2020

Reverse Genetic Analysis of *Ehd1* for an Association between Flowering Time and Seed Dormancy in Rice (*Oryza sativa* L.)

Yeter Karakoc
South Dakota State University

REVERSE GENETIC ANALYSIS OF *EHD1* FOR AN ASSOCIATION BETWEEN SEED DORMANCY AND FLOWERING TIME IN RICE (*Oryza sativa* L.)

BY

YETER KARAKOC

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Plant Science

South Dakota State University

2020

THESIS ACCEPTANCE PAGE

Yeter Karakoc

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

Xing You Gu
Advisor Date

David Wright
Department Head Date

Dean, Graduate School Date

ACKNOWLEDGMENTS

Foremost, I would like to express my humble gratitude to the Republic of Turkey, Ministry of Agriculture, and Forestry for providing the excellent opportunity and funding my research and educational training.

Throughout my life and time at South Dakota State University, I have fortunately had many great mentors. I give my thanks to my academic advisor, Dr. Xing-You Gu, for his support and academic knowledge.

I want to acknowledge Dr. Junwei Wang and Dr. Wirat Pipatpongpinyo at Dr. Gu' Seed Molecular Biology Lab for collaboration in this project.

I greatly appreciate my committee members, Dr. Anne Fennell, Dr. Brent Turnipseed, Dr. David Wright, and Dr. Huitian Lu for their continued advice, encouragement, guidance, toleration, and support throughout my master program.

I am genuinely thankful to Dr. Anne Fennell for her sincere love and endless support in all circumstances. It is ineffable to define how much gratitude I feel toward Dr. Fennell not only for being a precious mentor, but also for making me feel at home, being an inspiration, and a motherly figure.

Undoubtedly, I am most grateful to my family, who have wanted me to follow my dreams in science. They have always stood next to me and allowed me to do what I love. I am most appreciative of their unconditional love, guidance, and their never-ending encouragement, which has given me the tenacity of a worm who chooses to dive into the water and catch fish, rather than digging the soil. Thank you always for reminding me that I am capable of anything.

A huge hug and special recognition to Trevor Van Den Top, who has been the best friend and life-changing individual to me. He helped to make my life more comfortable with his precious presence and encouragement. He taught me to never give up since the END is not the end; it means Effort Never Dies. I am also indebted to Vincent Peta, Alex Soupir, and Trevor Van Den Top, who have been my role model for their self-assuredness, their passion for going further, and their determination.

Lastly, I am proud to express my infinite gratitude and would like to repeat my vow to MUSTAFA KEMAL ATATURK, who is the founder of the Republic of Turkey. On the path that you have paved, I swear to walk incessantly toward the aims that you have set.

-THANK YOU!

TABLE OF CONTENT

ABBREVIATIONS

aa	amino acid
ABA	abscisic acid
BC	backcross
bp	base pair
C	celsius
Cen	centimore
cDNA	complementary DNA
Chr	chromosome
CIM	composite interval mapping
cm	centimeter
cM	centiMorgan
CO	Arabidopsis CONSTANS
CTAB	cetyl trimethylammoniumbromide
DAR	days of after ripening
DTF	day to flowering
DOG	Delay of germination
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
dsRNA	double-stand RNAs
EDTA	ethylenediaminetetraacetic acid
FT	Flowering time
FLC	Flowering locus T and C

g	gram
GA	gibberellin
G-by-E	genotype by environmental interaction
GI	germination index
GP	germination percentage
GPt	arcsine transformed germination percentage
h	hour
Hy	Hygromycin-B
HR	Hygromycin resistance
HS	Hygromycin susceptible
IRS	inverted repeat sequence
IL	isogenic line
LDc	long-day-length
LOD	likelihood of odds
LR	likelihood ratio
kb	kilo-base
mM	mili-Molar
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
MIM	multiple interval mapping
NaCl	Sodium chloride
PYD	Physical dormancy
PD	physiological dormancy
PH	plant height
PCR	Polymerase chain reaction
PHS	pre-harvest sprouting
PS	photoperiod sensitivity
qRT-PCR	quantitative real time PCR
QTL	quantitative trait locus or loci
RFLT	Rice Flowering Locus T1
RNA	ribonucleic acid
RM	rice microsatellite
RNAi	RNA interference
s	second
SAM	shoot apical meristem
SD	seed dormancy
SDc	short-day-length
SIM	single interval mapping

SMA	single marker analysis
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
TAIL PCR	Thermal asymmetric interlaced polymerase chain reaction
TBE	tris-borate-EDTA
T-DNA	Transfer Deoxyribonucleic acid
TE	tris-EDTA
TEMED	tetrametylethylenediamine
TF	transcription factor

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

REVERSE GENETIC ANALYSIS OF *EHD1* FOR ASSOCIATION BETWEEN FLOWERING TIME AND SEED DORMANCY IN RICE (*Oryza Sativa* L.)

YETER KARAKOC

2020

This research aimed to understand the evolutionary mechanism of seed dormancy and flowering time and to provide basic knowledge for the manipulation of germination features in crop breeding.

Flowering time and seed dormancy are two adaptive traits for flowering plants. Seed dormancy (SD) helps regulate the timing of germination to promote the survival of seed-bearing plants in adverse environments. Cereal crops and weedy/wild relatives, such as Asian cultivated rice and weedy rice (*Oryza sativa* L.), have weak and strong seed dormancy, respectively. Thus, weedy and cultivated rice has been used as a model system to investigate mechanisms of seed dormancy in the previous research. Flowering time (FT) determines the timing of the seed set. Both seed dormancy and flowering time may associate with each other to complete the life cycle in natural ecosystems.

A cluster of quantitative trait loci (QTL) for SD/FT (*SD10/FT10*) was identified on chromosome 10, which was responsible for an SD-FT association in rice (*O. sativa* L.). Fine-mapping delimited *SD10/FT10* to a 200-kb region, which contains *Early heading1* (*Ehd1*). *Ehd1* encodes a Myb transcriptional factor (TF) and promotes flowering. Thus, *Ehd1* could be an underlying gene of *FT10*. However, *Ehd1* was not reported for an effect on SD. Thus,

the research was conducted to determine if *Ehd1* is an underlying gene for the QTL *FT10* only or both *FT10* and *SD10*. The first objective of this research was to evaluate RNA interference (RNAi)-mediated effects of *Ehd1* on FT and SD in generations of transgenic lines. An *Ehd1*-RNAi construct was used to transform the cultivar Nippponbare (*O. sativa*, ssp. *Japonica*), and then introduced into the genetic background of EM93-1 (*O. sativa*, ssp. *indica*), the donor of the flowering-promoting allele at *FT10/SD10* or *Ehd1*, by marker-assisted backcrossing (BC). Single plant-derived BC_2F_3 (selected from #14 and #47), BC_2F_4 and BC_2F_5 (selected from #81 and #84) lines were evaluated for the presence or absence of the *Ehd1*-RNAi transgene using Hygromycin-B testing, for time (days) from germination to flowering (DTF), and the degree of SD by standard germination testing. These generations of data demonstrated that there was one copy of the transgene segregating in the lines, silencing *Ehd1* delayed flowering and also enhanced seed dormancy, and there was a negative correlation between DTF and germination percentage. These results indicate that *Ehd1* has pleiotropic effects on both FT and SD.

The second objective of this research was to identify genes related by *Ehd1* to influences FT and SD. Quantitative trait analysis (QTL) was used to identify the possible genes in a mapping population of about 300 F_2 plants from a cross between an *Ehd1*-RNAi transgenic line and EM93-1. The mapping population was evaluated for DTF, SD, and the resist (R) or susceptible (S) response to Hygromicyn B, and genotyped for about 50 DNA markers distributed along the 12 chromosomes. A single copy of the RNAi construct (T-DNA) was mapped to chromosome 7, and the position was flanked by markers RM295 and RM3325. Large differences in both FT and SD between the R and S

groups were observed in the mapping population. Five QTL for FT and two QTL for SD were detected in the population. Both of the FT and SD QTL includes one mapped at the transfer DNA (T-DNA) insertion position of the *Ehd1*-RNAi transgene. All these QTLs were located on chromosomes 7 or 8, but not on chromosome 10 or near *Ehd1*. The QTL differed in effect on FT or SD between the R and S groups. These results suggested that *Ehd1* influences FT and SD at least partly by regulating expressions of genes underlying the QTL on chromosomes 7 and 8.

This research provided evidence that *Ehd1* has phenotypic effects on flowering time and seed dormancy, and *Ehd1* also regulates expressions of some other genes to influence the SD-FT association.

Chapter 1. Introduction and Literature Review

Seed dormancy and flowering time are adaptive traits of both ecological and agricultural importance as they regulate the timing of germination and seed set. This thesis project aimed to address genetic mechanisms underlying the association between seed dormancy and flowering time in rice (*Oryza sativa* L.) reported by Gu et al. (2018). This chapter starts with an introduction of concepts frequently used in the thesis, then reviews of research progress in the research area, and finally define the objectives of this project.

1.1 Introduction

1.1.1 Seed and dispersal unit

A seed in flowering plants (Angiospermae) is a mature ovule capable of germination to develop into a plant. Basic structures of a seed are the embryo, storage tissues, and testa (seed coat) tissues. The embryo is generated from a zygote (2n) produced by the fusion of a haploid egg cell (n) with a haploid sperm (n). A developed embryo contains radical, plumule, and one or two cotyledons. Plants are divided into two groups based on the number of cotyledons in a seed: monocotyledonous and dicotyledonous. Dicotyledonous plants have well-developed cotyledons, which serve as a storage tissue and provide nutrients for seed germination and seedling establishment (Bewley, 1997; Miller and Matthew, 2016; Tereshchenko et al., 2017).

The endosperm is developed from a primary endosperm cell (3n) produced by the fusion of two haploids (n) polar nuclei in the central cell with the other sperm (n). Based

on the persistence of the endosperm tissue at maturation, seeds can be divided into two groups: endospermic and non-endospermic. Endospermic seeds have a well-developed endosperm, which contains several cell types, including an aleurone layer and starchy endosperm. The aleurone layer consists of living cells that are capable of synthesizing hydrolysis enzymes to degrade the starchy endosperm. The liberated sugar by starch degradation for its growth is benefited by the embryo (Yan et al., 2014). The starch endosperm serves as a major nutrient reserve for germination and seedling growth. Seeds of grass species, including cereal crops, are endospermic. Whereas, for non-endospermic seeds, the nutrient in the endosperm is transferred to the cotyledons during seed development and a mature seed may have only one or a few cell layers of the endosperm tissue, such as seeds from *Arabidopsis thaliana*, or have non-endosperm, such as the common pea (*Pisum sativum*).

The testa is derived from the integuments (2n) or the inner cell layer of the ovule and one layer of the nucleus. Thus, the testa belongs to the maternal generation.

Seed, in some literature, often refers to a dispersal unit, which could be a pure seed enclosed by additional maternal tissue(s) to facilitate seed movement or dissemination. Depending on species, a dispersal unit can be a caryopsis, which is a seed enclosed within a pericarp (fruit coat), such as common wheat (*Triticum aestivum*) and maize (*Zea mays*). Dispersal units can also be a spikelet, such as rice and oats. A spikelet of rice is a caryopsis enclosed by the lemma and palea or hull (Figure 1.1).

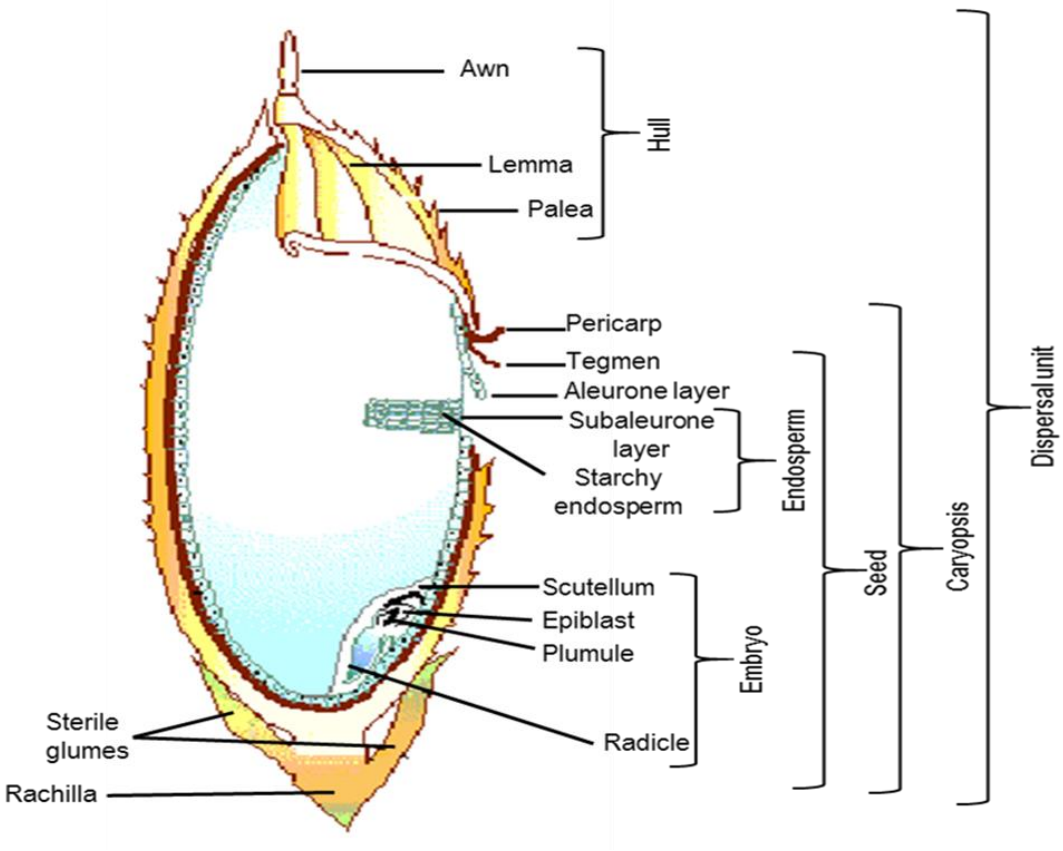

Figure 1.1 Structures of a dispersal unit. The diagram was modified from http://jeaheerice.cafe24.com/e_03_01.html to show structures of a seed, a caryopsis, and a spikelet from the rice plant.

1.1.2 Seed dormancy

A dormant seed lacks the capacity to germinate in a specified period under any combination of common physical environmental factors that are otherwise favorable for its germination (Baskin & Baskin 2004). Seed dormancy (SD) is a temporary failure of a viable seed to complete germination under favorable conditions (Bewley, 1997). Seed dormancy can be related to some adaptive benefits in consideration of gap detection, the spread of germination, or seasonal detection (Rodriguez et al., 2015).

The degree of seed dormancy determines the period between maturation and germination under favorable conditions. The conditions are complex combinations of water, light quality and quantity, mechanical restraint, temperature, gasses, hormone structure, and seed coats (Shu et al., 2016).

Seed dormancy, which is a genetically complex trait, is one of the most critical survival mechanisms in natural ecosystems and controlled by genetic and environmental elements. Dormant seeds can avoid germination in adverse conditions, such as frost, drought, and excessive moisture. Eventually, the seeds germinate in proper time, and the new plants can set seeds to complete the life cycle. Seed dormancy is also of agricultural importance. In cereal crops, weak seed dormancy can cause pre-harvesting sprouting (PHS), which is germination on the plant after maturation due to excessive moisture in the atmosphere and heavy or timeless rain, before harvest (Lin et al., 2016; Gu et al., 2004a). This case negatively affects grain quality and causes loss of yield (Gu et al., 2004a). Strong SD may cause the persistence of weeds in croplands and, reduced germination uniformity of crop cultivars, and feral plants; whereas weak or no SD may cause the pre-harvest sprouting (PHS) problem or vivipary (germination on the plant during seed development under some conditions such as frost, drought, and frost, drought, and excessive moisture) in crop production (Karssen, 2017). On the other hand, persistent seed dormancy can cause non-uniform germination or weed persistence in croplands.

Thus, knowledge about seed dormancy and germination can be used to model seasonal fluctuations of weed seed bank dynamics and population density, estimate

seedling rates in crop production, and manipulate germination capabilities in crop breeding.

Seed dormancy can be divided into the primary and secondary types, based on the time of the dormancy is developed or induced. Primary dormancy is developed on the plant before maturation and dispersal, which is influenced by environmental issues like humidity, temperature, and light (Gu et al., 2004a). Although the development of primary dormancy is induced by the plant hormone abscisic acid ABA, there seems no linear correlation between ABA content and the degree of dormancy. There are two types of primary dormancy, such as exogenous and endogenous dormancy. Germination is affected by external influences and inhibits the emergence of seed. This exogenous incident takes place due to seed coat dormancy, and tissues covering the embryo are also affected in this condition. The endogenous dormancy can be influenced by the internal condition of an embryo. ABA level during seed maturation in the mother plant is responsible for primary dominance (Song et al., 2019). Secondary dormancy is induced to the primary dormancy-released seeds by unfavorable environmental conditions for germination. In multiplexed cereals such as oat, wheat, and barley, the introduction of secondary dormancy happens by high temperature and hypoxia (Rodriguez et al., 2015). This research focused on primary dormancy.

Seed dormancy can also be divided into embryonic, endosperm-imposed, and coat-imposed types, based on seed component tissues that cause the dormancy. Embryo dormancy is enforced by germination inhibitors in the embryo and has been reported for many species, especially for wild oat and wheat (Debeaujon et al., 2018; Kelly et al., 992; Gu et al., 2004a). Endosperm-imposed dormancy is called weakness of endosperm,

which is related to coat dormancy release or germination progress. The ABA-GA ratio can prevent embryo regulation. GA induces endosperm weakening, even though there could be some blocked or limited mechanisms by ABA (Savage and Metzger, 2006). Coat-imposed dormancy is because the testa or the other maternal tissues such as palea hull or glumes, and in some circumstances endosperm restrict germination by blocking oxygen and water infusion into the embryo or releasing of toxic materials from the seed (Debeaujon et al., 2018; Kelly et al., 1992; Gu et al., 2004a). The plant hormone abscisic acid (ABA) induces seed dormancy and inhibits germination, while gibberellin (GA) promotes dormancy release and germination (Yamaguchi et al., 2018).

There is another type of classification of seed dormancy with five classes: physiological seed dormancy, morphological dormancy, morpho-physiological dormancy, physical dormancy, combinational dormancy. Among the five-dormancy types, physiological dormancy is the most popular one. There are three types of physiological dormancy (PD): PD deep, PD non-deep, and PD intermediate (Lu et al., 2017). In PD deep dormancy, the embryo controls germination but without the help of GA or Gibberellin. In intermediate and deep PD, GA promotes germination, and a seed coat with the tissues surrounding the embryo helps to control the condition. In non-deep dormancy, there are several types of classifications, and every type is dependent on different physiological effects of different temperatures. Morphological dormancy is effective in underdeveloped seeds and differentiated by cotyledons. Morphophysiological dormancy requires dormancy breaking treatments depending upon different temperatures (Zhou et al., 2015). PY or physical dormancy occurs in the seeds, which require prevention from water absorption.

1.1.3 Germination

Germination is one of the critical traits for the adaptability of plants. Germination starts with the absorption of water by a dry seed (imbibition) and terminates with the appearance of the embryonic axis, generally the radicle (Bewley, 1997; Amnuaysin et al., 2017).

Germination consists of the three phases in terms of water uptake, which are a rapid initial uptake (imbibition or Phase I), plateau phase in mitochondria synthesis (Phase II), and post-germination stage (Phase III) that radicle starts to grow.

The fundamental step, Phase I (imbibition), is the absorption of water by a dry seed, which causes swelling of the seed and breaks the seed coat by increasing the level of water so that radicle enables to come out in the form of the primary root. The start of Phase II (plateau phase) is related to the restriction of water uptake. Additionally, it is the stage in which physiological and biochemical events are observed, such as the synthesis of new mRNA and protein. The protuberance of the embryonic axes from the seed cover or testa, and maintenance of water uptake is observed in Phase III (Bewley et al., 1997).

Germination of seeds is a very complicated process that is regulated by different environmental factors like temperature, light, soil salinity, gibberellic acid (GA), and abscisic acid (ABA). GA is synthesized during germination and induces the synthesis of alpha-amylase, which promotes the degradation of starch.

Water plays an active role in enzyme activation, degradation, translocation, and use of spare storage material, and therefore is a fundamental requirement for germination.

Air consists of about 20% oxygen, 0.03% carbon dioxide, and about 80% nitrogen gas, in the course of seed germination, oxygen concentration, and respiration rise.

The response of germination to temperature depends on species, variety, growing region, quality of the seed, and duration of time from harvest. High-quality seeds can germinate under broader temperature ranges than low-quality seeds. The minimum temperature sometimes can not be detected throughout germination progress; however, the determination of germination at such a slow ratio is done before the actual germination is completed. The optimum temperature is identified as the temperature that gives the highest germination percentage in the shortest time. Correspondingly, the optimum temperature for most crops is considered as between 15 and 30 ^{o}C. The maximum temperature is evaluated at which denaturation of proteins essential for germination occurs. The maximum temperature for most species is between 30 and 40 ^{o}C. (McDonald, 2001; Shaban, 2013; Copeland et al., 1985; Simpson, 1990).

1.1.4 Flowering time

Flowering time is a feature of agricultural and biological importance since it regulates the time to set seeds under favorable conditions. It also supplies altitude and latitude consistency of domesticated and wild plants (Gu et al., 2007 and 2004b). Flowering occurs after the completion of gametophyte development when the panicle gradually emerges from the leaf sheath to prepare for double fertilization to produce the next generation of seeds in cereal crops. A life cycle of flowering plants starts with a fertilized egg or zygote (2n) and ends with the maturation of male and female gametophytes. Variation in the length of a life cycle is mainly determined by the period from seed to seedling (germination) and the period from vegetative to reproductive

development (flowering). Therefore, the seed dormancy and flowering time traits are essential for a locally adapted population to complete the life cycle (Garner 1933; Endo et al., 2016).

