 0.33-0.66, $p<0.001$) and days to flowering (r_f = 0.96-0.98, $p< 0.001$) in progeny lines of R40-05-03 and R52-29, which are heterozygous for a genomic region between markers RM25527 and InD29 and between markers InD47 and InD34, respectively (Fig. 2.4). The marker-trait correlation was not significant for both seed dormancy and flowering time in the progeny lines of R10-9 and R93-08-206-71, in which *qSD10* and *qFT10*-containing region are homozygous for the alleles from SS18-2 and EM93-1, respectively, for the marker InD47 to InD54 region (Fig. 2.4). Results from the progeny test revealed that *qSD10*/*qFT10* collocate on the narrowed region of <120 kb delimited by markers InD47 and InD54 (Fig. 2.4).

The R52-29 population, consisting of 82 plants, varied in germination (%) from 37% to 50%, which allele from EM93-1 reduced germination. The flowering time varied from 60 to 88 days, where allele from EM93-1 promoted flowering time (Table 2.2). In R52-29's progeny lines, *qSD10* additive effect could only be detected and accounted for 11% of the phenotypic variances. *qFT10* had additive effect and relatively small dominant effect, and accounted for a large proportion (96%) of the phenotypic variances (Table 2.2).

The R40-05-03 population, consisting of 72 plants, varied in germination (%) from 55% to 74% and DTF from 62 to 91 days (Table 2.2). The EM93-1-derived *qSD10*

segment reduced germination and promoted early flowering, while SS18-2-derived *qSD10* segment increased germination and delayed flowering (Table 2.2). In the progeny line of R40-05-03, the *qSD10* had additive effect and dominant effect, and accounted for 44% of the phenotypic variances while *qFT10* had additive effect and dominant effect, and accounted for a large proportion (93%) of the phenotypic variances (Table 2.2). A positive significant correlation between germination and flowering time of narrowed *qSD10* region was identified (Fig. 2.5). The linear relationship confirmed that early flowering plant tend to enhance strong degree of seed dormancy, while late flowering plant tend to reduce degree of seed dormancy (Fig. 2.5).

2.3.3 *qSD10/qFL10* candidate genes

The reference genome sequence corresponding to the narrowed *qSD10*/*qFT10* region contains 22 predicted genes (Appendix 2), annotated using the rice genome project database (Kawahara et al. 2013). These genes can be classified into five groups based on molecular functions of predicted proteins (Table 2.3): 1) function unknown (36%), 2) DNA binding (32%), 3) retrotransposon proteins (14%), 4) hydrolase activity (9%), and 5) stress response (9%). The locus *Os10g32600* in group 2 encodes a B type response regulator transcription factor (TF), which was reported to regulate flowering time, named as *Early Heading date 1* or *EHd1* (Doi et al. 2004). The narrowed *qSD10*/*qFT10* region also encompasses the locus *Os10g32810* in group 4 encoding Beta-amylase for abiotic stimulus response (Appendix 2). *Os10g32810* was located nearby InD54 in the narrowed region (Fig. 2.4).

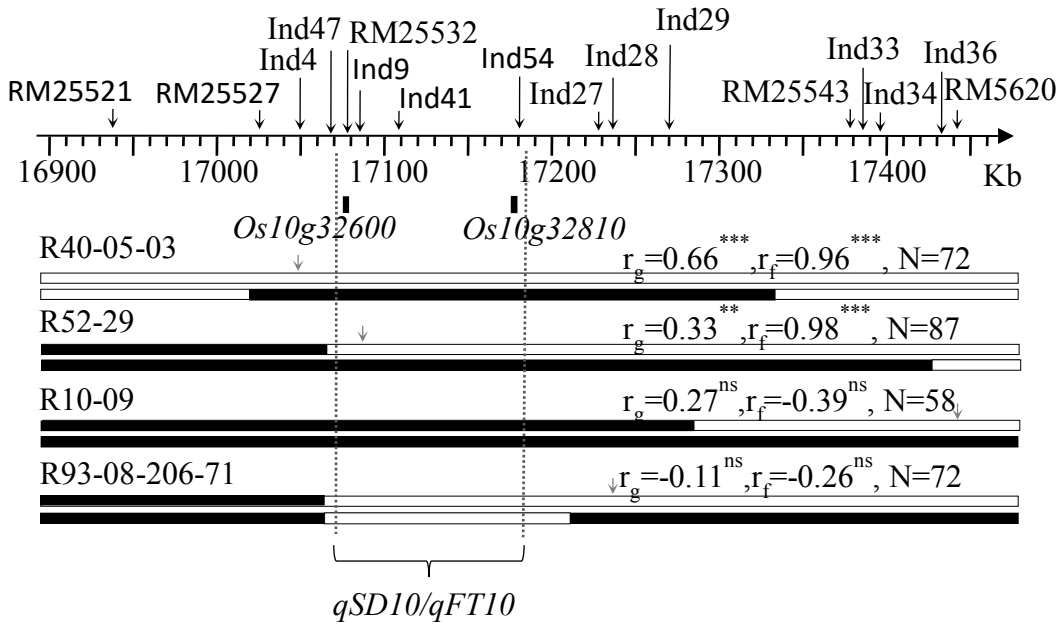

Figure 2.4 Fine mapping of *qSD10*. Physical map (top), selected recombinant and progeny tests of *qSD10.* Arrows indicates position of marker on reference genome (Nipponbare). The genotype of recombinants (R#) were represented by chromosomal segments from the parental lines SS18-2 (dark bar) and/or EM93-1 (empty bar). Each recombinant was used to evaluate for seed dormancy, and flowering time by progeny testing. The pointed grey arrow indicates marker used to genotype the progeny line derived from recombinant. Data shown are correlation coefficients (r) between marker (pointed grey arrow) genotypes and germination percentages (r_g) and, day to flowering (r_f) in the progeny line of N plants. The correlation significance at $P<0.05$, $P<0.01$, $P<0.001$, or not significant is shown as *, **,*** or ns. The vertical dotted line show the narrow *qSD10* region. The filled boxes are the predicted *SD10* candidate genes (*Os10g32600* and *Os10g32810*) based on the reference genome.

Table 2.2 Summary of genic effect of *qSD10* on germination and flowering time in progeny line in advanced mapping population.

Recombinant (marker)	Progeny line									
	Geno[a]	n	Germination (%)[b]				Days to Flowering			
			Mean ± SD	*a*	*d*	R^2	Mean ± SD	*a*	*d*	R^2
R40-05-03 (InD4)	EE	16	54.6 ± 12.8				62.0 ± 4.5			
	ES	36	47.0 ± 13.2	10.6^{***}	-18.3^{***}	0.44	67.9 ± 2.8	14.9^{***}	-9.9^{***}	0.93
	SS	19	74.2 ± 13.1				91.1 ± 1.9			
R52-29 (InD9)	EE	21	36.5 ± 19.5				59.9 ± 1.9			
	ES	51	49.2 ± 14.6	7.7^{**}	7.1^{ns}	0.11	65.4 ± 1.8	13.9^{***}	-5.4^{**}	0.96
	SS	14	50.4 ± 14.5				87.9 ± 2.1			
R10-09 (RM5620)	EE	11	48.9 ± 17.0	Fixed for			84.7 ± 2.1	Fixed for		
	ES	29	42.5 ± 10.7	SS18-2-like allele			82.4 ± 2.1	SS18-2-like allele		
	SS	18	50.3 ± 16.0				82.9 ± 2.2			
R93-8-206-71 (InD27)	EE	20	47.1 ± 9.6	Fixed for			60.9 ± 1.4	Fixed for		
	ES	27	44.6 ± 7.8	EM93-1-like allele			61.2 ± 1.7	EM93-1-like allele		
	SS	23	45.1 ± 10.1				60.3 ± 1.3			

[a] Genotype (Geno.) for alleles of the marker locus from the parents EM93-1 (E) and SS18-2 (S);

[b] Gene additive (*a*) and dominance (*d*) effects. A positive *a* or *d* value indicates the allele from SS18-2 increased germination or delayed flowering time. R^2 indicates the proportion of the variance explained by the marker. The superscripts denote the significance at the probability level of 0.05 (*), 0.01 (**), or 0.001 (***), or not significant (ns).

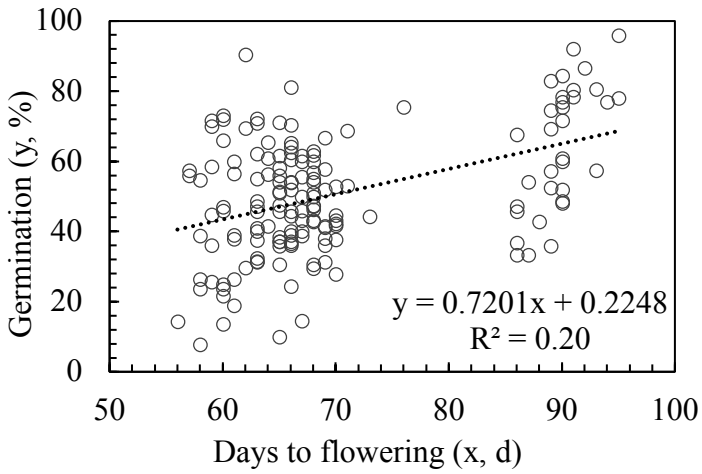

Figure 2.5 Scatter plot of seed dormancy and flowering time in the advanced mapping population. Seed dormancy was evaluated by germination percentage at 7 DAR. R^2 is a goodness-of-fit measure for linear regression model.

Table 2.3 Classification of the 22 predicted genes in the narrowed *qSD10/qFT10*.

Group	Annotated function	Number of genes
I	Unknown function protein	8
II	Protein with binding domain	7
III	Retrotransposon	3
IV	Hydrolase activity protein	2
V	Stress response protein	2

The number of gene and the molecular function were predicted based on MSU Rice Genome Annotation (Osa1) Release 7 (Kawahara et al. 2013).

2.3.4 Genotypic difference in seed moisture and germination ability

To determine the regulatory role of *qSD10* in seed maturation programs, such as dehydration and acquisition of desiccation tolerance, both seed moisture content and germinability were measured for a series of seed samples from the isogenic lines IL_{SD10}^{E} and IL_{SD10}^{S}. Developing seeds were harvested at 5-days interval starting from 20 to 40 DPA. The seed moisture content was 23% at 20 DPA and declined to 16% at 40 DPA in IL_{SD10}^{E}, and was 22% at 20 DPA and 14% at 40 DPA in IL_{SD10}^{S} (Table 2.4 and Fig. 2.6). Overall, the IL_{SD10}^{E} had 1% to 3% higher seed moisture content than IL_{SD10}^{S} during the 20-days period (Table 2.4 and Fig. 2.6). Moreover, IL_{SD10}^{E} gained about 2% germination at 30 DPA and 22% at 40 DPA. Whereas, IL_{SD10}^{S} achieved 5% germination at 25 DPA and 62% at 40 DPA. Overall, IL_{SD10}^{E} had 5% to 40% lower germination percentage than IL_{SD10}^{S} during 15 day period, and took a longer (>5d) period of development to gain a similar level of germinability as IL_{SD10}^{S} (Table 2.4 and Fig. 2.6). The relationship between germination capability and seed moisture was also determined, and found that the germination capability was negatively correlated with seed moisture content on both IL_{SD10}^{E} ($r= -0.94$) and IL_{SD10}^{S} ($r= -0.87$) isogenic lines (Table 2.4). The results suggested that *qSD10* may regulate seed dormancy via the acquisition desiccation tolerance mechanism.

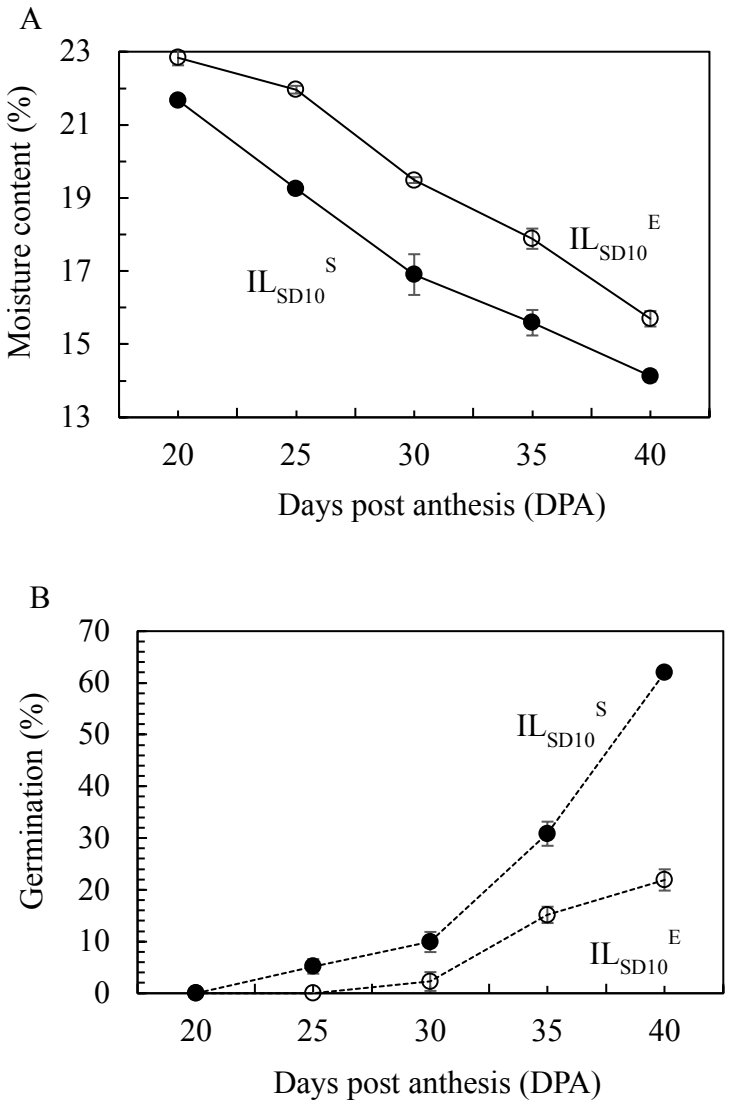

Figure 2.6 Temporal distributions of germination and seed moisture content of IL_{SD10}^{E} and IL_{SD10}^{S}. A, mean (and s.d.) of moisture content of 3 biological replications, 21 plant per replication. B, mean (and s.d.) of germination of 3 biological replications, 21 plant per replication. IL_{SD10}^{E} and IL_{SD10}^{S} are represented by open circles and filled circles, respectively.

