Identification of novel seed dormancy mutants in *Arabidopsis thaliana* and molecular and biochemical characterization of the seed dormancy gene *DOG1*

Melanie Schwab, 2008

Identification of novel seed dormancy mutants in *Arabidopsis thaliana* and molecular and biochemical characterization of the seed dormancy gene *DOG1*

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But seeds are invisible.
They sleep deep in the heart of the earth's darkness,
until someone among them is seized with the desire to awaken.
Then this little seed will stretch itself and begin – timidly at first –
to push a charming little sprig inoffensively upward toward the sun.

Antoine de St. Exupéry, Le petit prince (1943)

Abstract

Seed dormancy is defined as the failure of a viable seed to germinate under favorable conditions. In addition to having an adaptive role in nature by optimizing germination to the best suitable time, dormancy control is also important in crop plants. Seed dormancy is induced during seed maturation and can be released by after-ripening or stratification. The molecular mechanisms of the induction and the release of dormancy are largely unknown.

In this project novel seed dormancy mutants involved in the release of dormancy by stratification or after-ripening were identified in mutagenesis screens. Several mutants that fail to germinate under such conditions and additional novel non-dormant mutants have been isolated, which provide promising genetic material for follow up studies.

In addition, an in-depth molecular and biochemical characterization of a key dormancy gene, *DELAY OF GERMINATION 1 (DOG1),* was carried out. *DOG1* is an alternatively spliced gene and encodes a protein of unknown function. Mutant alleles of *DOG1* are completely non-dormant, indicating that *DOG1* is absolutely required for seed dormancy induction.

Promoter activity studies demonstrated that the *DOG1* promoter drives seed-specific expression in the endosperm and the embryo. The cell-specific expression pattern was studied by additional in-situ hybridization experiments, which showed that *DOG1* transcripts are localized in the vascular system and highly accumulated in the shoot apical meristem of the embryo. At the subcellular level, the *DOG1* protein variants are mainly localized in the nucleus.

Quantitative RT-PCR demonstrated that *DOG1* is maximally expressed during seed maturation, 14-17 days after flowering, and that the relative abundance of each splicing variant changes during the final stages of seed maturation. In addition, it was shown that *DOG1* expression and protein levels correlate with the degree of dormancy and that low temperatures during seed development cause an increase in *DOG1* transcript levels and seed dormancy.

The functionality of the different splicing variants was studied by transgenic complementation and overexpression approaches using the single splicing forms and a genomic sequence of the gene. Single splicing forms only induced dormancy when highly overexpressed, above a specific threshold. Below that threshold, a combination and probably also a specific ratio of the different splicing forms were required for dormancy function. DOG1 protein was only detected in the dormant overexpression lines, in which protein production probably outcompeted its degradation. These results strongly indicate that the function of DOG1 is determined by the presence and the ratio of all splicing forms, which is required to sustain protein stability.

Zusammenfassung

Samenruhe, auch Dormanz genannt, wird definiert als Ausbleiben der Samenkeimung eines wachstumsfähigen Samens bei optimalen Bedingungen. Dieser Anpassungsmechanismus an die Umwelt verhindert das vorzeitige Auskeimen des Samens zu einem ungünstigen Zeitpunkt. Die Steuerung von Dormanz ist auch bei Kulturpflanzen von Bedeutung. Dormanz wird während der Samenentwicklung induziert und kann durch Mechanismen wie Nachreife oder Stratifizierung aufgehoben werden. Die molekularen Mechanismen dieser Prozesse sind allerdings weitgehend unbekannt.

Um neue Regulatoren von Dormanz sowie Dormanz-brechenden Mechanismen zu identifizieren, wurden Mutagenese-Screens durchgeführt. Dabei wurden mehrere Mutanten isoliert, deren Dormanz nicht durch Nachreife oder Stratifizierung gebrochen werden kann. Zusätzlich wurden nicht-dormante Mutanten isoliert, die nicht allelisch zu bekannten Dormanz Genen waren.

Weiteres Ziel der Arbeit war es, das bisher unbekannte Schlüsselgen für Samenruhe, *DELAY OF GERMINATION 1* (*DOG1*), molekular und biochemisch zu charakterisieren. Dieses Gen wird alternativ gespleißt und kodiert ein Protein mit unbekannter Funktion. *DOG1*-Nullmutanten weisen keinerlei Dormanz auf, was auf eine essentielle Rolle von *DOG1* in der Dormanz Regulation hinweist.

Promotor-Reportergen Konstrukte zeigten, dass der *DOG1* Promotor samenspezifische Expression im Embryo und im Endosperm steuert. Zusätzliche in-situ Hybridisierungsexperimente verdeutlichten, dass *DOG1* mRNA im vaskulären System lokalisiert ist und im apikalen Sprossmeristem des Embryos akkumuliert. Auf subzellulärer Ebene konnte gezeigt werden, dass alle DOG1 Isoformen hauptsächlich nukleare Lokalisierung aufweisen.

Quantitative RT-PCR demonstrierte, dass das Expressionsmaximum von *DOG1* 14-17 Tage nach Blühbeginn während der Samenreife erreicht wurde. Am Ende dieser Phase trat eine Veränderung der relativen Menge der verschiedenen Transkripte ein. Das Expressionsniveau und die Menge an akkumuliertem DOG1 Protein korrelierten mit dem Dormanzgrad. Es konnte ebenfalls gezeigt werden, dass niedrige Temperaturen während der Samenentwicklung zu einer Erhöhung des *DOG1* Expressionsniveaus und zu stärkerer Dormanz führen.

Die Funktionalität der verschiedenen Spleiß-Varianten wurde mit Komplementations- und Überexpressionskonstrukten der einzelnen Spleiß-Varianten und eines genomischen *DOG1* Fragments untersucht. Eine einzelne Variante konnte in transgenen Pflanzen nur dann Dormanz induzieren, wenn diese stark überexprimiert war und einen bestimmten Grenzwert überschritt. Unterhalb dieser Schwelle ist eine Kombination und möglicherweise auch ein bestimmtes Verhältnis der Formen für die Dormanz Funktion notwendig. DOG1 Protein war nur in dormanten Überexpressionslinien nachweisbar, in denen vermutlich Degradationsprozesse den Überschuss an Protein weniger schwer beeinträchtigten. Dies deutet darauf hin, dass die verschiedenen DOG1 Varianten für die Bildung eines stabilen und funktionellen DOG1 Protein Komplexes notwendig sind. Alternatives Spleißen stellt dabei einen feinregulatorischen Mechanismus dar, der für die Funktion und Protein Stabilität von *DOG1* eine wichtige Rolle spielt.

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Abbreviations

%	Percent
°C	Degree Celcius
µl	Microliter
3'	Three prime end of a DNA fragment
35S	35S promotor from the Cauliflower Mosaic virus
5'	Five prime end of a DNA fragment
aa	Amino acid
ABA	Abscisic Acid
AGI	Arabidopsis genome initiative
attR1, attR2	Attachment sites for site-specific recombination during Gateway cloning
BCIP	5-Bromo-4-Chloro-3-Indoryl-Phosphate
bp	Base Pair(s)
BR	Brassinosteroids
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
cDNA	Complementary Deoxyribonucleic Acid
CDS	Coding sequence
Col	*Arabidopsis thaliana* Columbia accession
Cvi	Cape Verde Island accession
d	Days
DAF	Days after Flowering
DMF	N,N-Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
Dof	DNA-binding with one finger
DTT	Dithiothreitol
E. coli	*Escherichia coli*
e.g.	Exempli gratia [Lat.] for example
EDTA	Ethylenediamine Tetraacetic Acid
EMS	Ethyl-methane sulfonate
ER-GFP	Green Fluorescent Protein localized in the Endosplamatic Reticulum
EST	Expressed sequence tag
et al.	*Et alii / et aliae* [Lat.] and others
EtOH	Ethanol
F1, F2, F3	First, second, third... filial generation after a cross
GA	Gibberellin
GFP	Green Fluorescent Protein
GUS	ß-Glucoronidase
h	Hour(s)
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kas	Kashmir accession
kD	Kilo Dalton
LB	T-DNA left border
LB medium	Luria Bertani medium
L*er*	*Arabidopsis thaliana* Landsberg *erecta* accession
M	Molar
M1, M2, M3	First, second, third... filial generation after a mutagenesis treatment
mcs	Multiple cloning site
min	Minute
MPIZ	Max-Planck-Institut für Züchtungsforschung (MPI for Plant Breeding Research)
mRNA	Messenger Ribonucleic Acid
MS	Murashige-Skoog
n	Number

NaOCl	Sodiumhypochloride
NIL	Near Isogenic Line
ODx	Optical Density at Wavelength x
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate bufferd saline buffer
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
pGENE	Promoter sequence of a GENE
PVDF	Polyvinylidene difluoride
QPCR	Quantitative Real-time Polymerase Chain Reaction
QTL	Quantitative Trait Locus/Loci
RACE	Rapid amplification of cDNA ends
RB	T-DNA right border
RIL	Recombinant Inbred Line
RNA	Ribonucleic Acid
ROS	Reactive oxygen species
rpm	Rotations per minute
RT-PCR	Reverse transcription followed by a polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
ß-ME	Beta-Mercaptoethanol
T1, T2, T3	First, second, third transgenic generation after stable plant transformation
TAE	Tris Acetate EDTA
TB	Terrific Broth
T-DNA	Transferred DNA
TEMED	N,N,N',N'-tetramethylethylenediamine
TF	Transcription factor
TILLING	Targeting induced local lesions in genomes
Tris	Tris(hydromethyl)aminomethane
Tris/HCl	Buffer containing 2-amino-e-hydroxymethyl-1,3-propanediol and HCl
U	Units
UTR	Untranslated region
v/v	Volume/volume
w/v	Weight/volume
wt	Wild type
x	Crossed to (crosses are always indicated in the order: female x male)

Gene names

ABA	*ABSCISIC ACID DEFICIENT*
ABI	*ABSCISIC ACID INSENSITIVE*
AHG	*ABA-HYPERSENSITIVE GERMINATION*
ATS	*ABERRANT TESTA SHAPE*
BRI	*BRASSINOSTEROID INSENSITIVE*
CTS	*COMATOSE*
DAG	*Dof AFFECTING GERMINATION*
DET	*DE-ETIOLATED*
DOG	*DELAY OF GERMINATION*
DOGL	*DELAY OF GERMINATION-LIKE*
EIN	*ETHYLENE INSENSITIVE*
EPR	*EXTENSIN PROLINE-RICH*
ERA	*ENHANCED RESPONSE TO ABA*
ETR	*ETHYLENE RESPONSE*
FUS	*FUSCA*
GA	*GIBBERELLIN DEFICIENT*
GAI	*GIBBERELLIN INSENSITIVE*
GID	*GIBBERELLIN-INSENSITIVE DWARF*
HUB	*HISTONE MONOUBIQUITINATION*
HY	*LONG HYPOCOTYL*
LEA	*LATE EMBRYOGENESIS ABUNDANT*
LEC	*LEAFY COTYLEDON*
PHYA	*PHYTOCHROME A*
PHYB	*PHYTOCHROME B*
RDO	*REDUCED DORMANCY*
RGA	*REPRESSOR OF THE ga1-3 MUTANT*
RGL	*RGA-LIKE*
SLY	*SLEEPY*
TT	*TRANSPARENT TESTA*
TTG	*TRANSPARENT TESTA GLABRA*

The nomenclature for plant genes follows the Arabidopsis standard:
GENES are written in upper case italics, while mutant *genes* are indicated in lower case italics, PROTEINS are written in upper case regular letters, mutant proteins in lower case regular letters.

1 Introduction

1.1 *Arabidopsis thaliana* as a model plant

Arabidopsis thaliana, a small dicotyledonous species, is a member of the mustard family *Brassicacea*. Over the last 40 years, it has become the predominant model system in plant biology because of its suitability for genetic, molecular, biochemical and physiological studies (MEINKE ET AL., 1998; MEYEROWITZ, 2002). Arabidopsis has a very rapid life-cycle of 6 to 8 weeks, produces numerous self progeny, requires only limited space and can be easily grown in greenhouses or climate chambers. It possesses one of the smallest genomes among higher plants of approximately 130 megabases divided over 5 chromosomes. Its genome is nearly completely sequenced (ARABIDOPSIS GENOME INITIATIVE, 2000) and contains approximately 26 000 genes. Furthermore, an extensive spectrum of tools has been developed by a large research community over the last 20 years, including efficient mutagenesis and transformation technology; DNA, RNA, protein and metabolite isolation and detection methods; and knockout collections of nearly all Arabidopsis genes. Genetic and molecular data are publicly available in databases and sophisticated analysis tools provide a powerful and efficient basis to study the expression and regulation of genes, pathways and networks in a broad variety of contexts. Arabidopsis has a widespread natural distribution throughout the Northern hemisphere (HOFFMANN, 2002) from northern Scandinavia to Africa, including the Cape Verde Islands and has been found in a broad range of altitudes. Many different accessions have been collected from natural populations (ALONSO-BLANCO AND KOORNNEEF, 2000) and the naturally occurring variation among these different accessions comprise a complementing resource to the use of laboratory mutants and the standard laboratory accessions such as Landsberg *erecta* (L*er*) and Columbia (Col). Furthermore, *Arabidopsis thaliana* shows similar dormancy phenotypes and germination responses to those of many species and is therefore used as a model in seed research (KOORNNEEF ET AL., 2002).