1.1.5. Rice

Rice is one of the most crucial food crops in the world, particularly for Asia, Latin America, and Africa. Rice provides 35–60% of the food requirement consumed by nearly more than 3 billion people. China and India are the most abundant rice-producing and consuming countries in the world (Fageria, 2007). Besides, rice undoubtedly provided a very high level of compatibility to various ecological habitats, many of which are characterized by their unique hydrological state (Nguyen et al., 1997).

Rice has a significant advantage for seed dormancy in terms of genetic variation, having a small genome size, and sharing genome synteny with the other cereals in the grass family. Therefore, rice becomes an ideal model for research on seed dormancy in grasses (Ye et al., 2010). There are 23 species, including two cultivated species *Oryza. sativa* L. and *O.glaberrima* Steud, in the genus *Oryza* (Chopra and Prakash 2002). These species can be grouped into four distinct complexes: *O. sativa* (2n=AA=24), *O. officinalis* (2n=24-48), *O. meyeriana* (2n=24), and *O. ridleyi* (2n=48), based on genomic composition (Khush, 1997). The two cultivated species belong to the AA genome.

Asia cultivated rice originated from the wild rice *O. rufipogon,* which has perennial to annual types in tropic to subtropic areas (Khush and Brar, 2002). Thus, Asian cultivated rice is a short-day plant for flowering. Rice has two subspecies: indica and japonica (Kato et al., 1928). The Indica subspecies distributes in low latitudes and altitudes in tropical and subtropical regions. Whereas, japonica subspecies distributes

mostly in high latitude regions. The javanica type of rice distributes in some low latitude, high-grade mountainous regions such as southwest China, Laos, Myanmar and Vietnam, and many other Southeast Asian countries. Indica and japonica subspecies also differ in morphology and physiological-biochemical properties, such as plant height, seed length/width ratio, and starch types of grains (Lu et al., 2009).

The japonica subspecies includes temperate and tropical ecotypes, and the latter is also known as the javanica type (Morinaga, 1954). African cultivated rice originated from the wild species *O. barthii*. It is different from *O. sativa* by its panicle lacking secondary branches or short, rounded ligules (Khush and Brar, 2002; Oka, 1988).

1.2 Literature Review

1.2.1 QTLs controlling seed dormancy

Seed dormancy (SD) is controlled by multiple genes, which have been mapped as quantitative trait loci (QTL) in several species (oil, fruit, vegetable, and cereal) or model plants (rice, Arabidopsis). In Arabidopsis, SD QTLs are named as a series of *Delay of Germination* (*DOG*) like *DOG1, DOG2, DOG3, DOG6,* and *DOG7* by Alonso-Blanco et al. (2003) and Van der Schaar et al. (1997). In wheat (*Triticum aestivum*), by Osa et al., 2003, on the short and long arms of the chromosome 3A of *QPhs.ocs3A.1,* and *QPhs.ocs3A.2,* and the long arm of the chromosome 4A *QPhs.ocs4A. I*n barley (*Hordeum vulgare*), the major QTLs *SD1* on chromosome 7 (5H) and *SD2* on chromosome 7 (5H) by Han et al., 1999 and Gao et al., 2003 have been detected respectively.

Likewise, many SD QTLs were reported in wild, weedy and cultivated rice on the 12 chromosomes by Lin et al.,1998; Cai & Morishima 2000; Gu et al., 2004a, Gu et al., 2006, Gu et al., 2005b; Wan et al., 2006; Ye et al., 2010; Xie et al., 2011; Subudhi et al., 2012 by Lin et al.,1998; Cai & Morishima 2000; Gu et al., 2004a, Gu et al., 2006, Gu et al., 2005b; Wan et al., 2006; Ye et al., 2010; Xie et al., 2011; Subudhi et al., 2012 as summarized in Tables 1.1 and 1.2. For instance, Ye et al. (2010) identified three new seed dormancy QTLs which are *qSD1-2*, *qSD3* and *qSD10* from a backcross BC_1F_1 and BC_1F_3 populations obtained from the crossing of weedy (SS18-2) and cultivated rice line (EM93-1), and they are located on the long arm of chromosomes 1, 3 and 10, respectively. The dormancy-enhancing alleles of *qSD1-2* and *qSD10* have derived from EM93-1 and having pre-dominant additive (a) effect and small dominance (d) effect; correspondingly, the two new QTLs *qSD1-2* and *qSD10* seem to be co-dominant, and the QTL *qSD3* was a recessive locus. Having 42% of phenotypic variance indicated that the *qSD10* was detected the only known dormancy QTL and was segregating in the F_3 population, and the effect of *qSD10* might vary with genetic background in rice when dormancy-enhancing alleles of the QTLs *qSD1, qSD4, qSD7-1, qSD7-2, qSD8,* and *qSD12* were removed. Furthermore, it has been demonstrated that *qSD1-2* is identical to *sd1* (*semidwarf1*) (Ye et al., 20152010).

There are a large number of studies on the association between seed dormancy and plant height. The QTLs *qSD1-2* and *qSD7-2* were related to plant height, and *qSD7-2* was located close to the QTL *Sdr4*in different advanced generations by Gu et al., 2004a.. The isolated cluster of the *qSD7-2/qPH7* allele from SS18-2 could be used as an alternative gene for semidwarf varieties with increased resistance to pre-harvest sprouting

because this cluster has been found similar to *Sd1*. Besides, GA-sensitive (*qSD1-2* and *qPH1*) and GA-insensitive (*qSD7-2/qPH7*) natural mutants have been isolated by the researchers. In other words, *qSD1-2* and *qPH1*-underlying gene (*Sd1* or *OsGA20ox2*) has been able to regulate plant height and also has an influence on seed dormancy and germination ability by regulating GA biosynthesis in developing seeds. Furthermore, it is most likely to indicate that *qSD7-2/qPH7* could be a new gene to regulate the associated traits by GA signaling. (Ye et al., 2013).

Seed Dormancy1-2 (*qSD1-2*) was associated with endosperm-imposed dormancy and plant height, and also was indicated as the *semidwarf1* gene (*Sd1*, *OsGA20ox2* or Green Revolution gene) in rice. The 20 kb region of the *OsGA20ox2* (GA synthesis) gene, which is the only defined candidate gene for *qSD1-2*, has shown a pleiotropic effect on germination. Induced loss of the functional gibberellin (GA) synthesis gene causes seed dormancy along with the height reduction of rice plants. Expression gibberellin of the synthesis gene showed induced accumulation of gibberellin in the seed and thus enhances tissue morphogenesis with germination. The mutant allele (a loss-of-function mutation of the GA synthesis gene) found in the semi-dwarf cultivars significantly reduced the gibberellin accumulation and delayed tissue morphogenesis, abscisic acid (ABA) accumulation, seed maturation, physiological maturity, and subsequently enhanced the chance of seed dormancy (Ye et al., 2015).

The determination of the genetic functions and the traits has been developed through the quantitative trait loci (QTL) mapping the cloning of these loci in the cereal plants and the model plants as well as for the better assessment of the seed germination and dormancy mechanisms (Schatzki et al., 2013).

Table 1.1 A list of seed dormancy QTLs reported in rice

Name	Chr[a]	Marker[b]	R^2 (%)[c]	Donor[d]	Reference
NA	3	C1488	6	Kasalath	Lin et al.,1998
NA	5	R830	8	Kasalath	Lin et al.,1998
NA	7	R1440	11	Kasalath	Lin et al.,1998
NA	7	R1245	11	Kasalath	Lin et al.,1998
NA	8	C390	7	Nipponbare	Lin et al.,1998
qDOR-2	2	Amp1-RZ476	8-11	W1944	Cai &Morishima 2000
qDOR-3-1	3	G144-BCD454	13-17	W1944	Cai &Morishima 2000
qDOR-3-1	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., 2004a
qSD4	4	RM252	6-11	SS18-2	Gu et al., 2004a
qSD7-1	7	RM5672	7-18	SS18-2	Gu et al., 2004a
qSD12	12	RM270	48-54	SS18-2	Gu et al., 2004a
qSD7-2	7	RM346	7	SS18-2	Gu et al., 2004a
qSD8	8	RM135B	7	SS18-2	Gu et al., 2004a
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 et a;., 2010
qSD3	3	RM520	9-11	SS18-2	Ye et a;., 2010
qSD10	10	RM271	8-42	EM93-1	Ye et a;., 2010
qSD-1-1	1	RM23	12	cv.N22	Xie et al., 2011
qSD1-2	1	RM448	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 indicated.

[b] The nearest markers to the QTLs.

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

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

Several QTLs for SD were map-based cloned (Table 1.2). These include *qSD1-2*, *Seed dormancy4* (*Sdr4*) and *qSD7-1* (transcription factor gene) in rice (Ye et al., 2015; Sugimoto et al., 2010; Gu et al., 2011), *Delay of Germination1* (*DOG1*) in *Arabidopsis thaliana* (Betsink et al., 2006)**,** and *High-temperature germination6.1* (*Htg6.1*) gene in lettuce. Cloning and characterization of QTL underlying genes provided new knowledge about regulatory and evolutionary mechanisms of seed dormancy and manipulation.

Table 1.2 A list of some seed dormancy QTLs cloned from plant species.

QTL[a]	Molecular function[b]	Physiological function[c]	Species	Reference
DOG1	unknown protein	unknown	*Arabidopsis thaliana*	Betsink et al., 2006
Sdr4	unknown protein	unknown	*O. sativa*	Sugimoto et al., 2010
qSD7-1	bHLH transcription factor	ABA & flavonoid synthesis	*O. sativa*	Gu et al., 2011
MFT	Phosphatidylethanolamine-binding protein	unknown	*T. aestivum*	Liu et al., 2013
RD02	TFIIS Transcription elongation factor	Transcription elongation	*Arabidopsis*	Liu et al., 2011
qSD1-2	GA20-oxidase	Gibberellin synthesis	*O. sativa*	Ye et al., 2015
HUB1 (RD04)	C3HC4 ring finger	Transcription elongation	*Arabidopsis*	Liu et al., 2007

[a] name of the QTLs

[b] Molecular functions of cloned QTL

[c] Physiological function of cloned QTL

Genes controlling flowering time

1) QTLs associated with flowering time

Quantitative trait locus (QTL) mapping helps in the identification of particular genes responsible for genetic variation in complex traits (Miles and Wayne, 2008). QTL varies in different levels of polygenic effects, such as a product of multiple genes, and surrounding environments. The architecture of the plant body is dependent on the phenotypic trait of organisms (Luo et al., 2017). QTL helps in the detection of genes for both quantitative (height of a plant) and qualitative (smooth vs. wrinkled seeds) traits. Multiple phenotypic attributes can be regulated by a single gene. QTL can identify the pre-specific or candidate gene of a particular trait and defined a specific sequence in DNA as a marker-assisted technique. Quantitative Trait Loci (QTL) mapping is based on finding the relationship between a genetic marker and a known phenotype. One of the types called during the progress of the recombination inbred line population, traditional bi-parental of QTL mapping is limited by the recombination actions arranged over a few generations.

There are several approaches to QTL mapping, such as a single marker approach/analysis, single/ simple interval mapping (SIM), composite interval mapping (CIM), multiple interval mapping (MIM) (Bennewitz et al., 2018). The single-marker approach has several limitations as QTL detection decreases with an increase of distance between marker and QTL. The method cannot specify the relationship between markers and associated one or two QTLs. The effects of QTL are possibly ignored as the methods are perplexed with recombination frequencies. Simple/ Single Interval Mapping (SIM) evaluates the association between the attribute values and the genotype of a hypothetical QTL (at the specific QTL) multiple analysis facts between a pair of subsequent marker

loci (the appropriate interval). This has been the broadest method as it can be easily retrieved through statistical combinations such as MAPMAKER/QTL (Kadambari et al., 2018).

Composite Interval Mapping (CIM) for the single QTL in a definite time of interval with numerous regression analysis based on a marker oriented QTL. The multiple interval mapping (MIM**)** indicates the location of QTLs with the position between markers or probes. The method shows appropriate grants or the allowance for absent genotype data, and that helps to allow relations among QTLs to be identified. It can be said that a significant problem in multiple QTL methods is a model selection, and the main concerning factor is the formation of suitable criteria or baseline for comparing models or replicas (Kadambari et al., 2018).

Flowering time which furthers for adaptability of sexual reproduction under proper conditions has been studied, and flowering time which furthers for adaptability of sexual reproduction under proper conditions has been studied, and many QTLs for flowering time have been identified due to its agricultural importance. One of the identified major FT QTLs is *E1* from japonica type of varieties, which is important for the extremely early maturing cultivars. Moreover, the QTL *Se-1* on chromosome 6 has a major effect on flowering time; it has more than two alleles identified in different ecotypes. The QTL *Heading date1* (*Hd1*) was considered to be allelic to *Se-1* (Gu et al., 2004b).

Three QTLs with a significant effect on flowering time (FT) were mapped on chromosomes 7 ($Se_{7.1}$ and $Se_{7.2}$) and 8 (Se_8) in a backcross F_1 population from a cross between EM93-1 (day-neutral and extremely early flowering) and SS18-2 (sensitive to

photoperiod). The weedy rice SS18-2 has genes delaying flowering and sensitive to photoperiod sensitivity (PS) at $Se_{7.1}$, $Se_{7.2,}$ and Se_{8} under long day-length conditions. The three genes originated from wild rice (*O. rufipogon*) as the donor parent SS18-2 is the accession of wild-like weedy rice. SS18-2 may carry a mutant or promoting flowering allele at *Se-1* (*Hd1*) on chromosome 6 (Gu and Foley, 2007).

Table 1.3 Classical Mendelian genes and isolated genes for natural variation in flowering time in rice

Gene	Synonym	Effect on flowering	Chromosome	References
DTH2		LDc promotion	2	Wu et al., 2013
Ehd4		SDc/LDc promotion	3	Gao et al., 2013
Hd16	*ELI*	LDc repression	3	Dai and Xue, 2010; Hori et al., 2013; Kwon et al., 2014
DTH3	*OsMADS50*	SDc/LDc promotion	3	Lee et al., 2004; Bian et al., 2011
E3	*Hd6*	LDc repression	3	Syakudo and Kawase, 1953; Syakudo et al., 1954; Takahashi et al., 2001
E2	*Hd17, Ef7, OsELF3-1, OsELF3, Hd-q*	SDc/LDc promotion	6	Syakudo and Kawase, 1953; Syakudo et al., 1954; Matsubara et al., 2008a; Monden et al., 2009;

				Yuan et al., 2009; Matsubara et al., 2012; Saito et al., 2012
Se	*Sel, K, Lm, Hd1*	SDc promotion/LDc repression	6	Chandraratna, 1953-1955; Yokoo and Fujimaki, 1971; Yano et al., 1997-2000
Hd3a		SDc promotion	6	Kojima et al., 2002
RFT1		LDc promotion	6	Kojima et al., 2002; Ogiso-Tanaka et al., 2013
E1	*M, m-Ef1, Ghd7*	LDc repression	7	Syakudo and Kawase, 1953; Syakudo et al., 1954; Tsai and Oka, 1966; Tsai, 1976; Okumoto et al., 1992; Okumoto and Tanisaka, 1997; Xue et al., 2008
OsPRR37	*Hd2*	LDc repression	7	Koo et al., 2013

$Se_{7.1}$, $Se_{7.2}$		Photoperiod sensitivity	7	Gu et al., 2007
Hd18		SDc/LDc promotion	8	Shibuya et al., 2016
Se_{8}		Photoperiod sensitivity	8	Gu et al., 2007
DTH8	*Ghd8, LHD1, Hd5, LH8*	SDc promotion/LDc repression	8	Wei et al., 2010; Dai et al., 2012; Fujino et al., 2013; Chen et al., 2014
E	*Ef1*, *Ehd1*	SD/LD promotion	10	Tsai and Oka,1966; Tsai, 1976; Sato et al., 1988; Doi et al., 2004; Saito et al., 2009

Note: SDc, short daylength; LDc, long daylength; Se, photoperiod sensitivity; Hd, heading date.

Several progeny lines developed by the crossing of Nipponbare with Kasalath were used to identify 15 QTLs for flowering time. It was indicated that the allele of *Hd3* from Kasalath has no influence on photoperiod sensitivity (PS), but increases the effect of the Nipponbare alleles at *Hd1* and *Hd2*. *Heading date3a* (*Hd3a*) and *Rice Flowering Locus T1* (*RFLT1*) encode florigen (the floral stimulus) for the rice plants (Mulki et al., 2018). *RFLT1* is expressed under a short day and long day period condition. The non-functional *RFLT1* allele but the homozygous *Ehd1* and *Hd3a/RFLT1* are failed to influence the floral transition under long and short daytime. There is another florigen receptor called 14-3-3 proteins, which cannot relate to the mutated E105K with the non-functional state of *RFLT1* gene due to long or short daytime (Camoni et al., 2018).

Heading date1 (*Hd1*) was the first gene identified for flowering time in rice on chromosome 6 and significantly similar to Arabidopsis *CONSTANS (CO)* under short-day (SDc) conditions. *Hd1* represses flowering and functions antagonistically with *Ehd1* under long-day (LD) conditions. The arrangement of *Hd1/CO* flowering time (FT) group genes has a crucial role in the photoperiodism of short and long-day plants. The effect of *Hd6* from the Kasalath was observed only in the presence of the Nipponbare allele of *Hd2*. The allele of *Hd3a* enhances flowering under short-day (SDc) conditions, while *Hd3b* delays flowering under long-day (LDc) and natural-field conditions (Table 1.3) (Hori et al., 2016; Park et al., 2008; Doi et al., 2004).

Heading date (*Hd*) is an essential character of rice, which is related to many characters of agronomic importance, such as grain yield. Three QTLs for FT were mapped on chromosomes 1, 6, and 10 in a backcross F_1 population cross between the Africa cultivated variety *O.glaberrima*, which was the recurrent line as the donor parent

and, Asian cultivated rice variety Taichung 65 (T65). The QTLs in the heterozygous condition in *O. glaberrima* alleles around on chromosome 1 and 6 delayed heading date, while another allele/s on chromosome 10, which is known as *Ehd1* has the alleles from Taichung 65 (T65), promoted early heading (Doi et al., 2004).

Cloning of rice flowering time QTLs is important for understanding the flowering regulation but also to provide information on the pleiotropism of heading date genes on yield traits. *Grain Number Plant Height and Heading Date7* (*Ghd7)* has been the first cloned rice flowering QTL having an essential role in increasing grain productivity and yield potential. *Ghd1* is the fundamental gene in the regulatory network for *Ehd1-RFLT1* pathway, and also for principal gene connection the *Ehd1-RFLT1* pathway and the evolutionarily preserved *Hd1-Hd3a* pathway. Also, other similar flowering genes *DTH8/Ghd8* and *OsPRR37/Ghd7.1/DTH7/Hd2*, are significant regulators for the *Ehd1-RFLT1* and *Hd1-Hd3a* pathways, respectively, and the two central genes *Hd1* and *Ehd1* themselves (Zhu et al., 2017).

RFLT1 has a large effect on the grain productivity, which may be varied by different environments in rice. Besides, *RFLT1* has no response to the photoperiod. In that way, *RFLT1* is getting suitable to develop a great adaptation for different various eco-geographical conditions.

RFLT1 also has a changeable impact based on the genetic backgrounds and variable environmental conditions in the genetic control of grain yield in rice. Because of the case, it was similar to the variation of the *Ghd7*'s pleiotropic effect. It suggested that the pleiotropic effects of flowering genes depending on variable environmental conditions, and the genetic background can be a common case (Zhu et al., 2017).

According to ecogeographical adaptation, the yield potential of cultivated rice has also been reported in other studies to be considerably identified by the allelic combinations of *Ghd7*, *Ghd8,* and *Hd1*. *Ghd7* and *Ghd8* have a significant effect in terms of regulation of the *Ehd1-RFLT1* pathway, and also, *Hd1* acts as an *Ehd1* suppressor by interacting with the *Ghd7* protein (Zhu et al., 2017).

2) Early heading date 1 (*Ehd1*)

Early heading date 1 (*Ehd1*) is a foremost gene that acts as a signal integrator and persuades the expression of the two florigens (the floral stimulus) *Heading date 3a* (*Hd3a*) and *Rice Flowering Locus T1* (*RFLT1*), in rice. Under long-day and non-permissive conditions, *Ehd1* expression is compressed by *CONSTANS-like* (*COL*) proteins such as *COL4, Heading date 1* (*Hd1*), and *Grain yield and heading date 7* (*Ghd7*). However, *Hd1*, *OsMADS51*, and *OsINDETERMINATE 1* genes convince *Ehd1* to enhance flowering under permissive short-day conditions (Cho et al., 2016). *Grain yield and heading date 7 Ghd7* is one of the major repressors of *Ehd1* to function under long-day conditions (Lee et al., 2014).

Ehd1 functions are as a specific component in the flowering pathway in monocot plants. *Ehd1* has a significant role in promoting *Hd3a* and *RFT1*genes (Cho et al., 2016).

Ehd1 consists of the response regulator (RR) protein family in a cytokinin signaling pathway. One of the two type intermediary cytokinin signaling RRs, type-A RRs, has negative constituents that bind and prevents type-B RRs, which are transcription activators. Type-B RRs have a GARP DNA-binding (G) domain and a receiver (R) domain, whereas type-A RRs contain only the former. Cytokinin causes type-A RR genes

and balances their proteins, while type-B RR genes that have transcriptional activity are not stimulated by cytokinin (Cho et al., 2016).

The C-terminal region of the transcriptional activator *Ehd1* functions is similar to other type-B RR proteins in Arabidopsis. Only the G domain was located in the nucleus (Cho et al., 2016).

Ehd1 is one of the most prominent inducers of the floral stimulus or 'florigen' gene expression. It has also been found that the C-terminal portion of the protein expressed by the gene comprises the GARP DNA (G) binding domain, which promotes flowering during overexpression (Cho et al., 2016). Whereas the N-terminal portion of *Ehd1* develops a receiver (R) domain, which delays the flowering utilizing inhibiting *Ehd1* activity. Moreover, it has been seen that the *SNB* and *OsINDETERMINATE* 1 (*OsIDS1)* are the genes that show a negative effect on the expression of the *Ehd1* gene as these inhibit the flowering of rice plants. It has been found that mir172 is the gene that decreases the flowering time of the plants, and the level of mir172 rises among the older plants. The overexpression of the mir172 gene induces the action of the *Ehd1* and subsequently reduces the action of the SNB and *OsIDS1* gene and thus reduces the flowering time in the older plants.