Table 2.4 Genotypic differences in germination capability and seed moisture content in IL_{SD10}^{E} and IL_{SD10}^{S}.

Isogenic line	Statistic	Germination (%)					Moisture (%)					corr[a]
		20	25	30	35	40	20	25	30	35	40	
(1) IL_{SD10}^{E}	Mean	0.0	0.0	2.3	15.2	21.9	22.8	22.0	19.5	17.9	15.7	-0.94
	s.e.	0.0	0.0	1.8	1.6	2.1	0.2	0.1	0.1	0.3	0.2	
(2) IL_{SD10}^{S}	Mean	0.0	5.2	9.9	30.8	62.0	21.7	19.3	16.9	15.6	14.1	-0.87
	s.e.	0.0	1.4	2.0	2.3	0.9	0.1	0.2	0.6	0.4	0.2	
(1) – (2)		0.0	-5.2	-7.6	-15.7	-40.1	1.2	2.7	2.6	2.3	1.6	
T-test probability		n.d.	0.04	0.08	0.01	0.00	0.01	0.00	0.02	0.01	0.01	

[a] The correlation coefficient between germination (%) and seed moisture content (%) of IL_{SD10}^{E} and IL_{SD10}^{S}.

Data shown are means and standard error (s.e.).

2.3.5 Associations of the *qS10/qFT10* cluster with plant and leaf morphologies

Plant height, leaf and panicle length, and number of spikelets per panicle were measured in progeny line derived from five recombinants, which are heterozygous for *qSD10* – containing region. The marker-trait correlation was significant ($p < 0.0001$) for plant height in progeny line of all recombinants. The allele of *qSD10/qFT10* from EM93-1 reduced plant height (up to 11 cm.), and explained a large proportion (62% to 80%) of phenotypic variance (Table 2.5). The observed variation of leaf-length was inconsistent. For example, EM93-1-derived allele increased leaf length of progeny lines from R40-05-03, but decreased leaf length of progeny lines from R52-29 (Table 2.6). The correlation was also significant on panicle length, and EM93-1-derived allele increased the panicle length up to 1.8 cm. in progeny lines from R52-29 (Table 2.6). The spikelet per panicle was not different between EM93-1- and SS18-derived allele of *qSD10/qFT10* (Table 2.6). These results suggested that although the EM93-1-derived allele of *qSD10/qFT10* cluster tend to reduce plant height and panicle length but did not reduced number of spikelet.

Table 2.5 Summary of genic effect of *qSD10* on plant height in progeny line derived from selected recombinants.

Recombinant (marker)	Geno[a]	n	Plant height[b]			
			Mean ± SD	*a*	*d*	R^2
R86	EE	22	99.1 ± 5.0			
(rm5620)	ES	32	107.4 ± 6.5	10.6^{***}	-2.4^{ns}	0.78
	SS	12	120.3 ± 5.7			
R93	EE	7	106.1 ± 2.7			
(rm5620)	ES	19	113.2 ± 3.9	8.7^{***}	-1.7^{ns}	0.79
	SS	13	123.5 ± 7.2			
R24	EE	13	72.6 ± 6.5			
(InD9)	ES	35	85.6 ± 7.6	8.9^{***}	1.4^{ns}	0.73
	SS	17	92.7 ± 3.3			
R40-05-03	EE	16	90.7 ± 5.2			
(InD4)	ES	37	94.2 ± 5.6	6.1^{***}	-2.9^{ns}	0.62
	SS	19	102.6 ± 6.1			
R52-29	EE	22	83.4 ± 7.6			
(InD9)	ES	51	91.6 ± 5.3	10.6^{***}	-0.3^{ns}	0.77
	SS	14	105.4 ± 13.2			

[a] Genotype (Geno.) for alleles of the marker locus from the parents EM93-1 (E) and SS18-2 (S);

[b] Gene additive (*a*) and dominance (*d*) effects. A positive *a* or *d* value indicates the allele from SS18-2 increased plant height. R^2 indicates the proportion of the variance explained by the marker. The superscripts denote the significance at the probability level of 0.05 (*), 0.01 (**), or 0.001 (***), or not significant (ns).

Table 2.6 Summary of genic effect of *qSD10* on plant and leaf morphologies in three progeny lines.

Trait	Geno[a]	n	R24		R40-05-03		R52-29
			mean ± SD	n	mean ± SD	n	mean ± SD
	EE	13	29.9 ± 6.7	16	30.4 ± 5.9	22	30.5 ± 4.6
	ES	35	22.3 ± 3.8	37	29.1 ± 6.3	51	27.8 ± 3.5
LL[b]	SS	17	29.6 ± 3.9	19	23.6 ± 3.6	14	36.9 ± 4.5
(cm.)	*a*		-0.1^{ns}		-3.4^{***}		3.2^{***}
	d		-8.0^{***}		2.1^{ns}		-5.8^{***}
	EE	13	17.5 ± 1.1	16	19.9 ± 0.9	22	17.4 ± 1.0
	ES	35	17.6 ± 0.9	37	20.7 ± 1.3	51	17.9 ± 1.1
PL	SS	17	19.2 ± 0.8	19	20.8 ± 1.0	14	20.9 ± 0.9
(cm.)	*a*		0.8^{***}		0.5^{*}		1.8^{***}
	d		-0.76^{**}		3.1^{ns}		-1.2^{***}
	EE	13	124.4 ± 13.6	16	144.9 ± 16.3	22	140.3 ± 16.4
	ES	35	118.2 ± 13.3	37	181.0 ± 33.0	51	140.6 ± 14.6
SPP	SS	17	122.1 ± 11.5	19	135.5 ± 13.9	14	148.6 ± 30.3
(spikelet)	*a*		-0.1^{ns}		-4.7^{ns}		4.2^{ns}
	d		-5.1^{ns}		40^{***}		-3.9^{ns}

[a] Genotype (Geno.) for alleles of the marker locus from the parents EM93-1 (E) and SS18-2 (S);

[b] Gene additive (*a*) and dominance (*d*) effects. A positive *a* or *d* value indicates the allele from SS18-2 increased plant and leaf morphologies. R^2 indicates the proportion of the variance explained by the marker. The superscripts denote the significance at the probability level of 0.05 (*), 0.01 (**), or 0.001 (***), or not significant (ns).

LL: leaf length; PL: panicle length; SPP: spikelet per panicle.

2.4 Discussion

2.4.1 Map position and candidate genes of *qSD10*

A high-resolution map was developed by genotyping more than 4000 plants in advanced progeny lines. This map covers *qSD10* and its flanking regions of about 100 kb. There are 22 predicted genes in narrowed *qSD10/qFT10* region.

Of the 22 genes, *Os10g32600* and *Os10g32810* are most likely the candidate genes for seed dormancy. *Os10g32600* is a Myb TF gene, which is known to regulate flowering time. Seed dormancy genes, exhibiting pleiotropic effect on adaptive trait have been reported for plant height and *qSD1-2* in rice (Ye et al. 2013; Ye et al. 2015) or flowering time and *DOG1, HUB1*, and *RDO2* in *Arabidopsis* (Huo et al. 2016; Liu et al. 2011; Liu et al. 2007). Considering the pleiotropic effects of narrowed *qSD10* on both seed dormancy and flowering, and the location of *LOC_Os10g32600* in the narrowed region, *Os10g32600* gene may also be involved in the development of primary dormancy. Thus, *Os10g32600* was selected as *qSD10* candidate gene and used to confirm the molecular function on seed dormancy in Chapter 3.

The predicted beta-amylase gene *Os10g32810* could also be a candidate gene for *qSD10*. In rice, Beta-amylase is *de novo* synthesized in the endosperm aleurone layer during germination (Okamoto and Akazawa 1979; Wang et al. 1996). In barley, maize, wheat, and rye, Beta-amylase is synthesized and deposited in the endosperm during seed development (Wang et al. 1996). During germination, breaking down of the reserved starch requires an enzyme such as alpha and beta-amylase. Based on its predicted enzyme function and importance of potential enzymatic activity during germination, *Os10g32810* is a candidate gene for further study of *qSD10*.

2.4.2 Pleiotropic or linkage effects of the narrowed *qSD10* region

Isolation of a *qSD10* allele from weedy into cultivated rice identified associations of seed dormancy with flowering time and plant/leaf morphologies. These associations could arise from tight linkage of different genes in the 100-kb region, pleiotropic effects of a single gene, or physiological influence. *Ehd1* is a known gene for promoting flowering. Previous studies have only been discussed *Ehd1*'s effect on flowering (Doi et al. 2004; Doi and Yoshimura 1998) but did not report *Ehd1*'s effect on seed dormancy or germination. This dissertation discovered the relationship between seed dormancy and flowering time as confirmed by linear regression analysis (Fig 2.3 and Fig 2.5).

Furthermore, this dissertation detected variation in plant height and panicle length in lines EM93-1 and SS18-2, each with a different *qSD10/qFT10* allele. These differences were consistent across generations, in which the allele of *qSD10/qFT10* from SS18-2 (late flowering), tended to correlate positively with both plant height and panicle length. Difference in plant height and panicle length could also be a physiological effect of *Ehd1* where late flowering plants have longer period of vegetative growth resulting in increased plant height and panicle lengths.

2.4.3 Environmental influence on *qSD10*

qSD10 and *qFT10* influenced both germination and flowering in segregating populations. As a quantitative trait, seed dormancy is greatly influenced by many unknown environmental factors. In progeny tests, the larger contribution of *qSD10* to the total variance on -which trait- was found from 44% to 46% in R86- and R40-05-03, while relatively smaller contribution range from 11% to 28% was detected in R24, R93, and R52-29 (Table 2.1 and Table 2.2). It is possible that the large contribution observed

in R86- and R40-05-03 was partly caused by the synchronized genetic background. In addition, the large variation in the contribution across generations indicated that *qSD10* is greatly affected by environmental conditions. First, the plant materials were grown in different seasons or location (greenhouse). Second, environmental factors could also affect seed after-ripen, which also influence germinability. Third, the progeny tests on different recombinant lines from different generations (for instance, BC_1F_7-R24 and BC_1F_9-R40-05-03) were conducted in different experiments (time), where mature imbibed seed probably experienced different environmental factor during germination tests. Lastly, genotypic difference of *qSD10* in harvesting time (40 days after flowering) varied with generations or progeny lines. For instance, the difference in flowering time between allele of *qSD10/qFT10* from EM93-1 and SS18-2 was smaller in R86 (18 days) and R93 (19 days), and was larger (37 days) in R24. It is possible that the difference in harvesting date could also influence phenotypic evaluation.

2.4.4 Developmental mechanism of seed dormancy regulated by *qSD10*

This dissertation showed the allele of *qSD10* from EM93-1 promoted flowering, delayed the seed dehydration process, and reduced germination ability (strong dormancy) at maturation. On the other hand, the allele of *qSD10* from SS18-2 delayed flowering, accelerated the dehydration process, and promoted germination ability (weak dormancy) at maturation. In fact, the desiccation process plays a crucial role in switching seeds from the developmental to germination programs (Angelovici et al. 2010). This study found that EM93-1-derived *qSD10* allele had higher seed moisture content and required longer time to gain germination capability than SS18-2-derived *qSD10* allele. Based on the

dissertation observations, it can be speculated that *qSD10* regulates dehydration and desiccation in order to control seed dormancy.

Chapter 3 Molecular Characterization of *Os10g32600,* a Candidate Gene for *qSD10/qFT10*

3.1 Introduction

The quantitative trait locus (QTL) *qSD10* was collocated with a locus for flowering time (*qFT10*), and the *qSD10/qFT10* cluster was delimited to a genomic region of ~120 Kb (Chapter 2). There are 22 predicted genes in the ~120 Kb region (Appendix 2). One of the predicted genes is *Os10g32600*, which is annotated as a Myb family transcription factor (TF) gene. This TF gene was reported as the QTL for heading date, and named as *Early heading date 1* (*Ehd1*), in a cross between an accession of Asian cultivated rice (*Oryza sativa*) and an accession of African cultivated rice (*O. glaberrima*) (Doi et al. 2004; Doi et al. 1998). Thus, *Os10g32600* could be an underlying gene for *qFT10*, but not necessarily for *qSD10*. *Ehd1* was not reported for an effect on seed dormancy or germination (Doi et al. 2004; Doi et al. 1998). Therefore, *Os10g32600* was selected as the first candidate gene for *qFT10* and/or *qSD10* and to further determine if this TF gene is required for seed dormancy.

RNA interference (RNAi) -mediated gene silencing is a mechanism for post-transcriptional regulation of gene expression observed in many eukaryotic organisms. The mechanism is initiated by the presence of double-stranded RNAs (dsRNAs) or hairpin RNAs (hpRNAs), which are recognized and then cleaved by a dsRNA-specific RNAse called Dicer, or a Dicer-like enzyme complex, into 21- to 24 nucleotide RNA molecules called small interfering RNA (siRNA). The siRNA is then recruited by an argonaute protein into a RNA induced silencing complex (RISC) to trigger the degradation of a target mRNA to block its translation (Baulcombe 2004; Miki and

Shimamoto 2004; Watson et al. 2005). This process can be used to silence gene expression in organisms, and the RNAi technique has been used to silence seed dormancy genes cloned from weedy rice in our laboratory.

EM93-1 is an early maturation line of the *indica*-type cultivated rice and carries a *qSD10/qFT10* haplotype that enhances seed dormancy and promotes flowering. It was also reported that Nipponbare, a *japonica*-type cultivar frequently used as a recipient for transformation, carries a functional allele of *Os10g32600* that promotes flowering. Thus, the objectives of this research were: 1) to clone *Os10g32600* from weedy and cultivated rice to identify allelic variation between the isogenic lines of *qSD10*; 2) to develop transgenic lines for an *Os10g32600*-RNAi construct and evaluate the silencing effects in the genetic background of Nipponbare; and 3) to transfer the *Os10g32600*-RNAi transgene into the genetic background of EM93-1 and evaluate the silencing effects on seed dormancy and flowering time.