1.2 Seed development in *Arabidopsis thaliana*

The seeds of Arabidopsis are produced in slender fruit called siliques, which under optimal conditions contain 40 to 60 seeds. Seeds of the laboratory accession Landsberg *erecta* have an average length of approximately 0.5 mm at maturity and a dry weight of 20-30 µg (ALONSO-BLANCO ET AL., 1999). Seed development takes 20 days under standard conditions and can be divided into two general phases: embryogenesis and seed maturation. Embryogenesis is characterized as a cell-division and morphogenesis phase which leads to the formation of the fully developed embryo, the single-cell endosperm layer and the testa. This phase begins with a

double-fertilization event: one of two sperm cells fertilizes the haploid egg cell of the ovary and forms a diploid zygote, which develops into the embryo. The other sperm cell fuses with two haploid polar nuclei of the central cell of the embryo sac and results in a triploid endosperm cell. The two central cell maternal nuclei that contribute to the endosperm arise by mitosis from a single meiotic product. Therefore, the maternal contribution to the genetic constitution of the triploid endosperm is different from that of the embryo (FAURE ET AL., 2002). The seed coat is derived from the integuments of the ovule and, therefore, is of maternal origin (MAYER ET AL. 1991). At the end of embryogenesis, cell division in the embryo arrests (RAZ ET AL., 2001) and the seed enters the subsequent seed maturation phase. This phase begins with the switch from maternal to filial control (WEBER ET AL., 2005). The capacity for dormancy is initiated early during seed maturation and increases until the seed is mature (RAZ ET AL., 2001). Seed maturation is completed when storage reserves have accumulated, the water content of the seed has decreased and desiccation tolerance and primary dormancy have been established (Figure 1.1). ABA accumulation occurs during seed development in a biphasic manner. The first peak is derived from both zygotic and maternal tissue, while the second peak is derived from only zygotic tissue. (KARSSEN ET AL., 1983; HARADA, 1997, DEBEAUJON ET AL., 2007; HOLDSWORTH ET AL., 2008)

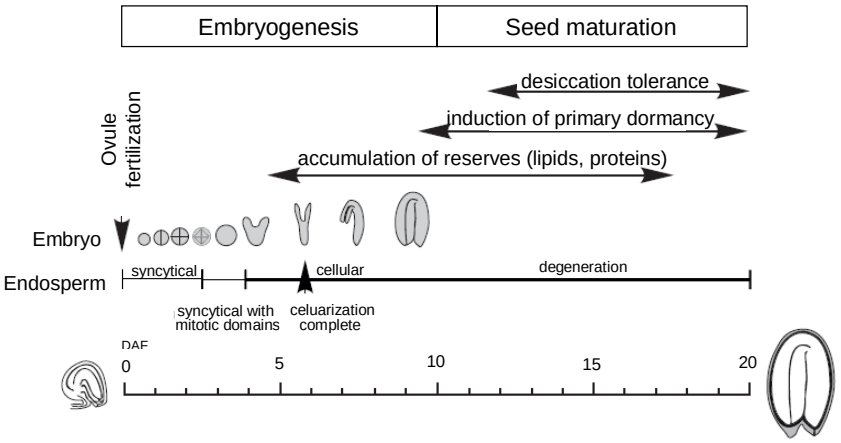

Figure 1.1: Processes during seed development in *Arabidopsis thaliana*.
Double fertilization results in the formation of a diploid embryo and a triploid endosperm. During embryogenesis the embryo development is completed and accumulation of reserves begins. During seed maturation induction of primary dormancy and onset of desiccation tolerance occurs. DAF, days after flowering. (Adapted from DEBEAUJON ET AL., 2007)

1.3 Seed dormancy and germination in *Arabidopsis thaliana*

1.3.1 Factors controlling seed dormancy

The seed functions as a dispersal unit, ensuring the survival of unfavorable conditions and the successful establishment of a new plant. A seed is desiccation tolerant and equipped with food reserves to sustain the growing seedling until it is established as an autotrophic organism.

An intact, viable, mature seed is considered to be dormant, when it is unable to germinate under conditions that are favorable for germination, including the presence of water, oxygen, appropriate temperature and light (BEWLEY, 1997a). When seed dormancy is released, germination begins with the water uptake by the quiescent dry seed and is completed by radicle protrusion through the testa and the endosperm, the structures surrounding the embryo.
Regulation of the timepoint of germination is critical: for example, winter annuals, which are the most common type in Arabidopsis, germinate in the autumn and require winter vernalization to accelerate flowering and seed production in the spring. Seed dormancy is then released during the summer, leading to germination in the fall. But if germination occurs in the spring, the conditions during summer will lead to demise of the vegetative plant and the onset of offspring will fail (DONOHUE, 2002). Seed dormancy acts as an adaptive trait to assure that germination occurs during the most suitable period for seedling establishment and life cycle completion. It is a survival strategy in which development is temporarily suspended and energy is conserved. (BEWLEY, 1997a)

Dormancy can be distinguished on the basis of the timing of dormancy, classified as primary dormancy and secondary dormancy. Primary dormancy is initiated during seed maturation and its maintenance in the mature seed is dependant on environmental and genetic factors. Once primary dormancy has been broken, exposure to unfavorable germination conditions that prolong inhibition of germination may induce secondary dormancy (CONE AND SPRUIT, 1983; DERKX AND KARSSEN, 1993). This inhibition may be due to active factors, such as endogenous ABA or secondary metabolites or passive factors, for example when imbibed seeds are exposed to relatively high temperatures in darkness (HILHORST, 1998).

The degree to which a seed is dormant can be determined by the embryo, the endosperm, and the seed coat. The three parts of a seed have different genetic compositions: the embryo and the endosperm originate from independent fertilizations, whereas the seed coat or testa is derived from maternal tissue (Chapter 1.2). Two types of seed dormancy have been recognized on the basis of the cause of the dormancy, coat-imposed dormancy and embryo dormancy. In the case of coat-imposed or coat-enhanced dormancy, inhibition of germination is conferred by the seed envelopes, the testa and the endosperm. Isolated embryos, where the physical barrier of the seed envelopes has been removed, are able to absorb water and nutrients, undergo cell expansion and finally germinate. There are six basic mechanisms of coat-imposed dormancy: prevention of water uptake (physical dormancy), mechanical restraint to radicle protrusion (mechanical

dormancy), interference with gas exchange, retention of inhibitors, inhibitor production (chemical dormancy) and light filtration.

In the case of embryo dormancy, the dormancy state is inherent in the embryo and not due to any influence of the seed coat or other surrounding tissues. In this type, the removal of the testa has no impact on the potential of the embryo to develop into a seedling (BEWLEY, 1997a; DEBEAUJON ET AL. 2007).

In addition, the growth potential of the embryo is important to overcome the constraints of the seed envelopes. The growth potential of the embryo has a major influence on the dormancy state of a seed (BENTSINK AND KOORNNEEF, 2002). It is influenced by the mother plant, maternal environmental conditions, and compounds produced by the embryo such as plant hormones, which are produced by the embryo itself (Figure 1.2) (DEBEAUJON ET AL., 2007; HILHORST, 2007). Such metabolic blocks are defined as physiologcal dormancy.

In Arabidopsis, the removal of the seed coat allows germination of non-germinating or strongly dormant phenotypes. Therefore it is classified as physiologically non-deep. In addition, seed dormancy in Arabidopsis is lost through stratification or after-ripening (BASKIN AND BASKIN, 2004).

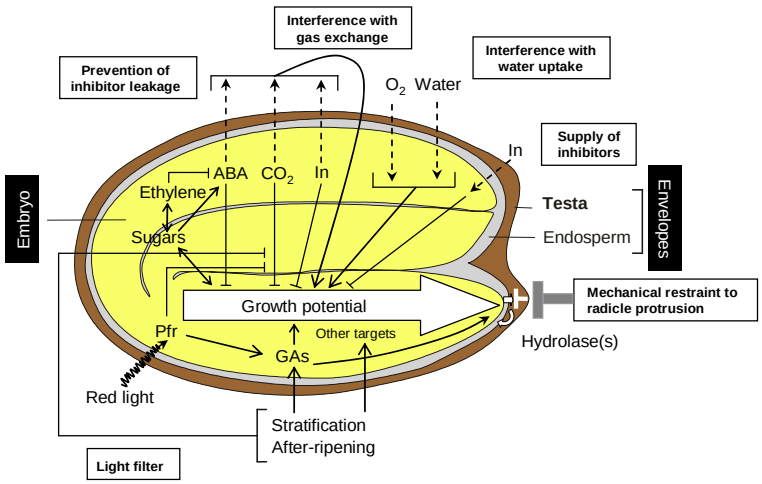

Figure 1.2: Interaction between the envelopes and embryo controlling seed dormancy and germination.
Radicle protrusion occurs when the embryo growth potential overcomes the constraints imposed by the envelopes. The main mechanisms of seed coat imposed dormancy are indicated in boxes. Hydrolase(s) secreted by the endosperm may contribute to the rupture of micropylar endosperm and testa. Full lines represent an action and dashed lines indicate diffusion or leakage. Sharp arrows stand for promotive actions, blunt arrows for inhibitory actions. (Adapted from DEBEAUJON ET AL., 2007; BENTSINK AND KOORNNEEF, 2002)

1.3.2 Promotion of germination and release of seed dormancy

The release of dormancy is a gradual process, determined by changes in the combined action of the restraints derived from the seed envelopes and the growth potential of the embryo. Dormancy can be overcome by exogenous factors that promote germination. Such exogenous factors can be

light and nitrate or the exogenous application of gibberellins and inhibitors of ABA biosynthesis. In addition seed dormancy is reduced during a prolonged dessication period of dry storage of mature seeds. This process is called after-ripening. Another dormancy breaking treatment is stratification in which seeds are exposed to cold temperatures under moist conditions. Many accessions require variable periods of after-ripening or stratification to release dormancy. In winter annuals after-ripening is the dormancy breaking process that occurs during summer, leading to germination in the fall, whereas in summer annuals dormancy is released by stratification during the cold winter, leading to germination in the the spring (BASKIN AND BASKIN, 1998).

The molecular mechanisms by which after-ripening and stratification contribute to the release of dormancy are not well understood. But recent studies have demonstrated the role of ABA catabolism in dormancy release (KUSHIRO ET AL., 2004; SAITO ET AL., 2004, FEURTADO AND KERMODE, 2007; FINKELSTEIN ET AL., 2008) and the interconnection of ABA and GA metabolism during seed germination (SEO ET AL., 2006; YAMAGUCHI ET AL., 2007). It has also been shown that the synergistic interaction of light and low temperatures regulates GA biosynthesis in imbibed seeds leading to germination (YAMAGUCHI ET AL., 1998, 2001; YAMAUCHI ET AL., 2004). Recent studies provided evidence that active transcription and translation, specific degradation of stored transcripts and accumulated proteins, enzymatic reactions and non-enzymatic reactions, often involving reactive oxygen species (ROS) function in the process of after-ripening (HOLDWORTH ET AL., 2008).

1.3.3 Genetic control of seed dormancy

Seed dormancy and germination are complex adaptive traits regulated by extensive interactions between environmental signals and endogenous developmental processes during the induction of dormancy, after-ripening, and imbibition. At present, about 50 genes (Figure 1.3, p. 10) are known to affect dormancy in Arabidopsis. Most of these were identified in mutagenesis screens and have been genetically and physiologically characterized. Mutants and the molecular and biochemical function of the respective genes are listed in Appendix A 1. Recently, the use of natural variation has provided a powerful additional tool to detect previously unknown genes of potentially ecological relevance. (BENTSINK AND KOORNNEEF, 2002; KOORNNEEF ET AL., 2002; BENTSINK ET AL., 2007)

1.3.3.1 Seed dormancy and germination mutants

Forward genetic screens have been used to identify mutations that lead to defects in dormancy and germination. Many of these mutants show pleiotropic phenotypes, which have allowed for

the identification of the underlying biochemical defects (e.g. dwarfness in GA-deficient mutants or reduced stomatal closure for ABA-deficient mutants). Many seed dormancy and germination mutants are affected in the biosynthesis or signalling pathways of plant hormones. Because plant hormones play multiple roles in the control of developmental processes, it is not surprising that these mutants show pleiotropy. Seed dormancy and germination mutants can be grouped into two classes, showing reduced dormancy or increased dormancy. Furthermore they can be classified into different groups including seed maturation mutants, plant hormone biosynthesis mutants, testa mutants and light response mutants (Figure 1.3, p. 10) as described by BENTSINK AND KOORNNEEF (2002).

Seed maturation mutants

After the completion of embryo morphogenesis, the fully developed embryo enters the seed maturation phase in which the mature embryo becomes arrested. This phase is genetically controlled by at least four major regulators: *ABA-INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*) and *LEAFY COTYLEDON 1* and *2* (*LEC1*, *LEC2*). Mutants of these genes were shown to be defective in controlling mid- and late seed development leading to defects in accumulation of storage proteins, onset of desiccation tolerance, and to premature germination. This non-dormant phenotype indicated that dormancy is induced during the later stages of seed maturation (RAZ ET AL.; 2001).