It has also seen that the SIP1 binds to the promoter of *Ehd1* and interacts with a chromatin remodeling factor *OsTrithorax* (*OsTrx1)* gene, and mutation of SIP1 leads to the late heading date of rice plants. The defective *SIP1* and *OsTrx1* leads to the reduced production of *H3K4me3* at *Ehd1* and results in reduced expression of *Ehd1* and thus late development of the flowers. Hence, it can be stated that the *Ehd1* gene is directly

responsible for flowering in rice, and, as mentioned above, the gene families also play crucial roles in the process of flowering (Jiang et al., 2018).

Flowering timing is determined by genetic pathways that combine endogenous and environmental signals, the transition to vegetative reproductive development in plants. Rice flowering time (heading date) has been a crucial agronomic feature for environmental adaptation, and well-timed flowering is essential for desirable grain production. There are two homologs of *Arabidopsis thaliana FLOWERING TIME LOCUS T (FT)*: *Heading date 3a* (*Hd3a*) and *RICE FLOWERING TIME LOCUS T1* (*RFLT1*) which have functioned as florigens under short-day (SDc) and long-day (LDc) conditions, respectively. Moreover, the genes expressed in the leaf phloem and transferred to the shoot apical meristem (SAM), to cause reproductive development. The positive and negative regulators of *Hd3a* and *RFT1* organize heading in rice. (Jiang et al., 2018).

Ehd1 is a B-type response regulator gene which independently promotes flowering time under SDc and weakly enhances flowering under LDc conditions and therefore works antagonistically with *Hd1* in rice (Doi et al., 2004; Park et al., 2008).

Ehd1 was identified as a QTL from a cross between Taichung 65 (T65) and African rice *O.glaberrima*. Taichung 65 (T65), which contains apparent adaptability in terms of insignificant response to photoperiod, and also has loss-of-function alleles of *Hd1* and *Ehd1*, was selected to understand the molecular mechanisms. The *O.glaberrima* line has a dominant allele promoting flowering, which was selected by the cross-combination of Nipponbare and T65. Nipponbare allele of *Ehd1* (*Ehd1-Nip*) also promotes early flowering. Since there was no major QTL found in Nipponbare x Kasalath

near the *Ehd1*, T65 most likely carries a loss-of-function allele of *Ehd1* (*ehd1-T65*). Nevertheless, *O.glaberrima,* Nipponbare, and Kasalath probably have functional *Ehd1* alleles (*Ehd1-gla, Ehd1-Nip, Ehd1-Kas*). The information from *Ehd1* encoding the B-type response regulator of 341 amino acid proteins was confirmed by the conversation of T65 with the corresponding Kasalath genomic fragment (Doi et al., 2004).

3) Association between seed dormancy and flowering time

The association between SD and FT can arise from the pleiotropic effects of a single gene. The genes *Flowering Locus T* and *C* (*FLC*) in Arabidopsis and *Perpetual flowering 1 (Pep1)* in *Arabis Alpina* was reported to have a pleiotropic effect on seed dormancy (Hughes et al., 2019). It has been found that the *Flowering Locus T* (FT) molecule is responsible for the flowering induction (Cho et al., 2016). Furthermore, it was reported that the *DOG1* transgene from Arabidopsis had a pleiotropic effect on flowering time in lettuce (Huo et al., 2016).

The *qSD1-1/ qFT1* and *qSD10/qFT10* haplotypes identified from the weedy rice line SS18-2 and the cultivated rice line EM93-1 were responsible, positive correlations between SD and FT; and the positive correlation may arise from pleiotropy (Gu et al., 2018).

The seed dormancy (SD) and flowering time (FT) traits may associate with each other to constitute a regulation for adaptation of plants. Both positive and negative correlations were detected in experimental populations of rice. The negative correlation is characterized by late-flowering plants tending to have strong SD. The linkage between SD and FT genes on chromosomes 7 and 8 accounted for the negative correlations in rice

by the late-flowering dormant genotype to have strong seed dormancy (Gu et al., 2018; Figure 3.8). However, the positive correlation is characterized by early-flowering dominant, and late-flowering recessive genotype plants tending to have strong SD.

Two sets of collocated QTLs SD and FT, *qSD1-1/qFT1* and *qSD10/qFT10*, were responsible for the positive correlations in rice. The significant positive correlation between seed dormancy and flowering time was evaluated by the germination percentage (Gu et al. 2018).

The association was delimited to a short segment of about 200 kb. In the narrowed region, there is a predicted gene which is called the *Early heading 1* (*Ehd1*) gene (Gu et al., 2018). Due to the nonconstant number of exons from parent T65 and Nipponbare (Figure 1.2), *Ehd1* was cloned from the parent line EM93-1 to determine if *Ehd1* has a function on seed dormancy.

To identify the negative correlation between SD and FT, and define the QTLs on the chromosomes 7 and 8, the BC_1F_1 population was analyzed. The close linkage between *qSD7-1* and *qFT7-1* expressed more to the negative correlation than the loss linkage between *qSD8* and *qFT8* (Gu et al., 2018).

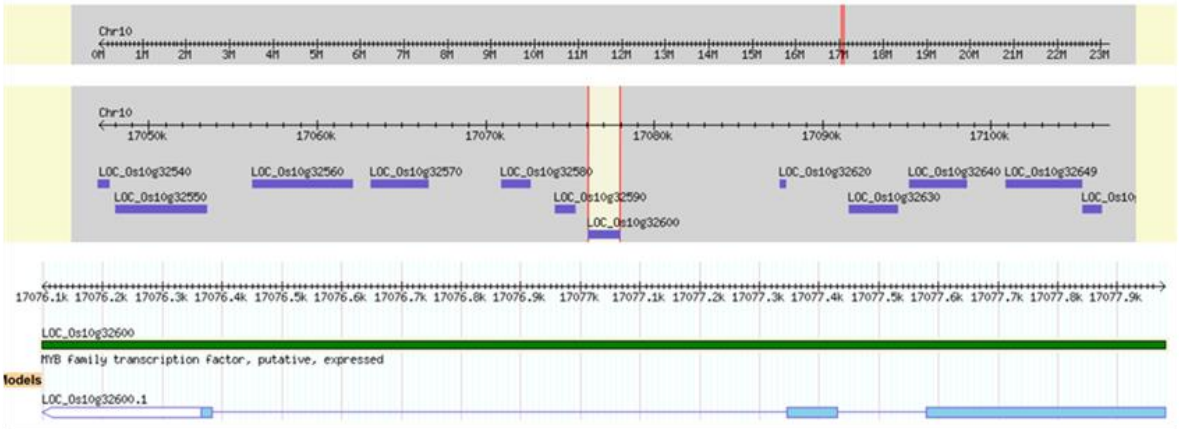

Figure 1.2 Structure of *Ehd1* from parent Nipponbare

(http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/#search)

1.2.3 RNA interference (RNAi)

1) Concept of RNAi

RNA interference (RNAi) is a biological mechanism involved in the regulation of gene expression at the post-transcriptional level (Cai et al., 2015). RNAi has many advantages centered on reverse genetics, such as rapid results, low cost, and heritability. RNAi can also cause knockdown of multiple homologous genes and obtain partial loss-of-function. Silencing can be forwarded towards a specific gene/s; thus, screening of large populations is not required. Moreover, stimulated phenotypes can be observed in the T1 generations. (Gilchrist and Haughn, 2010).

2) Development of the *Ehd1*-RNAi transgenic line

Os10g32600, which is regarding a gene model in the Gramene database, was forecasted to be 5,246 bp in length and contain five exons and four introns (www.gramene.org). In terms of the Gramene model, the PCR primers cEhd1-F and cEhd1-R were organized for cloning full-length cDNAs of *Os10g32600* from ILSD10E

and ILSD10S (Pipatpongpingyo, 2018). The cDNA sequences were aligned towards their genomic DNA to improve the gene structure, predict functional domains of the proteins extracted by the Conserved Domain Database (Marchler-Bauer et al. 2010) and by some bioinformatics programs including PROSITE (http://www.expasy.org/prosite/).

The *Ehd1*-RNAi-F and *Ehd*1-RNAi-R primers were regulated based upon *Os10g32600*'s coding sequence to enlarge a cDNA fragment of 317 bp from EM93-1 by PCR and then to constitute an inverted repeat sequence (IRS) for the RNAi experiment. After adding the 4-bp CACC to the 5' end of the forward primer for using 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 are related to LR Clonase reaction (Figure 1.3A).

The amplified fragment which linked to the cloning vector called Entry clone was transferred to pANDA- β destination, which has a *gus* linker vector located between two recombinant sites (*attR*) in the sense and antisense orientation and contains the kanamycin and hygromycin resistance genes for selection, by LR Clonase reaction to constitute the IRS between *attB1* and *attB2* flanking the g*us* linker (Figure 1.3B).

The pANDA- β vector was used to transform Nipponbare calli using an *Agrobacterium*-mediated transformation system (http://agronwww.agron.iastate.edu/ptf/protocol/rice.pdf). The transformed plantlets were selected using particular genes for resistances to hygromycin (Figure 1.3B) (Pipatpongpingyo, 2018).

1.3 Rationale and objectives of this research

The previous research identified a short chromosome segment containing the QTL cluster *qSD10/qFT10* for seed dormancy/flowering time on chromosome 10 from a cross between EM93-1 and SS18-2 (Gu et al. 2018; Pipatpongpinyo, 2018). EM93-1 is a pure line of indica type cultivated rice with an extremely short growth duration. SS18-2 is a line of tropic ecotype of weedy rice. This QTL cluster was responsible for a positive association between seed dormancy (SD) and flowering time (FT) and was narrowed to <200 kb, which contains *Early Heading Date1* (*Ehd1). Ehd1* is a gene known to promote flowering time in rice, and unknown for a function on seed dormancy. To determine if *Ehd1* is also involved in the regulation of seed dormancy, transgenic lines were developed for a transfer DNA (T-DNA) of *Ehd1*-RNAi in the genetic background of Nipponbare, a japonica-type of cultivated rice with a moderate length of growth duration, in the previous research. EM93-1 and Nipponbare are different not only in subspecies, flowering response to photoperiod (day length), and plant growth duration, but also likely in genotype for *qSD10/qFT10*. Thus, the first objective of this research was to introduce the *Ehd1*-RNAi T-DNA from the Nipponbare to the EM93-1 background by recurrent backcrossing to evaluate the gene-silencing effect on both flowering time and seed dormancy across generations. Data from the research for the first objective will be used to infer if *Ehd1* is the underlying gene of *qSD10, qFT10,* or both (i.e., pleiotropic effects).

Ehd1 is a regulatory gene encoding a Myb family transcription factor (Doi et al., 2004). It is essential to know what genes are regulated by *Ehd1* to influence the phenotypic variation for flowering time, seed dormancy, or both. Thus, the second

objective of this project was to identify quantitative trait loci (QTLs) regulated by the *Ehd1*-RNAi T-DNA in an F_2 population from a cross between EM93-1 and Nipponbare.

Chapter 2. Evaluation of *Ehd1*-RNA Interference Effects on Flowering Time and Seed Dormancy in Advanced Backcross Populations of Rice

2.1 Introduction

The previous research identified a cluster of quantitative trait loci (QTL) for seed dormancy (SD) and flowering time (FT), i.e., *SD10/FT10*, on chromosome 10 of rice (*Oryza sativa* L.) (Pipatpongpinyo, 2018). EM93-1 has weak dormancy and is an indica-type cultivated rice carrying the *SD10/FT10* haplotype, which increases seed dormancy (reduces germination) and promotes flowering. SS18-2 is a strongly dormant genotype of weedy rice (Ye et al., 2010). This cluster of QTLs was responsible for an SD-FT association.

Fine mapping can be used to verify a quantitative trait locus (QTL) on a small genomic region, as well as to distinguish a QTL underlying gene from closely linked genes for another trait. In a fine-mapping experiment, *SD10/FT10* was delimited to a 200-kb region, and this region was responsible for a positive correlation between seed dormancy and flowering time as evaluated by germination percentage. According to the positive correlation, seeds from early flowering plants cause lower germination or stronger dormancy (Gu et al., 2018). The narrowed region also contains the *Early heading date1 (Ehd1)* gene. *Ehd1* was first identified as a QTL for an early flowering time a cross between Asian cultivated (*Oryza sativa*), and African cultivated (*O.glaberrima*) rice. *Ehd1* has not been detected for an effect on seed dormancy in the previous research (Doi et al., 1998, 2004, 2015).

Ehd1 was reported to encode for a *Myb* family transcription factor (TF) involved in the regulation of flowering (Doi et al. 2004). It is unknown if the *Ehd1* TF also controls seed dormancy. Thus, transgenic lines for an inverted repeat sequence (IRS) of *Ehd1* were developed and used to silence the TF gene by RNAi (Pipatpongpinyo, 2018). The *Ehd1*-RNAi, transgene was used to transform the japonica cultivar Nipponbare, and the RNAi mediated gene silencing effects on flowering and germination were not stable in the transgenic T0 and T1 generations (Pipatpongpinyo, 2018). Thus, recurrent backcrosses (BC) were made to transfer the *Ehd1*-RNAi transgene into the background of EM93-1 to assess its effects on both seed dormancy and flowering time.

The objectives of the research were to develop the BC_2F_3, BC_2F_4, and BC_2F_5 plant populations; 1) to determine the copy number or segregation pattern of the *Ehd1*-RNAi transgene; 2) to evaluate effects of the transgene on seed dormancy and flowering time across the three generations; and 3) to evaluate the strength of correlations between seed dormancy and flowering time in multiple generations.

2.2 Materials and Methods

2.2.1 Parental lines and mapping population

Ehd1 is the only gene known to control flowering time in the narrowed *qSD10/qFT10* region. Thus, a sequence of the *Ehd1* cDNA from EM93-1 was selected as an inverted repeat sequence (IRS) to develop an *Ehd1-RNAi* transgene. The IRS was ligated into an intermediate vector containing a Hygromycin-B resistance gene as a selective marker, and introduced into the pANDA- β vector to transform the japonica cultivar Nipponbare.

A Nipponbare transgenic T_1 line (Nip $^{Ehd1\text{-RNAi}}$) was selected as a parent to cross with the indica line EM93-1 (Fig.1). EM93-1 was selected for the cross because it is an extremely early flowering genotype and carries a flowering-promoting allele at *qFT10/qSD10*. The hybrid F_1 plants from the Nip $^{Ehd1\text{-RNAi}}$ / EM93-1 cross were backcrossed with EM93-1 for two generations and then single plants selected for self-pollination in the following three generations.

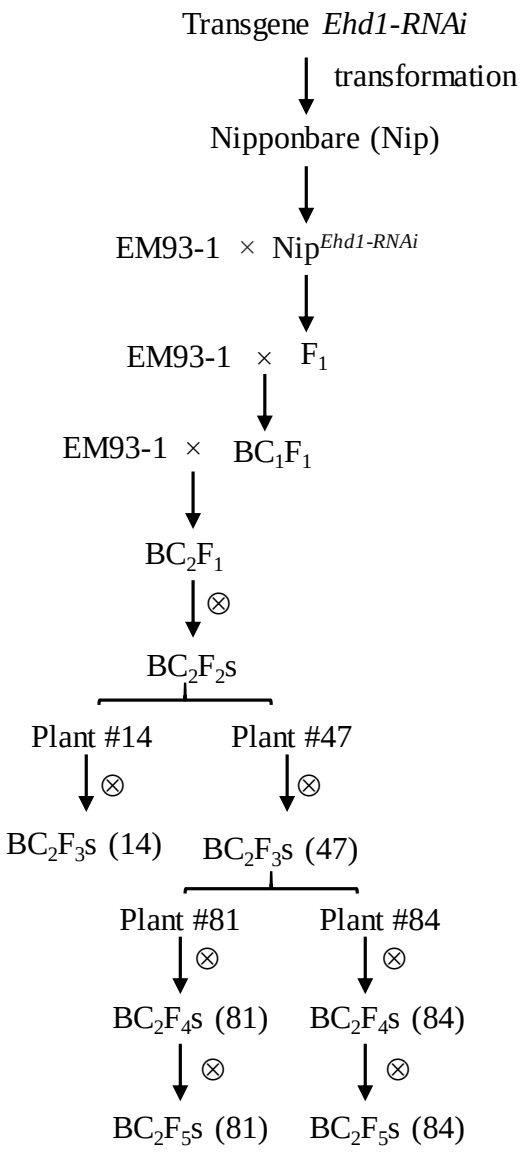

Figure 2.1 Breeding scheme used to develop advanced of backcrossing populations. *Ehd1*-RNAi is a transgene for an inverted repeat sequence from an *Ehd1* cDNA from the parent 'EM93-1' (*Oryza sativa* L. subspecies *indica*) RNAi analysis. 'Nipponbare' (*Oryza sativa* L. subspecies *japonica*) is the recipient of the transgene. The Nipponbare transgenic line (Nip$^{Ehd1\text{-RNAi}}$) was crossed with EM93-1 to develop the BC_2F_3 from plants #14 and #47, BC_2F_4 plants #81 and #84 and BC_2F_5 plants randomly selected from the BC_2F_4 lines #81 and #84.

2.2.2 Plant cultivation and seed harvest

The seeds of the BC_2F_3, BC_2F_4, and BC_2F_5 populations were air-dried in a greenhouse to break seed dormancy before germination at 30°C for seven days (d). The germinated seeds were transferred to a rice nutrition solution in 200-well Seed Starting Trays (Bootstrap Farmer) (Yoshida et al., 1976). Seedlings at 20 days old were assayed for the presence or absence of the *Ehd1*-RNAi transgene to using the method described in the following section.

Seedlings at four weeks old were transplanted into pots, with one plant per pot ($12\times12\times15$ cm^3). The pots were filled with a mixture of clay soil and SunShine medium (Sun Gro Horticulture). Temperatures in the greenhouse were set at 29/21°C for day/night, and day-lengths were natural. Supplementary lights were applied to maintain a minimum of 12-h light.

The plants were tagged for a flowering date when the first panicle of a plant first emerged from the leaf sheath. Plant height was measured at harvesting. Seeds were harvested at 40 days after flowering and dried in paper bags in the greenhouse for 3 d, then stored at -20 °C in a freezer to maintain any dormancy status (Pipatpongpinyo, 2018).

2.2.3 Detection of the *Ehd1*-RNAi transgene

A hygromycin B-resistant assay was used to identify the segregation of the *Ehd1*-RNAi transgene in the BC_2F_3, BC_2F_4, and BC_2F_5 generations. Two leaf segments of approximately 2 cm were sampled from each seedling at 20 days old and instantly placed on a medium in a closed Petri dish. The medium contains 1.5% Phytagel (Sigma-Aldrich

Co.), and 15 ug/mL Hygromycin B antibiotic (Invitrogen). The Petri dishes were sealed and kept in an incubator at 24°C and a photoperiod of 16-h light and 8-h dark for seven days. Pictures were taken on the fourth day and the seventh day to score responses of the leave samples to Hygromycin-B. Resistant (R) samples remained green, indicating that the plant contains the *Ehd1*-RNAi transgene, either homozygous (2 copies) or hemizygous (one copy). Whereas, susceptible (S) samples showed yellow-brown necrotic spots, indicating that the plant is a nullizygous (having loss-of-function for the identified gene) for the transgene.

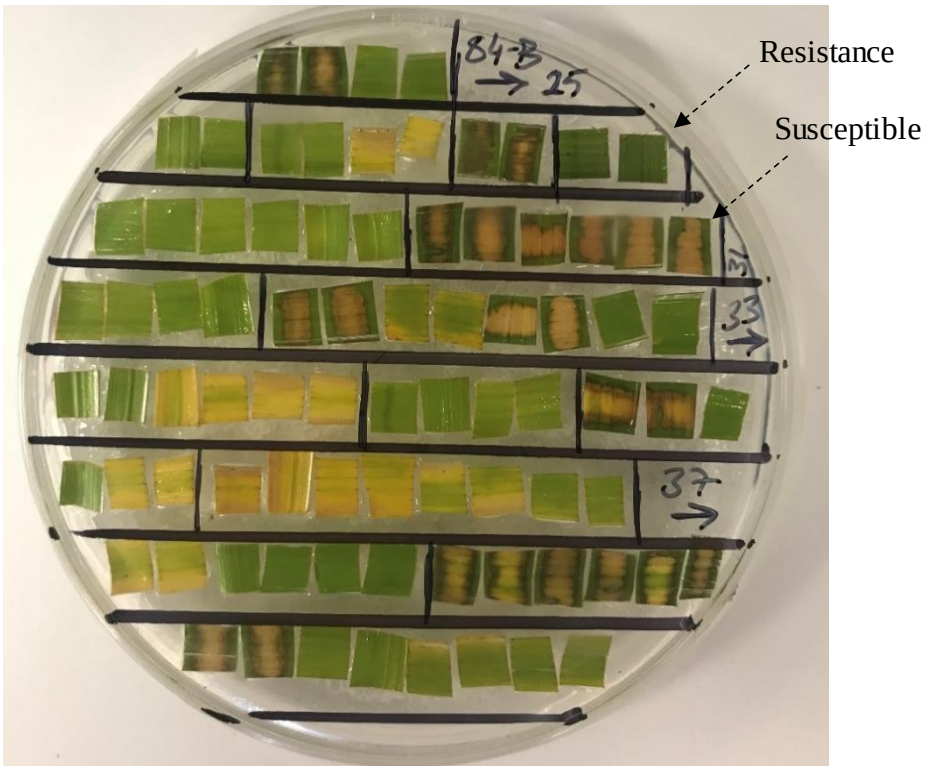

Figure 2.2 Phenotypic assessment for the *Ehd1*-RNAi T-DNA in the backcross populations. The image shows the genotypic difference in resistant (R) or susceptible (S) response to the Hygromycin-B test for BC_2F_5 plants $^{\#}$25 to #40. Two leaf segments from each BC_2F_5 seedling at 21 days old were incubated on a Hygromycin-B-containing medium and checked for the R or S responses at 4 and 7 days after the treatment.