3.2 Material and methods

3.2.1 Plant genotypes

Three genotypes were selected for this research: EM93-1, IL_{SD10}^{S}, and Nipponbare. EM93-1 (*O. sativa* L. subsp. *indica*) is the parental line that was used to map *qSD10* in the previous research. EM93-1 contains the allele at the *qSD10/qFT10* cluster that enhances seed dormancy (reduces germination) but promotes flowering. IL_{SD10}^{S} is isogenic to EM93-1 (IL_{SD10}^{E}) and contains the *qSD10/qFT10* allele that promotes germination but delays flowering from the weedy rice line SS18-2. Both EM93-1 (IL_{SD10}^{E}) and IL_{SD10}^{S} were sequenced to identify allelic variation of *Os10g32600*. Nipponbare (*O. sativa* L. subsp. *japonica*) is a cultivar that was reported to carry a

functional allele of *Os10g32600* or *Ehd1* to promote flowering (Doi et al. 2004). Nipponbare was used as a recipient for transformation.

3.2.2 Cloning and sequencing of genomic DNAs from the *Os10g32600* locus

The *Os10g32600* gene was isolated from EM93-1 (IL_{SD10}^{E}) and IL_{SD10}^{S} by PCR. Genomic DNA samples were prepared from young leaves using a CTAB method described in Chapter 2.2.4. The Phusion high-fidelity polymerase enzyme (Thermo Scientific) was used to amplify a large fragment of the genomic DNA. PCR primers were designed based on the reference genome (Nipponbare) for *Os10g32600* (Appendix 3) at Gramene database (http://www.gramene.org). The PCR reaction was performed in a volume of 50 μL containing 10 μL of 5× HF buffer, 100 ng DNA-template, 200 μM of dNTPs, 0.5 μM of forward and reverse primers, and 1 unit of Phusion DNA polymerase. The PCR reaction was conducted by an optimized protocol consisting of: 1) initial denaturation at 98°C for 30 s, 2) 35 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C at a rate of 15-30 s/kb, and 3) the final extension at 72° for 10 min. Amplified fragments were separated on 1.2% agarose gel and visualized under UV light. Expected fragments were excised from the gel under UV light and purified by a GelElute Extraction kit (5 Prime Inc., Gaitherburg, MD, USA).

The purified PCR fragments were directly sequenced with the PCR primers using a next-generation sequencing system at GenScript®, USA. The SeqMan program (DNASTAR®) was deployed to align and merge the sequencing data. The sequence assemblies were annotated with the program FGENESH (http://www.softberry) to predict gene structures. Finally, sequence assemblies of SD_{10}^{E} and SD_{10}^{S} were aligned by SeqMan program (DNASTAR®) to identify allelic variation.

3.2.3 Cloning and sequencing of full-length cDNAs of *Os10g32600*

Total RNA samples were prepared from flag leaves emerged on the flowering date using TRI Reagent (Sigma-Aldrich Co.). Four μg of the RNA was used to synthesize complementary DNAs (cDNAs) by reverse-transcription using the SuperScript III First-Strand synthesis system (Invitrogen Co.). Based on a gene model in the Gramene database, *Os10g32600* is predicted to be 5,246 bp in length and contain five exons and four introns (www.gramene.org). Thus, PCR primers cEHd1-F and cEHd1-R (Appendix 3) were designed based on the Gramene model to clone full-length cDNAs of *Os10g32600* from IL_{SD10}^{E} and IL_{SD10}^{S} by PCR. The PCR products were directly sequenced using the PCR primers (Appendix 3). The cDNA sequences were aligned against their genomic DNA to develop the gene structure, predict functional domains of the deduced proteins using the Conserved Domain Database (Marchler-Bauer et al. 2010) and other bioinformatics software including PROSITE (http://www.expasy.org/prosite/).

3.2.4 Development of an *Os10g32600*-RNAi vector for gene silencing

The primers EHd1-RNAi-F and EHd1-RNAi-R (Appendix 3) were designed based on *Os10g32600*'s coding sequence to amplify a cDNA fragment of 317 bp from EM93-1 by PCR and then to generate an inverted repeat sequence (IRS) for the RNAi experiment. The 4-bp CACC was added to the 5' end of the forward primer (Appendix 3) to use the topo cloning protocol (Invitrogen Co.). The amplified fragment was cloned into the Gateway pENTR/D-TOPO cloning vector (Invitrogen Co.) at the position between the recombination sites *attL1* and *attL2*, which is necessary for LR Clonase reaction. The structure of the amplified fragment that linked to the cloning vector called Entry clone (Fig3.1). The amplified fragment in the Entry clone was transferred to pANDA-β

destination vector by LR Clonase reaction. The pANDA-**β** destination vector carryies a g*us* linker located between two recombination sites (*attR*) in the sense and antisense orientation and contains the kanamycin and hygromycin resistance genes for selection (Fig 3.1). During the LR Clonase reaction, the amplified fragment from the Entry clone was inserted into pANDA-**β** vector to generate the IRS at the *attR* recombination site (between *attB1* and *attB2*) flanking the g*us* linker. The pANDA-**β** vector was used to transform Nipponbare calli using *Agrobacterium*-mediated transformation system (http://agron-www.agron.iastate.edu/ptf/protocol/rice.pdf). The transformed plantlets were selected using selective genes for resistances to hygromycin.

3.2.5 Purifying *Os10g32600*-RNAi transgenic lines and hybridization experiment

T_0 to T_3 generations in the Nipponbare background: The first generation (T_0) of plantlets from 10 transgenic events were grown in the greenhouse at conditions described before (Chapter 2.2.2). The T_0 plants were tested for the resistance (R) or susceptibility (r) to Hygromycin B. Single R T_0 plants were selected to develop individual T_1 lines by self-pollination and single-plant-selection techniques (Fig. 3.2).

All seedlings from each of the generations were tested for hygromycin resistant performance and sorted into the hygromycin resistant (R) and hygromycin susceptible (S) groups. Four T_2 lines, T149-8, T149-9, T149-13 and T149-33, derived from a T_1 plant (T149) were self-pollinated and seeds harvested for further evaluation. The two T_3 lines (T149-33-19 and T149-33-38) derived from T_2_T149-33 were selected based on the hygromycin resistant test. The T_3_T149-33-19 and T_3_T149-33-38 lines that classified into the R (IL_{Nip}^{RNAi}) and S (IL_{Nip}^{WT}) groups, respectively were used to evaluate for functions of the transgenes in the Nipponbare (japonica) background (Fig 3.2).

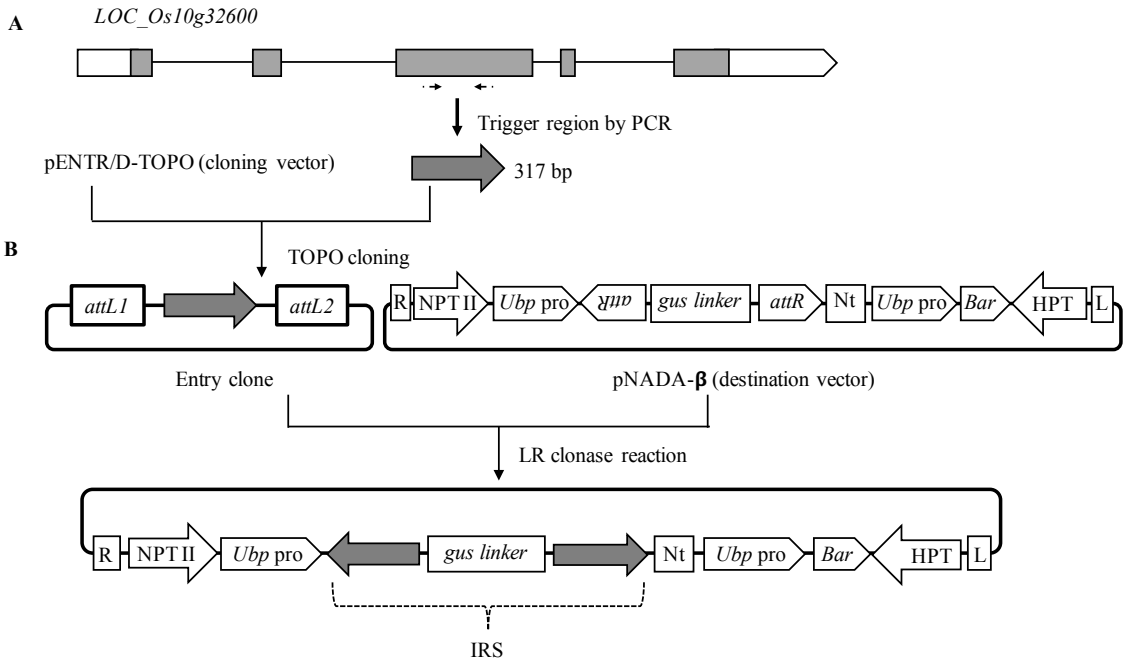

Figure 3.1 *Os10g32600*-RNAi vector construction and pANDA-**β** vector. A. Gene structure of the *Os10g32600* locus. Boxes and line fragments indicate exons(filled)/untranslated regions (open) and introns, respectively. Arrows indicate approximate positions of PCR primers used to amplify a DNA fragment from exon #3, which was selected to design an invert repeat sequence (IRS), trigger sequence (filled arrow), to silence the gene by RNA interference (RNAi). **B**. Vector construction to clone the IRS for RNAi. The trigger PCR fragment was cloned into pENTR/D-TOPO cloning vector, which carries two recombination sites (*attL1 and attL2*) for LR clonase reaction. In LR clonase reaction, the trigger fragment was transferred into pANDA-**β** vector, which contained R: border right; NPT II: Kanamycin resistance gene; Ubq pro.: Maize ubiquitin1 promoter; *gus* linker; Nt: NOS terminator, Bar: bialaphos resistance gene; HPT: Hygromycin resistance gene, and L: left border

Hybrid F_1 generation: the T_2_T149-33-4 line (R group), designated Nip^{RNAi} (Fig 3.2) was crossed with EM93-1 to develop the hybrid F_1 generation. The F_1 plants were tested for hygromycin resistance performance and sorted into the R and S groups.

BC_1F_1 and BC_2F_1 populations: to develop the BC_1F_1 (EM93-1//EM93-1/Nip^{RNAi}) generation, the F_1-15 line (R group), designated EM93/Nip^{RNAi} (Fig 3.2) was crossed with EM93-1. To develop the BC_2F_1 (EM93-1///EM-93-1//EM-93-1/Nip^{RNAi}) generation, the BC_1F_1-21 line (R group) (Fig 3.2) was crossed with EM93-1. Seedlings from BC_1F_1 and BC_2F_1 were sorted into the R and S groups. Two BC_2F_1 lines (BC_2F_1-17 and BC_2F_1-47) were selected to develop the BC_2F_2 populations to verify the function of the transgene.

Plant cultivation: A split-tiller technique was applied to increase the number of plants in the F_1 and BC_1F_1 generations.

To develop transgenic lines, seeds from selected transgenic plants were after-ripened and germinated at 30°C for 7 days. The seedlings were moved to 200-cell plug trays, with one seedling per cell, and cultured with the rice nutrition solution (Yoshida et al. 1976). After 2 to 3 weeks, the seedlings were transplanted into pots (12 cm× 12 cm × 15 cm dimensions) with one plant per pot. The pot was filled with a mixture of clay soil and Sunshine greenhouse medium #1 (Sun Gro Horticulture) in the 3:1 ratio. The day/night temperatures were set at 29°/21°C and the relative humidity was 60.8±11.1 % in the greenhouse. Flowering time was recorded as the date when the first panicle of a plant exerted from the leaf sheath. Seeds were harvested at 40 days after flowering, air dried in the greenhouse for 3 days, and stored at a -20°C freezer to maintain the status of primary dormancy

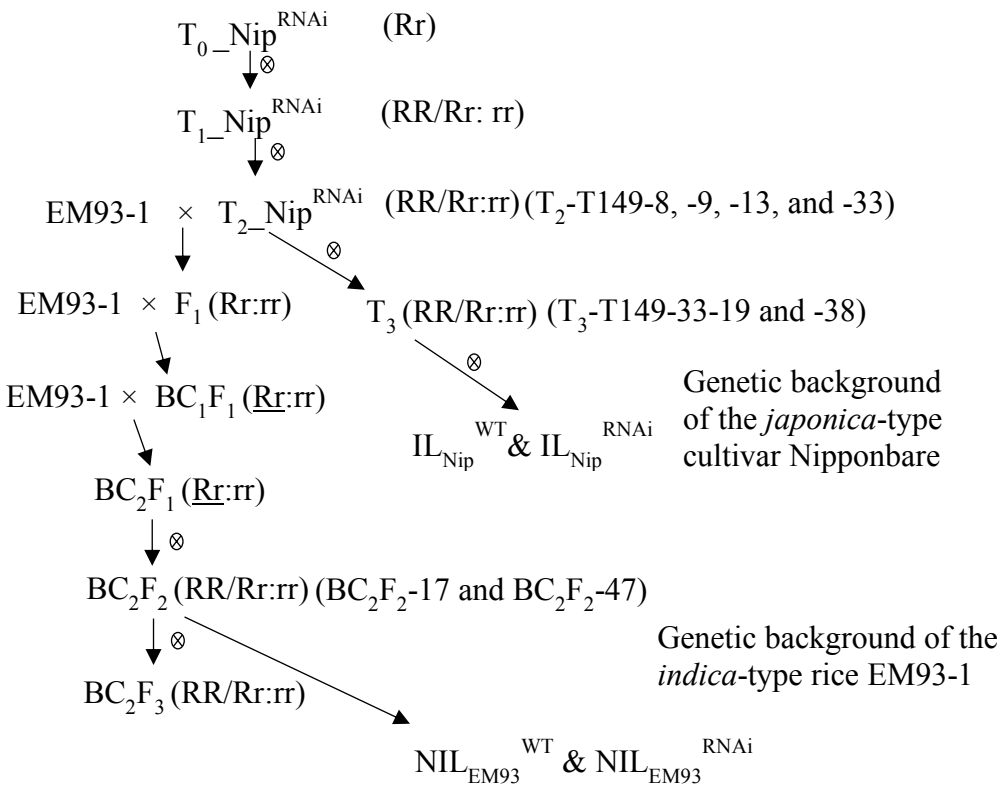

Figure 3.2 Breeding Scheme used to develop populations segregating for the *Os10g32600*-RNAi transgene. The transgene in the Nipponbare (Nip^{RNAi}) background was purified from the T_0 to T_3 generations. Plants with and without the transgene were identified by the resistant (R) and susceptible (r) responses to Hygromycin B, respectively. In addition, a T_2 R line was crossed with EM93-1 to introduce the transgene into the EM93-1 background by backcrossing. Near isogenic (transgenic) lines of EM93-1 S group ($NIL_{EM93}{}^{WT}$) and R group ($NIL_{EM93}{}^{RNAi}$) were selected from BC_2F_2 lines to test for responses on applied GA_3. All these generations of plants were evaluated for seed dormancy and flowering time.