Mutants with a role in biosynthesis or signaling pathways of plant hormones

Dormancy is controlled by plant hormones. Abscisic acid (ABA) and gibberellin (GA) are the main regulators. Brassinosteroids and Ethylene are involved to a lesser degree (KOORNNEEF ET AL., 2002, FINCH-SAVAGE AND LEUBNER-METZGER, 2006). ABA inhibits germination, whereas GA promotes germination. Therefore *ABA-deficient* mutants (*aba1, aba2, aba3*) are non-dormant (KOORNNEEF ET AL.; 1982, 1984, LÉON-KLOOSTERZIEL ET AL.; 1996a) and *GA-deficient* (*ga1, ga2, ga3*) mutants are not able to germinate (KOORNNEEF AND VAN DER VEEN, 1980; KOORNNEEF ET AL., 1998). Additional mutants were selected due to their reduced dormancy or enhanced germination phenotype in the presence of the germination inhibiting compound ABA and were characterized as *ABA-insensitive* (*abi1, abi2, abi3, abi, abi5*) mutants (KOORNNEEF ET AL., 1984; FINKELSTEIN, 1994). Conversely, non-germinating mutants were selected on low, in wild type non-inhibiting ABA concentrations. These mutants showed high sensitivity to ABA resulting in enhanced seed dormancy and were identified as ABA-supersensitive *enhanced response to ABA* (*era1, era2, era3*) mutants (CUTLER ET AL., 1996) and the *ABA-hypersensitive germination* (*ahg*) mutant (NISHIMURA ET AL., 2004). Through the use of GA biosynthesis

inhibitors such as paclobutrazol, mutants with defects in ABA biosynthesis (LÉON-KLOOSTERZIEL ET AL., 1996a) and ABA signal transduction (*abi3*; NAMBARA ET AL.; 1992) were identified. These ABA biosynthesis mutants were also found by screening for revertants of non-germinating GA biosynthesis mutants (KOORNNEEF ET AL., 1982). The hormonal action of ABA in plants is controlled by the precise balance between its biosynthesis and catabolism. The cytochrome P450 *CYP707A* gene family (*CYP707A1, CYP707A2, CYP707A3, CYP707A4*) encodes ABA 8'-hydroxylases which play a predominant role in ABA catabolism by converting ABA into the inactive compound phaseic acid (PA) (KUSHIRO ET AL., 2004; SAITO ET AL., 2004; OKAMOTO ET AL., 2006; SEO ET AL., 2006). The *cyp707a2* mutant exhibits hyperdormancy in seeds and accumulates a high ABA content, indicating that the *CYP707A* gene family is a major regulator of the level of ABA in plants.

The mechanism of GA signalling is now well established. It was shown by the analysis of mutants with defects in GA signal transduction that GA stimulates seed germination by triggering destruction of DELLA family proteins (GIBBERELLIN INSENSITIVE, GAI; REPRESSOR OF THE *ga1-3* MUTANT 1, RGA1; RGA-LIKE 1-3, RGL1-3). DELLA proteins are negative regulators of GA responses and RGL2 is the main DELLA protein repressing seed germination (PENG ET AL., 1997; LEE ET AL., 2002; SUN AND GUBLER, 2004; ARIIZUMI AND STEBER, 2007). In response to GA, DELLAs are rapidly degraded by the 26S proteosome. This is caused by the interaction of GID1 (GA-INSENSITIVE DWARF1) proteins with the F box protein SLY1, which is required for DELLA ubiquitination and subsequent degradation (STEBER, 2007; FINKELSTEIN ET AL., 2008). The *sleepy1* (*sly1*) mutant was selected in a screen for suppressors of the ABA insensitive *abi1-1* mutant. The non-germinating phenotype of *sly1* cannot be rescued by GA, indicating that *SLY1* is involved in GA perception (STEBER AT AL., 1998; MCGINNIS ET AL., 2003; STEBER, 2007; FINKELSTEIN ET AL., 2008).

Brassinosteroids (BRs), a group of plant steroid hormones, are also involved in the control of seed germination in Arabidopsis. STEBER AND MCCOURT (2001) have suggested that BRs are required to reverse ABA dependant inhibition of germination. The BR mutants *de-etiolated2* (*det2*) and *brassinosteroid-insensitive1* (*bri1)* show decreased germination, but are able to germinate without BR. This indicates that in contrast to GA, brassinosteroids are not absolutely required for germination.

The *ethylene insensitive2* (*ein2*) mutant, which was isolated as a suppressor of the *abi1* mutant, and the *ethylene response* (*etr*) mutants are hypersensitive to ABA and need a longer period of

after-ripening to germinate. The non-dormant phenotype of the double mutant *ein2 abi3-4* indicates that ethylene suppresses seed dormancy by inhibiting ABA action (BEAUDOIN ET AL., 2000; GHASSEMIAN ET AL.; 2000).

Seed coat or testa mutants

In Arabidopsis the seed coat defines a major germination constraint (Chapter 1.3.1, Figure 1.2). This has been confirmed by the isolation and identification of testa mutants, which show a reduced dormancy phenotype. Seed coat mutants consist of two major groups. One group, including the *transparent testa* (*tt*) and the *transparent testa glabra* (*ttg*) mutants, is affected in flavonoid pigmentation with seed colors ranging from yellow to pale brown. Mutants of the second group are affected in the testa structure. For example, *aberrant testa shape* (*ats*) mutants have only one integument, because they have lost the control of the differentiation between the inner and outer integuments (MESSNER MCABEE ET AL., 2006).

Mutants affected in response to light

Phytochrome plays a role in light-induced stimulation of seed germination. Therefore phytochrome-deficient mutants are affected in seed germination. Mutants lacking phytochrome B (*phyB*) are non-germinating and show a reduced sensitivity to red light, indicating that *PhyB* has a primary role in seed germination. Mutants lacking *PhyA* can only germinate after a prolonged imbibition phase, indicating that *PhyA* is only involved secondarily (SHINOMURA ET AL.; 1994). The *long hypocotyl* (*hy1, hy2*) mutants also show germination defects which are caused by aberrant photomorphogenesis due to defects in the chromophore leading to non-functional phytochromes (PARKS AND QUAIL, 1991; HUDSON, 2000). Analysis of the *pif3-like 5* (*pil5*) mutant in conjunction with *phyA* and *phyB* mutants demonstrated that PIL5, a bHLH transcription factor, interacts with the Pfr forms of PhyA and PhyB (OH ET AL., 2004). Red and far-red light signals lead to the degradation of PIL5 by the proteosome. In addition, PIL5 represses GA biosynthesis genes such as *GA3ox1* and *GA3ox2* and activates the GA catabolic *GA2ox* gene and genes encoding DELLA proteins (OH ET AL. 2006).

Miscellaneous dormancy mutants

Some mutants were directly selected for a reduced dormant phenotype (LÉON-KLOOSTERZIEL ET AL., 1996b; PEETERS ET AL., 2002). However, these mutant screens have been hampered by the low dormancy of standard lab strains such as Landsberg *erecta* (L*er*) and Columbia (Col), which reduces the likeliness of saturation of mutations in dormancy genes. Therefore, only a few mutants with reduced dormancy (*reduced dormancy 1-4*, *rdo1, rdo2, rdo3, rdo4*) have been

identified in L*er*. These mutants also show mild pleiotropic phenotypes. Recently *RDO4/HUB1* (*HISTONE MONOUBIQUITINATION 1*) was cloned (LIU ET AL.; 2007) and was shown to be involved in histone H2B monoubiquitination, revealing a role for chromatin remodeling in seed dormancy. *HUB2*, which is a homolog of *HUB1*, is also involved in this process and *hub2* mutants show a similar reduced dormancy phenotype.

Another mutant with reduced dormancy is the *Dof affecting germination* (*dag1*) mutant, which encodes a Dof (DNA-binding with one finger) transcription factor. In contrast to the *rdo* mutants, this phenotype is determined by the maternal genotype. *DAG1* may influence the import of compounds from the mother plant into the seed (PAPI ET AL., 2000). *DAG2* was isolated due to its similar expression pattern to *DAG1*, but the seed germination characteristics of the *dag2* mutants are opposite to those of *dag1* and show increased dormancy (GUALBERTI ET AL., 2002).

Mutations in the *COMATOSE* gene (*CTS*) lead also to a strong reduction in the germination potential and *cts* mutants are not able to respond to GA. *CTS* was also shown to affect the metabolism of stored lipids. *CTS* increases the germination potential and represses embryo dormancy and therefore acts as a major control point for the switch between the opposing developmental programmes of dormancy and germination. (RUSSEL ET AL., 2000; FOOTITT ET AL., 2002; PENFIELD ET AL., 2007; HOLDSWORTH ET AL., 2008).

A mutant that was selected based on a completely non-dormant phenotype, is the *dog1 (delay of germination 1)* mutant. To date, this is the only mutant that lacks seed dormancy and that has no obvious pleiotropic phenotype (Chapter 1.4) (BENTSINK, 2002).

1.3.3.2 Genetic analysis of natural variation for seed dormancy

Natural variation provides an additional genetic resource for identifying genes that are involved in the control of seed dormancy. Seed dormancy and germination are typical quantitative traits because they are highly influenced by environmental effects and controlled by many genes. Quantitative trait locus (QTL) analysis for seed dormancy was performed using recombinant inbred line (RIL) populations derived from crosses of the low dormant laboratory accession L*er* to accessions with different levels of seed dormancy (VAN DER SCHAAR ET AL., 1997; ALONSO-BLANCO ET AL., 2003; CLERKX ET AL. 2004; BENTSINK ET AL., IN PREP.). This revealed more than 12 regions with seed dormancy QTL, which were called *DELAY OF GERMINATION* (*DOG*). Some QTL were detected only in crosses between low dormant accessions, whereas others were

identified as major QTL in the progeny of crosses between accessions with low and high dormancy.

The differences of dormancy levels between Arabidopsis accessions are analyzed by characterizing their germination phenotypes during after-ripening. The low dormant accession L*er* germinates 100% after three to six weeks of storage, whereas the strong dormant accession Cvi requires 15-25 weeks, depending on the maternal growth conditions. The near isogenic line NIL *DOG1*, which carries the Cvi allele of the dormancy QTL *DOG1* in L*er* background, shows an increased dormancy level compared to L*er* and reaches 100% germination only after 12 weeks. The loss of function allele of *DOG1* (*dog1*) shows a completely non-dormant phenotype (Figure 1.4). The dormancy QTL mapped in the RIL population of L*er* and Cvi (*DOG1-7*) are shown in Figure 1.3. Many of them do not colocate with known dormancy loci. Therefore cloning of the genes underlying the QTL might lead to the identification of novel genes involved in the regulation of seed dormancy and germination.

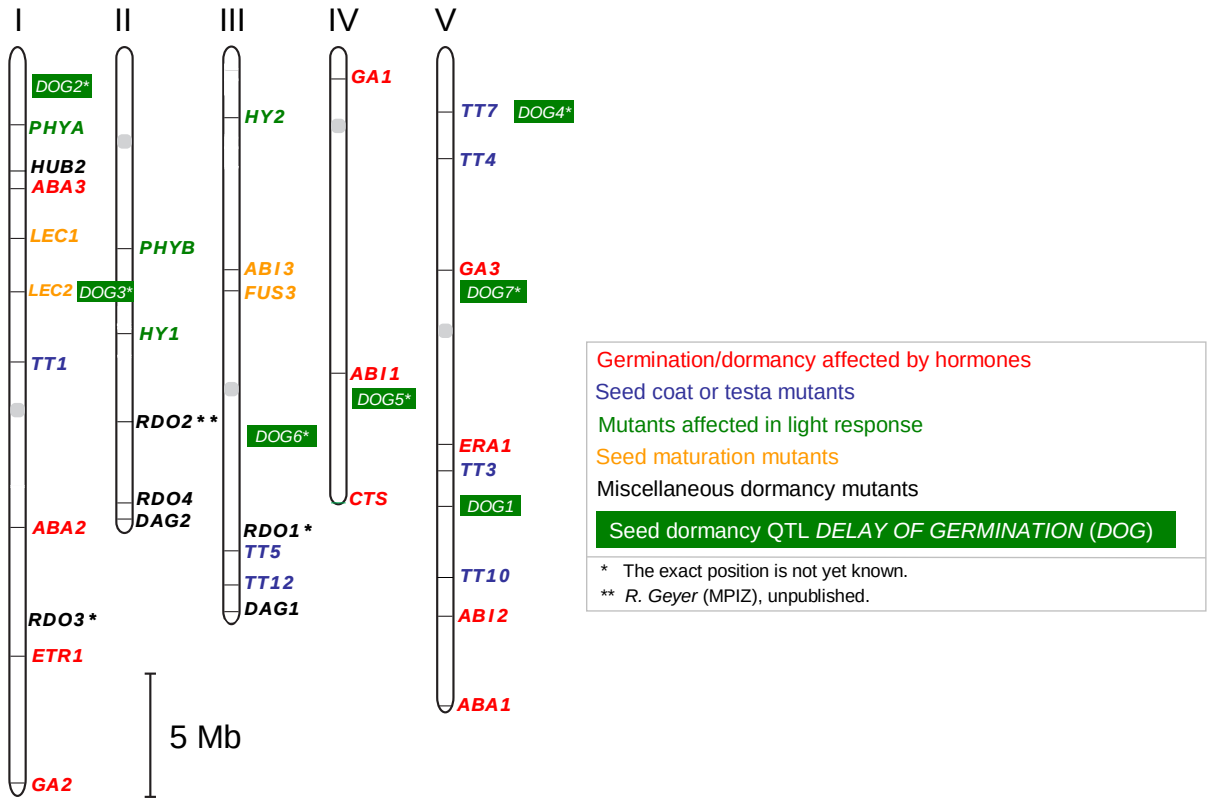

Figure 1.3: Arabidopsis physical map of seed dormancy and germination loci.
Mutants are divided in groups, which are shown in different colors as indicated in the legend. The seed dormancy QTL *DELAY OF GERMINATION* (*DOG*) were mapped in L*er*/Cvi RILs (ALONSO-BLANKO ET AL., 2003) and are shown in green blocks. The physical position of cloned genes is marked with a horizontal line. Genes, whose position is not yet known, are indicated with (*). (**) indicates the recently cloned gene *RDO2* (R. Geyer, unpublished). (Adapted from BENTSINK ET AL., 2007)

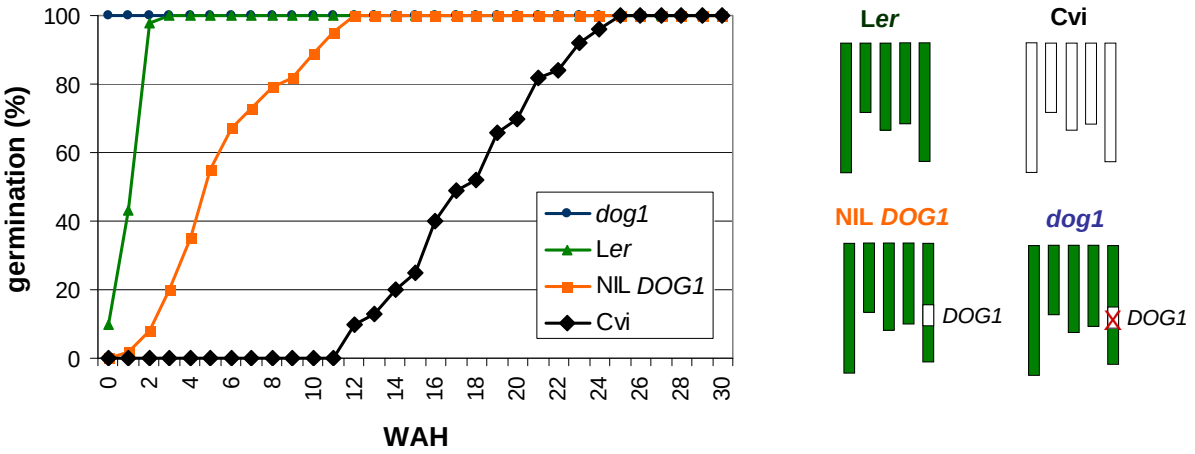

Figure 1.4: Germination phenotype of the Arabidopsis accessions L*er*, Cvi, the near isogenic line NIL *DOG1* and the *dog1* mutant at different timepoints after seed harvest.
The genotypes of L*er*, Cvi, NIL *DOG1* and the loss of function mutant *dog1* are illustrated on the right side of the graph. WAH, weeks after harvest.