2.2.4 Seed dormancy assessment

Seed dormancy was evaluated for individual plants by germination testing using the protocol developed by the lab (Gu et al., 2003). Before germination, seed samples were partially after-ripened at room temperature (25°C) for seven days. Three seed samples from the backcross populations were prepared. Each sample of 50 well-developed seeds was distributed on a filter paper in a 9-cm Petri dish, which was soaked with 8 mL water, and germinated in an incubator set at 30°C, and 100% relative humidity and dark condition for seven days. Germinated seeds were determined according to the radicle emergence from the hull by >3mm, and counted daily from the second day. Germination was counted daily for seven days to calculate. Germination percentage (GP);

$$GP = \Sigma ni/N\ (\%)$$

Where, ni is the number of germinated seeds at the day i (i=1, 2, 3… 7), and N is the total number of seeds in a sample.

2.2.5 Data analysis

One-way analysis of variance was first used to test the significance of differences in time (d) to flowering (DTF) and germination levels between the R and S groups in each of the BC_2F_3 to BC_2F_5 lines. Linear correlation analysis was conducted to quantify the strength of the correlation between DTF and germination levels in individual lines. The analysis was performed using RStudio statistical program.

2.3 Results

2.3.1 Segregation pattern of the *Ehd1*-RNAi transgene in the BC_2F_3 and BC_2F_4 lines

Segregation for hygromycin resistant (HR) and susceptible (HS) responses was observed in individual lines of the BC_2F_3 to F_5 generations. Table 2.1 lists the segregating data for the two BC_2F_3 lines and the two F_4 lines. The observed segregation ratios of HR: HS in the four lines fit Mendelian's expectation of 3:1 for a dominant gene. Segregation of HR and HS was also observed in the BC_2F_5 populations, but the segregation ratios were not tested for fitness as the plants were randomly selected from all the BC_2F_4 plants. In any event, the HR phenotype was conferred by the selective gene (Pipatpongpinyo, 2018) on the vector for the *Ehd1*-RNAi transgene. Thus, the above observations demonstrated that this transgene has a single copy segregating in the advanced backcross generations.

Table 2.1 Fitness tests for segregation ratios of hygromycin resistant (HR) to susceptible (HS) plants in the BC_2F_3 and F_4 lines.

Line[a]	Observed number		Expected number[b]			Chi-square value[c]
	HS	HR	Total	HS (1/4)	HR (3/4)	
BC_2F_3_14	13	25	38	9.5	12.7	0.0002
BC_2F_3_47	10	37	47	11.75	15.7	<0.0001
BC_2F_4_81	21	71	92	23	30.7	<0.0001
BC_2F_4_84	19	68	87	21.75	29.0	<0.0001
Total	63	201	264	66	88.0	<0.0001

[a] Refer to Fig. 2.1 for pedigrees of the lines.

[b] Expected based on one dominant gene

[c] Probability of the chi-square test computed by R studio.

2.3.2 Effects of *Ehd1*-RNAi transgene on seed dormancy and flowering time in the BC_2F_3 generation

The HR and HS groups in each of the two BC_2F_3_14 and 47 lines were different in both seed dormancy and flowering time. Seed dormancy was evaluated by germination percentage at the 7 and 14 days of after-ripening (DAR). For the BC_2F_3_14 line, the HR group had a lower germination level than the HS group at both 7 and 14 DAR (Fig. 2.3A). However, for the BC_2F_3_47 line, the germination level was lower in the HR than in the HS group at 7 DAR but was a little higher in the HR than in the HS group at 14 DAR (Fig. 2.3B). These ANOVA test results indicate that silencing *Ehd1* by RNAi

enhanced the degree of seed dormancy, and this enhancing effect could be detected best at 7 DAR. Since the two lines were derived from different plants, the difference in the germination level at 14 DAR suggests that the BC_2F_3 plants #14 and 47 may differ in the genetic background.

Flowering time varied considerably between the HR and HS groups. The time (days) from germination to flowering (DTF) was about 120 days for the HR plants or about 60 days for the HS group in both BC_2F_3_14 and 47 lines (Figs. 2.3C and D). These results indicate that silencing *Ehd1* by RNAi delayed flowering.

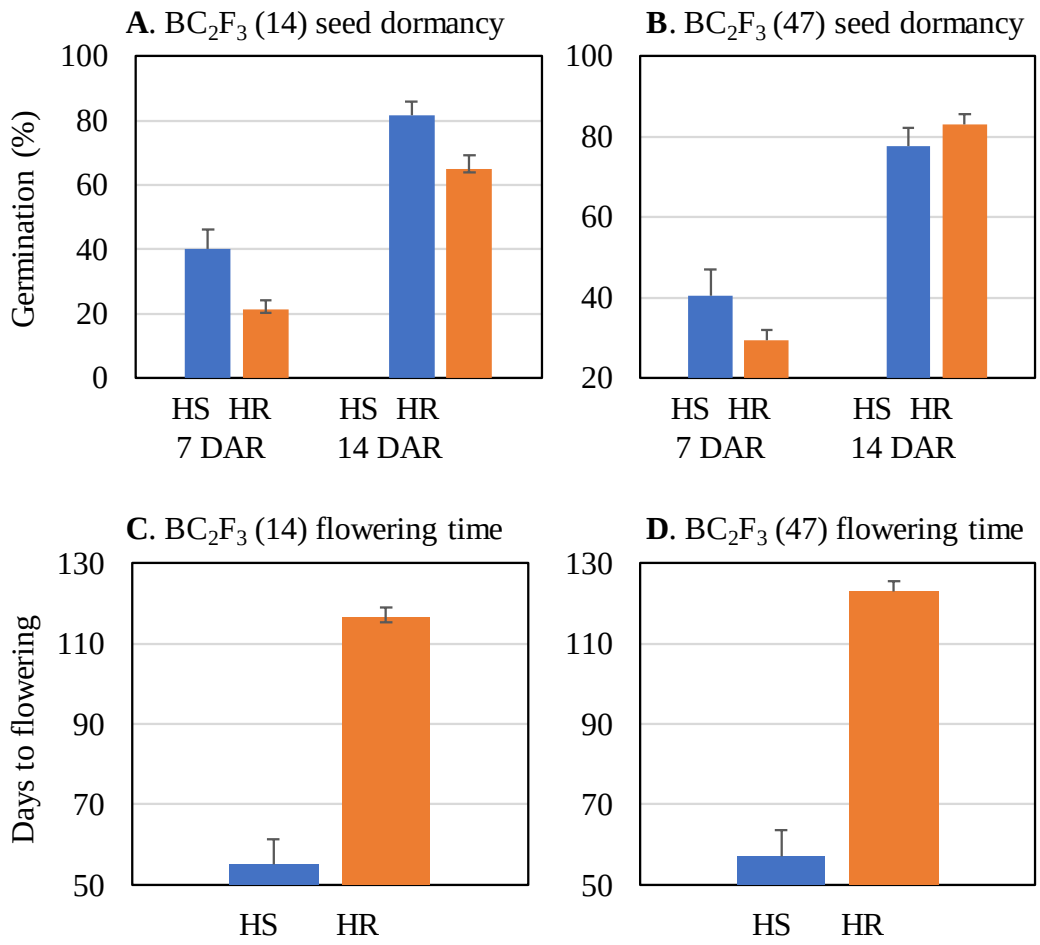

Figure 2.3 Genotypic differences were indicated by using ANOVA in seed dormancy and flowering time between the hygromycin resistant (HR) and susceptible (HS) groups in two BC_2F_3 lines. The lines BC_2F_3 (14) and (47) were derived from single plants. Seed dormancy was evaluated by germination percentages at 7 and 14 days of after-ripening (DAR). Flowering time was quantified by days from germination to flowering (DTF). Columns (bars) represent means (s.e.) of the HR or HS groups.

2.3.3 Effects of *Ehd1*-RNAi transgene on flowering time and seed dormancy in the BC_2F_4 generation

The two BC_2F_4 lines were derived from the single plants (#81 and 84) in the BC_2F_3_47 line. The BC_2F_4_81 and 84 lines were replicated in two successive seasons (summer and fall) to infer whether the *Ehd1*-silencing effects on seed dormancy and flowering time are influenced by a genotype by environmental (G×E) interaction. Fig. 2.4 shows genotypic differences in both seed dormancy and flowering time. For seed dormancy that was evaluated by germination percentage at 7 DAR, the two lines were consistent in the genotypic distribution pattern, which varied with the seasons. For example, the mean germination level was higher and lower in the HR than in the HS group for seeds harvested in the summer and fall seasons, respectively (Figs. 2.4A and B). These results indicate that silencing *Ehd1* caused genotypic variation for seed dormancy, and also suggests that the silencing effect varied with the seasons most likely because of a G×E interaction.

For flowering time, the BC_2F_4_81 and 84 lines displayed similar patterns for the genotypic difference in DTF between the HR and HS groups in the summer and fall seasons (Figs. 2.4C and D). These results confirmed the observation in the BC_2F_3 generation that silencing *Ehd1* by RNAi delayed flowering, and also suggest that *Ehd1* may have a greater effect on flowering time than on seed dormancy.

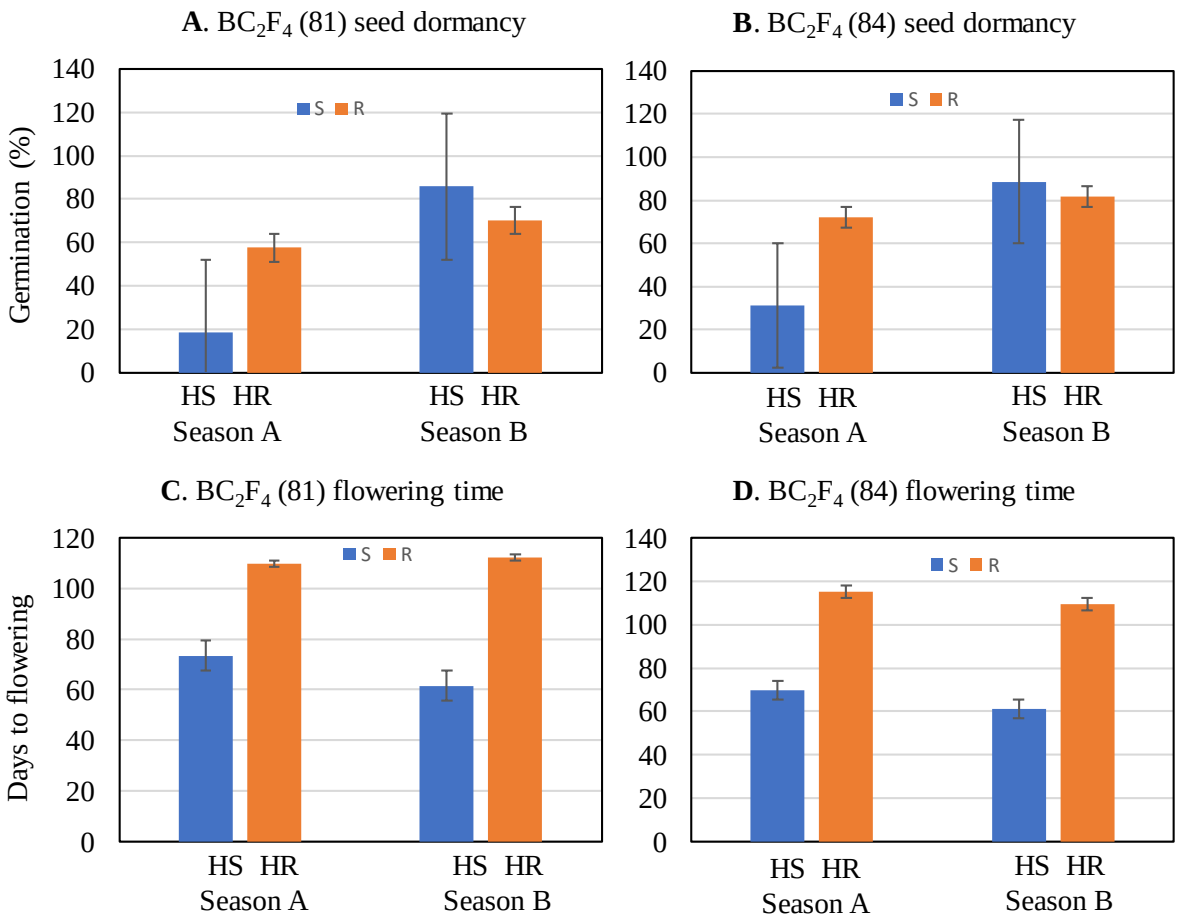

Figure 2.4 Genotypic differences were indicated by using ANOVA in seed dormancy and flowering time between the hygromycin resistant (HR) and susceptible (HS) groups in two BC_2F_4 lines. The lines BC_2F_4 (81) and (84) were derived from single plants. Seed dormancy was evaluated by germination percentages for two seasons, 7 days of after-ripening (DAR). Flowering time was quantified by days from germination to flowering (DTF). Columns (bars) represent means (s.e.) of the HR or HS groups.

2.3.4 Effects of *Ehd1*-RNAi transgene on flowering time and seed dormancy in the BC_2F_5 lines

The BC_2F_5_81 and 84 populations were germinated on May 17th, 2019, and the plants matured in the fall to winter seasons. These two populations displayed similar genotypic distribution patterns for both seed dormancy and flowering time. For example, the HR group had a lower germination level, or took a longer time to flowering, than the HS group (Figs. 2.5A and B). These results confirmed significant observations in the BC_2F_3 and BC_2F_4 generations, namely, silencing *Ehd1* by RNAi decreased the germination ability (increased seed dormancy) and delayed flowering.

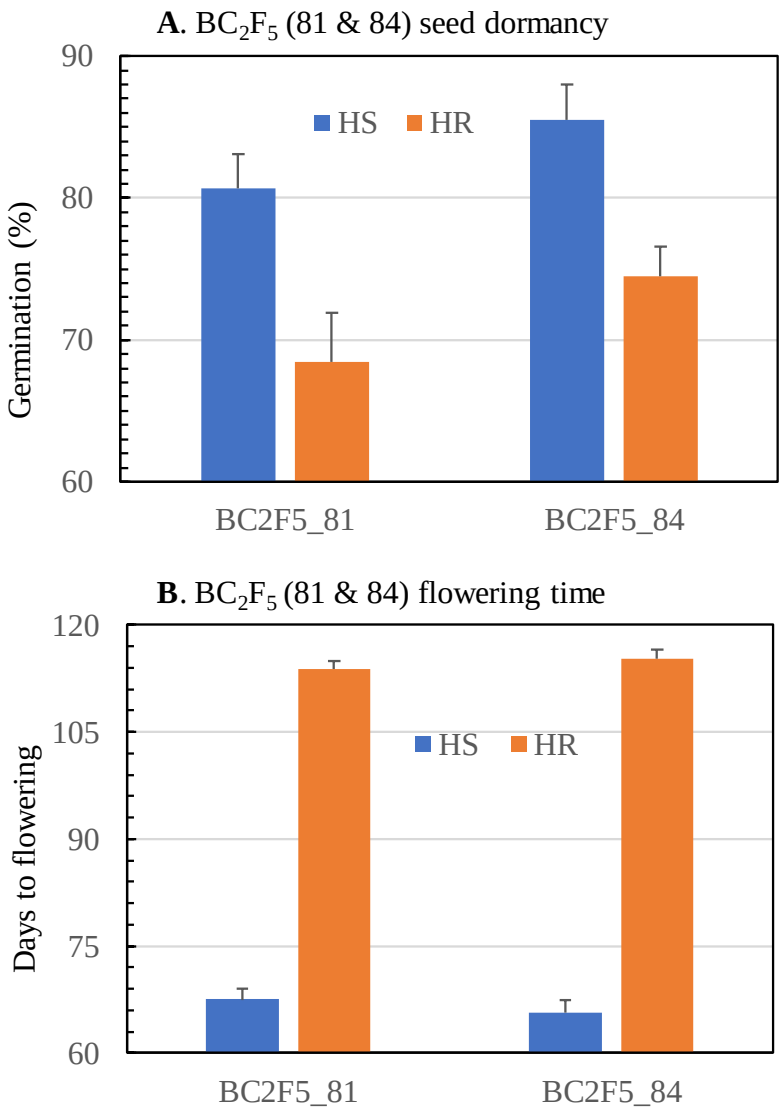

Figure 2.5 Genotypic differences were indicated by using ANOVA in seed dormancy (**A**) and flowering time (**B**) between hygromycin resistant (HR) and susceptible (HS) groups in two BC_2F_5 populations. Seed dormancy was evaluated by germination percentage at 7 days of after-ripening. Flowering time was evaluated by days from germination to flowering.

2.3.5 Correlations between hygromycin resistance, flowering time and seed dormancy in the BC_2F_3 to BC_2F_5 generations

The data from the BC_2F_3 to BC_2F_5 generations presented in Figs. 2.3 to 2.5 demonstrated that *Ehd1* has pleiotropic effects on seed dormancy and flowering time. Thus, a linear correlation analysis was used to evaluate the strength of the correlation between these two effects. Table 2.2 summarized the analytic results for correlations between two of the three traits/variables: hygromycin response (Hy=0 for HS or 1 for HR), DTF, and germination percentage (GP) in the BC_2F_3 to BC_2F_5 generations. Hy was correlated strongly and positively with DTF (r=0.66-0.96) in all the generations, and negatively with GP in the BC_2F_3 and BC_2F_5 generations (r=-0.31 to -0.35). The correlations between DTF and GP were negative in the BC_2F_3 and BC_2F_5 lines and the BC_2F_4 line in the fall season, which were weak but statistically significant (r=-0.3; Table 2.2). The correlations between DTF and GP were positive (r=0.78) in the BC_2F_4 lines in the summer season, but HY and GP were not consistent in the BC_2F_4 lines. Therefore, results from the correlation analysis confirmed that *Ehd1* has a demonstrable and constant effect on flowering time but has a relatively small and environmentally-sensitive effect on seed dormancy.

Table 2.2 Summary of correlations between tested traits in the BC_2F_3 to BC_2F_5 generations

Correlation	Statistics	BC_2F_3	BC_2F_4_A	BC_2F_4_B	BC_2F_5
Hy and DTF	N	85	86	93	384
	r	**0.958**	**0.940**	**0.661**	**0.864**
	Probability	<.0001	<.0001	<.0001	<.0001
Hy and GP	N	82	86	93	183
	r	**-0.351**	**0.784**	**-0.100**	**-0.308**
	Probability	0.0010	<.0001	0.3398	<.0001
DTF and GP	N	85	86	93	182
	r	**-0.275**	**0.781**	**-0.258**	**-0.279**
	Probability	0.0110	<.0001	0.0126	0.0001

Note: Hy, Hygromycin-B test.; DTF, Day to Flowering; GP, Germination Percentage; BC_2F_4_A and B, and BC_2F_5_A and B seed maturation in summer and fall seasons, respectively.

2.4 Discussion

2.4.1 Copy number and segregation pattern of the *Ehd1*-RNAi transgene

The research detected only one copy of the *Ehd1*-RNAi transgene in the BC_2F_3 to BC_2F_5 lines. The transgene behaved as a dominant Mendelian factor, as confirmed by the three generations of segregation data (Table 1). Further research is needed to map the transgene insertion site on the genome of rice.

2.4.2 Transgenic effects on seed dormancy and flowering time across generations

This research detected *Ehd1*-silencing impact on flowering time and seed dormancy. The three generations of data (Figs. 2.3 to 2.5) provide sound evidence that the *Ehd1* transcription factor gene controls not only the genotypic variation in flowering time but also regulates the genotypic variation in seed dormancy.

The *Ehd1*-silencing effect on DTF was >40 days (d) in the advanced backcross generations, which was greater than the observation of 10 d in the BC_1F_1 and BC_2F_1 populations (Pipatpongpinyo, 2018). This backcross was made using Nipponbare as the donor parent and EM93-1 as the recurrent parent. It is expected that two generations of backcross followed by generations of self-pollination and single plant selections for the EM93-1-like plants helped synchronized the genetic background toward the recurrent parent. EM93-1 had a shorter duration of growth than Nipponbare in our greenhouse conditions, indicating that the two parents differentiated in genes for flowering time and seed dormancy. Thus, the differences in the effect size between this and the previous research could be explained by the different number of genes for seed dormancy and

flowering in the genetic backgrounds (Gu et al., 2018). Further research is needed to confirm the effect size hypothesis and map the genes differentiated between EM93-1 and Nipponbare.

2.4.3 Correlations between seed dormancy and flowering time in multiple generations

In a majority of the advanced backcross lines, DTF was negatively correlated with GP, with late flowering plants tending to have strong seed dormancy (Table 2.2). Positive and negative correlations were also observed in the previous research by previous research (Pipatpongpinyo, 2018). This current work and previous information suggest that there could be some G×E interaction involved in the regulation of the association between SD and FT.

The G×E interaction inferred by data from this research was related to *Ehd1*'s pleiotropy. Pleiotropy is the feature of a gene that influences two or more traits so that a mutation in the gene could cause variations for different characters simultaneously (Falconer and Mackay, 1996). Both flowering time and seed dormancy are essential traits for crop breeding. Further research is needed to identify the physiological and molecular mechanisms underlying the GxE interaction.