3.2.6 Transgenic plant identification by the Hygromycin resistance

The *Os10g32600* RNAi transgene was constructed with the hygromycin resistance gene *HPT* in a vector for transformation (Fig. 3.1). Thus, the hygromycin resistance was used to determine if a plant carries the transgene in each of the generations.

A fresh leaf fragment at about 2 cm from the leaf tip was excised from a seedling and immediately placed on the medium in a sealed Petri dish. The hygromycin medium, contained 15 ug/mL Hygromycin B (invitrogen) and 1.5% Phytagel (Sigma-Aldrich Co.). The Petri dish containing lined leaf fragments from a population was placed in an incubator set at 24°C and 16/8 h of light/dark for 7 days. After 7 days, the response of a leaf fragment to the hygromycin medium was recorded as resistant (R) for the green leaf color, or as susceptible (S) for yellow-brown necrotic spots. The R plants were positive for the transgene, while the S plants are wild-type, or negative for the transgene. Seedlings were then sorted into the hygromycin resistant (R) and hygromycin susceptible (S) groups.

3.2.7 Genotyping of transgenic lines

To verify the hybrid's genotype, all seedlings of F_1, BC_1F_1, and BC_2F_1 were genotyped with the marker InD11. This marker was designed for a 219-bp fragment from the 4th intron of *Os10g32600* (Appendix 3), and using gDNA of EM93-1 and Nipponbare as control. DNA extraction and PCR reaction was performed following the methods described in Chapter 2.2.4.

3.2.8 Phenotypic identifications

Phenotypic assessments for seed dormancy and time (days) to flowering were conducted following the methods described in chapter 2.2.3. The degree of seed dormancy was measured by germination percentages of partially after-ripened seeds. The after-ripening treatment was conducted by storing seed samples at the room temperature (25°C) for 7 or 14 days. The time periods (d) of after-ripening for a progeny line or a segregating population was determined when the mean germination percentage was 40-60 % in a preliminary test. Germination testing was conducted using a standard protocol (Gu et al. 2003). A sample of about 50 well-developed seeds were placed in a 9-cm Petri dish, which was lined with a filter paper and soaked with 8 mL water. The Petri dishes with soaked seeds were placed in an incubator for 7 days under conditions of 30°C, 100% relative humidity in dark. Germinated seeds were determined based on the radicle protrusion from the hull at least 3 mm. Germination percentages of the three replicates from each of the plants in a progeny line were averaged to represent the degree of seed dormancy for the plant at the days of after-ripening (DAR).

Flowering time and plant height of individual lines were determined following methods described in Chapter 2.2.3.

3.2.9 Statistic analysis and transgene effect evaluation

To verify whether a R line was carrying one (Hemizygous or Rr) or two (Homozygous or RR) copies of the *Os10g32600*-RNAi construct, seedlings from a single plant-derived progeny line were determined for segregation patterns of hygromycin resistance and susceptibility. Based on the hygromycin test, T_2-T149-13 and T_2-T149-33 were grouped into three groups: wild type or nullizygous (rr), which was susceptible to hygromycin and did not carry RNAi construct, hemizygous (Rr), and homozygous (RR).

Linear correlation analysis was deployed to estimate genic effect by the marker-trait association in each of the progeny transgenic lines.

Data from the T_2-R149-13 and T_2-R149-33 lines, were used to estimate additive and dominance effects by the linear regression model indicated in chapter 2.2.5.

$$y_{ij} = \mu + ax + dz + \varepsilon_{ij} \quad \text{Equation 3.1}$$

Where, y_{ij} is the trait value for the j^{th} plant of the i^{th} marker genotype; μ is the model mean; x is the dummy variable for the additive component and was coded as -1, 0, and 1 for i = 1 (hygromycin susceptible or rr), 2 (hygromycin resistant heterozygote or Rr), and 3 (hygromycin resistant homozygote or RR), respectively; z is the dummy variable for the dominance component and was coded as 0.5 for $i = 2$, or -0.5 for $i = 1$ or 3; a and d are regression coefficients and estimates of the additive and dominance effects, respectively; and εij is the error term of the model.

The Student T-test was used to determine the significance of difference between the R and S groups in the progeny lines except for the above-described T_2-R149-13 and T_2-R149-33 lines. A significant difference indicates that the transgene was functional or the RNAi silencing system worked in the plants.

Chi-Square test was used to determine whether an observed R and S segregation ratio followed Mendelian's expectation of the 3:1 or 1:1 ratio. The results from the Chi-Square test was also used to infer the copy number of the RNAi construct in a transgenic line.

3.2.10 Transcriptional analysis

Quantitative Real Time PCR (qRT-PCR) was conducted to quantify transcriptional profile of *Os10g32600* between wild type (S group) and RNAi-induced mutant plants (R

group). In the Nipponbare background T_3 generation, the S group (wild-type or Nip^{WT}) was compared with the R group (Nip^{RNAi}). For the BC_1F_1 population that had hybrid genetic background, the hybrid S was compared with the hybrid R group. Total RNA was extracted from the flag leaves on the date of flowering, with three biological replications for each of the groups. PCR primers were designed based on the *Os10g32600* sequence (qPCR-2_EHd1_F and qPCR-2_EHd1_R) or the *Actin* sequence (the experimental control) (Appendix 3). The qRT-PCR reaction was performed in 20 µL containing 2 µL cDNA, 10 µL 2× Power SYBR® Green PCR Master Mix (AppliedBiosystems Co.), 2 µL of each 1.25 µM forward and reverse primers, and 4 H_2O. qRT-PCR was performed using an ABI 7600HT Fast Real-time PCR system. The qRT-PCR conditions were 1 cycle of 50°C for 2 min, then an auto increment step for 10 min at 95°C, followed by 40 cycles of 15 second at 95°C, 30 second at 60°C, and 30 second at 72°C. The final cycle threshold (C_T) of target gene was used to normalize against the C_T of A*ctin*. The relative expression of *Os10g32600* or *EHd1* is calculated as $2^{-\Delta CT}$

$$\Delta C_T = C_{T,\ EHd1} - C_{T,\ actin} \qquad \text{Equation 3.2}$$

3.2.11 Germination responses to exogenous GA

To determine the possible relation of *Os10g32600* with the plant growth hormone gibberrelin (GA) during the development of seed dormancy and germination, near isogenic (transgenic) lines of EM93-1 S group ($NIL_{EM93}{}^{WT}$) and R group ($NIL_{EM93}{}^{RNAi}$) (Fig 3.2) were tested for responses on applied GA_3 (Acros Organic, NJ).

Previous studies showed involvement of GA in regulation of both seed germination and flowering characteristic. For instance, rice' GAMYB gene, a positive transcriptional regulator of GA-dependent α-amylase in the aleurone cells, was reported

to be important for floral organ and pollen development (Kaneko et al. 2004). The result in chapter 2 of this dissertation revealed that the allele of *Os10g32600* from EM93-1 (IL_{SD10}^{E}) delayed germination and promoted flowering time. The hypothesis of this experiment was that *Os10g32600* may regulate germination by disrupting GA biosynthesis or signal transduction. To prove the hypothesis, a germination test was conducted using seeds from S (NIL_{EM93}^{WT}) and R (NIL_{EM93}^{RNAi}) group of BC_2F_1 (Fig 3.2) with GA_3 solution.

For germination testing, mature dried seeds without an after ripening treatment were soaked with the GA_3 solution at difference concentrations 0 (control), 0.1, 1.0, and 10 μM, then incubated for 7 days at 30°C and 100% relative humidity in the dark. The germination test was conducted by using three replications of 50 well-developed seeds of 12 plants from each of the NIL_{EM93}^{WT} and NIL_{EM93}^{RNAi}.

3.3 Results

3.3.1 Allelic difference of *Os10g32600* in genomic DNA (gDNA) sequence

In the initial experiment, we sequenced the 1883 bp gDNAs from IL_{SD10}^{E} and IL_{SD10}^{S}, based on a gene model of *Os10g32600* at MSU Rice Genome Annotation (Osa1) Release 7 (Kawahara et al. 2013). These two sequences represent the alleles of *Os10g32600* from the parental lines EM93-1 and SS18-2, designated $SD10^{E}$ and $SD10^{S}$, respectively. It was predicted based on the program FGENESH (www.softberry.com) that both alleles consist of three introns and four exons including 5' and 3' untranslated regions (UTR) and designated gene model #1 (Fig 3.3). The sequence comparison showed seven single nucleotide polymorphism (SNP) between the two alleles (Table 3.1). Of the 7 SNPs, only the G/A substitution is present in Exon #1 (Fig 3.3). This

mutation occurs at the site 319, where *SD10*^{E} is the nucleotide G while *SD10*^{S} is the nucleotide A. Thus, the G/A substitution is likely a functional mutation between *SD10*^{E} and *SD10*^{S}.

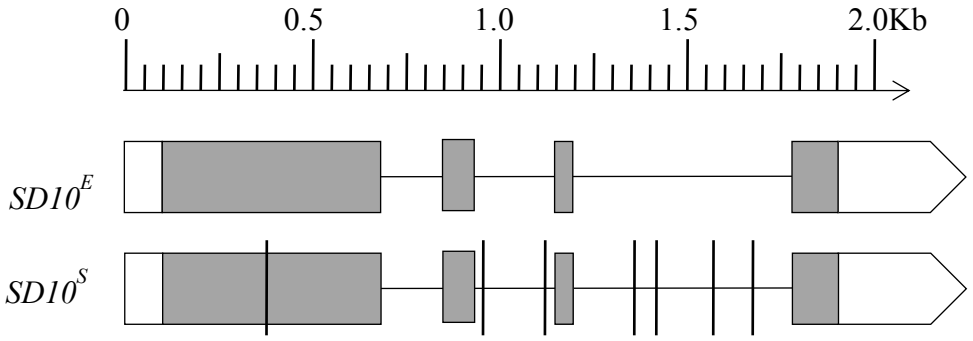

Figure 3.3 Gene model #1 for *SD10*^{E} and *SD10*^{S} alleles of *Os10g32600*. The model is predicted based on genomic DNA sequences from EM93-1 (*SD10*^{E}) and SS18-2 (*SD10*^{S}). Boxes and line segments indicate exons (filled) or UTRs (empty) and introns, respectively. Vertical lines indicate point mutations at *SD10*^{S}.

Table 3.1 List of SNPs at the *Os10g32600* locus between EM93-1 and SS18-2.

Mutation No.	Site[a]	EM93-1	SS18-2[b]	exon/intron
1	319	G	A	Exon #1
2	903	T	G	Intron #2
3	1095	T	-	Intron #2
4	1370	A	G	Intron #3
5	1421	T	C	Intron #3
6	1503	A	-	Intron #3
7	1603	G	T	Intron #3

[a] The number of nucleotides from the start codon (Fig. 3.3).

[b] A dash represents a deletion.

3.3.2 Allelic difference of *Os10g32600* in cDNA sequence

Full-length cDNAs (Fl_cDNAs) of *Os10g32600* were cloned from IL_{SD10}^{E} (*SD10*^{E}) and IL_{SD10}^{S} (*SD10*^{S}), based on a gene model in the Gramene database. These two cDNA clones were similar in the size of 1026 bp, as shown on the electrophoresis gel image (Fig. 3.4).

The cDNA sequences from *SD10*^{E} and *SD10*^{S} showed a high similarity, and both are also similar to the predicted cDNA sequence from Nipponbare (Appendix 4). A gene structure of *Os10g32600* was predicted based on the cDNA sequences of *SD10*^{E} and *SD10*^{S}, and gDNA and cDNA sequence from Gramene database (www.gramene.org) (Fig 3.5) and designated as model #2. It was predicted that both alleles consist of four introns and five exons (Fig 3.5). A Single Nucleotide Polymorphism (SNP) in the coding region was identified in exon #3 of the gene model #2 where *SD10*^{E} contained nucleotide G and *SD10*^{S} contained nucleotide A (Fig 3.5). The SNP identified in cDNAs was same as the SNP found in the gDNA sequences.

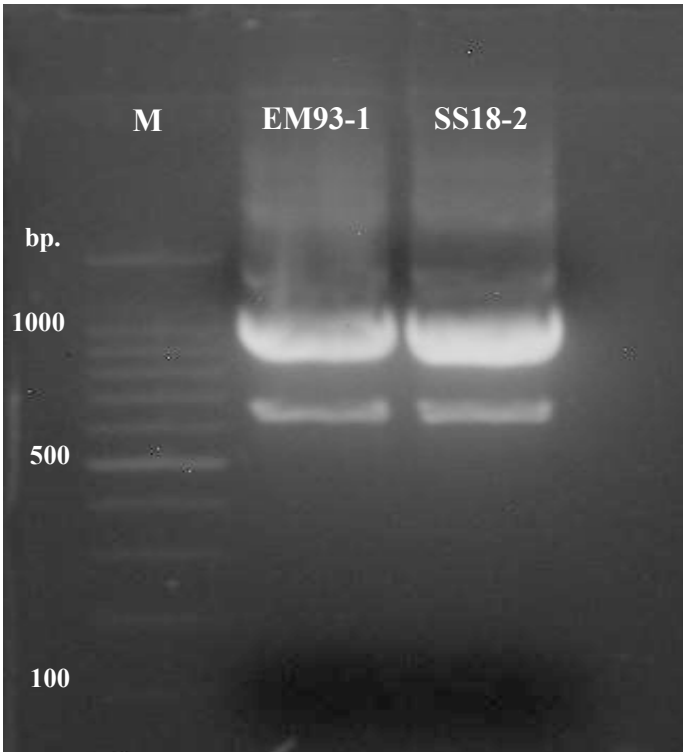

Figure 3.4 Gel image showing cDNA clones from EM93-1 (*SD10*^{E}) and SS18-2 (*SD10*^{S}).