1.4 The seed dormancy gene *DOG1*

DOG1 is the first seed dormancy specific gene cloned by exploiting natural genetic variation in dormancy. *DOG1* was shown to be the major locus that determines the genetic variation for seed dormancy between the accessions L*er* and Cvi. *DOG1*, for which the Cvi allele increased the level of dormancy (Figure 1.4), explained 12% of the variance observed in the recombinant inbred line population of L*er* and Cvi. The underlying gene of the *DOG1* QTL was identified and cloned by high-resolution mapping of the QTL and by identification of a loss-of-function allele (*dog1-1*) which resulted in loss of dormancy (BENTSINK ET AL., 2006). This indicates that *DOG1* is absolutely required for the induction and maintenance of seed dormancy.

GA requirement and ABA sensitivity of the different *DOG1* alleles

It was shown that *dog1-1* mutant seeds still require light-induced GA-biosynthesis to overcome inhibition by ABA for germination, but to a lesser degree compared to L*er* and NIL *DOG1*. In addition, *dog1-1* mutants showed normal sensitivity to ABA, which indicates that *DOG1* functions independent from the ABA signal transduction pathway. Combining NIL *DOG1* with *aba1-1* resulted in a non-dormant phenotype. This suggests that ABA is required for achieving dormancy. (BENTSINK ET AL., 2006)

DOG1 is exclusively expressed in seeds

Expression analysis revealed that *DOG1* shows a seed-specific expression pattern, unlike other genes involved in dormancy, which have a general role in the plant and show a broader expression pattern during development (Figure 1.5, A). *DOG1* is highly expressed during the

maturation phase (Figure 1.5, B), whereas the expression disappears during imbibition. This suggests that *DOG1* plays an important role during seed maturation (BENTSINK ET AL., 2006). *DOG1* expression has also been shown to be higher in NIL *DOG1*, which is carrying a Cvi introgression of *DOG1* in L*er* background, compared to L*er*. There was no detectable *DOG1* expression in *dog1-1* (Figure 1.5, *C*). This demonstrates that the level of *DOG1* expression correlates with the degree of dormancy.

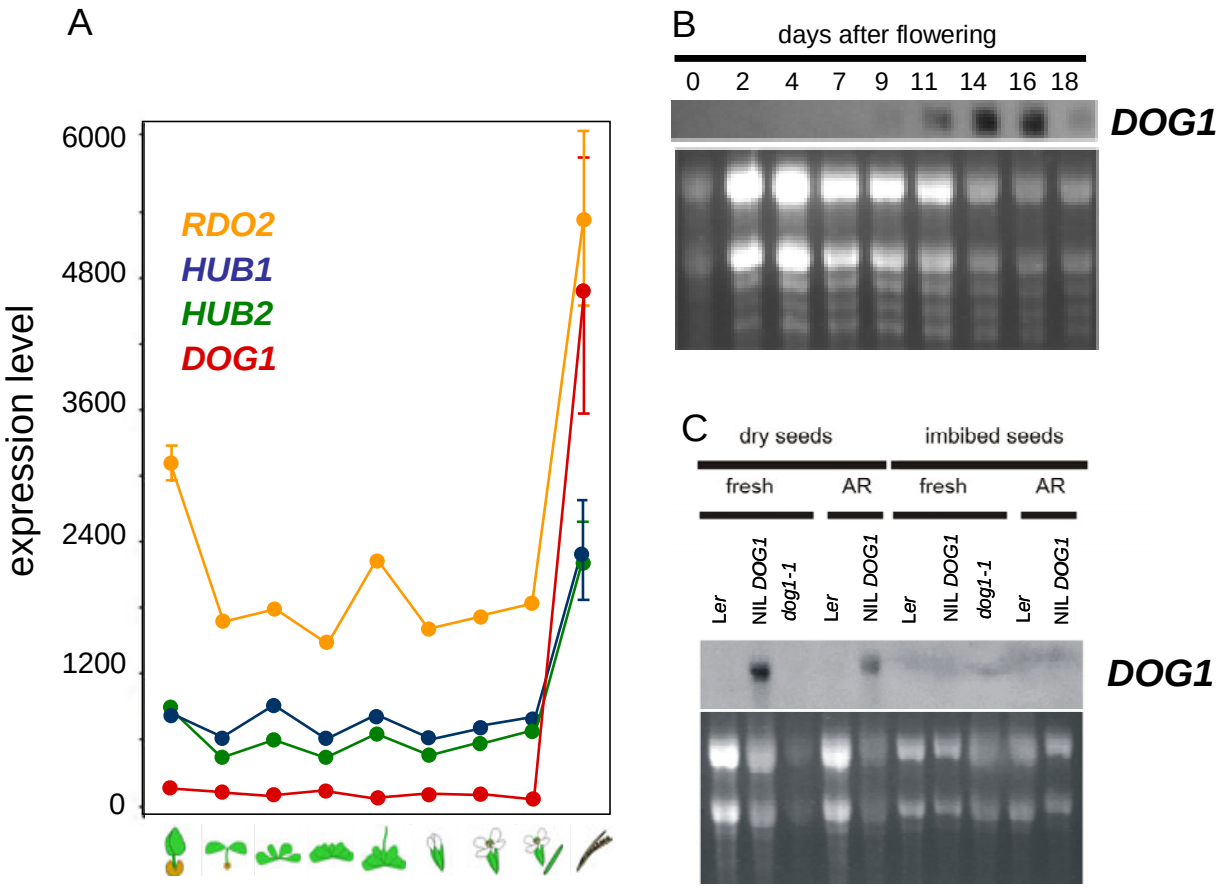

Figure 1.5: Expression profile of *DOG1*.
(A) *DOG1* expression in comparison to seed dormancy genes (*RDO2*, *HUB1* and *HUB2*), which are not exclusively expressed in seeds (www.genevestigator.ethz.ch), (B) Northern blot analysis of *DOG1* expression in developing siliques of NIL *DOG1* during seed maturation (BENTSINK ET AL., 2006), (C) Northern Blot analysis of *DOG1* expression in fresh, dry and imbibed seeds of L*er*, NIL *DOG1* and *dog1* (L. Bentsink, unpublished). (B+C) Upper: Autoradiograph, lower: RNA loading stained with ethidium bromide. Blot was probed with *DOG1* fragment.

DOG1 is alternatively spliced

Molecular cloning and sequence analysis of cDNAs encoding *DOG1* revealed that *DOG1* is alternatively spliced. The gene structure of *DOG1* consists of three exons and two introns. By the use of alternative 5' donor and 3' acceptor sites in the second intron, a set of four different transcripts is generated (α, β, γ, δ), which encode for three protein isoforms, because the β and γ transcripts both carry a stop-codon at the same position. The *dog1-1* mutant has a 1 bp deletion

in the second exon, which causes a frameshift and an early stop-codon (Figure 1.6, Appendix A 10). It is not yet known, whether all of the transcripts are translated into peptides. (BENTSINK ET AL., 2006)

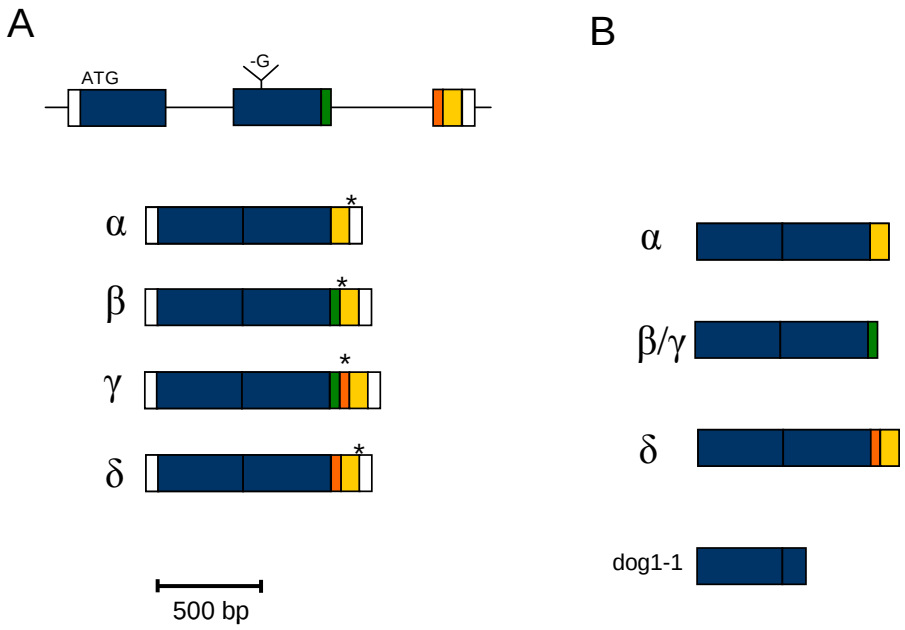

Figure 1.6: Schematic representation of *DOG1* gene structure, transcripts from alternative splicing and corresponding protein variants.
(A) Structure of the *DOG1* gene and position of the *dog1-1* mutation (top) and the four different splicing forms (α to δ). The position of the mutation in the *dog1-1* mutant (-G) is indicated on top of the genomic structure, the position of the stop-codon located in the different slicing forms is indicated with *. The white boxes indicate the 5' and 3' UTR. (B) Structure of the three protein isoforms resulting from alternative splicing and the potential truncated dog1-1 mutant protein.

Sequence and expression diversity of *DOG1* in different accessions of *Arabidopsis thaliana*

To analyze the allelic differences of *DOG1*, several accessions were sequenced. The sequence polymorphisms in the coding regions of *DOG1* alleles did not correlate with the dormancy level of the accessions. In addition, there were several polymorphisms, insertions and deletions (indels) found in the 5' upstream cis-regulatory region. Analysis of the *DOG1* expression diversity of accessions with distinct promoter alleles indicated that the variation in dormancy phenotypes might be caused by functional variation in the *DOG1* cis-regulatory region. However, the polymorphisms in the cis-regulatory region that are responsible for the variation in *DOG1* expression have not yet been determined (BENTSINK ET AL., 2006).

DOG1 encodes a protein with unknown function

DOG1 is encoded by the gene At5g45830 and is a member of a novel plant-specific gene family with presently unknown molecular functions. Based on the gene structure, five additional genes in the Arabidopsis genome show high similarity to *DOG1*, called *DOG1-Like 1-5* (*DOGL1-5*)

(Table 1.1, A). T-DNA insertion lines that are presumed to be null-mutants of *DOGL1-4* did not reveal dormancy phenotypes (BENTSINK ET AL., 2006), but it is possible that they have redundant functions. The DOG1 protein consists of three highly conserved protein domains of unknown function, which are in part also present in *DOGL1-5* (Table 1.1, B). Three potential *DOG1* homologues were identified in different *Brassicaceae* species, *Lepidium sativum*, *Brassica rapa* subspecies *pekinensis* and *Brassica napus* (Table 1.1, C; Figure 1.7).

The putative *DOG1* promoter region (2 349 bp upstream of the ATG) contains seed-specific motifs such as an RY repeat (CATGCA), which is required for seed-specific expression and two ABRE motifs (TACGTGTC) that are known to be involved in ABA response. This agrees with the seed-specific expression pattern. Because the *DOG1* sequence does not show any domains with known function, no conclusions can be drawn from *in silico* analysis. Therefore, functional information can only be gained experimentally.

Table 1.1: Sequence similarities of *DOG1* gene family members and potential homologues of *DOG1* in other species.

A	Plant-specific gene family	Similarity with *DOG1*		Annotation	AGI code
	DOG1	DNA	AA	unknown protein	At5g45830
	DOG1-like 1	59%	52%	unknown protein	At4g18660
	DOG1-like 2	52%	40%	hypothetical protein	At4g18680
	DOG1-like 3	52%	49%	hypothetical protein	At4g18690
	DOG1-like 4	46%	22%	transcription factor-related protein	At4g18650
	DOG1-like 5	44%	23%	DNA binding protein-related	At3g14880
B	**Three conserved protein domains in DOG1***	**Also present in**		**Annotation**	**Accession number**
	PD870616	*A. thaliana*		DOG1L1-5, unkown function	as mentioned above
	PD004114	*A. thaliana*		D bZIP TF, but DOG1 shows only homology between annotated regions	ABI34670.1
	PD388003	*A. thaliana*		DOGL5, unknown function	as mentioned above
		Nicotiana glauca x Nicotiana langsdorffii		tumor related protein like, unknown function (31% global identity)	BAA05470.1
		Oryza sativa		unnamed protein product (22% global identity)	BAB08196
C	**Potential *DOG1* homologues in other species**	**Local identity (%)**		**Annotation**	**Accession number**
	*Lepidium sativum***	93% (165 bp)		not annotated	not public
	Brassica rapa subsp. *pekinensis*	81% (393 bp)		not annotated, BAC clone	AC189537
	Brassica napus EST	82% (394 bp)		not annotated, cDNA clone from embryo library	CN827162

* Defined by ProDom (http://prodom.prabi.fr/prodom/current/html/home.php).
** Partial cDNA, Leubner-Metzger, unpublished.