Chapter 3. Mapping of *Ehd1*-Regulated Genes Controlling Seed Dormancy and Flowering Time in Rice

3.1 Introduction

The QTL cluster *qSD10/FT10* for seed dormancy/flowering time was delimited to a short genomic region of ~200 kb in previous research (Gu et al. 2018). There are 22 predicted genes in the ~200 kb region, including *Os10g32600,* which is annotated as a Myb family transcription factor (TF) gene underlying the early heading QTL *Ehd1* in rice (Doi et al., 1998, 2004). To determine if *Ehd1* has a pleiotropic effect on seed dormancy, a reverse genetic strategy was used to silence the TF gene by RNA interference (*Ehd1*-RNAi) in the previous research (Pipatpongpinyo, 2018). Data from the BC_2F_3, BC_2F_4, and BC_2F_5 populations segregating for the *Ehd1*-RNAi construct revealed: 1) silencing *Ehd1* delayed flowering and also enhanced seed dormancy; and 2) effects of *Ehd1* on seed dormancy and flowering time varied with genetic backgrounds. For example, *Ehd1* had a much greater effect on flowering time in the EM93-1 (>40 d) than in the Taichung 65 and Nipponbare (<10 d) backgrounds (Doi et al., 1998, 2004; Chapter 2). Therefore, it is hypothesized that *Ehd1* may also regulate some other genes influencing seed dormancy and flowering time.

The *qSD10/FT10* cluster was detected in the genetic background of EM93-1, an indica-type line with a short growth duration and weak seed dormancy. The *Ehd1*-RNAi transgenic line was developed in the genetic background of Nipponbare, a japonica type cultivar with a moderate growth period and weak seed dormancy. This research used a QTL mapping strategy to identify genes regulated by *Ehd1* in an F_2 population from a cross between EM93-1 and the Nipponbare *Ehd1*-RNAi transgenic line. The objectives

of this research were: 1) to map the transfer DNA (T-DNA) of *Ehd1*-RNAi on the rice genome; 2) to identify QTLs associated with seed dormancy and flowering time in the F_2 population, and 3) to compare differences of the QTLs effects between subpopulations with and without the T-DNA to infer genes regulated by *Ehd1*.

3.2 Materials and Methods

3.2.1 Parental lines and mapping population

Since *Ehd1* is the only gene known to control flowering time in a narrowed *qSD10/qFT10* region, a sequence of *Ehd1*'s cDNA from 'EM93-1' was selected as an inverted repeat sequence (IRS) for RNAi (*Ehd1*-RNAi). The IRS was ligated into containing a Hygromycin-B resistance gene as a selective marker, and the vector (pANDA-β vector) was used to transform to the japonica cultivar Nipponbare. A Nipponbare transgenic T_1 line (Nip $^{Ehd1\text{-RNAi}}$) was selected as a parent to cross with the indica line EM93-1 (Fig.2.1; Fig.3.1). EM93-1 was chosen for the cross because it is an extremely early flowering genotype and carries a flower-promoting allele at *qSD10/qFT10* and *Ehd1*. Hybrid F_1 plants from the Nip$^{Ehd1\text{-RNAi}}$/EM93-1 cross were self-pollinated to develop an F_2 population as a mapping population.

3.2.2 Plant cultivation and seed harvesting

The F_2 seeds were air-dried at room temperature (25 °C) for several months to break seed dormancy before germination in an incubator of 30 °C. Germinated seeds were cultured in a nutrition solution (Yoshida et al., 1976) for four weeks. Seedlings were identified for the presence or absence of the *Ehd1*-RNAi transgene before transplantation into pots, with one plant per pot. The pots were filled with a mixture of soil and SunShine

medium (Sun Gro Horticulture) and placed in a greenhouse. The greenhouse was set at 29/21°C for the day/night temperatures, and day-lengths of >12 h. Plants were tagged for a flowering date when the panicle of a plant first emerged from the leaf sheath. Plant height was measured at harvest. Seeds were harvested at 40 days after flowering, dried in paper bags in the greenhouse for 3 days, and stored at -20 °C in a freezer to maintain any dormancy status.

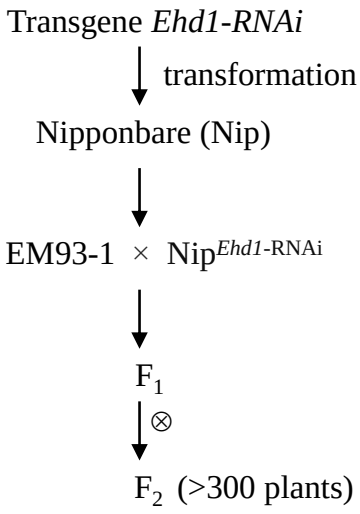

Figure 3.1 Breeding scheme used to develop a mapping population**.** *Ehd1*-RNAi is a transgene for an inverted repeat sequence from an *Ehd1* cDNA form the 'EM93-1' (*Oryza sativa* L. subspecies *indica*). 'Nipponbare' (*Oryza sativa* L. subspecies *japonica*) is the recipient of the transgene. The Nipponbare transgenic line, designated Nip$^{Ehd1\text{-RNAi}}$, was crossed with EM93-1 to develop an F_2 population for QTL mapping.

3.2.3 Transgenic analysis

A hygromycin B resistant test was used to identify the segregation of the *Ehd1*-RNAi transgene in the F_2 population. Two fresh and young leaves segments of approximately 2 cm from a seedling were transferred onto a medium of 1.5% Phytagel (Sigma-Aldrich Co.) and 15 ug/ml Hygromycin B antibiotic (Invitrogen) in a closed Petri dish. The Petri dishes were sealed and kept in an incubator, which was set at 24°C and 16-h light and 8-h dark for seven days. Pictures were taken on the fourth day and the seventh day to score responses of the leave samples to Hygromycin-B. Resistant (R) samples remained green, indicating that the plant contains the *Ehd1*-RNAi transgene, either homozygous (2 copies) or hemizygous (one copy). Whereas, susceptible (S) samples showed yellow-brown necrotic spots indicating that the plant is nullizygous (having two loss-of-function alleles for the same gene) for the transgene.

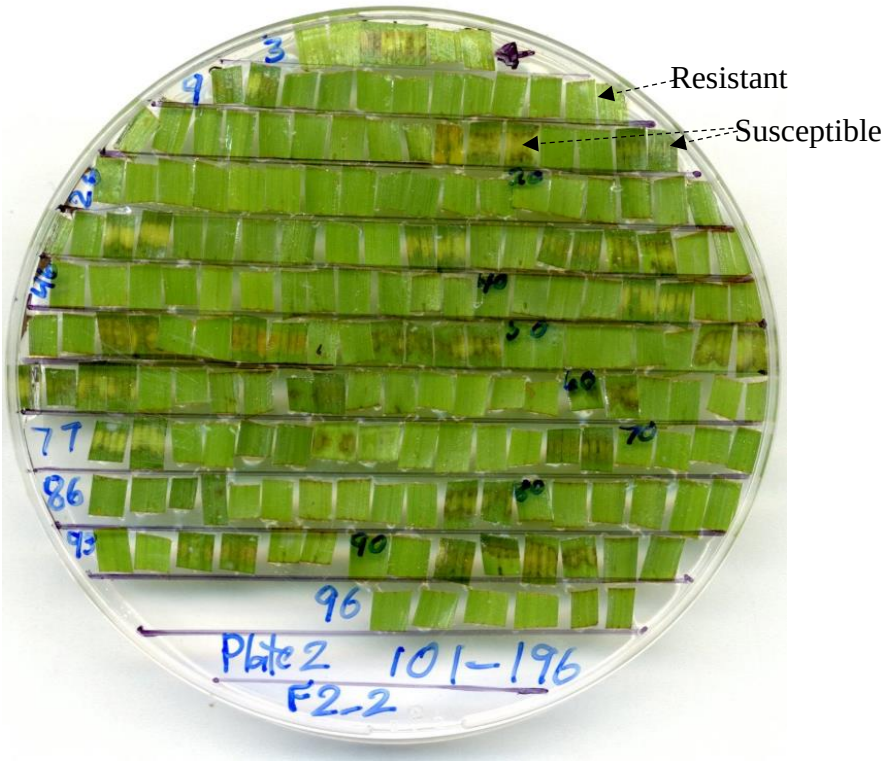

Figure 3.2 Phenotypic assessment for the *Ehd1*-RNAi T-DNA in the F_2 plants. The image shows the genotypic difference in resistant (R) or susceptible (S) response to the Hygromycin-B test for F_2 plants #101 to 196. Two leaf segments from each F_2 seedling at 21 days old were incubated on a Hygromycin-B-containing medium and checked for the R or S responses at 4 and 7 days after the treatment.

3.2.4 Phenotypic identification for seed dormancy

Seed dormancy was evaluated by germination percentage. Prior to germination testing, seed samples were moved from the freezer to room temperature (25°C) for seven days for partial after-ripening. The germination testing was conducted using three replicates from each of the F_2 plants. A replicate of about 50 well-developed seeds was

distributed on a filter paper in a 9-cm Petri dish, which was soaked with 8 mL water, and germinated in an incubator set at 30°C, 100% relative humidity and dark condition for seven days. Germinated seeds were determined according to the radicle emergence from the hull by >3mm, and counted daily from the second day. Two parameters were used to quantify the difference among plants: germination percentage and index. Germination percentage (GP) at the 7th day was calculated as;

$$GP = \Sigma ni/N\ (\%)$$

Where, Σni is the number of germinated seeds at the day i (i=1, 2, 3… 7), and N is the total number of seeds in each sample. Germination index (GI) was calculated as;

$$GI = (7\times n1+6\times n2+5\times n3+4\times n4+3\times n5+2\times n6+1\times n7)/7\times N(\%)$$

Where ni (i=1 to 7) is the number of germinated seeds at the day, i, and N is the total of seeds in a sample. Thus, GI is a weighted mean of germination percentages for 7 days, which gives early germinated seeds with larger weight (Gu et al., 2005a).

3.2.5 Marker genotyping

1) Marker selection

Simple sequence repeat (SSR), or rice microsatellite (RM), markers were selected based on the information in the Gramene database (www.gramene.org) to genotype the F_2 populations. The selected markers are shown on physical maps of the 12 chromosomes (Figure. 3.6).

2) DNA extraction

The leaf tissue was sampled from 20 days old seedling. A leaf segment of about 10 cm from each seedling was collected in a 1.5 ml microcentrifuge tube, and ground into powder in liquid nitrogen. The DNA extraction was conducted using the CTAB (Hexadecyl trimethyl-ammonium bromide) method. The leaf powder was incubated in 750µl of 2xCTAB extraction buffer (2% Hexadecyl trimethyl-ammonium bromide, 20 mM EDTA, 100 mM pH8.0 Tris-HCl, 0.2% β-Mercaptoethanol, and 1.4 M NaCl). The cationic detergent in the buffer assists in lysis of cell membranes and forms complexes with nucleic acids, in a water bath at 65^{o}C for 30 min. During the process, the samples were shaken every 10 minutes. Afterward, 750 µl of chloroform- isoamyl alcohol was added to the tubes and mixed gently for 3-5 minutes in order to separate proteins and polysaccharides, and then centrifuged at 12000 rpm at 4 ^{o}C for 20 minutes. The supernatant liquid was transferred to a new tube and mixed with 60 µl ice-cold isopropanol to precipitate the DNA by centrifuge at 12000 rpm at 4 ^{o}C for 20 minutes. The supernatant liquid was discharged attentively, and the pellet was air-dried and dissolved with 70 µl 70% ethyl alcohol to purify the DNA pellet. The air-dried DNA pellet was dissolved in order to remove the rest of the ethanol, in 1000 µl of 0.5xTE buffer (10 mM Tris pH 8). The DNA solution was checked for quantity and quality using a spectrophotometer (Nano Drop^{TM}-1000 V3.8.1) and polyacrylamide gel electrophoresis, relatively. The DNA samples were stored at -20 ^{o}C for marker genotyping.

3) Polymerase chain reaction (PCR)

Markers were amplified by PCR in the machine (C1000Touch^{TM} Thermal Cycler). The reaction's volume is 15-20 µl, which consists of 50 ng DNA-template, 200 µM of dNTPs which are grown DNA strand by the DNA polymerase (Fisher BioReagentsTM Nucleotides), 4 µl of 5× Green Go Taq® reaction buffer (Promega, Madison, WI), 20 µM of forward and reverse (F/R) primers which are short fragments of single DNA that are complementary to DNA sequences that flank the target region of interest, and 0.2 unit, of *Taq* polymerase.

The PCR procedure consisted of 1) initial denaturation at 95°C for three min., 2) 42 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds, 3) final extension at 72°C for 10 min., and 4) storage at 10°C for overnight.

4) Electrophoresis and gel imaging

The PCR products were examined for the molecular size by electrophoresis on a 6% non-denature polyacrylamide gel. The gel was prepared with 0.1% APS, 6% polyacrylamide, 0.5× TBE buffer, 0.01% TEMED, and ethidium bromide. Electrophoresis was run at ~300 volts for 2-3 hours. The electrophoresis gel pictures were taken under UV light and annotated with the AlphaEaseFCTM (Alpha Innotech) gel imaging system.

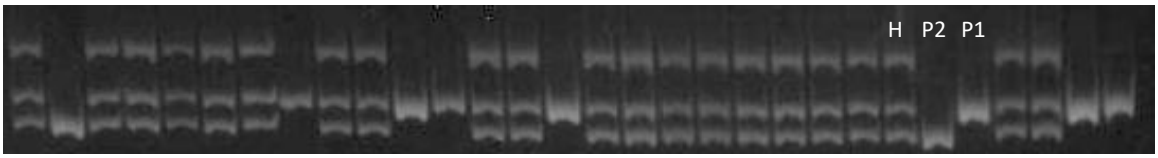

Figure 3.3 Segregating pattern of a marker in the F_2 population. The gel image shows a marker that is polymorphic between the parents EM93-1 (P1) and Nip$^{Ehd1\text{-RNAi}}$ (P2) and segregation patterns for P1-like, P2-like, and heterozygous (H).

3.3.6 Linkage map construction

Marker genotyping data from the F_2 population were used to construct a framework linkage map using the software MAPMAKER/EXP 3.0. Markers were grouped at 3.0 LOD (likelihood of odds) and 40 cM. Markers were ordered based on their physical positions (Figure 3.6). Map distances were centiMorgan (cM) converted using Kosambi's mapping function (Lincoln et al., 1992).

3.3.7 Mapping of the *Ehd1*-RNAi T-DNA insertion

Two methods were used to map the T-DNA insertion site: 1) thermal asymmetric interlaced polymerase chain reaction (Tail-PCR), and 2) linkage mapping.

1) Tail-PCR

Tail-PCR is a quick method to locate a T-DNA in the large genome (Liu and Chen, 2007). The first reaction was in a volume of 15 µl, which consists of a 50 ng DNA-template, 5.2 µl ddH_2O, 0.5 µl dNTPs (Fisher BioReagentsTM Nucleotides), 3 µl of 5× Green Go Taq® reaction buffer (Promega, Madison, WI), 0.5 µl of forwards and reverse primers, and 0.8 unit of *Taq* polymerase.

The PCR procedure started at 93°C for 2 min., and 95°C for 1 min., followed by 46 cycles of 95 °C for 30 s, 62 °C for 40 s, 72°C for 2.5 min., 95°C for 30 s, 25°C for 2.5 min., 72°C for 3 min., 72°C for 3 min., 95 °C for 20 s, 58°C for 40 s, at 72°C for 2.5 min., and ended with 72°C for 5 min, and 4°C afterward**.** Before starting the second reaction, the PCR product was diluted with a 1:100 TAIL reaction 40 µl by transferring 1 µl PCR products to 39 µl of ddH_2O. Unlike the first reaction, the 20 µl volume consisted with 2 µl of 10× EX- Taq® reaction buffer, 14.35 µl ddH_2O, 1.6 µl dNTP**s**, 0.4 µl primers**,** and 0.25 unit of EX-*Taq* polymerase. The PCR procedure consisted of at 95°C for 20 s, 65°C for 40 s, 72°C for 2.5 min., 95°C for 20 s, 68°C for 40 s, 72°C for 2.5 min., 95°C for 20 s, 50°C for 40 s, 72°C for 2.5 min., 72°C for 5 min, and at 4°C for 30 s. The PCR product from the 2nd reaction was diluted to 20 µl (1 µl + 19 µl) for the third reaction. The total volume contained 2.5 µl of 10× EX- Taq® reaction buffer, 17.75 µl ddH_2O, 2 µl of dNTP**s**, 0.75 µl of primers**,** and 0.25 unit of EX-Taq polymerase. The PCR procedure consisted of 95°C for 20 s, 68°C for 40 s, 72°C for 2.5 min., 95°C for 20 s, 68°C for 40 s, at 72°C for 2.5 min., 95°C for 20 s, 50°C for 40 s, 72°C for 2.5 min., 72°C for 3 min, and at 4°C for 30 s.

2) Linkage mapping

Linkage mapping was used as an alternative method, in case the Tail-PCR failed to map the T-DNA insertion site. Linear correlation analysis was conducted for each of the polymorphic markers (Figure 3.3) and the resistance/susceptible (R/S) responses. The marker with the highest correlation coefficient was further tested for the degree of linkage disequilibrium by Chi-square testing. Besides, the R and S phenotypes were also used for QTL mapping described in the following section.

3.3.8 QTL analysis

1) Data transformation

The frequency distribution for the germination data (x_i) was skewed to the low germination end. Thus, the arcsine ($x_i^{-0.5}$) transformation was used to improve the normality of the original data before QTL analysis.

2) Single marker analysis

Single marker analysis (SMA) was conducted for the initial set of markers to detect the T-DNA insertion site and QTLs associated with seed dormancy and flowering time on each of the 12 chromosomes. SMA was performed using the linear model:

$$y_{ij} = \mu + bx_i + \varepsilon_{ij} \quad \text{(Eq. 1)}$$

where; y_{ij} is the phenotypic value of the jth plant for the ith marker genotype; μ is the model mean; x_i is the dummy variable for the marker genotypes, with i = -1 for the EM93-1-like homozygote, 0 for a heterozygote, and 1 for the Nipponbare-like homozygote; b is the regression coefficient and the estimate of the marker effect, and ε_{ij} is the random error and effects that cannot be explained by the model.

3) Composite interval mapping

Composite interval mapping (CIM) was performed to map the QTL detected by SMA and to precisely estimate the positions and effects of the QTLs using data from the whole F_2 population. The mapping software was Windows QTL Cartographer (v2.5_009; NC State University). Parameters for CIM were set at 1-cM walking speed (add other

parameters selected for mapping) to calculate likelihood ratios (LR), additive (*a*) and dominance (*d*) effects, and proportion of the variance explained (R^2).

To determine the QTL that could be regulated by the *Ehd1* gene, the CIM analysis was also conducted for each of the resistant (R) and susceptible (S) subpopulations. The estimates for the *a*, *d*, and R^2 were compared between the R and S groups. If a QTL effect was significantly greater in the R than the S subpopulation, the QTL underlying gene is believed to be downregulated by *Ehd1* because the increased effect occurred when *Ehd1* was silenced by the RNAi. However, if a QTL effect was significantly lower in the R than the S subpopulation, the QTL underlying gene is believed to be upregulated by *Ehd1* because the increased effect occurred when *Ehd1* was functional.

3.3 Results

3.3.1 Segregation pattern of the *Ehd1*-RNAi transgene in the F_2 population

To specify the copy number of the transgene segregating in the mapping population, 297 F_2 plants were evaluated for the resistance (R) or susceptibility (S) by hygromycin B, which is conferred by the selective gene of the transgene. Of the 297 plants, 223 were resistant, and the remaining 74 were susceptible. The observed segregation ratio of 223 R:74 S fits the Mendelian expectation of 3:1 for a dominance gene. This observation indicates that the *Ehd1*-RNAi (T-DNA) transgene has a single copy in the genome.

Table 3.1 Fitness test for segregation ratio of HR-HS observed in the F_2 population

Item	No. of HR plants	No. of HS plants	Total number
Observed	223	74	297
Expected (3:1)	222.8	74.3	297
Chi-square value		0.0011	

Note: HR, Hygromycin resistant; HS, Hygromycin susceptible. The ratio of 3:1 is expected for a dominance gene. The threshold is 3.84 for one degree of freedom at the probability level of 0.05.

3.3.2 Map position of the *Ehd1*-RNAi transgene

The Tail-PCR experiment was run two times and failed to yield a quality gel image to infer the map position of the T-DNA. Thus, the linkage mapping method was used to locate the T-DNA insertion site.

Correlation analysis for about 50 polymorphic markers identified that RM295 correlated with the R and S responses, with r=0.775 (Table 3.3). The fitness test indicates that RM295 and the T-DNA site are at a high level of linkage disequilibrium (Table 3.2). The chi-square statistic of 253 exceeds 11.07 for the null hypothesis for independence between the marker locus and the T-DNA insertion site. This result reveals that the transgene in the parent Nipponbare was inserted near the end chromosome 7 (Figure 3.8).

Table 3.2 Fitness test for the marker RM295 and T-DNA joined distribution in the F_2 population.

RM295	Observed plants		Expected plants		x^2 value
	S	R	S (0.25)	R (0.75)	(1:2:1:3:6:3)
EE (0.25)	69	5	18.375	55.125	253^{***}
EN (0.5)	3	135	36.75	110.25	
NN (0.25)	0	82	18.375	55.125	

Note: R and S represent plants resistant or susceptible to Hygromycin-B, respectively. Marker genotypes of RM295 are indicated by the allele from the parents EM93-1 (E) or Nipponbare$^{Ehd1\text{-RNAi}}$ (N), or the heterozygous (EN). The values in the parentheses are expected genotypic frequencies based on Mendelian ratios for a dominant trait (R/S) or the marker. The superscript to the chi-square value indicates that the observed ratio for the RM295 and T-DNA does not fit the expectation for two independently inherited loci, as it was more significant than the threshold of 16.05 for the 5 degrees of freedom at the probability level of 0.005.

3.3.3 Segregation pattern for flowering time

A broad range of variation for flowering time was observed in the F_2 population, which varied from 55 to 185 days from germination to flowering or DTF (Figure 3.4).

There was a significant difference in the distribution pattern by using ANOVA between the S and R subpopulations. In the S sub-population, plants varied in DTF from about 55 to 155 days, with the mean value 95±25 and standard deviation. However, in the R sub-population, plants varied in DTF from 95 to 185 days, with the mean±standard deviation of 128±22 and standard deviation. The difference in DTF between the subpopulations was 33 d, indicating that silencing *Ehd1* by RNAi delayed flowering remarkably. There could be other genes also responsible for the variation within the R and S subpopulations.