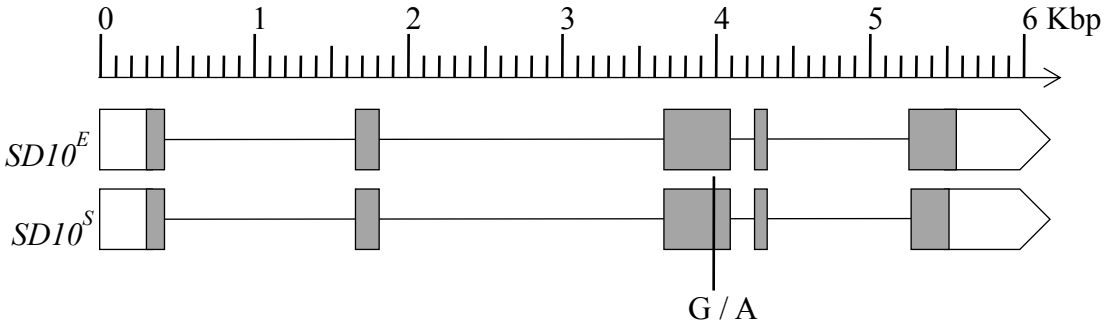

Figure 3.5 Gene model #2 for the *Os10g32600* alleles from EM93-1 (*SD10*^{E}) and SS18-2 (*SD10*^{S}). The structure was predicted based on cDNA sequences of *Os10g32600* from EM93-1 (*SD10*^{E}) and SS18-2 (*SD10*^{S}). Boxes and line segments indicate exons (filled) or UTRs (empty) and introns (line fragment), respectively. Vertical lines indicate point mutations at *SD10*^{S}. The nucleotide before and after slash indicated a point mutation.

3.3.3 Allelic difference of *Os10g32600* in protein sequence

The Fl-cDNA sequences of *Os10g32600* from EM93-1 and SS18-2 were used to deduce protein sequences and to predict molecular functions. The deduced proteins consist of 341 amino acid (aa) residues and both contain a response regulatory domain and a Myb-type helix-turn-helix (H-T-H) domain (Fig 3.6). One amino acid substitution was detected at the 195th residue in the Myb-type H-T-H domain, where the Aspartic acid (D) residue in $SD10^{E}$ was substituted by the Asparagine (N) residue in $SD10^{S}$ (Fig 3.6). The change in aa from D to N is corresponding to the G/A substitution. This also suggests that this G/A substitution is a functional mutation.

```
                                   Response regulatory  domain
SD10E MDHRELWPYGLRVLVIDDDCSYLSVMEDLLLKCSYKVTTYKNVREAVPFILDNPQIVDLV     60
SD10S MDHRELWPYGLRVLVIDDDCSYLSVMEDLLLKCSYKVTTYKNVREAVPFILDNPQIVDLV     60
      ************************************************************

SD10E ISDAFFPTEDGLLILQEVTSKFGIPTVIMASSGDTNTVMKYVANGAFDFLLKPVRIEELS     120
SD10S ISDAFFPTEDGLLILQEVTSKFGIPTVIMASSGDTNTVMKYVANGAFDFLLKPVRIEELS     120
      ************************************************************

SD10E NIWQHIFRKQMQDHKNNNMVGNLEKPGHPPSILAMARATPATTRSTATEASLAPLENEVR     180
SD10S NIWQHIFRKQMQDHKNNNMVGNLEKPGHPPSILAMARATPATTRSTATEASLAPLENEVR     180
      ************************************************************
                                   H-T-H myb domain
SD10E DDMVNYNGEITDIRDLGKSRLTWTTQLHRQFIAAVNHLGEDKAVPKKILGIMKVKHLTRE     240
SD10S DDMVNYNGEITDIRNLGKSRLTWTTQLHRQFIAAVNHLGEDKAVPKKILGIMKVKHLTRE     240
      **************:*********************************************

SD10E QVASHLQKYRMQLKKSIPTTSKHGATLSSTALDKTQDHPSRSQYFNQDGCMEIMDYSLPR     300
SD10S QVASHLQKYRMQLKKSIPTTSKHGATLSSTALDKTQDHPSRSQYFNQDGCMEIMDYSLPR     300
      ************************************************************

SD10E DDLSSGSECMLEELNDYSSEGFQDFRWDSDKQEYGPCFWNF     341
SD10S DDLSSGSECMLEELNDYSSEGFQDFRWDSDKQEYGPCFWNF     341
      ***************************************
```

Figure 3.6 Alignment of *Os10g32600* protein sequences from $IL_{SD10}{}^{E}$ and $IL_{SD10}{}^{S}$. The sequences were deduced based on cDNA sequences, and contain a response regulatory domain and a Myb-type helix-turn-helix (H-T-H) DNA-binding domain (underlined). The bold letter indicates a change in amino acid residue.

3.3.4 Effects of the *Os10g32600*-RNAi transgene on seed dormancy and flowering in the Nipponbare background

1) Segregation ratios of the transgenes

T_2, seedlings were sorted into two groups, based on resistance (R) or susceptibility (S) to the selective reagent Hygromycin B. The R:S ratio in the progeny T_2_T149-8, -9, -13 and -33 was 71:29, 81:19, 64:22, and 64:18, respectively, which followed Mendelian's expectation of a 3:1 ratio (Table 3.2). The result indicated that these four lines (T_2_T149-8, -9, -13, and -33) carried one copy (hemizygote or Rr) of the RNAi construct.

Table 3.2 Fitness tests for segregation ratios of the *Os10g32600*-RNAi transgene in four T_2 lines.

Group[a]	Number of plants in the line			
	T149-8	T149-9	T149-13	T149-33
Resistant	71	81	64	64
Susceptible	29	19	22	18
χ^2 value	0.85	1.92	0.01	0.40

[a] Plants were grouped based on resistant or susceptible responses to Hygromycin B, which was a selective marker for the transgene. The chi-square values were calculated based on the 3:1 expectation for a dominance gene. The chi-square threshold for two degree of freedom at the significant level of 0.05 is 5.99.

2) Effects of the transgenes on seed dormancy and flowering time

The T_2 generation: Four lines of T_2 generation were divided into 2 group where group 1 lines (T_2_T149-13 and T_2_T149-33) have three genotypes, where the R plants were determined for homo (RR) and heterozygous (Rr) by progeny testing for the response to hygromycin and group 2 lines (T_2_T149-8 and T_2_T149-9) consisted of only R (RR+Rr) and S (rr) plants.

In group 1, the genotype differences in germination and flowering time were observed in both T_2_T149-13 and T_2_T149-33 lines (Table 3.3). The variation in germination ranged from 54% to 62% in the line T_2_T149-13 (n = 40) and from 60% to 69% in the line T_2_T149-33 (n = 42) with the presence of the RNAi construct reducing germination or enhancing seed dormancy (Table 3.3). Only dominant effect was detected in group 1 lines, and accounted up to 34% of the phenotypic variances. The variation in flowering time ranged from 135 days to 138 days in the line T_2_T149-13 and from 131 days to 137 days in the line T_2_T149-33, where the presence of the RNAi construct delayed flowering time. Only additive effect on flowering time was detected in T_2_T149-13 and accounted for a relatively small proportion (18%) of phenotypic variance, while additive and dominance effect were detected in T_2_T149-33 and accounted for a large proportion (83%) of phenotypic variance (Table 3.3). Because of the large variation in both seed dormancy and flowering time of T_2_T149-33 line, one R (#19) and one S (#38) plant was selected to be developed as T_3 RNAi silencing generation (Fig 3.2) to confirm the results.

In Group 2, The difference in germination or flowering time between the R and S groups was also significant ($P< 0.01$ or 0.001) in the lines T_2_T149-8 and T_2_T149-9

(Table 3.3). The variation in germination ranged from 42% to 64% in the line T_2-R149-8 (n = 66) and from 18% to 33% in the line $T_2$149-9 (n = 43), with the R group reduced germination (Table 3.3). The variation in flowering time ranged from 74 days to 75 days ($P<0.001$) in the line T_2_T149-8 and from 82 days to 86 days ($P< 0.01$) in the line T_2_T149-9, where R group delayed flowering time (Table 3.3).

The T_3 generation: Two T_3 lines were derived from two T_2 plants that were homozygous (RR) and nullizygous (rr) for the RNAi transgene (RR) in the T_2_T149-33 line. A significant difference ($p< 0.001$) in germination (%) was found between the T_3 R and S groups, where R group reduced 40% of germination (%) and accounted for a large proportion (67%) of the phenotypic variance (Table 3.3). Flowering time was also significantly different ($p< 0.001$) between the T_3 R (133 days) and T_3 S (107 days) group where R group delayed flowering time (26 days) and accounted for 84% of the phenotypic variance (Table 3.3).

Consistency of results from both T_2 and T_3 generation indicated that the candidate gene *Os10g32600* had effect on both seed dormancy and flowering time in Nipponbare background.

Table 3.3 Summary of genic effects of the *Os10g32600*-RNAi transgene on germination and flowering time in T_2 or T_3 lines.

Line	Genotype[a]		N[b]	Germination (%)[c]				Days to flowering			
				Mean ± SD	*a*	*d*	R^2	Mean ± SD	*a*	*d*	R^2
	Nip^{WT}	(rr)	24	61.5 ± 12.6				135.5 ± 2.2			
T_2_T149-13	Nip^{RNAi}	(Rr)	12	44.1 ± 12.1	-3.64^{ns}	-13.78^{**}	0.30	137.2 ± 2.2	1.36^{*}	0.26^{ns}	0.18
	Nip^{RNAi}	(RR)	4	54.2 ± 13.5				138.3 ± 1.7			
	Nip^{WT}	(rr)	15	69.1 ± 9.4				131.1 ± 1.5			
T_2_T149-33	Nip^{RNAi}	(Rr)	19	52.7 ± 12.6	-4.66^{ns}	-11.72^{**}	0.34	138.3 ± 1.6	3.16^{***}	3.99^{***}	0.83
	Nip^{RNAi}	(RR)	8	59.8 ± 6.6				137.4 ± 1.5			
T_2_T149-8	Nip^{WT}	(rr)	20	63.6 ± 11.6			0.31	73.9 ± 1.9			0.20
	Nip^{RNAi}	(RR/Rr)	46	41.7 ± 16.6	(***)	-		75.3 ± 1.0	(***)	-	
T_2_T149-9	Nip^{WT}	(rr)	13	33.4 ± 7.7			0.35	82.2 ± 2.1			0.19
	Nip^{RNAi}	(RR/Rr)	30	18.1 ± 10.5	(***)	-		85.6 ± 3.6	(**)	-	
T_3_T149-33-38	Nip^{WT}	(rr)	23	53.0 ± 13.7			0.67	107.0 ± 3.6			0.84
T_3_T149-33-19	Nip^{RNAi}	(RR)	44	13.0 ± 13.5	(***)	-		133.0 ± 6.4	(***)	-	

[a] Genotypes for the transgene were determined based on resistant (R) or susceptible (r) responses to Hygromycin B. Nip^{WT} represents the nullizygote (rr) without the transgene in the Nipponbare (Nip) background. The homozygous RR and the hemizygous Rr were detected by progeny testing.

[b] The number of plants in the group.

[c] Additive (*a*) and dominance (*d*) effects or their combination (*a*+*d*); positive values indicates the transgene reduced germination or delayed flowering time. R^2 indicates the proportion of the variance explained by the transgene. Superscripts indicate that the effect *a*, *d*, or (*a*+*d*) was significant at the probability level of 0.05 "*", 0.01"**", or 0.001"***", or not significant (ns).

3) Transcription profile of *Os10g32600* in the transgenic lines

The transcription level of *Os10g32600* was quantified for the T_3 R (Nip^{RNAi}, homozygous (RR)) and T_3 S (Nip^{WT}), genotypes for the presence and absence (wild type) of the RNAi-construct or nullizygous (rr), respectively. The transcription level of *Os10g32600* was significantly ($p< 0.01$) lower in the Nip^{RNAi} line than in the Nip^{WT} line (Fig 3.7). This result indicated that the RNAi construct worked for *Os10g32600* and silenced the gene expression, resulting in reduced germination and delayed flowering.

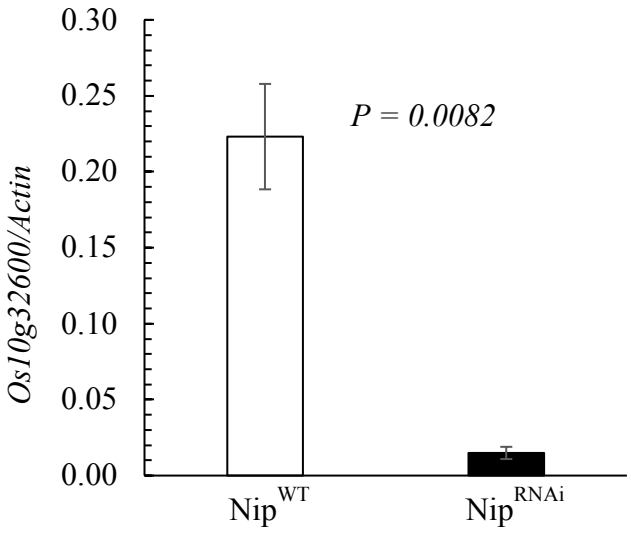

Figure 3.7 Genotypic difference in the transcript abundance of *Os10g32600* between the Nip^{RNAi} (homozygous or RR) and Nip^{WT} (nullizygous or rr) groups.
Nip^{RNAi} is the genotype that has the *Os10g3260*-RNAi construct in the Nipponbare (Nip) background (Nip^{WT}). The total RNA samples were prepared from the flag leaves. The data showed mean transcription level (s.e.), relative to the *Actin* control, for three biological replications and three technical replications.

3.3.5 Effects of the *Os10g32600*-RNAi transgene on seed dormancy and flowering in the EM93-1 genetic background

1) The hybrid F_1 generation

The hybrid F_1 plants from the EM93-1/ T_2_T149-33-4 (Nip^{RNAi}) cross segregated into the R and S groups, with the segregating ratio fitting the 1:1 Mendelian's expectation (Table 3.4). This result indicates that the parent plant T_2_T149-33-4 was hemizygous (Rr) for the RNAi transgene.