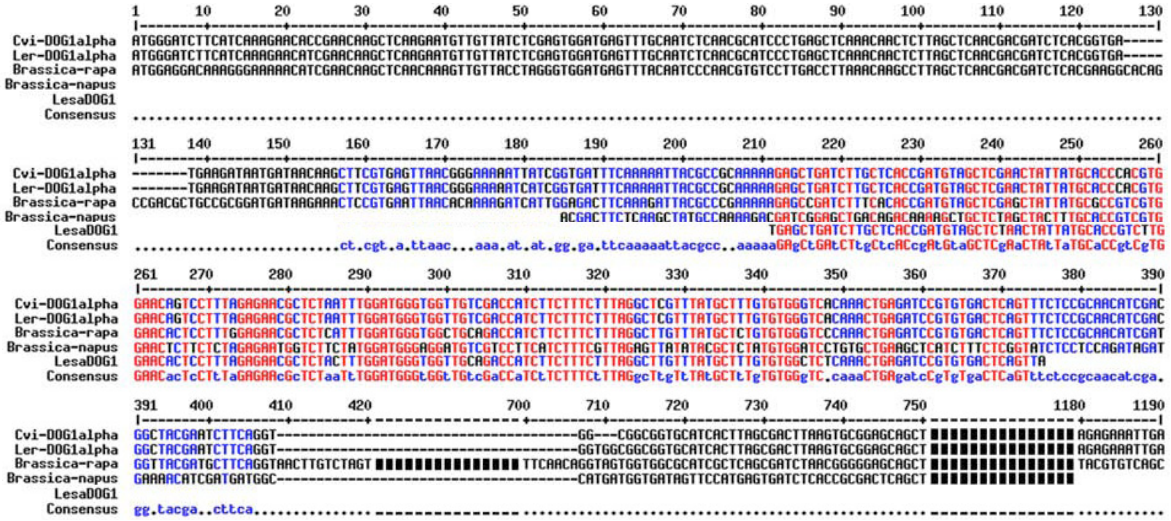

Figure 1.7: Potential *DOG1* homologues in *Brassica rapa*, *Brassica napus* and *Lepidium sativum*.
The two *DOG1*alpha cDNAs represent the splicing variants of the *A. thaliana* accessions Cvi and L*er* (BENTSINK ET AL. 2006). The two Brassica sequences are a *B. rapa* genomic DNA (AC189537, NCBI database) and a *B. napus* EST (CN827162, NCBI EST database). *LesaDOG1* is a 165 bp partial cDNA of *L. sativum* that was cloned by RT-PCR from seed RNA (Leubner-Metzger, unpublished). The sequences of the alpha *DOG1* splicing variant from Cvi and L*er* are presented from the 5' to the 3' ends; black boxes and dashed lines mark sequence omissions; red and blue color indicate homologous nucleotides, black color indicates sequence polymorphisms.

1.5 Alternative splicing in plants

Alternative RNA splicing of pre-mRNAs is a powerful posttranscriptional regulatory mechanism, which is employed by all eukaryotes. It can affect quantitative control of gene expression and contribute to transcriptome and proteome diversity. Through the selection of alternative splicing sites in the pre-mRNA of a single gene, multiple mRNAs can be generated that encode structurally and functionally distinct protein isoforms (KRIVENTSEVA ET AL., 2003). Differentially spliced RNAs can be generated by following various patterns including exon skipping and intron retention, but very often by the use of alternative 5’ donor sites or 3’ acceptor sites (KAZAN, 2003; LAREAU ET AL., 2004; WANG AND BRENDEL, 2006; LOPEZ, 1998).

Alternative splicing is commonly detected by two different approaches: on a genome-wide scale by aligning ESTs to the genome or to each other and the subsequent analysis of structural diversity, or on a locus-specific level by RT-PCR of a particular gene and looking for alternate isoforms. Genome wide comparative analysis of alternative splicing among different eukaryotes showed that 20% of Arabidopsis genes undergo alternative splicing, which is a lower fraction of genes compared to mouse and human. Exon-skipping is the least prevalent type of alternative splicing in *A. thaliana*, much less than in other organisms (Figure 1.8) (KIM ET AL., 2006). The lower fraction of alternatively spliced genes in Arabidopsis might in part be explained by the lower EST/cDNA coverage to date. WANG AND BRENDEL (2006) showed that based on

EST/cDNA aligments, most of the identified alternatively spliced genes in Arabidopsis (67%) have only two isoforms and that the frame of translation is altered in 62% of the alternative splicing isoforms. In 42% of these frame shifted alternative splicing forms, a premature stop-codon was introduced (>50 bp of upstream of the last exon-exon junction), which makes them possible candidates for non-sense-mediated decay.

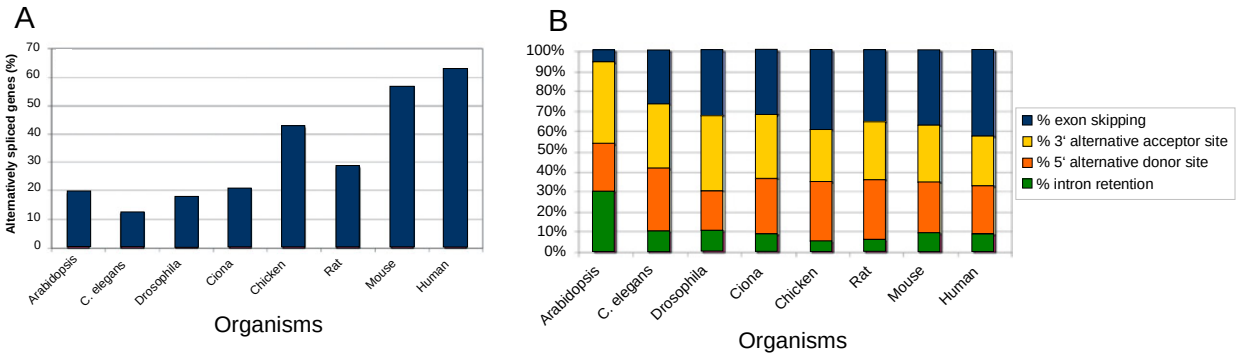

Figure 1.8: Alternative splicing among different eukaryotes.
(A) Percentage of alternative spliced genes among eight different organisms. Percentages were calculated based on the analysis of UniGene clusters using all reliable data available, (B) relative prevalence of the different types of alternative splicing in different species. (KIM ET AL., 2006)

Alternative splicing can cause the deletion or modification of protein activity and can affect RNA stability and translational efficiency (SMITH ET AL., 1989). It can be constitutive, leading to stable ratios of transcript variants, or dynamic and tightly regulated in response to developmental, physiological and biochemical cues (LOPEZ, 1998). To date, differentially spliced transcripts have been experimentally detected in a small number of plant genes, including genes involved in transcription, flowering regulation, disease resistance, enzyme activities and even in splicing itself (KAZAN, 2003). Many splice variants are produced in an organ-specific manner (LAZARESCU ET AL., 2006; HRADILOVA AND BRZOBOHATY, 2007;), show differential subcellular localizations (KOO ET AL., 2007) or are dependent on the developmental stage or on environmental conditions such as light (MANO ET AL., 2000), temperature (SABLOWSKI AND MEYEROWITZ, 1998; LAZAR AND GOODANN 2000) or environmental stresses (MARRS AND WALBOT, 1997; TANABE ET AL., 2006).

These recent computational and experimental studies indicate that alternative splicing emerges as a major regulatory mechanism. But it is still largely unknown, how this process itself is regulated and how it is involved in signal transduction pathways. Although the number of recent examples of alternatively spliced genes is increasing, it is still a major challenge to study the functional relevance and the distinct molecular and biochemical functions of alternative splicing variants.

The present study was focused on the molecular and functional characterization of *DOG1*, a key gene involved in the regulation of seed dormancy and a newly-discovered example of an alternatively spliced gene (Figure 1.6). Therefore one focus was to study the functional relevance of the alternative splicing of *DOG1* and how alternative splicing is linked with seed dormancy.

1.6 Objectives of the thesis

The work described in this thesis aimed to contribute to a better understanding of the molecular mechanisms of seed dormancy in *Arabidopsis thaliana*. In particular, the taken approaches focused on the selection of novel mutants with a role in seed dormancy and on the study of the recently identified dormancy specific gene *DOG1*.

Identification of novel seed dormancy mutants

Because the number of genes known to affect the release of seed dormancy is still limited, this project intended to identify additional genes, using a mutant approach with strongly dormant accessions. This has not been done before and represents a promising strategy to enlarge the number of dormancy mutants and thus underlying genes. Former mutagenesis screens were not saturating due to the use of low dormant accessions. The main aim was to identify and characterize mutants that fail to germinate under dormancy breaking conditions such as stratification and after-ripening, as well as to screen for novel non-dormant mutants.

Molecular and biochemical characterization of the dormancy gene *DOG1*

To make progress in understanding the mechanisms of seed dormancy it is important to unravel unknown players and regulatory pathways, in particular key genes that are specific for dormancy. The recently identified gene *DOG1* is such a gene. It was shown to be specific and essential for dormancy and it is not directly involved in the hormonal regulation of seed dormancy, rather its function is still unknown.

This thesis aimed to characterize the regulation of the *DOG1* gene and protein and to uncover its molecular function by using molecular biological and biochemical approaches, including expression analysis, protein analysis and production and characterization of transgenic plants. *DOG1*, also newly-discovered to be alternatively spliced, allowed for the study of the functional relevance of alternative splicing of *DOG1* and its contribution to the regulation of seed dormancy.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and antibiotics

The chemicals and antibiotics (Table 2.1) were purchased from the following suppliers: Merck (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), Carl Roth (Karlsruhe, Germany), Difco Laboratories (Detroit/Michigan, USA), Duchefa (Haarlem, Netherlands) and Sigma (Deisenhofen, Germany).

Table 2.1: Antibiotics.

Antibiotics	Stock Conc. (mg/ml)	Solvent	Final Conc. for selection on LB or YEB medium (mg/l)	
			E. coli	*A. tumefaciens*
Ampicillin (Amp)	100	H_2O	100	-
Carbenicillin (Carb)	50	Ethanol	-	50
Chloramphenicol (Cam)	50	Ethanol	25	-
Gentamycin (Gent)	10	H_2O	10	10
Hygromycin (Hyg)	50	H_2O	50	50
Kanamycin (Kan)	50	H_2O	50	25
Rifampicin (Rif)	50	DMSO	-	50
Spectinomycin (Spec)	100	H_2O	100	100
Tetracyclin (Tet)	10	H_2O	10	10

2.1.2 Buffers and Culture Media

Stock solutions of the following buffers and culture media were prepared as described by SAMBROOK AND RUSSELL (2001): BSA, DNA loading buffer, PBS, PCR Buffer, SDS, TAE, LB, YEB and TB medium. All media, buffers and aqueous solutions were made with highly purified Milli-Q-water (Millipore, Bedford, USA). Whenever required, the solutions were autoclaved for 20 min at 121°C.

2.1.3 Restriction enzymes, nucleic acid modifying enzymes and kits

Restriction enzymes were used from New England BioLabs® (Schwalbach/Taunus, Germany), and Roche (Mannheim, Germany). 10x Buffers for restriction enzymes were supplied by manufacturers.

Nucleic acid modifying enzymes were used from:

Klenow fragment exo- (MBI Fermentas, St. Leon-Roth, Germany)
Lysozyme (Roche, Mannheim, Germany)
Platinum®*Pfx* DNA-Polymerase (Invitrogen, Karlsruhe, Germany)
Ribonuclease Inhibitor (Roche, Mannheim, Germany)

RNase A (DNase-free) (Qiagen, Hilden, Germany)
RNase H (Promega, Mannheim, Germany)
SuperScript™ II reverse transcriptase (Invitrogen, Karlsruhe, Germany)
Taq DNA Polymerase (Roche, Mannheim, Germany)
T4 DNA ligase (Invitrogen, Karlsruhe, Germany)

The following commercial reagents and kits were used:

Bio-Safe™ Coomassie G-250 stain (BIORAD, Hercules, USA)
BP-Clonase (Invitrogen, Heidelberg, Germany)
First Strand cDNA Synthesis Kit (Invitrogen, Karlsruhe, Germany)
High Pure PCR Product Purification Kit (Roche, Mannheim, Germany)
HisTrap FF crude Kit (GE Healthcare, Freiburg, Germany)
LR-Clonase (Invitrogen, Karlsruhe, Germany)
MAXIscript® In vitro transcription kit (Ambion, Cambridgeshire, UK)
Miniprep® Kit (Qiagen, Hilden, Germany)
pENTR™ Directional TOPO Cloning Kit (Invitrogen, Karlsruhe, Germany)
Protein assay (BIORAD, Hercules, USA)
RealMasterMix SYBR ROX (5Prime, Hamburg, Germany)
RNAqueous RNA extraction Kit and plant RNA isolation aid (Ambion, Cambridgeshire, UK)
RNeasy Plant Mini® Kit (Qiagen, Hilden, Germany)
Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen, Karlsruhe, Germany)

2.1.4 Oligonucleotides

Synthetic oligonucleotides were purchased from Invitrogen (Karlsruhe, Germany). The primers that were used in this study are shown in tables 2.2 to 2.5.