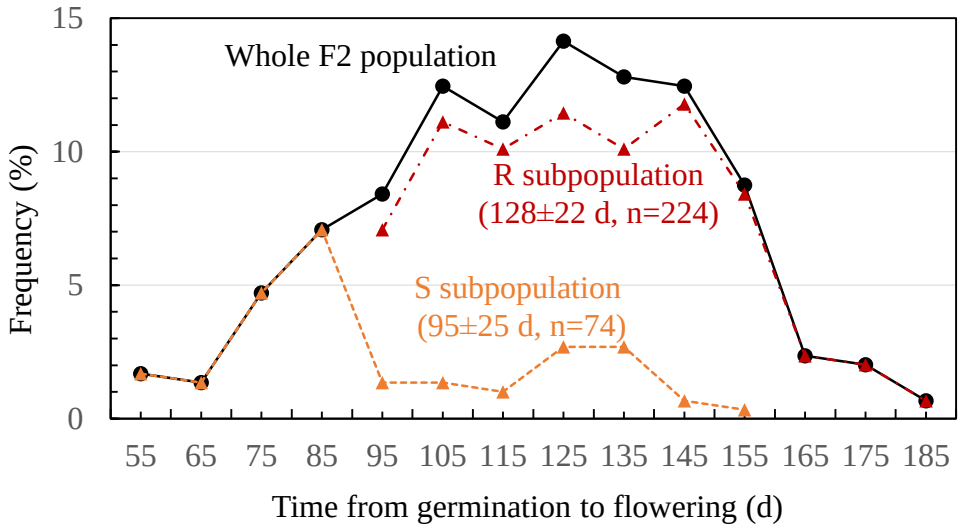

Figure 3.4. Frequency distributions of time to flowering in the F_2 population. The population was divided into two subpopulations, based on plants that were resistant/susceptible to hygromycin B. The values calculated by using ANOVA in the parentheses are means and standard deviations for the subpopulations.

3.3.4 Segregation pattern for seed dormancy

Variation in seed dormancy was observed for the F_2 population, as shown by variation in germination percentage arcsine-transform (GPt) from 0.5 to 1.35, or germination index arcsine-transform (GIt) from 0.05 to 0.95 percentage (Figure 3.5A-B). Figure 3.5A illustrates the difference between S and R subpopulation based on germination percentage and mean value. In the R sub-population, plants varied in percentage by 0.34 %. Whereas, in the S sub-population, plants had a 0.51 % average.

The frequency distribution does not follow a normal distribution, as is shown in the Figure 3.5A-B, to obtain an approximate accurate assumption, some forms of transformations such as logarithmic, square-root, arcsine, or Box-Cox transformations methods should be used. Thereby, the germination percentage and germination index data had to be transformed by using the arcsine method which is;

$$= \text{Arcsine } [(GP/100)^{0.5}]$$

The variation in the germination index (Figure 3.5B) between the two subpopulations was from 0.05 to 0.95. The difference in the germination index between the subpopulation was 12% on average, indicating that silencing *Ehd1* by RNAi reduced germination remarkably. There could be other genes also responsible for the variation with the subpopulations.

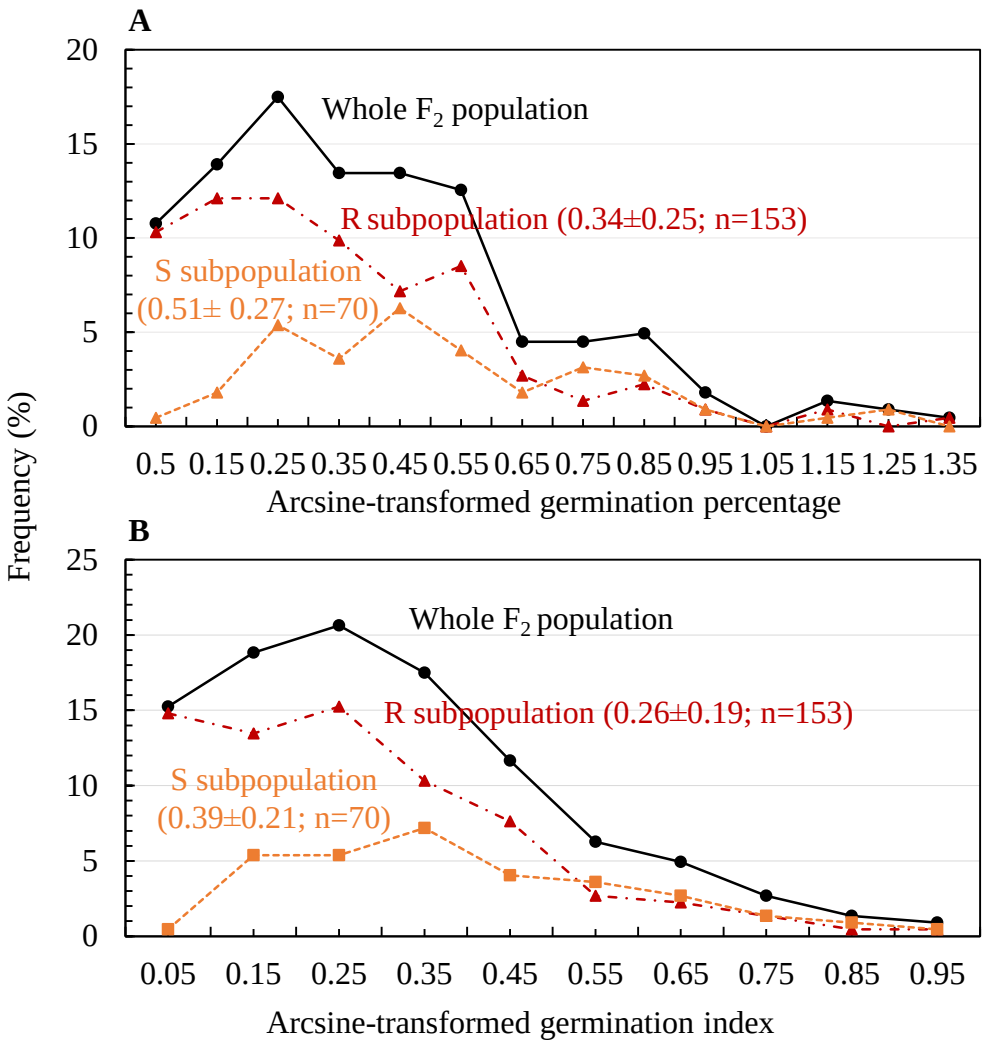

Figure 3.5 Frequency distributions of the germination percentage in the F_2 population. **A.** Arcsine-transformed germination percentage. **B.** Arcsine-transformed germination index. The population was divided into two subpopulations, in terms of plants resistant (R) /susceptible (S) to Hygromycin-B.

3.3.5 QTLs associated with flowering time and seed dormancy

1) QTLs detected by single marker analysis

The F_2 population was first genotyped with about 50 polymorphic markers across the 12 chromosomes (Fig. 3.6). The marker genotyping data were used to scan for QTLs by single-marker analysis. Table 3.3 lists the parameters obtained from the analysis. For the resistance/susceptibility to hygromycin, the marker RM295 on chromosome 7 had the most considerable effect. Since the genetic analysis detected only one insertion site for the *Ehd1*-RNAi T-DNA, it can be concluded that the T-DNA insertion site locates near the end of chromosome 7.

For flowering time, a vast majority of the markers on chromosomes 7 and 8 were highly correlated with days to flowering (Table 3.3). This result indicates that QTLs for flowering time are mainly located on the two chromosomes. For seed dormancy measured by germination percentage and germination index, several markers on chromosomes 1, 4, 7, and 8 had low but significant correlations (Table 3.3). These results suggest that there could be QTLs on the named chromosomes.

The correlation coefficients of selected markers with flowering time and seed dormancy in the F_2 population indicated according to the markers which located in the T-DNA insertion site on chromosome 7 and chromosome 8, like RM295, RM3325 or RM427, there is a negative correlation between the two variables, the marker RM295 had the highest correlation with the R and S responses, with r=0.775 (Table 3.3).

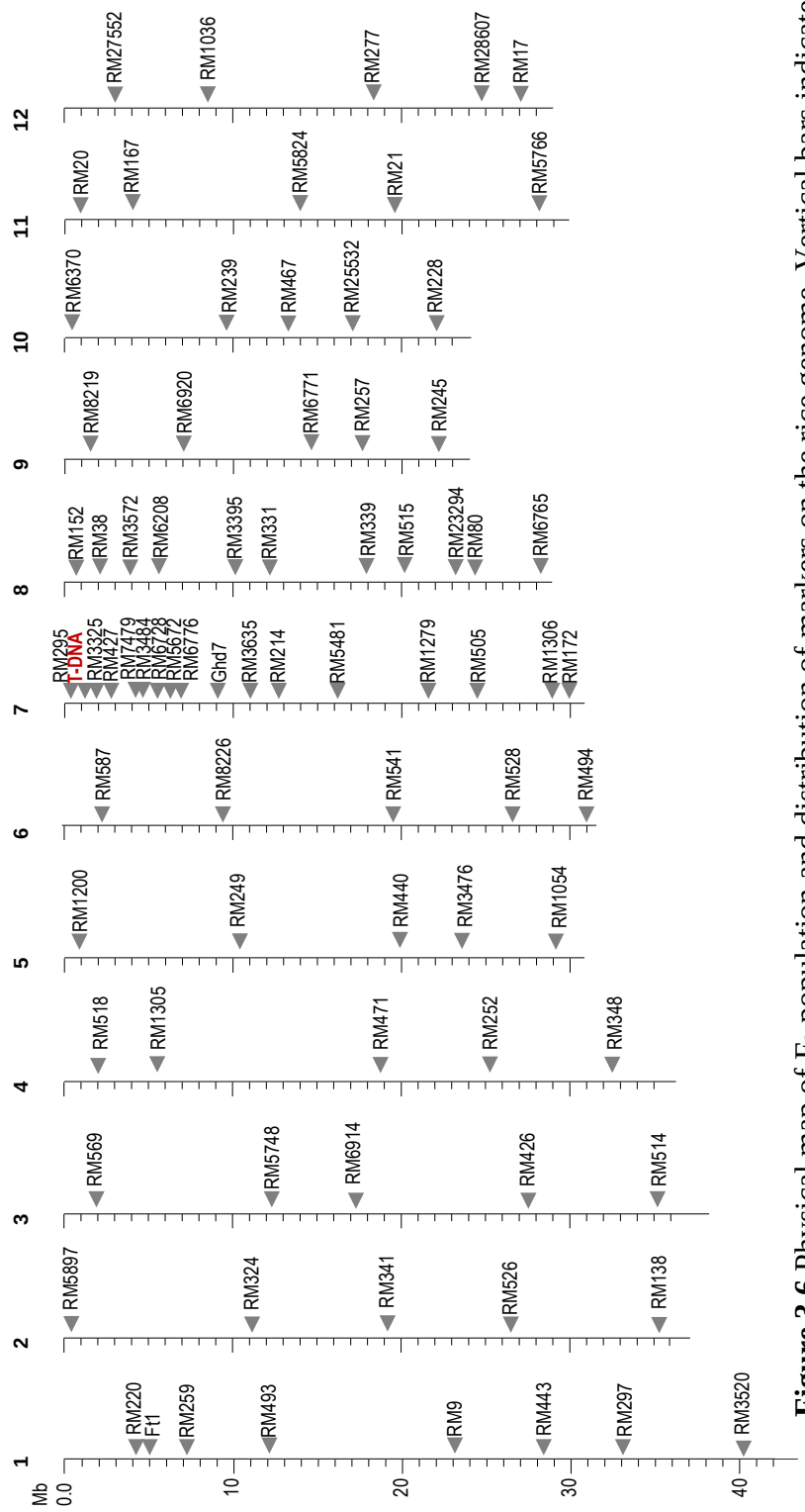

Figure 3.6 Physical map of F_2 population and distribution of markers on the rice genome. Vertical bars indicate chromosomes 1 to 12. The physical map in mega base (Mb) was developed based on data from the rice genome browser (http://rice.plantbiology.msu.edu/). Markers, placed on the right side of the chromosomes, were selected to map the F_2 population

Table 3.3. Summary of correlation coefficients of selected markers with flowering time and seed dormancy in the F_2 population.

Marker	Chr	Hy	DTF	GI	GPt
RM220	1	-0.047	-0.060	-0.052	-0.071
RM259	1	-0.036	-0.088	0.081	0.068
RM443	1	-0.265*	-0.135	0.206**	0.190*
RM297	1	-0.078	-0.021	0.092	0.088
RM324	2	-0.008	-0.048	0.130	0.147
RM526	2	-0.089	-0.039	-0.068	-0.036
RM569	3	0.033	-0.124	0.008	-0.001
RM514	3	0.100	-0.036	-0.101	-0.095
RM518	4	-0.023	-0.139	-0.008	-0.008
RM252	4	0.073	0.118	-0.144	-0.180*
RM1200	5	0.003	-0.123	0.039	0.041
RM440	5	-0.087	-0.075	0.147	0.137
RM587	6	0.156	0.026	0.068	0.039
RM8226	6	-0.061	-0.133	0.095	0.082
RM541	6	-0.066	-0.075	0.031	0.020
RM528	6	0.024	-0.033	0.162	0.164
RM295	7	0.775***	0.401***	-0.189*	-0.209*
RM3325	7	0.736***	0.408***	-0.140	-0.154
RM427	7	0.684***	0.394***	-0.101	-0.110
RM3484	7	0.451***	0.376***	-0.125	-0.120

(on the next page)

(Continued)

Marker	Chr	Hy	DTF	GI	GPt
RM214	7	0.178	0.410***	-0.153	-0.172
RM1279	7	0.110	0.367***	0.051	0.044
RM172	7	0.053	0.244***	0.049	0.040
RM152	8	-0.107	0.045	-0.027	-0.011
RM6208	8	0.061	0.319***	-0.192*	-0.187*
RM331	8	0.022	0.219***	-0.171	-0.188*
RM3572	8	0.023	0.278***	-0.169	-0.166
RM6920	9	0.038	-0.012	0.058	0.088
RM245	9	-0.075	-0.010	0.032	0.020
RM239	10	0.057	0.040	-0.071	-0.071
RM467	10	0.107	0.119	-0.138	-0.138
RM21	11	0.103	0.117	-0.082	-0.081
RM167	11	0.071	0.070	-0.079	-0.071
RM27552	12	0.059	0.034	-0.166	-0.194
RM28607	12	0.031	-0.046	-0.069	-0.093
RM17	12	0.049	-0.021	0.027	0.029

Note: Hy, T-DNA insertion; DTF, day to flowering time; GI, germination index; and GPt; arcsine transformed germination percentage, and significant levels.

3) Genome-wide scan of QTLs by composite interval mapping

The F_2 population was then genotyped with additional markers, which were selected primarily for chromosomes 7 and 8, to increase mapping resolution of FT and SD QTLs using composite interval mapping (CIM). Fig. 3.7 shows distributions of likelihood ratios (LR) for days to flowering and arcsine-transformed germination percentage along the genome of 12 chromosomes. Based on the LR distribution patterns and peak values, QTLs with a relatively significant effect on FT or SD were located on chromosomes 7 and 8. These QTLs are named as *qFT7-3*, *qFT7.1*, *qFT7.2*, and *qFT8* for flowering time, or *qSD7* and *qSD8* for seed dormancy. Both *qFT-3* and *qSD7* are collocated at the T-DNA insertion site (Fig. 3.7), indicating that these two QTLs are pleiotropic effects of the *Ehd1* silencing by RNAi.

The genetic effects of four QTLs on flowering time and two QTLs on germination ability are summarized in Table 3.4. The *Ehd1*-RNAi T-DNA had the most considerable influence on days to flowering (DTF, R^2=21%) and a moderate effect on germination (R^2=12%). Besides, the remaining three FT QTLs (*FT7.1*, *FT7.2*, and *FT8*) contributed 16%, 13%, and 14% to the total phenotypic variance in DTF, respectively. For these three FT QTLs, alleles from the parents EM93-1 and Nipponbare promoted and delayed flowering, respectively. The QTL for SD on chromosome 8 is named as *qSD8-2* because it is located on a different position from the previously mapped *qSD8* (Gu et al., 2018). *qSD8-2* contributed 15% to the total phenotypic variation in germination percentage in the F_2 population. The allele at *qSD8-2* from Nipponbare reduced germination, as shown by the negative values of gene additive and dominance effects (Table 3.4). Figure 3.8 shows map positions and one-LR support regions for the four FT and two SD QTLs.

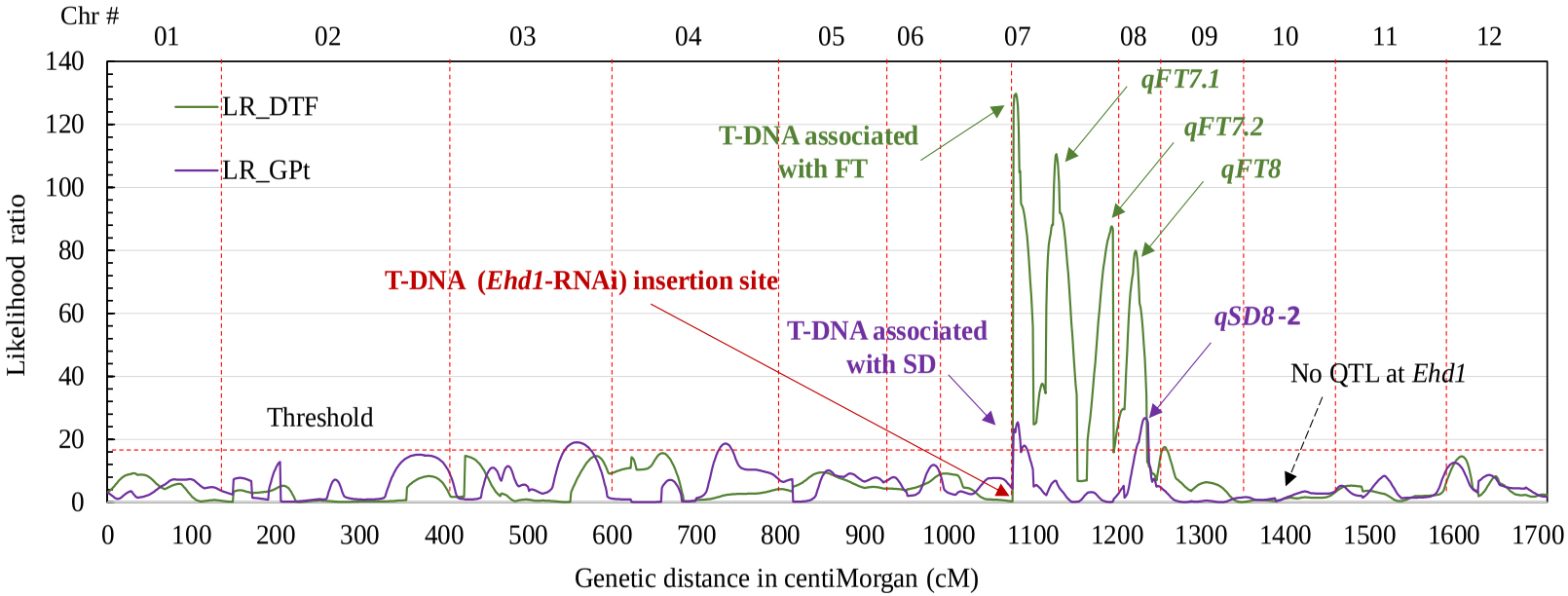

Figure 3.7 Distributions of likelihood ratios for flowering time (FT) and seed dormancy (SD) on the rice genome. The genome is aligned from the top of chromosome 1 to the end of chromosome 12 chromosomes (Fig. 3.6), which are separated by vertical dotted lines. The red arrow indicates the T-DNA insertion site, and the black arrow states that there is no QTL at *Ehd1* site on chromosome 10. The green color peaks are indicators of the QTLs for flowering time and the purple peaks are showing the QTLs of seed dormancy. The threshold is indicated by the red horizontal line and likelihood ratio values were derived by WinQTLCart composite interval mapping.