Genotypic differences in germination (%) and flowering time between the F_1 R (EM93/Nip^{RNAi}) and S (EM93/Nip^{WT}) groups were observed where the difference was 17% in germination and 14 days in flowering time (Table 3.5). The R group had significantly lower germination (18%) than S group (35%) and R group required longer period to flowering (131 days), while S group required less (117 days) (Table 3.5). The result suggested that *Os10g32600*-RNAi transgene had effect on both seed dormancy and flowering time in hybrid F_1.

2) The BC_1F_1 generation

The phenotypic ratio of R:S also fitted to Mendelian's expectation of 1:1 ratio (Table 3.4). The result showed significant difference in both germination ($p < 0.0001$) and flowering time ($p < 0.0001$), where the difference was 33% in germination and 39 days in flowering time (Table 3.5). The R group had lower germination (16%) than S group (49%) and R group also required longer time to flower (110 days) compared to S group (71 days) (Table 3.5).

qRT-PCR analysis for the R and S groups of BC_1F_1 plants demonstrated that the RNAi construct significantly decreased ($p < 0.05$) the expression level of *Os10g32600* in RNAi silencing line (R group) compared to wild type lines (S group) (Fig 3.8). Above results indicated that loss-of-function mutation of *Os10g32600* enhanced seed dormancy and delayed flowering time (Table 3.5).

Above results showed silencing *Os10g32600* gene in the BC_1F_1 generation delayed flowering and enhanced seed dormancy, which was consistent with the observation in the F_1 generation and was also consistent with the observations in the T_2 and T_3 generations of the Nipponbare transgenic lines. However, the association between seed dormancy and flowering time was different in direction between the fine mapping and RNAi experiments.

Table 3.4 Fitness tests for the *Os10g32600*-RNAi transgene in the F_1, BC_1F_1 and BC_2F_1 generations.

	Number of plants in the line		
Group[a]	F_1	BC_1F_1	BC_2F_1
Resistant	13	9	24
Susceptible	9	14	40
χ^2 value	0.7273	1.0870	4.0000

[a] Plants were grouped based on resistant or susceptible responses to Hygromycin B, which was a selective marker for the transgene. The chi-square values were calculated based on the 1:1 expectation for a dominance gene. The chi-square threshold for one degree of freedom at the significant level of 0.05 is 3.84.

Table 3.5 Progeny testing for the *Os10g32600*-RNAi transgene in the F_1 (EM93-1 × Nip^{RNAi}) and BC_1F_1 (EM93-1//EM93-1/Nip^{RNAi}) generations.

Population	Group[a]	Statistic	Germination (%)	Days to flowering
F_1	(1) Resistant	mean (26)	18.1	130.6
		s.e.	1.5	0.4
	(2) Susceptible	mean (18)	34.7	116.9
		s.e.	4.6	0.8
	Effect: (1) - (2)		-16.6	13.7
	T-test probability		0.0003	<0.0001
BC_1F_1	(1) Resistant	mean (18)	16.1	110
		s.e.	2.3	3.3
	(2) Susceptible	mean (14)	49	70.8
		s.e.	4.4	1.8
	Effect: (1) - (2)		-32.9	39.2
	T-test probability		<0.0001	<0.0001

[a] Plants were grouped based on resistant or susceptible responses to Hygromycin B, which was a selective marker for the transgene. Data shown are means (plant number) and standard error (s.e.).

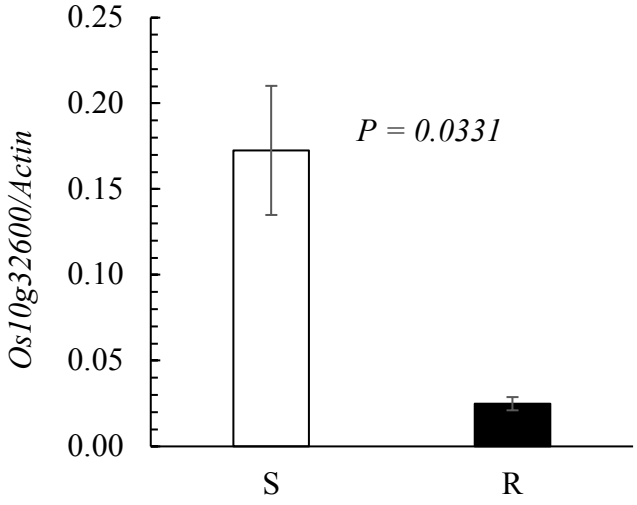

Figure 3.8 Genotypic difference in the transcript abundance of the *Os10g32600* between R and S groups of the BC_1F_1 plants.

The BC_1F_1 plants were classified into two groups based on the resistant (R) or susceptible (S) responses to Hygromycin B, the selective marker for the *Os10g32600* RNAi transgene. The total RNA samples were prepared from the flag leaves. The data shown are mean transcription levels (s.e.), relative to the *Actin* control, for three biological replications and three technical replications.

3) The BC_2F_1 generation

The BC_2F_1 population was developed by backcrossing a hybrid between EM93-1 and a BC_1F_1 plant with the *Os10g32600*-RNAi construct. The BC_2F_1 population consisted of 64 plants, which could be divided onto two groups based on their resistance (R) or susceptibility (S) to the selective reagent hygromycin. The R:S ratio fitted the 1:1 Mendelian's expectation (Table 3.4). Based on marker genotype experiment, all of 64

plants carried functional allele of *Os10g32600* gene from EM93-1 (Appendix 5). The of phenotypic difference in both germination (26%, P <0.0001) and flowering time (42 days, p < 0.0001) between the R and S groups were significant (Table 3.6)., R group promoted germination (54%) and delayed flowering time (109 days), while S group reduced germination (28%) and required less time to flower (67 days) (Table 3.6). The BC_2F_1 population has purer genetic background than the BC_1F_1 population. The result from BC_2F_1 population verified that the knock down of the *Os10g32600* gene reduced the degree of seed dormancy and delayed flowering time in the EM93-1 background. This observation was consistent with the effects of *qSD10/qFT10* on seed dormancy and flowering time in the fine mapping experiment shown in Chapter 2. Moreover, above finding can also confirmed the pleiotropic effect of *Os10g32600* gene on seed dormancy and flowering time in *indica*-type background.

Table 3.6 Summary effects of the *Os10g32600*-RNAi transgene on germination and flowering time in the BC_2F_1 (EM93-1///EM93-1//EM93-1/Nip^{RNAi}) population.

Genotype[a]	Statistic	Germination (%)	Days to flowering
(1) Resistant	mean (24)	53.5	109.0
	s.e.	17.3	4.1
(2) Susceptible	mean (40)	27.9	67.0
	s.e.	9.7	3.6
Effect: (1) - (2)		25.6	42.0
T-test probability		0.0003	<0.0001

[a] Plants were grouped based on resistant or susceptible responses to Hygromycin B, which was a selective marker for the transgene. The number of plants in the group is indicated in parenthesis. Data shown are means (plant number) and standard error (s.e.).

3.3.6 Genotypic difference in the response of germination to applied GA_3

It is hypothesized that *Os10g32600* may be involved in the regulation of germination by influencing GA signaling in imbibed seeds. If the hypothesis was correct, a GA application must promote germination and diminish the genotypic difference in GA response of germination of seeds from the plants that are nullizygous for the *Os10g32600*-RNAi transgene in the BC_2F_1 population. To prove this hypothesis, seed samples from the BC_2F_1 R ($NIL_{EM93}{}^{RNAi}$) and S ($NIL_{EM93}{}^{WT}$) plants were germinated in the buffer with 0.0 (control), 1.0, and 10.0 μM GA_3. There was a significant difference in

germination percentage in each of the 0.0, 1.0, and 10.0 uM GA_3 solutions, with germination percentage being higher in the R than in the S group plants (Table 3.7). These results indicate that the genotypic difference of the *Os10g32600* locus in seed dormancy could not be eliminated by a GA application. Thus, it is suggested that *Os10g32600* may regulate germination through some new mechanisms, rather than the GA signaling pathway.

Table 3.7 Genotypic differences in germination response to the GA_3 treatment between nearly isogenic lines (NIL) for the *Os10g3260*-RNAi transgene.

Genotype[a]	Statistic	Germination (%) in the buffer containing GA_3		
		0.0 μM	1.0 μM	10.0 μM
(1) NIL_{EM93}^{RNAi}	Mean (12)	68.5	81.9	90.9
	s.e.	4.5	3.1	2.8
(2) NIL_{EM93}^{WT}	Mean (12)	48.9	63.9	79.3
	s.e.	3.6	3.7	2.3
(1) – (2)		19.6	18.0	11.6
T-test probability		0.0029	0.0016	0.0039

[a] NIL_{EM93}^{RNAi}, plants that were homozygous (RR) for the *Os10g3260*-RNAi transgene selected from the BC_2F_1 (EM93-1///EM930-1//EM93-1/Nip^{RNAi}) population; NIL_{EM93}^{WT}, plants that were nullizygous (rr) for the *Os10g3260*-RNAi transgene selected from the BC_2F_1. Data shown are means (plant number) and standard error (s.e.).

3.4 Discussion

3.4.1 Novel amino acid residue is crucial for the function of the *EHd1* gene

EHd1 promotes flowering time by inducing FT-like gene expression only under short-day condition (Doi et al. 2004). The *EHd1* allele from the *japonica* cultivar Taichung T65 (*ehd1*^{T65}) was a loss-of-function mutation causing delayed flowering, while another allele from the *japonica* cultivar Nipponbare (*EHd1*^{Nip}), the indica cultivar Kasalath (*EHd1*^{kas}), and the African rice *O. Glaberrima* (*EHd1*^{gla}) contain functional alleles. It was predicted that ehd1^{T65} differs from the functional alleles in the deduced protein sequence only at the 219th residue, which changed from Glycine (Gly) to Arginine (Arg) in T65 (Doi et al. 2004). Thus, the 219th residue seems to be crucial for the late-flowering phenotype in T65.

This research identified a different functional mutation, which caused a change in the 195th amino acid residue in the 3rd exon (Gene model #2 (Fig 3.5)). The 195th residue is Aspartic acid (Asp) in EM93-1 and is Asparagine (Asn) in SS18-2. This mutation caused the variation in both germination ability and flowering time. Doi et al (2004) reported that mutation at the 219th residue of *EHd1*caused the varitation in flowering time. However, this research found the 219th residue is Gly in the alleles from the two parents EM93-1 and SS18-2. Based on these finding, it can indicate that at least there are two candidates of functional point mutation at the *Ehd1* locus in rice.

3.4.2 Functions of *Os10g32600* in the *japonica*- and *indica*-type backgrounds

The observed results in two generation (T_2 and T_3) of RNAi silencing experiment indicated that wild-type (functional) allele of *Os10g32600* not only promoted flowering time but also reduced seed dormancy in the *japonica* background. Interestingly, this

result was different from the result in the fine-mapping experiment, in which that the *Os10g32600* allele from EM93-1 promoted flowering and enhanced seed dormancy in the *indica* background.

The opposite direction of the association between seed dormancy and flowering time suggested that 1) the *Os10g32600* alleles of EM93-1 and Nipponbare have similar function in flowering time but different function in seed dormancy, where EM93-1 allele promoted both early flowering time and seed dormancy level while Nipponbare allele also promote early flowering but reduced seed dormancy level. 2) Some factor(s) from background of Nipponbare could alter the TF gene's function in seed dormancy. Regardless of the opposite direction of the association, this research showed concrete evidence to confirm that other than control flowering time *EHd1* (*Os10g32600*) had a novel function to regulate germination.

3.4.3 About the functions of *Os10g32600* in the advanced backcross generation

The BC_2F_1 population appear to have purer *indica*-type genetic background when considering both the *Os10g32600* allele of BC_2F_1-derived seedling and plant morphology such as plant height and flowering time. The result from marker genotype performed to verify the *Os10g32600* allele demonstrated that all BC_2F_1-derived seedling carried EM93-1 allele of *Os10g32600* (Appendix 5). EM93-1 is semidwarf (65 cm) in plant height and require 60 days to flower. The BC_2F_1 plants were similar to recurrent parent EM93-1 in plant height and the time to flowering (Table 3.6 and Appendix 6).

The result from BC_2F_1 population showed silencing *Os10g32600* reduced the degree of seed dormancy and also delayed flowering time, which is consistent with the result in the fine-mapping experiment. These results 1) indicated that the *Os10g32600* allele from

weedy/wild SS18-1 line is a loss-of function allele which cause delay in flowering time and promote germination; 2) verify the pleiotropic effect of *Os10g32600* (*EHd1*) on seed dormancy and flowering time.

To further confirm whether *Os10g32600* is a *qSD10* candidate gene and has pleiotropic effect, the BC_2F_2 segregation populations were developed to estimate genic effect and to quantify the transcription level of *Os10g32600* gene in different seed tissue such as embryo and endosperm tissue.

In fact, the two BC_2F_2 population were developed from BC_2F_1-14 and BC_2F_1-47 lines and cultivated in the greenhouse condition on December 2017. BC_2F_1-14 and BC_2F_1-47 populations consisted of 48 and 55 plants which were sorted into R and S groups. The R:S phenotypic ratio of both BC_2F_2 followed Mendelian's expectation of 3:1 ratio (Appendix 7). These two lines (BC_2F_1-14 and BC_2F_1-47) carried only one copy (hemizygous or Rr) of the RNAi construct and the seedlings from each line were supposed to have three genotypes (RR, Rr, and rr) which would be appropriate to estimate genic effect of *Os10g32600* on both seed dormancy and flowering time.

Chapter 4 Conclusion and discussion

4.1 Project Summary

This research was centered on rice QTL *qSD10* and generated a wide range of phenotypic, genotypic and molecular data to characterize it. These data are original and valuable to elucidate evolutionary and developmental mechanisms of seed dormancy in rice. This chapter summarizes the main results from this four-year dissertation and discusses the biological and agricultural implications of the major conclusions from the experimental data.

4.1.1 Phenotypic association and the *qSD10/qFT10* cluster

Seven experiments were conducted to 1) delimit *qSD10* to a short genomic region and identify its candidate genes; 2) infer *qSD10* pleiotropic effects on other adaptive traits utilizing a fine mapping approach; and 3) determine physiological mechanisms for the development of seed dormancy as regulated by *qSD10*.

A high-resolution map of 1.6 Mb for a *qSD10*-containing region was developed with 22 of polymorphic markers. With this map, nine rare recombinants between the markers were selected by genotyping more than 4000 plants from advanced generations of progeny lines. *qSD10* was narrowed from 3 Mb to a genomic segment of ~100 kb.