Table 2.2: Primers used for molecular cloning of PCR products (classical and gateway cloning).

Name	Primer sequence in 5' to 3' orientation
ABA1_3-SpeI	CCCCACTAGTTTATCGGTCACATAGGTTCCGTGT
ABA1_5-ApaI	CCCCGGGCCCATGGGTTCAACTCCGTTTTGCTAC
ABA1_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTTCAACTCCGTTTTGCTAC
ABA1_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAAGCTGTCTGAAGTAATTTATC
antF_DOG1	AACATATGGGATCTTCATCAAAGAA
antR_DOG1	AACTCGAGCACATTGATTTTAGCTAGCTGCT
DOG1_3'SpeI	CCCCACTAGTTCAATTTCTCTCATTATTTGTCGTCT
DOG1_5'ApaI	CCCCGGGCCCATGGGATCTTCATCAAAGAACATC
DOG1_5'HindIII	CCCCAAGCTTATGGGATCTTCATCAAAGAAC
DOG1beta-withoutSTOP_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTTTCCTTCCTCTCCTCCGGCATT
DOG1delta_withoutSTOP_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCATTTCTCTCATTATTTGTCGTCTC
DOG1F_TOPO	CACCATGGGATCTTCATCAAAGAACATCG
DOG1-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGATCTTCATCAAAGAACATC
DOG1-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCCACGTAAACACACAAATC
DOG1prom_F_BspHI	CCCCTCATGACCATGAACAAGAACGATTCTCTCTCCT
DOG1prom_R_BamHI	CCCCGGATCCGATCTCTTTTGGTTTGCGTGTTTG
DOG1prom_GW_F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAACAAGAACGATTCTC
DOG1prom_GW_R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGATCTCTTTTGGTTTGCGTGTTTG
ER-GFP_F_BspHI	CCCCTCATGAAGATATAACAATGAAGACTAATCTTTTTC
ER-GFP_R_BamHI	CCCCGGATCCTTAAAGTTCATCTTTGTATAGTTCATCC
GFP_3'SpeI	CCCCACTAGTTTACTTGTACAGCTCGTCCATGCC
GFP_R_gw	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTTGTACAGCTCGTCCATGCC
strepII_3'SpeI	CCCCACTAGTTTATTTTTCAAATTGAGGATGAGACC
strepII_R_GW	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATTTTTCAAATTGAGGATGAGACC

Table 2.3: Primers used for colony-PCR as well as for sequencing of constructs and PCR products.

Name	Primer sequence in 5' to 3' orientation
35S-promoter	CAATCCCACTATCCTTCGC
ABA_F2	CCATGCAATGGCGAGGATGG
ABA_R2	TTCATACCATTTGGAGCATCAG
ABA1_F1	TTATACCAGCGGATATCGAGTC
ABA1_R1	CCATCAACGAGACCGTTAATC
Basta_R	CAGTCGTAGGCGTTGCGTGCCTTC
DOG1_1R	CTTGTACCGGAGATAGAATC
DOG1_3'UTR	CTCCTAGCTGACTTGTCGAGACGAG
DOG1_F1	TAGAGAACGCTCTAATTTGG
DOG1F_exon2	GACCAAGAAAGTCTCAAGCCTAC
DOG1promoter_1F	ACCATGAACAAGAACGATTC
DOG1promoter_2F	GATCACCACCACTACTATAC
DOG1promoter_3F	GTGTCGAACTATCCTCATAC
DOG1promoter_4F	GTACAATCCGCTGTCTCAGGACATC
DOG1promoter_5F	GGAACAACAACTCGCACTCTC
DOG1promoter_6F	GACATTTGTCATTGTTTCCC
DOG1promoter_F	GGCCACCTATCCGGTCATATATC
DOG1promoter_R	GGCCACCTATCCGGTCATATATC
F1-GW_seq	ATACACAGCCAGTCTGCAGGTCG
GFP_R	TTATTTGTATAGTTCATCCATGCC
GUS_R	CAGGTGTTCGGCGTGGTGTAGAG
gwR1-seq	GCCGCCATAGTGACTGGATATG
M13_F	GTAAAACGACGGCCAG
M13_R	CAGGAAACAGCTATGAC
nosTerm_R	GCAAGACCGGCAACAGGATT
pat_F	GCTTCAAGAGCGTGGTCGCTGTC
pat_R	GAAGTTGACCGTGCTTGTCTCG
pGreen_RB_R1	CAATACGCAAACCGCCTCTCC
psoup_F	CTATCGACTACGCGATCATGGC
psoup_R	CTCCCAATCAGGCTTGATCC
R1-ER-GFP-Seq	TCCCTCAGGCATGGCGCTC
SelA	TCGCGTTAACGCTAGCATGGATCTC
SelB	GTAACATCAGAGATTTTGAGACAC
smGFP_R	CCCCTTATTTGTATAGTTCATCCATGCC
strepII_R	TTATTTTTCAAATTGAGGATGAGACCAACC
T35S_R	TCTGGGAACTACTCACAC

Table 2.4: Primers used for quantification of real-time PCR products in SYBR® Green based detection assays.

Name	Primer sequence in 5' to 3' orientation
Actin8_F	CTCAGGTATTGCAGACCGTATGAG
Actin8_R	CTGGACCTGCTTCATCATACTCTG
alpha_short	CCACTATTCACAGTTGTACATGCATCGAATATTACTTC
beta_short	CCACTATTCACAGTTGTACATGCATCGAATATTACTATAG
beta_transgene	CTTTCCTTCCTCTCCTCCGGCATTACCTTC
delta_short	CGCAAAATGCCACGACGTGAATAAACTTC
exon2_qRT_F	GGATTCTATCTCCGGTACAAGGAGCGGATTTC
gamma_short	CGCAAAATGCCACGACGTGAATAAACTATAG

Table 2.5: CAPS marker used for detection of *dog1-1* specific mutation.
The primer combination produces a 220 bp product in wt and a 193 bp product in *dog1-1* on digestion with SfuI.

Name	Primer sequence in 5' to 3' orientation
dog1 sfu_F	TCCCATCGCCACTGTGGCTTACGAGTTCGA
dog1 sfu_R	CTCATGCATCGAAAGATGAAG C

Table 2.6: Primer used for synthesis of antisense probes for RNA in-situ hybridization by *in vitro* transcription.

Name	Primer sequence in 5' to 3' orientation
DOG1_F_in situ	GAGCTGATCTTGCTCACCGATGTAG
DOG1_R_in situ_T7	CCCCTAATACGACTCACTATAGGGAGAGGCTCTCCGACATTCTCCATCTCGTAAG

2.1.5 Plasmids

Plasmids used for the generation of constructs described in this thesis are listed below.

Table 2.7: List of vectors used for molecular cloning.

Vector	Supplied / provided by	Resistance
pAM-PAT 35S GW GFP Terminator	Sandra Noir, Ralf Panstruga (MPIZ, Cologne, Germany)	amp^R
pBAT B GFP	Klaus Richter, Joachim Uhrig (MPIZ, Cologne, Germany)	$spec^R$
pBAT-B GW ER-GFP	Melanie Schwab, cloned during this work	$spec^R$
pCR® -BluntII-TOPO®	Invitrogen (Heidelberg, Germany)	kan^R
pDONR™ 201	Invitrogen (Heidelberg, Germany)	kan^R
pENTR™ /D-TOPO®	Invitrogen (Heidelberg, Germany)	amp^R, kan^R
pET-21a(+)	Novagen (Madison, USA)	amp^R
pGJ1029	Guido Jach (MPIZ, Cologne, Germany)	amp^R
pGreen DOG1promoterCvi GW	Melanie Schwab, cloned during this work	kan^R
pGreen GW GUS	Franziska Turck (MPIZ, Cologne, Germany)	kan^R
pGreen GW mcs	Hailong An, Hugo Konijn, Franziska Turck (MPIZ, Cologne, Germany)	kan^R
pGWB3 GUS	Kazumi Nakabayashi (MPIZ, Cologne, Germany)	hyg^R, kan^R
pLeela GW	Klaus Richter, Joachim Uhrig (MPIZ, Cologne, Germany)	amp^R
pMD GWY strepII	Shahid Mukhtar, Laurent Deslandes, Imre Somssich (MPIZ, Cologne, Germany)	$spec^R$
pXCSG strepII	Jane Parker (MPIZ, Cologne, Germany)	amp^R

2.1.6 Bacterial strains

For standard cloning chemical competent or electrocompetent cells of the *Escherichia coli (E. coli)* strain DH5alpha were used (HANAHAN, 1983). The DB3.1 strain, which is resistant to *CcdB*, was used for the Gateway Entry, Donor and Destination vectors. The BL21(DE3) plysS strain was used for antigen overexpression in *E. coli* (2.2.5).

DH5alpha: F- *end* A1 *hsd*R17 (rk-, mk+) *gyr*A96 *rel*A1 *sup*E44 L- *rec*A1 80dlacZM15 D (*lac*ZY AargF) U196

DB3.1: F-*gyr*A462 *end* A1 D(sr1-*rec*A) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rsp*L20(Smr) *xyl*-5 l- *leu mtl*-1

BL21(DE3) plysS: F– *dcm ompT hsdS*(rB– mB–) *gal* λ(DE3) [pLysS Camr]

Agrobacterium tumefaciens was used for plant transformation. Depending on the particular binary vectors (Appendix A 9), the following Agrobacteria strains were used:
GV3101, GV3101+psoup, GV3101+pMP90RK (KONCZ AND SCHELL, 1986; KONCZ ET AL. 1990; HELLENS ET AL., 2000).

2.1.7 Plant material

Arabidopsis plants used in this study were derived from the accessions Landsberg *erecta* (L*er*) originating from Poland (RÉDEI, 1992), Cvi (N8580), from the Cape Verde Islands (LOBIN, 1983) and Kashmir 2 (Kas-2, N1264, EL-LITHY ET AL., 2006) from India. In addition the recombinant inbred line NIL *DOG1* was used, which has an introgression of the Cvi allele of *DOG1* at the bottom of chromosome V in L*er* background. Seeds of the non-dormant mutant allele of *DOG1* (*dog1-1*) were obtained from L. BENTSINK (2006) and are in the NIL *DOG1* background. A non-dormant mutant line C 3-7 in Columbia background was found in a mutant screen by Raz (1999) and was confirmed in this study to be an additional mutant allele of *dog1* (*dog1-2*). Seeds of the mutants *aba1-1*, *aba1-3*, *aba1-5*, *aba2-1* and *ga4-1* in L*er* background (KOORNNEEF ET AL., 1982; LÉON-KLOOSTERZIEL ET AL. 1996a; KOORNNEEF AND VAN DER VEEN, 1980) were provided by M. Koornneef.

2.2 Methods

2.2.1 Plant work

2.2.1.1 Germination of seeds and plant growth conditions

Arabidopsis thaliana seeds were germinated directly on soil or sown on water-soaked filter paper (Macherey & Nagel, Düren, Germany) in 6 cm Petri dishes and incubated in a climate room (25°C, 16 h light/day) for 7 days. In some cases seeds, were kept on moist filter paper at 4°C in the dark for 4 to 7 days prior to germination under the above mentioned conditions to synchronize germination.
Seeds that were not able to germinate, were rescued by the isolation of the embryo, which was transferred onto germination medium (0.5x MS-salts, 0.8% Daishin agar, pH 5.7).
Plants were grown in an air-conditioned greenhouse between 18-25°C, supplemented with additional light providing a day length of minimum 12 h (will be cited in this study as ‘standard greenhouse conditions’). When more precisely controlled conditions were required, plants were

grown in climate chambers (Pervival Scientific Inc., Perry, USA; Elbanton BV, Kerkdriel, Netherlands) under long day conditions with a 16/8 h light/dark cycle at two different temperature conditions: 22°C day temperature and 16°C night temperature or constantly at 15°C.

2.2.1.2 Seed surface sterilization

Seeds were surface sterilized by a 1 min incubation in 70% Ethanol followed by a 10 min incubation in a freshly made NaOCl solution (3-3.5% NaOCl, 0.25% SDS). Afterwards the seeds were rinsed three times with absolute Ethanol. After drying, the seeds were sown on MS-Agar plates under sterile conditions (1x Murashige-Skoog salts (MS), 1% sucrose, 0.8% Daishin agar, pH 5.7 and selective antibiotics dependent on the experiment).

2.2.1.3 Seed dormancy measurements and germination assays

To measure the degree of dormancy, the percentage of germinating seeds of each genotype was determined during a time-course of seed storage. The curves describing germination percentage during the time of storage represented the kinetics of seed dormancy. Between 100 and 150 seeds of each genotype were evenly sown on filter paper soaked with 0.5 ml demineralized water in a 6 cm Petri dish. Petri dishes were placed in moisture chambers containing a filter paper saturated with tap water and closed with transparent lids. The moisture chambers were stored as described before. After 7 days the total number and the number of germinating seeds were scored using a dissecting microscope (MZ6 and MZ12.5, Leica, Wetzlar, Germany) and the percentage of germinating seeds was calculated.

2.2.1.4 Crossing of plants

To prepare the pistil as a pollen acceptor, young flower buds were selected at a stage when they were still closed and before the pollen of the anthers was ripe. Closed flower buds were opened with fine forceps and the anthers of the acceptor flower were removed, the sepals and petals were kept as shelter for the pistil. All remaining older and younger flowers from the inflorescence were removed. The stigma of the carpel was pollinated with pollen from recently opened flower buds from the donor plant. Unless otherwise mentioned, reciprocal crosses were made in each case.