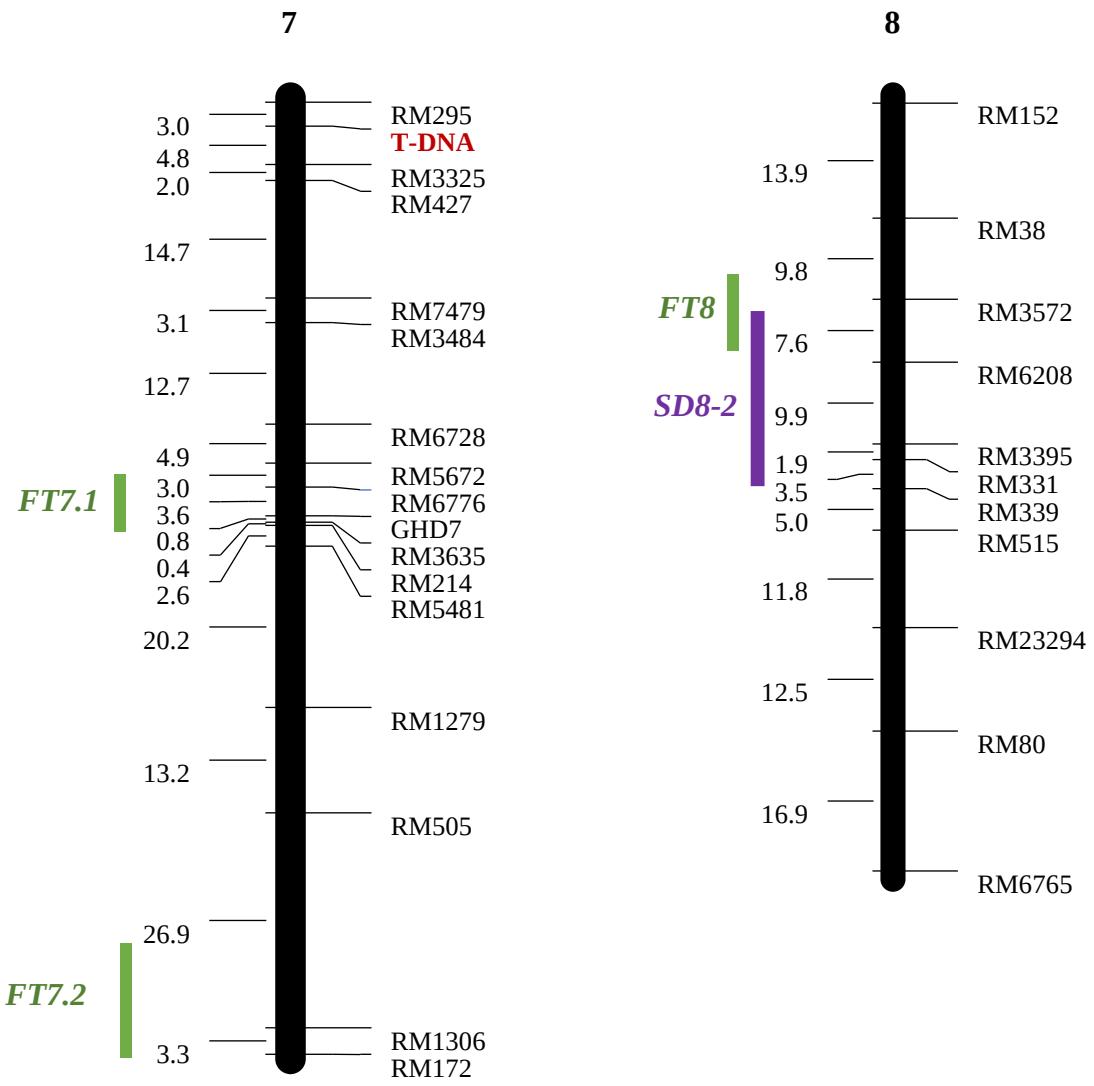

Figure 3.8 Map positions of the T-DNA insertion site and QTLs associated with flowering time (FT) and seed dormancy (SD) on chromosomes 7 and 8. The genetic map in centiMorgan (cM) was placed on the left side and developed using the software MAPMAKER/EXP 3.0. Markers, situated on the right side of the chromosomes, were selected to map the F_2 population. Markers were grouped at the threshold of 3.0 likelihood ratio (LR). The green and purple columns on the left side indicate the QTLs for flowering time and seed dormancy, respectively.

Table 3.4 Summary of QTLs associated with flowering time (FT) and seed dormancy (SD) in the whole F_2 population

QTL[a]	Marker[b]	Chr	cM[c]	LR[d]	a^d	d^d	R^{2d}
Flowering time evaluated by days to flowering							
Ehd1-RNAi	T-DNA	7	4	129.84	13.71	20.86	0.21
qFT7.1	GHD7	7	52	110.61	8.36	13.37	0.16
qFT7.2	RM1306	7	118	87.63	12.11	2.07	0.13
qFT8	RM3572	8	27	79.87	5.67	8.09	0.14
Seed dormancy evaluated by germination percentage							
Ehd1-RNAi	T-DNA	7	3	25.45	-0.04	-0.19	0.12
qSD8-2	RM6206	8	62	26.75	-0.06	-0.10	0.15

[a] *Ehd1*-RNAi refers to the *Ehd1*-RNAi T-DNA insertion site.

[b] The marker nearest to the QTL.

[c] Genetic distance in CentiMorgan from the QTL to the top of the chromosome (chr).

[d] Likelihood ratio (LR), additive (*a*) and dominance (*d*) effect, and the proportion of the variance explained by the QTL (R^2) computed by composite interval mapping. The positive and negative *a* value indicates that the allele from the parent and Nipponbare delayed flowering and reduced germination, respectively.

3.3.6 Differences in effects of QTLs associated with flowering time and seed dormancy between the F_2 subpopulations

Composite interval mapping of the distribution of likelihood ratio was conducted for the R and S subpopulations, and the data are summarized in Figure 3.9 and Table 3.5. For flowering time, the QTL peak value was reduced for *qFT7.1* but increase for *qFT7.2* and *qFT8* in the R subpopulation, where *Ehd1* was silenced by RNAi (Fig. 3.9). This result indicates that *Ehd1* promoted the effects of *qFT7.1* but inhibited the impact of the remaining two FT QTLs. The promoting or inhibiting effect was most extensive for *qFT7-1*, as it contributed much more to the phenotypic variance in the S (R^2=58%) than in R (R^2=11%) subpopulation (Table 3.5). Interestingly, silencing *Ehd1* increased additive effects, but reduced dominance effects for both *qFT7.2* and *qFT8* (Table 3.5).

For seed dormancy, *qSD8-2* was significant in the S, but not significant in the R subpopulation (Fig. 3.9). *qSD8-2* contributed 50% to the phenotypic variance in the S subpopulation (Table 3.5). This result suggests that *qSD8-2* was inhibited by *Ehd1*-RNAi.

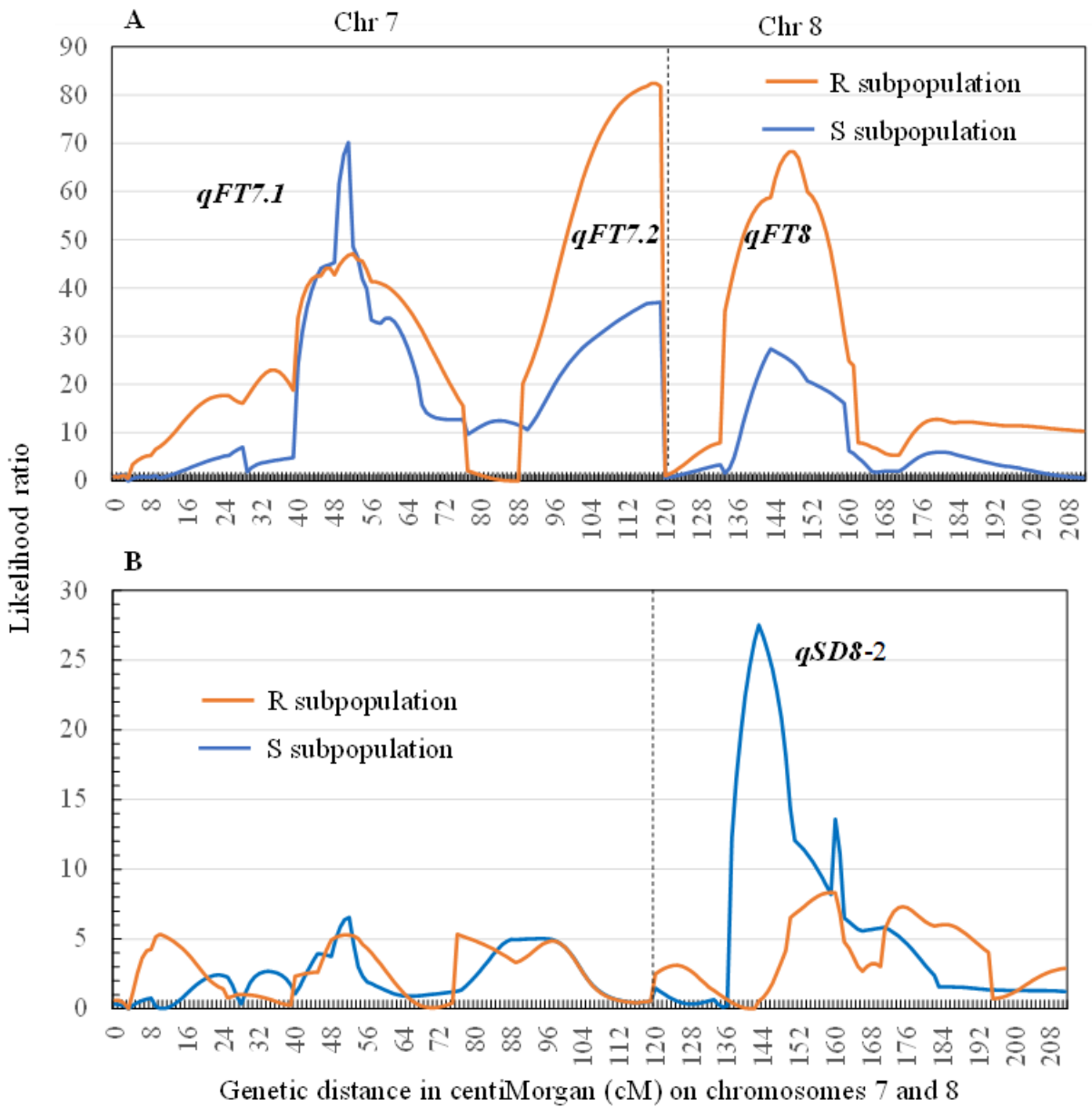

Figure 3.9 Distributions of likelihood ratios (LR) for flowering time (A) and seed dormancy (B) on chromosomes 7 and 8 in the R and S F_2 subpopulations. The flowering time (FT) was measured by days from germination to flowering and seed dormancy (SD) measured by germination percentage. The figures show differences in peak values for individual FT or SD QTLs between the R and S subpopulations.

Table 3.5 Summary of QTLs associated with flowering time (FT) and seed dormancy (SD) in the R and S F_2 subpopulations.

QTL[a]	Subpopulations[b]	LR[c]	*a*[c]	*d*[c]	R^2 [c]
qFT7.1	S	76.5	29.2 d	31.8 d	0.58
	R	44.2	5.3 d	7.8 d	0.11
qFT7.2	S	43.8	9.9 d	13.5 d	0.22
	R	81.7	14.2 d	-1.5 d	0.24
qFT8	S	34.8	4.3 d	10.3 d	0.18
	R	73.0	7.8 d	6.1 d	0.25
qSD8-2	S	29.9	-0.02	-0.21	0.50
	R	Not significant			

[a] *Ehd1*-RNAi refers to the *Ehd1*-RNAi T-DNA insertion site.

[b] The resistance (R) and susceptible (S) subpopulations.

[c] Likelihood ratio (LR), additive (*a*) and dominance (*d*) effect, and the proportion of the variance explained by the QTL (R^2) computed by composite interval mapping. The positive and negative *a* value indicates that the allele from the parent Nipponbare delayed flowering and reduced germination, respectively.

3.4 Discussions

3.4.1 Map position of the *Ehd1*-RNAi T-DNA and pleiotropy of *Ehd1*

This research identified one copy of the *Ehd1*-RNAi T-DNA based on the segregation pattern for the R and S responses to the selective agent hygromycin B. Two methods were used to map the T-DNA insertion site, but only one method was successful. Using linkage analysis, we located the T-DNA on the top of chromosome 7. RM295 is the marker nearest to the *Ehd1*-RNAi T-DNA. Therefore, RM295 can be used to tag the transgene in future research.

The Myb transcription factor gene *Ehd1* was reported as a QTL for heading date on chromosome 10 of rice by using a cross between the *japonica* cultivar Taichung T65 ($ehd1^{T65}$) and a line of African rice (*O. glaberrima*) (Doi et al., 2004). The *O. glaberrima* allele of *Ehd1* ($ehd1^{Gla}$) promotes flowering time by regulating FT-like gene expression only under short-day conditions. *Ehd1* was also detected in a cross between Taichung T65 and Nipponbare, and the Nipponbare allele of *Ehd1* ($ehd1^{Nip}$) confers early heading. It was believed that T65 might have a loss-of-function allele at *Ehd1* ($Ehd1^{T65}$), which delays flowering (Doi et al. 2004). The current study provided evidence that silencing *Ehd1* delays flowering for more than 30 days, which is two times greater than the effect of $Ehd1^{T65}$ in the *O. glaberrima* and Nipponbare backgrounds. Therefore, it is possible that $Ehd1^{T65}$ is a change-of-function, not a loss-of-function mutation.

More importantly, this research provided evidence that *Ehd1* has a pleiotropic effect on seed dormancy. Specifically, the functional allele at *Ehd1* promotes flowering

and reduces seed dormancy. This is because silencing *Ehd1* delayed flowering and reduced germination ability.

3.4.2 QTLs associated with seed dormancy and flowering time in the F_2 population

Four QTLs for flowering time (FT) and two QTLs for seed dormancy (SD) were detected in the F_2 population. One of the QTLs is the *Ehd1*-RNAi T-DNA, which had pleiotropic effects on both FT and SD. The wide range of flowering time and the observation of seed dormancy indicate that there could be more QTLs with a relatively small effect on FT or SD segregating in the mapping population.

The *qSD10/FT10* cluster was detected in the genetic background of EM93-1, an indica-type line with a short growth duration and weak seed dormancy (Pipatpongpinyo, 2018). Whereas, the *Ehd1*-RNAi transgenic line was developed in the genetic background of Nipponbare, a *japonica*-type cultivar with a moderate growth period and weak seed dormancy. The current research did not detect *qSD10/FT10* in the F_2 population from a cross between EM93-1 and a Nipponbare *Ehd1*-RNAi transgenic line. The result of the present study suggests that there is no functional differentiation at *qSD10/FT10* and *Ehd1* between the parent lines EM93-1 and Nipponbare.

Several QTLs for FT and SD were mapped on chromosomes 7 and 8 of the rice genome. *qFT7.1*, *qFT7.2,* and *qFT8* mapped in this research are located in the same genomic regions as those detected in the other populations (Gu et al., 2006). Only *qSD8-2* discovered in the current research was mapped onto a different position that was not reported by the previous research, namely *qSD8* in Gu et al. 2004a. Thus, we named the QTL detected in this research as *qSD8-2* (Figure 3.8).

3.4.3. Genes for flowering time or seed dormancy regulated by *Ehd1*

The current research detected several QTLs regulated by *Ehd1*. In terms of the likelihood ratio (LR) distribution patterns and peak values calculated by composite interval mapping, the QTLs with a relatively significant effect on FT or SD were located on chromosomes 7 and 8.

For flowering time, the effect of *qFT8* was increased and, the effects of *qFT7.1* were reduced when *Ehd1* was silenced by RNAi. This indicated that *Ehd1* promoted the effect of *qFT7.1* but inhibited the effect of *qFT8*. The promoting or inhibiting effect was most extensive for *qFT7.1*, as it contributed much more to the phenotypic variance in the S (R^2=58%) than in R (R^2=11%) subpopulation. Interestingly, silencing *Ehd1* increased additive effects, but reduced dominance effects for both *qFT7.2* and *qFT8*.

For seed dormancy, *qSD8-2* was significant in the S subpopulation only. QTL *qSD8-2* contributed 50% to the phenotypic variance in the S subpopulation. This suggested that the underlying gene *qSD8-2* was inhibited by silencing *Ehd1*.

Together these results indicated that *FT7.1* and *qSD8-2* were upregulated by *Ehd1* and that silencing *Ehd1* reduced the effect of *FT7.1* but increased the effect of *qFT7.2*, and *qFT8* (Figure 3.10). The FT QTLs were mapped in the same positions as those reported in the previous positions (Gu et al., 2007). Further research is needed to confirm *qSD8-2* with different populations and to collect more information to verify the model in Figure 3.10.

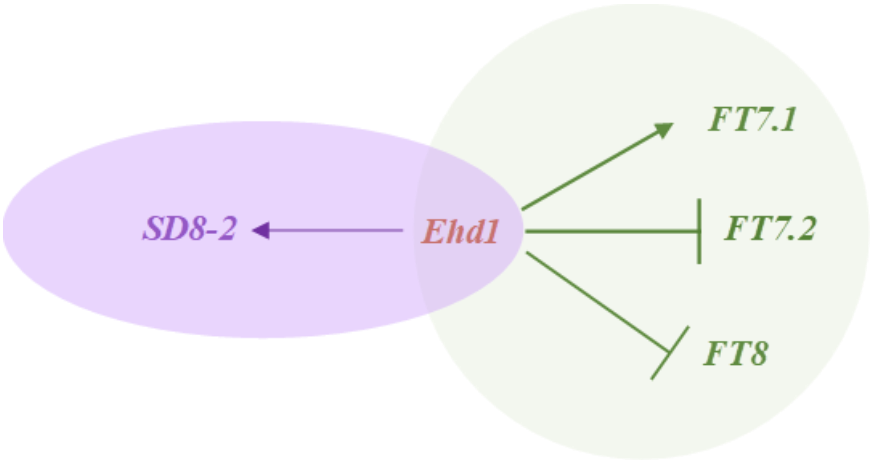

Figure 3.10 A putative model to explain how *Ehd1* regulates the QTLs for flowering time or seed dormancy. The promoting or inhibiting relations in the figures were inferred by data from QTL analysis for the R and S F_2 subpopulations (Table 3.5).

References

Carlos A-B, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. Genetics 164:711-729.

Amnuaysin N, Korakotchakorn H, Chittapun S, Poolyarat N (2018) Seed germination and seedling growth of rice in response to atmospheric air dielectric-barrier discharge plasma. Songklanakarin J. Sci. Technol. 40 (4), 819-823.

Baskin JM, Baskin CC (2004) A classification system for seed dormancy. Seed Science Research 14:1-16.

Bennewitz S, Bergau N, Tissier A (2018) QTL mapping of the shape of type VI glandular trichomes in tomato. *Frontiers in plant science* 9: 1421.

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. Proceedings of the National Academy of Sciences of the United States of America 103:17042-17047.

Bewley JD (1997) Seed germination and dormancy. The plant cell, 9(7), 1055.

Bian XF**,** Liu X, Zhao ZG, Jiang L, Gao H, Zhang YH, Zhang M, Chen LM, Liu SJ, Zhai HQ, Wan JM (2011) Heading date gene, *dth3* controlled late flowering in *O.glaberrima* Steud by down-regulating *Ehd1*. Plant Cell Rep 30:2243–2254.

Cai HW, Morishima H (2000) Genomic regions affecting seed shattering and seed dormancy in rice. Theoretical and Applied Genetics 100:840-846.

Cai S, Jiang G, Ye N, Chu Z, Xu X, Zhang J, Zhu G. (2015) A key ABA catabolic gene, OsABA8ox3, is involved in drought stress resistance in rice. *PLoS One* 10, no. 2: e0116646.

Camoni L, Visconti L, Aducci P, Marra M (2018) 14-3-3 proteins in plant hormone signaling: doing several things at once. *Frontiers in plant science* 9:297.

Chandraratna MF (1953) A gene for photoperiod sensitivity in rice linked with apiculus color. Nature 171:1162–1163.

Chandraratna MF (1955) Genetics of photoperiod sensitivity in rice. J Genet 53:215–223.

Chen J, Li X, Cheng C, Wang Y, Qin M, Zhu H, Zeng R, Fu X, Liu Z, Zhang G (2014) Characterization of epistatic interaction of QTLs *LH8* and *EH3* controlling heading date in rice. Sci Rep 4:4263.

Cho L-H, Yoon J, Pasriga R, An G (2016) Homodimerization of *Ehd1* is required to induce flowering in rice. *Plant Physiology* 170, no. 4: 2159-2171.

Chopra VL, Prakash S. (2002) Evolution and adaptation of cereal crops. Science Publishers.

Copeland LO, McDonald MB (1985) Principle of seed science and technology. 4th. EditionSB117.C73 2001.

Dai X, Ding Y, Tan L, Fu Y, Liu F, Zhu Z, Sun X, Gu P, Cai H, Sun C (2012) *LHD1*, an allele of *DTH8*/*Ghd8*, controls late heading date in common wild rice (*Oryza rufipogon*). J Integr Plant Biol 54:790–799.

Dai C, Xue HW (2010) Rice *early flowering1*, a CKI, phosphorylates DELLA protein SLR1 to regulate gibberellin signaling negatively. EMBO J 29:1916–1927.

Yan D, Duermeyer L, Leoveanu C, Nambara E (2014) *Plant and Cell Physiology*, Volume 55, Issue 9, Pages 1521533, https://doi.org/10.1093/pcp/pcu089 .

Debeaujon I, Lepiniec L, Pourcel L, Routaboul JM (2018) Seed coat development and dormancy. *Annual Plant Reviews online*, 25-49.

Doi K, Izawa T, Fuse T, Yamanouchi U, Kubo T, Shimatani Z, Yano M, Yoshimura A (2004) *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hd1*. Genes & Development, 18:926 936.

Doi K., Takeshi Izawa, Takuichi Fuse, Utako Yamanouchi, Takahiko Kubo, Zenpei, Shimatani, Masahiro Yano, and Atsushi Yoshimura (2015) *Ehd1*, a B-type response regulator in rice, confers short-day promotion of flowering and controls *FT-like* gene expression independently of *Hd1*. Genes Dev. 18(8): 926–936.

Doi K, Yoshimura A, Iwata N (1998) RFLP mapping and QTL analysis of heading date and pollen sterility using backcross populations between *Oryza sativa* L. and *Oryza glaberrima* Steud. Breeding Science 48:395-399.

Endo M, Araki T, Nagatani A (2016) Tissue-specific regulation of flowering by photoreceptors. *Cellular and molecular life sciences* 73, no. 4: 829-839.

Fageria NK (2007). Yield physiology of rice. Journal of Plant Nutrition, 30(6), 843-879.

Falconer DS, Mackay Trudy FC (1996) Introduction to Quantitative Genetics. Fourth edition.

Fujino K, Yamanouchi U, Yano M (2013) Roles of the *Hd5* gene controlling heading date for adaptation to the northern limits of rice cultivation. Theor Appl Genet 126:611–618.

Gao W, Clancy JA, Han F, Prada D, Kleinhofs A, and Ullrich SE (2003) Molecular dissection of a dormancy QTL region near the chromosome 7 (5H) L telomere in barley. *Theoretical and Applied Genetics*, *107*(3), 552-559.

Gao H, Zhang X-M, Fei G, Chen J, Jin M, Ren Y, Wu W, Zhou K, Sheng P, Zhou F, Jiang L, Wang J, Zhang X, Guo X, Wang J-L, Cheng Z, Wu C, Wang H, Wan J-M. (2013) *Ehd4* encodes a novel and *Oryza*-genus-specific regulator of photoperiodic flowering in rice. PLoS Genet 9:e1003281.

Garner WW (1933) Comparative responses of long-day and short-day plants to the relativelength of day and night. Plant Physiology 8: 347–356.