This dissertation discovered a positive correlation between germination percentage and time to flowering in the recombinants R24, R86, R93, R10-09, and R40-05-03 being consistent in two advanced mapping populations (chapter 2.3.1 and chapter 2.3.2). The positive correlation indicates that early flowering plants tended to have

stronger seed dormancy than the late-flowering plants. The narrowed *qSD10* also showed pleiotropic effect on plant height and panicle length, but not on leaf length and the number of spikelet per panicle. The *qSD10* allele from the parent line (EM93-1) enhanced seed dormancy, delayed flowering time, and reduced plant height and panicle length.

There are 22 candidate genes in the narrowed *qSD10* region. Among these genes, *Os10g32600* and *Os10g32810* are the promising candidate genes of *qSD10*. *Os10g32600* is a predicted Myb-type H-T-H transcription factor and was reported to regulate flowering time (Doi et al. 2004). *Os10g32810* is a predicted Beta-amylase gene. Beta-amylase genes were predicted to regulate the response to abiotic stimulus in rice (by MSU database (Kawahara et al. 2013)) and was reported to be involved during the breakdown of the reserved starch in cereal (Sun and Henson 1991; Wang et al. 1996)

Physiological experiments were conducted to determine the relationship between seed moisture content and seed germinability during a period from 20 to 40 days post anthesis using the isogenic lines IL_{SD10}^{E} and IL_{SD10}^{S}. The results revealed that IL_{SD10}^{E} had a higher seed moisture content and lower germination percentage than IL_{SD10}^{S}, indicating that *qSD10* control the development of seed dormancy by regulating the acquisition of desiccation tolerance.

4.1.2 Functional analysis of *Os10g32600,* a candidate gene for *qSD10*/*qFT10*

Eleven experiments were performed to: 1) identify the sequence variation of *Os10g32600* between the alleles from EM93-1 and SS18-2; 2) develop transgenic lines for an RNAi construct to silence *Os10g32600* in the Nipponbare genetic background; and

3) evaluate the effects of the *Os10g32600*-RNAi transgene on seed dormancy and flowering time in the EM93-1genetic background.

Both gDNA and cDNA of *Os10g32600* were cloned from the parental lines EM93-1 ($SD10^E$) and SS18-2 ($SD10^S$). Sequence analyses revealed that *Os10g32600* contained five exons and four introns. Seven point mutations were detected between $SD10^E$ and $SD10^S$, and one (a G/A substitution) of the seven mutations occurred in the protein coding region. The protein sequence deduced from the cDNA sequence contains 341 amino acid (aa) residues and is predicted to have two conserved domains: a response regulatory domain and a Myb-type H-T-H DNA binding domain. The G/A substitution in the cDNA sequence causes a non-synonymous codon, resulting in an aa alteration in Myb-type H-T-H DNA binding domain from Aspartic acid in $SD10^E$ to Asparagine in $SD10^S$. This alteration may modify the binding ability of the transcription factor. This allelic difference of *Os10g32600* might account for the variation in germination and flowering time.

In the Nipponbare background, *Os10g32600*-RNAi transgene enhanced seed dormancy and delayed flowering time. However, in the EM93-1 background, the RNAi silencing lines reduced seed dormancy and delayed flowering time. This dissertation also found that the inhibitory effect of *Os10g32600* on seed germination could not be compensated by applied GA hormone, indicating that *Os10g32600* controls seed dormancy with a mechanism different from the GA signaling pathway.

4.2 Discussions on Implications

4.2.1 About coadaptation between seed dormancy and flowering time

Flowering plants require multiple developmental transitions to complete their life cycle. The first transition occurs at germination, going from seed to seedling. The second transition occurs at the time of flowering, when transitioning from vegetative to reproductive stage (Huijser and Schmid 2011).

Both seed dormancy and flowering time are quantitative traits controlled by multiple genes and influenced by environmental conditions. Previous studies have explored coadaptation of seed dormancy and flowering time at both phenotypic and molecular levels (Blair et al. 2017; Chiang et al. 2009; Huo et al. 2016; Ritland 1983; Springthorpe and Penfield 2015; Taylor et al. 2017). This dissertation identified positive association between flowering time and seed dormancy, confirming previous observations of coadaptation of these traits. The association of both traits could arise from a linkage between genes for different traits or pleiotropic effects of single genes. The result from chapter 3 of this dissertation discovered pleiotropic effect of a single gene, *Os10g32600,* on both seed dormancy and flowering traits in rice. Some research had identified genes that have pleiotropic effect on both seed dormancy and flowering traits such as *HUB1* (Liu et al. 2007), *FLC* (Chiang et al. 2009), *RDO2* (Liu et al. 2011), and *DOG1* (Huo et al. 2016).

Recent research intent to explain the relationship between flowering time, seed dormancy and seasonal cues. The timing of flowering is critical because the difference in flowering time could mean that seed development and primary seed dormancy are established at different temperatures and could influence seed dormancy statue. For

instance, a cryptic function of flowering time coincides with a temperature-sensitive switch in seed dormancy, where *Arabidopsis* seeds that developed below 14°C became more dormant than those developed above 15°C (Springthorpe and Penfield 2015). This suggested that the degree of seed dormancy is influenced by temperature at flowering time. Similarly, Vidigal et al. (2016) found that altitude and temperature were the major geographical and climatic factors associated with the temporal control of germination and flowering, and that these are the factors driving the coadaptation of seed dormancy and flowering traits.

Despite many years of research to elucidate the association between seed dormancy and flowering time, little is known about genetic bases underlying their coadaptation. Further research is required to underlying mechanisms of the coadaptation, which is essential for breeders to manipulate crop cultivars for germination capability (PHS resistant verities) and growth duration (short vegetative phase) by using seed dormancy and/or flowering time genes.

4.2.2 Origin of *Ehd1*'s variants

Rice originated from the ancestor *O. rufipogon* in the tropical to subtropical areas. The parent EM93-1 is a photoperiod insensitive, *indica*-type breeding line with an extremely short growth duration (about 55-60 days from germination to flowering) and weak seed dormancy (Gu et al. 2004b). Whereas, the parent SS18-2 is a tropical ecotype of weedy rice originated from Southern Thailand, and is moderately sensitive to photoperiod with strong in seed dormancy (Gu et al. 2003; Suh et al. 1997). This research discovered the *qSD10/qFT10* cluster and the phenotypic contributions of the parental alleles. The *qSD10/qFT10* haplotype from EM93-1 enhances seed dormancy and

promotes flowering. It is possible that the haplotype in EM93-1 originated from mutants from the wild ancestor in tropical areas, where the genotype matured in the wet season and required a given degree of seed dormancy to survive the coming dry season. Furthermore, artificial selection contributed for the *EHd1* gene to be maintained in cultivar EM93-1. The farmer select early flowering plants for short duration of growth to increase the number of crops per year resulting in an indirect selection of a strong seed dormancy allele due to its pleotropic effect. The *qSD10/qFT10* haplotype from SS18-2 promote germination but delay flowering. Natural selection retained the SS18-2 haplotype was likely because the weed ecotype contains several QTL with a major effect on seed dormancy, such as *qSD1-1, 4, 7-1* and *12* (Gu et al. 2004b).

4.2.3 Biological and agricultural importance of the *qSD10/qFT10* cluster

It is assumed that genotypes of a low degree of seed dormancy and delayed flowering may offer more variable reproduction (Ritland 1983). Several rice gene have been reported to influence yield-related traits such as *Gn1a* gene that increase grain number per panicle (Ashikari et al. 2005) or the combination of *Ehd1* and *Hd1* genes that was reported to reduce number of spikelet per panicle (Endo-Higashi and Izawa 2011). However, the dormancy-enhancing allele of *qSD10/qFT10* promoted flowering, but did not influence other yield-related traits (leaf length and spikelet per panicle).

Early flowering varieties are less tolerant to pre-harvest sprouting in general. The narrowed *qSD10/qFT10* cluster can be used to manipulate germination capability and to develop early flowering phenotypes. The *qSD10/qFT10* allele from EM93-1 reduced germination and promoted flowering which makes it useful for a breeding program to improve the resistance to PHS, and it also can be used to develop short growth varieties.

4.2.4 Working model for the genetic control of seed dormancy by *qFT10*

The *qSD10/qFT10* cluster showed a pleiotropic effect on flowering time and seed dormancy. This suggest that flowering time and seed dormancy coevolved and share portions of their regulatory pathway, with a divergence occurring at some point downstream. However, the precise point the pathway diverge is still not clear. This dissertation provided two possible models to explain the divergence point and related molecular mechanism of *qFT10* control of seed dormancy.

In model #1, *qFT10* (*EHd1*) interacts with X (unknown) factor to regulate flowering time, subsequently flowering time influence seed dormancy. In this model *qFT10* control both traits through the same pathway and the divergence occurs after flowering (Fig 4.1). Some research showed *EHd1* interact with other genes such as rice *Indeterminate 1* or *Osld1* (Park et al. 2008), *OsMADS51* (Kim et al. 2007), *Hd3a*, and *OsMADS14* (Doi et al. 2004) to regulate time to flowering, but none of them have reported the effect on seed dormancy.

In model #2, It is possible that divergent control of these two traits occur earlier than in model #1, considering that the transition from vegetative to reproductive phase occurs before induction of seed dormancy at the seed maturation period. In model #2 *qFT10* controls flowering time and seed dormancy through independent pathways (Fig 4.1).

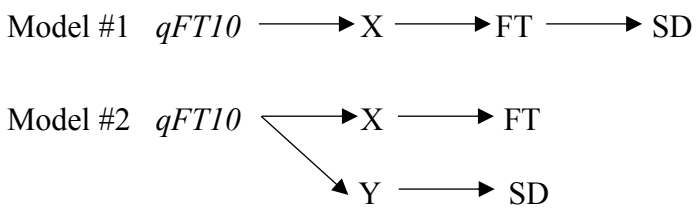

Figure 4.1 Two working models used to explain *qFT10* regulation of seed dormancy (SD) and flowering time (FT). X and Y represent unknown portions of the pathways.

4.2.5 Hypothesis of molecular mechanism for the regulation of seed dormancy by *qSD10*

This dissertation proposes a working model (Fig. 4.2) to explain the development mechanism through which *qSD10* regulate primary seed dormancy in rice. The functional allele of *qSD10* from EM93-1 promotes early flowering and delayed the seed dehydration processes at maturation, resulting in reduce germination ability (strong dormancy). A non-functional allele of *qSD10* from SS18-2 delays flowering time and accelerates the dehydration processes at maturation, consequently promoting germination (weak dormancy). This model considers the desiccation process, which has an important role in the transition of seeds from the developmental to the germination regime (Angelovici et al. 2010). Furthermore, recent research showed the involvement of desiccation mechanisms on regulation of seed dormancy development by *qSD1-2* (Ye et al. 2015).

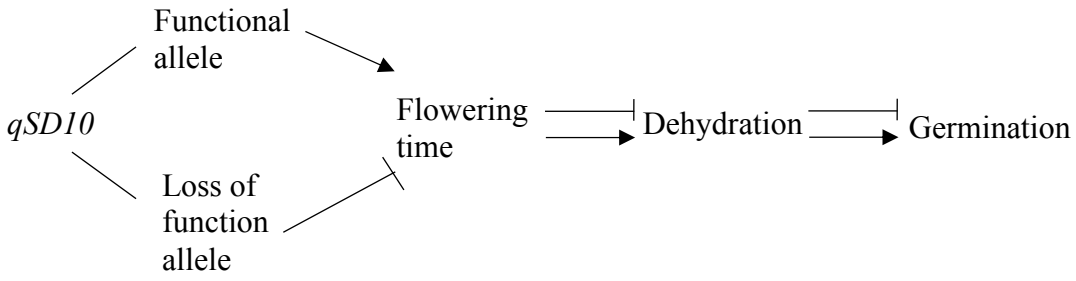

Figure 4.2 Working model explaining the developmental mechanism of primary seed dormancy regulation by *qSD10*.

APPENDICES

Appendix 1. List of newly designed PCR primers used for fine mapping experiment.

Primer name	Sequences (5'-3')	Physical position
InD4 F	TGGCTACATGGTCATCGAGAA	17054558
InD4 R	CATTTGGACGAAAACCTTACCA	
InD9 F	CGTCCCATGAAAAATAAACTAGTACC	17085007
InD9 R	TGATGATGTGCCCCTCCATA	
InD27 F	TGTTAAGGTGGCAGCTTCTG	17230696
InD27 R	CACAAAAGGACGACGGATG	
InD28 F	TCGCAATATAGGTTTCGAATAACA	17231224
InD28 R	TCTGGCAAAGGAATTATACGC	
InD29 F	TTGGGAGAGCTAATGGTGAAA	17269946
InD29 R	ATTCGCACGCAATCTCTACC	
InD33 F	ATTGTGTGCTTCACCCATCT	17385978
InD33 R	TTTCTGAAGCCTGAAATTTTATATG	
InD34 F	CCCAACTCTTTAGGCCATGC	17388396
InD34 R	ACGAAGTTCAGGGATGCAAA	
InD36 F	GCATGAATCCATCCATCGAA	17454171
InD36 R	AAGCTTTTGCCACATCGTCT	
InD41 F	TACTAGCCAATCCCCACCAG	17109357
InD41 R	CAAGTGGTTCAAGCAATGATG	
InD47 F	GGCATCCAATTCGTGAGTG	17071321
InD47 R	GCAGCCTCTCCTGGTACTCC	
InD54 F	CTAATGTCCAGCTGCCAAGC	17183902
InD54 R	TGGCTCGATTACGTGTCTTG	

Forward and reverse primer indicated as F and R, respectively. The physical positions are determined by alignment primers against the Nipponbare genome sequence (Gramene Database, www.gramene.org).

Appendix 2. List of predicted gene underlying the narrowed *qSD10* region.