2.2.1.5 Plant transformation

Plants were transformed using the Floral Dip method (CLOUGH AND BENT, 1998). Plants were grown at a density of five plants/pot (9 x 9 cm).To obtain strong plants, they were first grown

under short day conditions (8/16 h light/dark cycle at 22°C) until the first bolts appeared. The plants were then transferred to standard greenhouse conditions, the first shoots were clipped to break apical dominance and to encourage growth of multiple secondary shoots. When the first flowers appeared on shoots of approximately 10 cm length (5-10 days after clipping) the plants were used for transformation. Three days before plant transformation an Agrobacteria liquid culture or glycerol stock was streaked onto plates with YEB medium containing the selective antibiotics and incubated for 2-3 days at 28°C. The bacteria were collected from the plates by scraping and were resuspended in 30 ml YEB medium. Before dipping the flowers, 120 ml of a 5% sucrose solution containing 0.03% of Silwet L-77 was added to the culture. The inflorescences of the plants were dipped into the Agrobacteria solution for 5-10 seconds with gentle agitation, then placed horizontally in a moist chamber and finally transferred to the greenhouse on the following day.

2.2.1.6 Transient transformation of *Nicotiana benthamiana* leaves

Overnight cultures or glycerol stocks of Agrobacteria carrying the binary constructs *p35S::alphaDOG1::smGFP-Term*, *p35S::betaDOG1::smGFP-Term* and *p35S::deltaGFP:: smGFP-Term* were plated and grown in high density on plates with YEB medium containing selective antibiotics and incubated at 28°C for 2 to 3 days. Bacteria were scraped from the plate and resuspended in 3 ml induction medium (10mM $MgCl_2$, 10mM MES pH 5.6 (KOH), 0.15mM Acetosyringon (in DMSO)). The culture was incubated for 2 h at 28°C in the dark and diluted with induction medium to an OD_{600} of 0.5. Healthy, fresh-looking leaves of young *Nicotiana benthamiana* plants were infiltrated with a needleless syringe on the underside. Leaf material was harvested for analysis, 72 h after infiltration.

2.2.1.7 Selection of transformants

The seeds of transgenic plants carrying a T-DNA insertion containing Kanamycin and Hygromycin resistance were selected on MS-agar plates with the corresponding antibiotics. Transgenic plants containing the BASTA resistance gene were grown on soil for 10 to 15 days. The seedlings were sprayed twice with 200 mg/l Glufosinat (BASTA, Hoechst, Germany) with a 7 day interval.

2.2.2 Microscopy and cytological methods

2.2.2.1 Microscopy

Light microscopy was performed with an Axiosphot microscope with Differential Interference Contrast (Nomarsky)-Optiks (Zeiss, Heidelberg, Germany) or Leica PTC-200 (Wetzlar,

Germany). The DISKUS software package (Carl H. Hilgers-technisches Büro, Königswinter, Germany, version 4.30.19) was used to take digital photos. Confocal-laser-scanning microscopy was performed with Leica TCS SP2 AOBS or LSM 510 META (Zeiss, Heidelberg, Germany). For visualization of GFP fluorescence, excitation was at 488 nm, emission was detected at 505-530 nm.

2.2.2.2 GUS staining

GUS activity was assayed according to SESSIONS AND YANOFSKY (1999) using seedlings, leaves, inflorescences, siliques, isolated embryos, and isolated endosperm. The tissues were submerged in 0.5 to 1 ml GUS staining buffer (0.2 % Triton X-100, 50mM $NaPO_4$ pH 7.2, 2mM $K_4Fe(CN)_6*H_2O$, 2mM $K_3Fe(CN)_6$, containing 2 mM X-Gluc), subsequently vacuum infiltrated for 1 h to allow complete penetration of the X-Gluc solution and incubated at 37°C for 18h. The tissues were cleared in increasing concentrations of ethanol (30-100% EtOH) until the chlorophyll was bleached.

2.2.2.3 Ruthenium red staining of mucilage

Seed mucilage was examined with the cationic dye ruthenium red (Sigma, Deisenhofen, Germany). Ruthenium red was dissolved in de-ionised water and used at a final concentration of 200µg/ml. Seeds were imbibed on wet filterpaper for 1 h, followed by incubation in the dye solution for 10 min and de-staining by washing with de-ionised water before examination (WILLATS ET AL., 2001).

2.2.2.4 RNA in-situ Hybridization

The *DOG1* probe contained nucleotides 197 to 575 relative to the ATG of the cDNA. The probe was synthesized by PCR and was used as template for T7-polymerase driven *in vitro* transcription (Ambion, Cammbridgeshire, UK).

Siliques of the accession Cvi at 14 DAF were used as samples. Sample preparations and in-situ hybridizations of 8 mm sections were done as described by COEN ET AL. (1990) with the following modifications. Tween-20 (0.03%) was added to the fixative and dehydration of the fixed material was done without NaCl. Plant material was embedded in Paraplast+ (Kendall, Mansfield, USA) in an ASP300 tissue processor (Leica, Wetzlar, Germany). Probes were not hydrolyzed. After the color reaction, slides were mounted in 30% glycerol and photographed using differential interference contrast microscopy.

2.2.3 EMS-mutagenesis of Arabidopsis seeds

Approximately 10 000 seeds each of L*er*, NIL *DOG1* and Cvi were wrapped in miracloth and imbibed for 14 h in 50 ml 0.1% KCl at 4°C on a tumbler. The seeds were washed with 100 ml distilled demineralized water and then imbibed in a freshly prepared 30mM EMS solution (Sigma, Deisenhofen, Germany) for 17h on a rocking table at room temperature in the dark. The EMS solution was carefully removed and the seeds were washed twice with 100 ml of 100mM sodium thiosulfate for 15 min to decontaminate residual EMS. The seeds were then rinsed three times with demineralized water for 30 min and mixed with a 0.15% water agar solution which was then pipetted directly onto the soil. About 30 seeds per tray were sown and grown under normal greenhouse conditions. The seeds from each tray were harvested as a pool.
The success of the mutagenesis was analysed in the M1 population by counting plants showing discolored clonal sectors on leaves indicating mutations in components of the photosynthesis apparatus. Approximately 2% of the M1 population showed such clonal sectors, indicating the successful efficiency of the mutagenesis experiment. Since the M1 uncovers only dominant mutations, the screening for novel dormancy mutants was carried out in the M2 population. The different selection strategies are described in detail in chapter 3.1.

2.2.4 Molecular biological methods

If not indicated otherwise, the methods applied in this study were taken from SAMBROOK AND RUSSEL (2001) and AUSUBEL (1994) using standard procedures.

2.2.4.1 Genomic DNA extraction from plant tissue

In order to extract plant genomic DNA, a method from DELLAPORTA ET AL. (1983) was used. Two to three frozen young leaves or inflorescences were ground in a 2 ml reaction tube with a metal bead by shaking for 1 min at a high frequency using a Mixer Mill (Type MM 300, Qiagen, Hilden, Germany). After adding 750 µl of extraction buffer (50mM Tris pH 8, 10mM EDTA pH 8, 0.1M NaCl, 1% SDS) the mixture was vortexed and incubated at 65°C for 10 min. 200 µl of 5M/3M potassium acetate solution was added and the mixture was incubated for 20 min on ice, followed by centrifugation for 10 min at 13 000 rpm. The supernatant was transferred into a new tube and centrifugation was repeated (10 min, 13 000 rpm). The supernantant was transferred into a new tube and mixed with an equal volume of isopropanol followed by centrifugation for 10 min at 13 000 rpm. The precipitated DNA was washed with 80% ethanol and air-dried. 50 µl demineralized water was used to dissolve the pellet, 1 µl of RNase A

(1 mg/ml) was added to eliminate RNA. 1 µl of this solution was used as a template for standard PCR reactions.

2.2.4.2 Plasmid DNA isolation from bacteria

Small scale plasmid isolation from *E. coli* was performed using the column-based Plasmid Isolation Mini kit (Qiagen) according to the manufacturer's protocol.

2.2.4.3 Separation of DNA fragments by agarose gel electrophoresis

DNA fragments were mixed with DNA loading buffer and analyzed by agarose gel electrophoresis. The agarose concentration depended on the size of fragments to be resolved. Electrophoresis was performed at 5 V/cm using TAE buffer. 1kb ladder DNA size marker (Invitrogen) was used to estimate the size of DNA fragments. After electrophoresis, DNA was visualized on a transilluminator under UV light (254 nm).

2.2.4.4 Purification of PCR products and gel-extracted DNA fragments

PCR products, gel-extracted PCR products and gel-extracted restriction digest products were purified using the Roche High Pure PCR Purification Kit following the manufacturer's protocol.

2.2.4.5 Total RNA extraction from leaf tissue

Total RNA from leaves was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol.

2.2.4.6 Total RNA extraction from siliques and seeds

Extraction of total RNA from siliques, dry seeds and imbibed seeds was performed as established by KUSHIRO AND OKAMOTO ET AL. (2004) using RNAqueous columns with Plant RNA Isolation Aid (Ambion, Cammbridgeshire, UK). 40 mg of silique or seed material was ground into a fine powder in liquid nitrogen with a mortar and pestle. 4x 800 µl of lysis/binding buffer was added directly into the mortar. When the buffer thawed, the solution was distributed into 4 x 2 ml tubes and 200 µl of Plant RNA Isolation Aid was added to each tube, followed by centrifugation for 10 min at 13 000 rpm. The supernatant was carefully transferred into a new 2 ml tube and an equal volume of 64% ethanol was added. This mixture was applied to the RNAqueous filter cartridge (700 µl each time) and centrifuged at 13 000 rpm for 1 min; and the flow-through was discarded. In case of green siliques, 20 mg original tissue was loaded per column, for yellowish siliques and dry seeds, which are more difficult tissues due to high lipid

content, only 10 mg of original tissue was loaded onto one column. After loading all the sample mixture, 700 µl of wash solution 1 was applied to the filter and centrifuged at 13 000 rpm for 1 min and the flow-through was discarded. This was followed by a second and third washing step, adding each time 500 µl of wash solution 2/3 to the filter, centrifuging at 13 000 rpm for 1 min (2 min at the 3rd washing step) and discarding the flow-through. After the final washing step the dry filter cartridge was put into a fresh 1.5 ml tube and 50 µl of preheated (95°C) elution solution was added to the center of the filter and the eluate was recovered by centrifugation at 13 000 rpm for 10 min. This was repeated by adding a second amount of 50 µl preheated elution solution. At the end of the column-based purification, 100 µl of eluted RNA was obtained per column. All of the columns from one original sample were then combined (200 to 400 µl). The absorbance was checked using the Nanodrop ND-1000 spectrophotometer (Peclab, Erlangen, Germany) to calculate the appropriate elution volume after the high salt precipitation step.

RNA was further purified through additional precipitation steps, a high salt precipitation to remove polysaccharides and the precipitation of high molecular weight RNA with lithium chloride. The RNA solution was adjusted to a total volume of 1 ml by adding RNase free water. 250 µl of isopropanol and 250µl of a high salt precipitation solution (1.2M sodium citrate, 0.8M sodium chloride) were added, mixed and kept on ice for 2 h. The RNA was recovered by centrifugation at 13 000 rpm for 15 to 30 min at 4°C. The supernatant was removed and the pellet was rinsed with 70% ethanol and centrifuged again at 13 000 rpm for 5 min at 4°C. After removing the ethanol, the pellet was air-dried and dissolved in an appropriate volume of RNase free water to achieve a minimum concentration of 200 ng/µl.

For the precipitation of high molecular weight RNA with lithium chloride 0.5 volume of 5M LiCl was added to the eluted RNA. The solution was mixed and kept on ice over night. The RNA was recovered by centrifugation at 13 000 rpm for 20 to 30 min at 4°C and the supernatant was removed afterwards. The pellet was rinsed with 70% ethanol and centrifuged for 5 min at 13 000 rpm at 4°C. The ethanol was removed; the pellet was dried and dissolved with RNase free water in a final volume of 10 µl. The concentration and quality of the RNA was determined by measuring the ratios of absorbance A_{260nm}/A_{280nm} which should be between 1.8 and 2.0 and A_{260nm}/A_{230nm} which should be between 2 and 3. The integrity of RNA was tested by running 500 ng of total RNA that was mixed with 5 µl of loading buffer with formaldehyde and incubated at 60°C for 10 min on a 1% agarose gel. Total RNA was stored at -80°C.

2.2.4.7 cDNA synthesis

For first strand cDNA synthesis 2.2 µg of total RNA was used to synthesize cDNA by reverse transcription using 200 U of Superscript™ II Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) per reaction and oligo(dT)$_{16-18}$ primers (500 ng) as priming method. cDNA synthesis was performed according to the manufacturer's protocol. After RNase H treatment at 37°C for 20 min, the mixture was adjusted to a final volume of 35 µl. 2.5 µl of the synthesized first-strand cDNA was used subsequently for a qPCR reaction.

2.2.4.8 Standard PCR reaction

All PCR reactions were done with a MJ Research DNA Engine Tetrad®2 peltier thermal cycler (Biozym, Hess. Oldendorf, Germany). For standard reactions (Colony-PCR, genotyping) *Taq*-DNA Polymerase was used. For high accuracy PCR reactions (Cloning), the proof-reading Platinum® Pfx-Polymerase was applied as described in the manufacturer's protocol. A standard PCR reaction was performed as follows:

Table 2.8: Standard PCR reaction.