Gilchrist E, Haughn G 2010 Reverse genetics techniques: engineering loss and gain of gene function in plants. Briefings in functional genomics. Vol 9. No 2. 103-110.

Gu X-Y, Chen Z-X, Foley ME (2003) Inheritance of seed dormancy in weedy rice. Crop Science 43:835-843.

Gu, X-Y, Foley, M. E., and Chen, ZX (2004b). A set of three genes regulates photoperiodic responses of flowering in rice (Oryza sativa). Genetica, 122(2), 127-140.

Gu X-Y, Foley ME (2007) Epistatic interactions of three loci regulate flowering time under short and long daylengths in a backcross population of rice (*Oryza sativa*). Thedor Appl Genet 114:745-754 DOI 10.1007/s00122-006-0475-z.

Gu X-Y, Foley ME, Horvath DP, Anderson JV, Feng J, Zhang L, Mowry CR, Ye H, Suttle JC, Kadowaki K, Chen Z (2011) Association between seed dormancy and

pericarp color is controlled by a pleiotropic gene that regulates abscisic acid and flavonoid synthesis in weedy red rice. Genetics 189:1515-1524.

Gu X-Y, Kianian SF, Foley ME (2004a) Multiple loci and epistases control genetic variation for seed dormancy in weedy rice (*Oryza sativa*). Genetics 166:1503-1516.

Gu X-Y, Kianian S, Foley M (2006) Isolation of three dormancy QTLs as Mendelian factors in rice. Heredity 96:93-99.

Gu X-Y, Kianian SF, Foley ME (2005b) Phenotypic selection for dormancy introduced a set of adaptive haplotypes from weedy into cultivated rice. Genetics 171:695-704.

Gu X-Y, Kianian SF, Hareland GA, Hoffer BL, Foley ME (2005a) Genetic analysis of adaptive syndromes interrelated with seed dormancy in weedy rice (*Oryza sativa*). Theoretical and Applied Genetics 110:1108-1118.

Gu X-Y, Pipatpongpingyo W, Zhang L, Zhou Y, Ye H, Feng J (2018) Two constructing patterns and underlying genes for coadaptation of seed dormancy and flowering time in rice (*Oryza sativa* L.).Scientific Reports 8:16813.

Han F, Ullrich SE, Clancy JA, Romagosa I (1999). Inheritance and fine mapping of a major barley seed dormancy QTL. *Plant Science*, 143(1), 113-118.

Hori K, Matsubara K, Yano M (2016) Genetic control of flowering time in rice: integration of Mendelian genetics and genomics. Theor Appl Genet DOI 10.1007/s00122-016-2773-4.

Hughes PW, Soppe WJJ, Albani MC (2019) Seed traits are pleiotropically regulated by the flowering time gene PERPETUAL FLOWERING 1 (PEP1) in the perennial Arabis Alpina. *Molecular Ecology* 28, no. 5: 1183-1201.

Hori K, Ogiso-Tanaka E, Matsubara K, Yamanouchi U, Ebana K, Yano M (2013) *Hd16*, a gene for casein kinase I, is involved in the control of rice flowering time by modulating the day-length response. Plant J 76:36–46.

Huo HQ, Wei SH, Bradford KJ (2016) *DELAY OF GERMINATION 1* (*DOG1*) regulates both seed dormancy and flowering time through microRNA pathways. Proceedings of the National Academy of Sciences of the United States of America 113:2199-2206.

Jiang P, Wang S, Zheng H, Li H, Zhang F, Su Y, Xu Z, Lin H, Qian Q, Ding Y (2018) SIP 1 participates in the regulation of flowering time in rice by recruiting OsTrx1 to Ehd1. *New Phytologist* 219, no. 1: 422-435.

Karssen CM (2017) Hormonal regulation of seed development, dormancy, and germination studied by genetic control. In *Seed development and germination*, pp. 333-350. Routledge.

Kato S, Kosaka H, Hara S (1928). On the affinity of rice varieties, as shown by the fertilityof hybrid plants (in Japanese). Bull. Sci. Fac. Agric. Kyushu Univ 3:132-147.

Kelly KM, Staden JV, Bell V (1992) Seed coat structure and dormancy Plant Growth Regulation volume 11, pages 201–209.

Khush GS (1997). Origin, dispersal, cultivation, and variation of rice. *Plant molecular biology*, 35(1-2), 25-34.

Khush GS, and Brar DS (2002) Rice book. International Rice Research Institute, DAPO. Box 7777, Metro Manila, Philippines.

Kojima S, Takahashi Y, Kobayashi Y, Monna L, Sasaki T, Araki T, Yano M (2002). *Hd3a*, a rice ortholog of the Arabidopsis *FT* gene, promotes the transition to

flowering downstream of *Hd1* under short-day conditions. Plant Cell Physiol 43:1096–1105.

Koo BH, Yoo SC, Park JW, Kwon CT, Lee BD, An G, Zhang Z, Li J, Paek NC (2013) Naturalvariation in *OsPRR37* regulates heading dateand contributes to rice cultivation at a wide range of latitudes. Mol Plant 6:1877–1888.

Kwon CT, Yoo SC, Koo BH, Cho SH, Park JW, Zhang Z, Li J, Li Z, Paek NC (2014). Naturalvariation in *Early flowering1* contributes to early flowering in *japonica* rice under long days. Plant, Cell Environ 37:101–112.

Lee S, Kim J, Han J-J, Han M-J, An G (2004) Functional analyses of the flowering time gene *OsMADS50*, the putative *SUPPRESSOR OF OVEREXPRESSION OF CO 1/AGAMOUS LIKE 20* (*SOC1*/*AGL20*) ortholog in rice. Plant J 38:754–764.

Lee Y-S, Lee D-Y, Cho L-H, and An G (2014) Rice miR172 induces flowering by suppressing OsIDS1 and SNB, two AP2 genes that negatively regulate expression of *Ehd1* and florigens. *Rice* 7, no. 1: 31.

Lin M, Zhang D, Liu S, Zhang G, Yu J, Fritz A K, Bai G (2016) Genome-wide association analysis on pre-harvest sprouting resistance and grain color in US winter wheat. *BMC Genomics* 17, no. 1: 794.

Lin SY, Sasaki T, Yano M (1998). Mapping quantitative trait loci controlling seed dormancy and heading date in rice, Oryza sativa L., using backcross inbred lines. *Theoretical and Applied Genetics*, *96*(8), 997-1003.

Liu YG and Chen Y (2007) High-efficiency thermal asymmetric interlaced PCR for amplification of unknown flanking sequences. *BioTechniques 43:649-656* Doi 10.2144/000112601.

Lincoln S, Daly M and Lander E (1992) *Constructing genetic maps with MAPMAKER/EXP* 3.0, Ed. 3. Whitehead Institute, Cambridge, MA.

Liu S, Sehgal SK, Li J, Lin M, Trick HN, Yu J, Gill BS, and Bai G (2013) Cloning and Characterization of a Critical Regulator for Preharvest Sprouting in Wheat. e Genetics Society of America DOI: 10.1534/genetics.113.152330.

Liu Y, Geyer R, van Zanten M, Carles A, Li Y, Harold A, van Nocker S, Soppe WJ (2011) Identification of the *Arabidopsis REDUCED DORMANCY 2* gene uncovers a role for the polymerase associated factor 1 complex in seed dormancy. PloS One 6:e22241.

Liu Y, Koornneef M, Soppe WJ (2007) The absence of histone H2B monoubiquitination in the *Arabidopsis hub1* (*rdo4*) mutant reveals a role for chromatin remodeling in seed dormancy. The Plant cell 19:433-444.

Lu BR, Xingxing C, Jin X (2009) Efficient indica and japonica rice identification based on the InDel molecular method: Its implication in rice breeding and evolutionary research. National Natural Science Foundation of China and the Chinese Academy of Sciences. Published by Elsevier Limited and Science in China Press. All rights reserved. DOI:10.1016/j.pnsc.2009.01.011.

Lu, JJ, Tan DY, Baskin CC, and Baskin JM. (2017) Effect of seed position on a parental plant on the proportion of seeds produced with nondeep and intermediate physiological dormancy. *Frontiers in plant science* 8: 147.

Luo M, Zhao Y, Zhang R, Xing J, Duan M, Li J, Wang N, Wang W, Zhang S, Chen Z, Zhang H, Shi Z, Zhao J (2017) Mapping of a major QTL for salt tolerance of mature field-grown maize plants based on SNP markers. *BMC plant biology* 17, no. 1: 140.

Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH (2010) CDD: aConserved Domain Database for the functional annotation of proteins. Nucleic Acids Research 39:D225-D229.

Matsubara K, Kono I, Hori K, Nonoue Y, Ono N, Shomura A, Mizubayashi T, Yamamoto S, Yamanouchi U, Shirasawa K, Nishio T, Yano M (2008) Novel QTLs for photoperiodic flowering revealed by using reciprocal backcross inbred lines from crosses between *japonica* rice varieties. Theor Appl Genet 117:935–945.

Matsubara K, Ogiso-Tanaka E, Hori K, Ebana K, Ando T, Yano M (2012) Natural variation in *Hd17*, a homolog of Arabidopsis *ELF3* that is involved in rice photoperiodic flowering. Plant Cell Physiol 53:709-716.

McDonald, Miller B (2001) Physiology of Seed Germination (https://pdfs.semanticscholar.org/)

Miller, Matthew D (2016) The Modifiable Conceptual Unit Problem demonstrated using pollen and seed dispersal. Global ecology and conservation 6: 93-104.

Miles CM, and Wayne M (2008) Quantitative trait locus analysis Nature education 1(1):208.

Morinaga, T (1954) Classification of rice varieties on the basis of affinity. In Reports of the 5th Meeting of International Rice Commission's Working Party on Rice Breeding. Ministry of Agriculture and Forestry, Tokyo, pp. 1-14.

Monden Y, Naito K, Okumoto Y, Saito H, Oki N, Tsukiyama T, Ideta O, Nakazaki T, Wessleer S R, Tanisaka T (2009) High potential of a transposon mPing as a marker system in japonica × japonica cross in rice. DNA Res 16:131–140.

Mulki, MA, Bi X, and Korff Mv (2018) FLOWERING LOCUS T3 controls spikelet initiation but not floral development. *Plant Physiology* 178, no.3: 1170- 1186.

Murty G, Vemireddy L, Srividhya A, Nagireddy R, Jena SS, Gandikota M, Patil S, Veeraghattapu R, Deborah DAK, Reddy GE, Shake M, Dasari A, Puram VRR, Durgarani ChV, Neeraja CN, Siddiq EA, Sheshumadhav M (2018) QTL-Seq-based genetic analysis identifies a major genomic region governing dwarfness in rice (Oryza sativa L.). *Plant cell reports* 37, no. 4): 677-687.

Nguyen HT, Ranganathan C, Blum A (1997) Breeding of drought resistance in rice: Physiology and molecular genetics considerations. Crop Sci. 37:1426-1434

Ogiso-Tanaka E, Matsubara K, Yamamoto S, Nonoue Y, Wu J, Fujisawa H, Ishikubo H, TanakaT, Ando T, Matsumoto T, Yano M (2013) Natural variation of the *RICE FLOWERING LOCUS T1* contributes to flowering time divergence in rice. PLoS ONE 8:e75959.

Oka, HI (1988) Origin of cultivated rice. *Japan Scientific Societies Press.*

Okumoto Y, Yoshimura A, Tanisaka T, Yamagata H (1992) Analysis of a rice variety Taichung 65 and its isogenic early-heading lines for late-heading genes *E1*, *E2*, and *E3*. Jpn J Breed 42:415–429 (in Japanese with English summary).

Okumoto Y, Tanisaka T (1997) Trisomic analysis of a strong photoperiod-sensitivity gene *E1* in rice (*Oryza sativa* L.). Euphytica 95:301–307.

Osa M, Kato K, Mori M, Shindo C, Torada A, Miura H (2003) Mapping QTLs for seed dormancy and the *Vp1* homolog on chromosome 3A in wheat. Theoretical and Applied Genetics 106:1491-1496.

Park SJ, Kim SL, Lee S, Je BI, Piao HL, Park SH, Kim CM, Ryu CH, Park SH, Xuan YH, Colasanti J, An G, Han CD (2008) Rice Indeterminate1 (OsId1) is necessary for the expression of Ehd1 (Early heading date 1) regardless of photoperiod. The Plant Journal56, 1018-1029.

Pipatpongpinyo, W (2018) Map-Based cloning and molecular characterization of the 63 seed dormancy 10 locus in rice (Oryza sativa L.).

Rodriguez MV, Barrero JM, Corbineau F, Gubler F, Benech-Arnold RL (2015) Dormancy in cereals (not too much, not so little): about the mechanisms behind this trait. Seed Science Research, 25(2), 99-119.

Saito H, Ogiso-Tanaka E, Okumoto Y, Yoshitake Y, Izumi H, Yokoo T, Matsubara K, Hori K, Yano M, Inoue H, Tanisaka T (2012) *Ef7* encodes an ELF3-like protein and promotes rice flowering by negatively regulating the floral repressor gene *Ghd7* under both short and long-day conditions. Plant Cell Physiol 53:717–728.

Saito H, Yuan Q, Okumoto Y, Doi K, Yoshimura A, Inoue H, Teraishi M, Tsukiyama T, Tanisaka T (2009) Multiple alleles at *Early flowering 1* locus making a variation in the basic vegetative growth period in rice (*Oryza sativa* L.). Theor Appl Genet 119:315–323.

Savage WEF, Metzger GL (2006) Seed dormancy and the control of germination. DOI: 10.1111/j.1469-8137.2006.01787.x · Source: PubMed.

Sato S, Sakamoto I, Shirakawa K, Nakasone S (1988) Chromosomal location of an earliness gene *Ef1* of rice, *Oryza sativa* L. Jpn J Breed 38:385–396.

Schatzki J, Burkhard S, Wolfgang E, Cornelia H, Ivo Fe, Becker HC, and Christian M (2013) Mapping of QTL for seed dormancy in a winter oilseed rape doubled haploid population. *Theoretical and applied genetics* 126, no. 9: 2405-2415.

Shaban M (2013) Effect of water and temperature on seed germination and emergence as a seed hydrothermal time model. International Journal of Advanced Biological and Biomedical Research Volume 1, Issue 12, 2013: 1686-1691.

Shibuya T, Hori K, Ogiso-Tanaka E, Yamanouchi U, Shu K, Kitazawa N, Shomura A, Ando T, Ebana K, Wu J, Yamazaki T, Yano M (2016) *Hd18*, encoding histone acetylase related to *Arabidopsis FLOWERING LOCUS D*, is involved in the control of flowering time in rice. Plant Cell Physiol. DOI:10.1093/pcp/pcw105.

Shu K, Liu XD, Xie Q, He ZH (2016). Two faces of one seed: hormonal regulation of dormancy and germination. *Molecular Plant*, *9*(1), 34-45.

Simpson, GM (1990) Seed dormancy in grasses. Cambridge University Press, Cambridge.

Song Y, Jiaojun Z, Qiaoling Y (2019) Roles of abscisic acid and gibberellins in maintaining primary and secondary dormancy of Korean pine seeds. Journal of Forestry Research: 1 12.

Subudhi PK, Parco A, Singh PK, DeLeon T, Karan R, Biradar H, Cohn MA, Brar DS, Sasaki T (2012) Genetic architecture of seed dormancy in US weedy rice in different genetic backgrounds. Crop Science 52:2564-2575.

Sugimoto K, Takeuchi Y, Ebana K, Miyao A, Hirochika H, Hara N, Ishiyama K, Kobayashi M, Ban Y, Hattori T, Yano M (2010) Molecular cloning of *Sdr4*, a regulator involved in seed dormancy and domestication of rice. Proceedings of the National Academy of Sciences of the United States of America 107:5792-5797.

Syakudo K, Kawase T (1953) Studies on the quantitative inheritance (11) A. Rice (*Oryza sativa* L.) (d) Inheritance of the heading duration and the quantitative function of the causal genes *E1*, *E2,* and *D1*. Jpn J Breed 3:6–12 (in Japanese with English summary).

(*Oryza sativa* L.) (d) Inheritance of the heading period and the quantitative function of

the genes *E3*, *E4*, and *E5*. Jpn J Breed 4:83–91 (in Japanese with English summary).

Syakudo K, Kawase T, Yoshino K (1954) Studies on the quantitative inheritance (13) A. Rice (*Oryza sativa* L.) (d) Inheritance of the heading period and the quantitative function of the causal genes in its determination. (2) On the quantitative function of the genes *E3*, *E4*, and *E5*. Jpn J Breed 4:83–91 (in Japanese with English summary).

Takahashi Y, Shomura A, Sasaki T, Yano M (2001) *Hd6*, a rice quantitative trait locus involved in photoperiod sensitivity, encodes the alpha subunit of protein kinase CK2. Proc Natl Acad Sci USA 98:7922–7927.

Tereshchenko N, Olga Z, Boris M, Aleksandra K, Valeryi S, Ivan L, Albina Z, Lyudmila P, and Tatyana Y (2017) The influence of silicon oxide nanoparticles on morphometric parameters of monocotyledons and dicotyledons in soil and climatic conditions of western Siberia, as well as on microbiological soil properties. *BioNanoScience* 7, no. 4: 703711.

Tsai KH, Oka HI (1966) Genetic study of yielding capacity and adaptability in crop plants. 2. Analysis of genes controlling heading time in Taichung 65 and other rice varieties. Bot Bull Acad Sin 7:54–70.

Tsai KH (1976) Studies on earliness genes in rice, with special reference to the analysis of, is alleles at the *E* locus. Jpn J Genet 51:115–128.

Van der Schaar W, Alonso-Blanco C, Léon-Kloosterziel KM, Jansen RC, Van Ooijen JW, Koornneef M (1997) QTL analysis of seed dormancy in *Arabidopsis* using recombinant inbred lines and MQM mapping. Heredity 79:190-200.

Wan J, Jiang L, Tang J, Wang C, Hou M, Jing W, Zhang L (2006) Genetic dissection of the seed dormancy trait in cultivated rice (*Oryza sativa* L.). Plant Science 170:786-792.

Wei X, Xu J, Guo H, Jiang L, Chen S, Yu C, Zhou Z, Hu, P, Zhai H, Wan J (2010) *DTH8* suppresses flowering in rice, influencing plant height, and yield potential simultaneously. Plant Physiol 153:1747–1758.

Wu W, Zheng XM, Lu G, Zhong Z, Gao H, Chen L, Wu C, Wang HJ, Wang Q, Zhou K, Wang JL, Wu F, Zhang X, Guo X, Cheng Z, Lei C, Lin Q, Jiang L, Wang H, Ge S, Wan J. (2013) Association of functional nucleotide polymorphisms at *DTH2* with the northward expansion of rice cultivation in Asia. Proc Natl Acad Sci USA 110:2775–2780.

Xue W, Xing Y, Weng X, Zhao Y, Tang W, Wang L, Zhou H, Yu S, Xu C, Li X, Zhang Q (2008) Natural variation in *Ghd7* is an important regulator of heading date and yield potential in rice. Nat Genet 40:761–767.

Xie K, Jiang L, Lu B, Yang C, Li L, Liu X, Zhang L, Zhao Z, Wan J (2011) Identification of QTLs for seed dormancy in rice (*Oryza sativa* L.). Plant Breeding 130:328-332.

Yamaguchi S, Kamiya Y, Nambara E. (2018). Regulation of ABA and GA levels during seed development and germination in Arabidopsis. Annual Plant Reviews online, 224 247.

Yano M, Harushima Y, Nagamura Y, Kurata N, Minobe Y, Sasaki T (1997) Identification of quantitative trait loci controlling heading date in rice using a high-density linkage map. Theor Appl Genet 95:1025–1032.

Yano M, Katayose Y, Ashikari M, Yamanouchi U, Monna L, Fuse T, Baba T, Yamamoto K, Umehara Y, Nagamura Y, Sasaki T (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the Arabidopsis flowering time gene *CONSTANS*. Plant Cell 12:2473–2484.

Ye H, Beighley DH, Feng J, Gu X-Y (2013) Genetic and physiological characterization of two clusters of quantitative trait Loci associated with seed dormancy and plant height in rice. G3: Genes, Genomes, Genetics 3:323-331.

Ye H, Foley ME, Gu X-Y (2010) New seed dormancy loci detected from weedy rice-derivedadvanced populations with major QTL alleles removed from the background. Plant Science 179:612-619.

Ye H, Feng J, Zhang L, Zhang J, Mispan MS, Cao Z, Beighley DH, Yang J, and Gu X-Y (2015) Map-based cloning of seed dormancy1-2 identified a gibberellin synthesis gene regulating the development of endosperm-imposed dormancy in rice. *Plant Physiology* 169, no. 3: 2152-2165.

Yoshida S, Forno Da, Cock JH, Gomez KA (1976) Laboratory Manual for Physiological Studiesof Rice. The International Rice Research Institute, Los Baños, Philippines.

Yokoo M, Fujimaki H (1971) Tight linkage of blast-resistance with late maturity observed in different *indica* varieties of rice. Jpn J Breed 23:35–39 (in Japanese with English summary).

Yuan Q, Saito H, Okumoto Y et al. (2009) Identification of a novel gene *ef7* conferring an extremely long basic vegetative growth phase in rice. Theor Appl Genet 119:675 684.

Zhang L, Lou J, Foley ME, Gu X-Y (2017) Comparative Mapping of Seed Dormancy Loci Between Tropical and Temperate Ecotypes of Weedy Rice (*Oryza sativa* L.). G3: Genes, Genomes, Genetics 7:2605-2614.

Zhou YM, Lu JJ, Tan DY, Baskin CC, Baskin JM (2015) Seed germination ecology of the cold desert annual Isatis violascens (Brassicaceae): two levels of physiological dormancy and role of the pericarp.\ *PloS one* 10, no. 10: e0140983.

Zhu YJ, Fan YY, Wang K, Huang DR, Liu WZ, Ying JZ, Zhuang JY (2017) Rice Flowering Locus T1 plays an important role in heading date influencing yield traits in rice. Scientific Reports | 7: *4918* | DOI:10.1038/s41598-017-05302-3.