Gene#	Locus Name	Molecular function	Gene product	aa. size
1	Os10g32580	hydrolase activity	GDSL-like lipase/acylhydrolase	391
2	Os10g32590	universal stress response	Adenine nucleotide alpha hydrolases-like superfamily protein	207
3	Os10g32600	DNA binding	MYB family transcription factor	168
4	Os10g32620	unknown	unknown	103
5	Os10g32630	unknown	unknown	821
6	Os10g32640	retrotransposition	retrotransposon protein, Ty3-gypsy subclass	911
7	Os10g32649	retrotransposition	retrotransposon protein, Ty3-gypsy subclass	1292
8	Os10g32658	retrotransposition	retrotransposon protein, Ty3-gypsy subclass	309
9	Os10g32670	unknown	unknown	147
10	Os10g32680	unknown	unknown	365
11	Os10g32690	unknown	unknown	126
12	Os10g32700	protein binding	hypersensitive-induced response protein	293
13	Os10g32720	unknown	unknown	185
14	Os10g32730	hydrolase activity	haloacid dehalogenase-like hydrolase domain	398
15	Os10g32740	protein binding	zinc finger family protein	720
16	Os10g32750	protein binding	zinc finger family protein	607

(To be continued)

(Appendix 2 continued)

Gene#	Locus Name	Molecular function	Gene product	aa. size
17	Os10g32760	protein binding	zinc finger family protein	647
18	Os10g32770	DNA binding	WD-40 repeat-containing protein	247
19	Os10g32780	protein binding	RING zinc finger ankyrin protein	104
20	Os10g32790	unknown	unknown	223
21	Os10g32800	unknown	unknown	429
22	Os10g32810	response to abiotic stimulus	beta-amylase	536

Locus name is the predicted gene that locate in narrowed (<100 kb.) qSD10 region. Function is the predicted function of the above mentioned gene based on MSU database (Kawahara et al. 2013). Gene product is the type of predicted protein of gene.

Appendix 3. List of PCR primers used for experiments in chapter 3.

Primer name	Sequences (5'-3')	Position
For cloning cDNA		
5'-UTR_F	CGGAGAAGCAGATGTAGTCG	17081379
3'-UTR2_R	CAAAAGTTGGTTTCCTCGACA	17075767
For cloning gDNA		
EHd1-1F	TGCAAGGATATATGCATGAAGAA	17081678
EHd1-1R	TTAGTGCATATTTGACCTAGTTCCT	17079710
EHd1-2F	CCTCCAAGTTTGGCATACCTAC	17079826
EHd1-2R	TTCCGAGGTCTCGTATGTCC	17077651
EHd1-3F	ATCTCGAAAAACCCGGTCAT	17077799
EHd1-3R	GGGAAATTGTAGTCGTAAAATCCA	17075724
Ehd1-4F	GACGAGCCATCCCTTTGAAC	17078285
Ehd1-4R	ACCTGCAGATGACTGGCAAC	17077352
Ehd1-5F	GCAGAGGCAGTTCCAAAGAAG	17077434
Ehd1-5R	CGGCCAGAAATTTTCGGTAA	17076717
Ehd1-6F	GTCGGGGGTGAACGAAATTA	17076753
Ehd1-6R	TCAGAAAGTCAATTGCAGGTATCA	17076025
For RNAi		
EHd1-RNAi _F	caccGGATCGAAGAGCTGAGCAAC	17077880
EHd1-RNAi _R	ACTGCTGCAATGAACTGACG	17077599
For genotyping		
InD11-F	CCGATGGGGTAAATAAATGG	17076860
InD11-R	CATGCTCAAATCTCGCCTAA	17076641
For transcriptional analysis by qRT-PCR		
qPCR-2_EHd1_F	GTTGCACCGTCAGTTCATTG	17077607
qPCR-2_EHd1_R	AGAGCGGTGGATGACAAAGT	17076309
Actin F	AGGAATGGAAGCTGCGGGTAT	29075144
Actin R	GCAGGAGGACGGCGATAACA	29075607

Forward and reverse primer indicated as "F" and "R", respectively. The physical position was determined by alignment primer against the Nipponbare genome sequence (Gramene Database, www.gramene.org).

Appendix 4 Alignment of cDNA sequences of *Os10g32600* from EM93-1 ($SD10^{E}$), SS18-2 ($SD10^{S}$), and Nipponbare ($SD10^{N}$).

```
SD10^N      ATGGATCACCGAGAGCTGTGGCCTTATGGACTAAGAGTTCTGGTCATCGATGACGACTGT 60
SD10^E      ATGGATCACCGAGAGCTGTGGCCTTATGGACTAAGAGTTCTGGTCATCGATGACGACTGT 60
SD10^S      ATGGATCACCGAGAGCTGTGGCCTTATGGACTAAGAGTTCTGGTCATCGATGACGACTGT 60
            ************************************************************

SD10^N      TCATACTTGTCAGTCATGGAAGATTTACTTCTGAAGTGCAGCTACAAGGTTACAACGTAT 120
SD10^E      TCATACTTGTCAGTCATGGAAGATTTACTTCTGAAGTGCAGCTACAAGGTTACAACGTAT 120
SD10^S      TCATACTTGTCAGTCATGGAAGATTTACTTCTGAAGTGCAGCTACAAGGTTACAACGTAT 120
            ************************************************************

SD10^N      AAGAACGTCAGAGAAGCTGTGCCTTTCATATTGGACAATCCACAAATAGTTGACCTAGTA 180
SD10^E      AAGAACGTCAGAGAAGCTGTGCCTTTCATATTGGACAATCCACAAATAGTTGACCTAGTA 180
SD10^S      AAGAACGTCAGAGAAGCTGTGCCTTTCATATTGGACAATCCACAAATAGTTGACCTAGTA 180
            ************************************************************

SD10^N      ATCAGTGATGCGTTCTTTCCTACCGAAGATGGTTTGCTCATTCTGCAAGAAGTAACCTCC 240
SD10^E      ATCAGTGATGCGTTCTTTCCTACCGAAGATGGTTTGCTCATTCTGCAAGAAGTAACCTCC 240
SD10^S      ATCAGTGATGCGTTCTTTCCTACCGAAGATGGTTTGCTCATTCTGCAAGAAGTAACCTCC 240
            ************************************************************

SD10^N      AAGTTTGGCATACCTACAGTGATTATGGCTTCAAGTGGAGACACAAATACAGTGATGAAA 300
SD10^E      AAGTTTGGCATACCTACAGTGATTATGGCTTCAAGTGGAGACACAAATACAGTGATGAAA 300
SD10^S      AAGTTTGGCATACCTACAGTGATTATGGCTTCAAGTGGAGACACAAATACAGTGATGAAA 300
            ************************************************************

SD10^N      TATGTTGCAAATGGCGCTTTTGATTTCCTGCTAAAACCTGTGAGGATCGAAGAGCTGAGC 360
SD10^E      TATGTTGCAAATGGCGCTTTTGATTTCCTGCTAAAACCTGTGAGGATCGAAGAGCTGAGC 360
SD10^S      TATGTTGCAAATGGCGCTTTTGATTTCCTGCTAAAACCTGTGAGGATCGAAGAGCTGAGC 360
            ************************************************************

SD10^N      AACATTTGGCAGCACATATTCCGAAAGCAAATGCAAGATCACAAGAACAATAACATGGTT 420
SD10^E      AACATTTGGCAGCACATATTCCGAAAGCAAATGCAAGATCACAAGAACAATAACATGGTT 420
SD10^S      AACATTTGGCAGCACATATTCCGAAAGCAAATGCAAGATCACAAGAACAATAACATGGTT 420
            ************************************************************

SD10^N      GGAAATCTCGAAAAACCCGGTCATCCTCCATCAATATTAGCCATGGCTCGTGCTACTCCG 480
SD10^E      GGAAATCTCGAAAAACCCGGTCATCCTCCATCAATATTAGCCATGGCTCGTGCTACTCCG 480
SD10^S      GGAAATCTCGAAAAACCCGGTCATCCTCCATCAATATTAGCCATGGCTCGTGCTACTCCG 480
            ************************************************************

SD10^N      GCTACCACGAGATCAACGGCCACCGAAGCTTCGCTAGCGCCTCTAGAAAATGAGGTGAGA 540
SD10^E      GCTACCACCAGATCAACGGCCACCGAAGCTTCGCTAGCGCCTCTAGAAAATGAGGTGAGA 540
SD10^S      GCTACCACCAGATCAACGGCCACCGAAGCTTCGCTAGCGCCTCTAGAAAATGAGGTGAGA 540
            ************************************************************

SD10^N      GATGACATGGTCAACTACAATGGCGAGATCACGGACATACGAGACCTCGGAAAGTCCAGG 600
SD10^E      GATGACATGGTCAACTACAATGGCGAGATCACGGACATACGAGACCTCGGAAAGTCCAGG 600
SD10^S      GATGACATGGTCAACTACAATGGCGAGATCACGGACATACGAAACCTCGGAAAGTCCAGG 600
            ****************************************** ****************

SD10^N      CTGACCTGGACCACGCAGTTGCACCGTCAGTTCATTGCAGCAGTGAACCACCTCGGAGAA 660
SD10^E      CTGACCTGGACCACGCAGTTGCACCGTCAGTTCATTGCAGCAGTGAACCACCTCGGAGAA 660
SD10^S      CTGACCTGGACCACGCAGTTGCACCGTCAGTTCATTGCAGCAGTGAACCACCTCGGAGAA 660
            ************************************************************
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SD10N      GACAAGGCAGTTCCAAAGAAGATACTAGGGATAATGAAGGTCAAACATTTGACAAGAGAG 720
SD10E      GACAAGGCAGTTCCAAAGAAGATACTAGGGATAATGAAGGTCAAACATTTGACAAGAGAG 720
SD10S      GACAAGGCAGTTCCAAAGAAGATACTAGGGATAATGAAGGTCAAACATTTGACAAGAGAG 720
           ************************************************************


SD10N      CAAGTTGCCAGTCATCTGCAGAAATACAGGATGCAACTGAAGAAATCGATTCCAACAACA 780
SD10E      CAAGTTGCCAGTCATCTGCAGAAATACAGGATGCAACTGAAGAAATCGATTCCAACAACA 780
SD10S      CAAGTTGCCAGTCATCTGCAGAAATACAGGATGCAACTGAAGAAATCGATTCCAACAACA 780
           ************************************************************

SD10N      AGCAAACACGGAGCGACTTTGTCATCCACCGCTCTCGACAAAACACAAGACCACCCTTCA 840
SD10E      AGCAAACACGGAGCGACTTTGTCATCCACCGCTCTCGACAAAACACAAGACCACCCTTCA 840
SD10S      AGCAAACACGGAGCGACTTTGTCATCCACCGCTCTCGACAAAACACAAGACCACCCTTCA 840
           ************************************************************

SD10N      AGATCGCAGTATTTCAATCAAGACGGATGCAAGGAAATCATGGACTACTCTTTACCGAGA 900
SD10E      AGATCGCAGTATTTCAATCAAGACGGATGCATGGAAATCATGGACTACTCTTTACCGAGA 900
SD10S      AGATCGCAGTATTTCAATCAAGACGGATGCATGGAAATCATGGACTACTCTTTACCGAGA 900
           ****************************** ***************************

SD10N      GATGACCTCTCAAGTGGCTCAGAGTGCATGCTTGAAGAACTGAACGATTACTCATCCGAA 960
SD10E      GATGACCTCTCAAGTGGCTCAGAGTGCATGCTTGAAGAACTGAACGATTACTCATCCGAA 960
SD10S      GATGACCTCTCAAGTGGCTCAGAGTGCATGCTTGAAGAACTGAACGATTACTCATCCGAG 960
           ************************************************************

SD10N      GGTTTCCAAGATTTCCGATGGGATTCAGACAAACAGGAATATGGACCATGTTTTTGGAAT 1020
SD10E      GGTTTCCAAGATTTCCGATGGGATTCAGACAAACAGGAATATGGACCATGTTTTTGGAAT 1020
SD10S      GGTTTCCAAGATTTCCGATGGGATTCAGACAAACAGGAATATGGACCATGTTTTTGGAAT 1020
           ************************************************************

SD10N      TTCTAG 1026
SD10E      TTCTAG 1026
SD10S      TTCTAG 1026
           ******
```
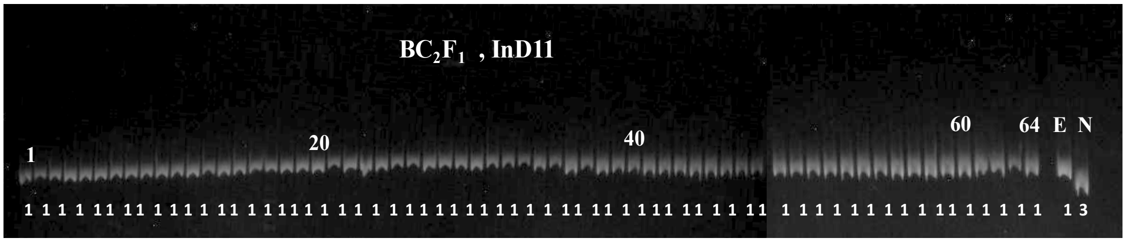

Appendix 5. Gel images showing genotypes of *OS10g32600* in the BC_2F_1 (EM93-1///EM93-1//EM93-1/Nip^{RNAi}) population.



Note: InD11 is an *OS10g32600* gene-based marker. E, EM93-1-like genotype coded as 1; N, Nipponbare-like genotype coded as 3. The image shows that all the 64 BC_2F_1 plants were synchronized by the EM93-1-derived allele at *OS10g32600.*

Appendix 6. Summary of *Os10g32600*'s effect on plant height in the BC_2F_1 (EM93-1///EM93-1//EM93-1/ Nip^{RNAi}) population.

Population	Genotype[a]	N[b]	Statistic	Plant height (cm.)
BC_2F_2	(1) Resistant	24	mean	82.5
			s.e.	0.7
	(2) Susceptible	40	mean	62
			s.e.	0.5
		Effect: (1) - (2)		20.5
		T-test probability		<0.0001

[a] Genotypes were grouped based on resistant or susceptible responses of plants to Hygromycin B.

[b] The number of plants in the group.

Appendix 7. Fitness tests for segregation ratios of the *Os10g32600*-RNAi transgene in two BC_2F_2 lines.

Group[a]	Number of plants in BC_2F_2 line	
	BC_2F_2-14	BC_2F_2-47
Resistant	34	45
Susceptible	14	10
χ^2 value	0.44	1.36

[a] Plants were grouped based on resistant or susceptible responses to Hygromycin B, which was a selective marker for the transgene. The chi-square values were calculated based on the 3:1 expectation for a dominance gene. The chi-square threshold for two degree of freedom at the significant level of 0.05 is 5.99.

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