Concentration	Reagent	Volume
10 mM	dNTPs	1
10 pmol/µl	Primer 1	1
10 pmol/µl	Primer 2	1
10x	PCR buffer	5
50-200 ng/µl	DNA template (genomic DNA, plasmid DNA, cDNA, bacterial colony)	1
	DNA polymerase (*Taq* 0.025 - 0.05 U/µl; *Pfx* 2.5 U/µl)	1
	H_20	adjust to 50 µl
Annealing Temperature	55°C - 65°C	
Elongation Time	1 minute/1 kb	
Number of cycles	35	

2.2.4.9 Quantitative PCR

Quantitative PCR was performed using a Mastercycler® ep *realplex* (Eppendorf, Hamburg, Germany). To ensure that the primer combinations did not produce any undesired PCR fragments or primer dimers, a SYBR Green qPCR with melting curve analysis was performed (60°C to 95°C with a heating rate of 0.1°Cs^{-1} and continuous fluorescence measurements) using the RealMasterMix SYBR ROX (5Prime, Hamburg, Germany). To avoid genomic DNA amplification the primers were either located in different exons or across exon-exon boundaries.
Plasmid DNA of *pDONR201_alphaDOG1, pDONR201_betaDOG1* and *pDONR201 _deltaDOG1* was used to produce a dilution series with the concentrations 10^{-1} to 10^{-8} fmol/µl. Absolute quantification was performed based on calibration curves of these DNA standard

molecules. The quantification of the normalization gene *ACTIN8* was calculated based on a 10-fold dilution series of a reference sample. Plant material for quantitative expression analysis was grown in two independent biological replicates. For each data point siliques from 5 to 6 independent plants were collected for each biological replicate. QPCR reactions of each of the biological replicates were done in technical duplicates.

Table 2.9: Reaction setup for qPCR.

Concentration	Reagent	Volume
1x, 0.1x, 0.01x or 0.001x	cDNA	2.5
2.5x	RealMasterMix	10
20x	SYBR Green	1.25
10 pmol/µl	Primer 1	2
10 pmol/µl	Primer 2	2
	H_20	fill to 25 µl

Table 2.10: PCR program for quantitative real-time PCR of alpha, beta, gamma and delta *DOG1*.

Step	Temperature	Time (min:sec)	cycles (#)
Denaturation and Heat activation of Hotstart Polymerase	95°C	2:00	
Denaturation	95°C	00:15	
Annealing	64°C	00:15	50
Extension	68°C	00:30*	
melting curve analysis			

* single fluorescent measurement

Table 2.11: PCR program for quantitative real-time PCR of the normalization gene *ACTIN8*.

Step	Temperature	Time (min:sec)	cycles (#)
Denaturation and Heat activation of Hotstart Polymerase	95°C	2:00	
Denaturation	95°C	00:15	
Annealing	60°C	00:15	40
Extension	68°C	00:15*	
melting curve analysis			

* single fluorescent measurement

2.2.4.10 Molecular cloning of constructs for production of transgenic plants

Unless otherwise described, cloning strategies performed in the course of this thesis included directional TOPO cloning reactions and BP reactions for cloning of PCR products into *pENTR* or *pDONR201* vector and LR reactions for production of the final destination vectors. All reactions were performed following the manufacturer's protocols. All constructs were verified by sequencing. C-terminally fused and tagged proteins were confirmed to be in frame with the protein. After transformation steps into *E. coli* and *A. tumefaciens*, the presence of the transformed plasmid was tested via colony-PCR using a primer that was located in the backbone

of the vector and a second primer being localized within the two Gateway recombination sites (Table 2.3, p. 21; Appendix A 2 – A 8).

Two new binary vectors were designed in the course if this work: *pBAT-B GW ER-GFP* and *pGreen DOG1promoterCvi GW*. The binary vector *pBAT-B GW ER-GFP* was produced to fuse *DOG1* promoter sequences from L*er*, Kas and Cvi with the reporter gene GFP localized in the endoplasmatic reticulum (ER-GFP). The sequence of ER-GFP was amplified from the vector *pGJ1029* using the primers ER-GFP_F_BspHI and ER-GFP_R_BamHI and the PCR product was cleaved with the corresponding restriction enzymes. The binary vector *pBAT-B* GFP was digested with BspHI and BamHI to cut out the Gateway cassette and the GFP sequence of the original vector, followed by ligation of the linearized vector with the digested PCR product. The new fusion vector pBAT-B_ER-GFP was again cut with BspHI at the 5' end of ER-GFP, the sticky ends were filled using Klenow fragment followed by blunt ligation of the Gateway cassette (attR1-CmR-*ccdB*-attR2).

To produce a binary vector for fusions of the *DOG1* promoter from the strongly dormant accession Cvi with any desired gene or cDNA, the vector *pGreen DOG1promoterCvi GW* was designed. First, the vector *pDONR201_DOG1promoterCvi* and the binary vector *pGreen GW mcs* were combined by LR reaction. The fusion vector *pGreen DOG1promoterCvi mcs* was then linearized in the mcs with HindIII, the sticky ends were filled using Klenow fragment followed by blunt ligation of the Gateway cassette (attR1-CmR-*ccdB*-attR2) to the *DOG1* promoter sequence.

The cloning strategy for the C-terminally *strepII*- and *GFP*-tagged DOG1 protein constructs (Table 2.12, marked with **) driven by the *DOG1* promoter from Cvi was as follows. First the alpha, beta or delta *DOG1* splicing forms (in *pDONR201*) were combined with *pXCSG strepII* and *pAM-PAT 35S GW smGFP-Term* via LR reaction. This was followed by a PCR step using the primer combinations DOG1F_TOPO and smGFP_R or DOG1F_TOPO and strepII_R. The PCR products were then combined with the *pENTR* vector via directional TOPO cloning, adding attL1 and attL2 sites to the *DOG1* cDNAs tagged with *strepII* or fused to *GFP*. Finally, these *pENTR* clones were combined with the binary vector *pGreen DOG1promoterCvi GW* by performing LR reactions.

All of the constructs generated in the course of this thesis are indicated and described in the table below. A complete list including selection markers in *E. coli*, *A. tumefaciens* and plants of each construct can be found in the Appendix A 9. Vector maps of representative clones for each group of transgenic designation are shown in Appendix A 1 – A 8.

Table 2.12: Overview and description of generated constructs.

	Transgenic designation	Description of construct	Transformed into
Localization studies	**native promoter GUS fusions**		
	pDOG1_Cvi::GUS *pDOG1_Kas::GUS* *pDOG1_Ler::GUS*	Expression of a *DOG1* promoter driven coding sequence of GUS using Gateway cloning into *pGreen GW GUS* and *pGWB3*	*dog1-1* , L*er* , Cvi
	native promoter ER-GFP fusions		
	pDOG1_Cvi::ER-GFP *pDOG1_Kas::ER-GFP* *pDOG1_Ler::ER-GFP*	Expression of a *DOG1* promoter driven coding sequence of GFP localized in the endoplasmatic reticulum, using Gateway cloning into *pBAT-B GW ER-GFP*	*dog1-1*
	localization of DOG1 protein		
	pDOG1_Cvi::alphaDOG1::smGFP* *pDOG1_Cvi::deltaDOG1*::smGFP*	Expression of a 2686 bp *DOG1* promoter (derived from Cvi) driven alpha or delta *DOG1* cDNA, C-terminally fused to coding sequence of *smGFP*, using Gateway cloning into *pGreen DOG1promoter Cvi GW***	*dog1-1*
	p35S::alphaDOG1::smGFP-Term* *p35S::betaDOG1*::smGFP-Term* *p35S::deltaDOG1*::smGFP-Term*	Constructs for transient expression in tobacco leaves, expressing *35S* promoter driven alpha, beta or delta *DOG1* cDNA, coding sequence of *smGFP* and terminator sequences, using Gateway cloning into *pAM-PAT 35S GW smGFP Terminator*	*N. benthamiana*
Analysis of *DOG1* splicing variants	**Complementation lines**		
	*pDOG1_Cvi::alphaDOG1** *pDOG1_Cvi::betaDOG1** *pDOG1_Cvi::deltaDOG1**	Expression of a 2686 bp *DOG1* promoter (derived from Cvi) driven alpha, beta or delta *DOG1* cDNA, produced by Gateway cloning into *pGreen DOG1promoterCvi GW*	*dog1-1*
	Overexpression lines		
	*p2x35S::alphaDOG1** *p2x35S::betaDOG1** *p2x35S::deltaDOG1**	Expression of a double *35S* promoter driven alpha, beta or delta *DOG1* cDNA, produced by Gateway cloning into *pLeela*	*dog1-1* , L*er*
	p2x35S::GF_DOG1	Plants expressing double *35S* promoter driven 2.8 kb *DOG1* genomic sequence between the partial 5' and 3' UTR regions, based on Cvi sequence and produced by classical cloning into *pLeela*	L*er*
	Inducible expression		
	*pMD::alphaDOG1** *pMD::betaDOG1** *pMD::deltaDOG1**	Expression of a strong ß-estradiol inducible p*G10-90* promoter driven alpha, beta or delta *DOG1* cDNA, produced by Gateway cloning into *pMD GWY strepII*	*dog1-1*
	pMD::ABA1_Col	Expression of a strong ß-estradiol inducible p*G10-90* promoter driven *ABA1* cDNA from Col, produced by Gateway cloning into *pMD GWY strepII*	*aba1-1, aba1-3, aba1-5*
Analysis of DOG1 protein	**StrepII-tagged DOG1 protein**		
	p35S::alphaDOG1::strepII* *p35S::deltaDOG1*::strepII*	Expression of the *35S* promoter driven alpha or delta *DOG1* cDNA in frame with a *strepII* coding sequence, using Gateway cloning into *pXCSG strepII*	*dog1-1*
	pDOG1_Cvi::alphaDOG1::strepII* *pDOG1_Cvi::deltaDOG1*::strepII*	Expression of the 2686 bp *DOG1* promoter (derived from Cvi) driven alpha or delta *DOG1* cDNA in frame with a *strepII* coding sequence, using Gateway cloning into *pGreen DOG1promoter Cvi GW***	*dog1-1*

* Cvi and L*er* alleles were used for the constructs of each *DOG1* splicing variant.
** Cloning strategy as described in text above.

2.2.4.11 Sequencing

DNA sequencing was performed by the MPIZ DNA core facility on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377, 3100 and 3730 sequencers using BigDye-terminator v.3.1 chemistry. Premixed reagents were from Applied Biosystems. Sequences were analyzed with the software Chromas PRO version 1.33 (share-it, Cologne, Germany).

2.2.5 Biochemical methods

2.2.5.1 Antigen production

A region unique to the DOG1 protein, which corresponds to amino acid residues 1-151, was amplified by PCR using the primer antDOG1_F with *NdeI* site in conjunction with the primer antDOG1_R with *XhoI* site. The DNA fragment was cut out with *NdeI* and *XhoI* and subcloned into the corresponding sites in the expression vector *pET21a* for histidine tagged antigen

overexpression in *E. coli*. The histidine tagged protein was purified with an optimized procedure based on the manufacturer's protocol and described as follows. An over-night culture of the *E. coli* strain BL21(DE3) plysS was inoculated 1:10 in 2x 250 ml TB medium including ampicillin (100 mg/l), grown at 37°C until the OD_{600} reached 0.6 and then induced with 1.5mM IPTG. After 2 h the cells were harvested by centrifugation at 5 000 rpm for 20 min at 4°C, the pellet was resuspended in 25 ml binding/lysis buffer (20mM phosphate, 500mM NaCl and 20mM imidazole) and lysozyme (1mg/ml) was added. After incubating on ice for 30 min, the cell suspension was centrifuged for 15 min at 5 000 rpm. The pellet was resuspended in 25 ml binding/lysis buffer containing 1% Tween20 and 1% NP-40 followed by several freeze/thaw cycles, sonicated five times for 15 sec each and harvested by centrifugation at 15 000 rpm for 15 min. IPTG induced expression of the truncated DOG1 protein fragment resulted in the production of inclusion bodies in *E. coli*, therefore the protein was purified under denaturing conditions. The insoluble protein in the pellet was dissolved in 25 ml binding/lysis buffer containing 8M urea. The mixture was kept on ice for 2h and after centrifugation for 10 min at 14 000 rpm at 4°C the denatured histidine-tagged antigen was purified by applying the supernatant to the Ni^{2+} column of the HisTrap FF crude Kit. The antigen was eluted with buffer containing 20mM phosphate, 500mM NaCl and 300mM or 500mM imidazole. The eluted protein was separated on 15% SDS-PAGE using CBB staining (Bio-Safe™ Coomassie G-250 stain, BIORAD, Hercules, USA). Gel slices containing the 19.4 kD antigen were used to raise a polyclonal antibody in rabbit (Eurogentec, Seraing, Belgium).

2.2.5.2 Protein extraction from seed and leaf tissue

Protein from seeds was extracted following an adapted protocol from MEYER ET AL. (1988). 10 mg of freshly harvested dry seeds were frozen in liquid nitrogen and homogenized in 500 µl of an solution of 50% (w/v) phenol in 0.1M Tris-Cl (pH 8.0) supplemented with 5% ß-ME, mixed for 30 min at 4°C and centrifuged at 10 000 rpm for 10 min at 4°C. The phenol phase was re-extracted for 15 min at 4°C with one volume of 0.1 Tris-HCl (pH 8.0) saturated with phenol. The phenol phase was separated by centrifugation (10 000 rpm, 10 min, 4°C) and proteins were precipitated by 4 volumes of 0.1M ammonium acetate in methanol at -30°C for 4 h. The precipitate was collected by centrifugation and washed 6 times with 0.1M ammonium acetate in methanol and air-dried. The precipitates were dissolved in 1x SDS sample buffer (20-25 µl), heated to 98°C for 10 min and centrifuged.

Total leaf protein was extracted under denaturing conditions. 100 mg of rosette and cauline leaves were frozen and homogenized in liquid nitrogen. 100 µl of extraction buffer

(100mM Tris-Cl (pH 7.5), 6M urea, 2M thiourea, 0.2% (v/v) Triton X-100, 0.2% (w/v) N-Lauroylsarcosyl, 2mM DTT) was added and mixed gently for 30 min at 4°C. The supernatant was centrifuged twice at 14 000 rpm for 15 min at 4°C. The pellet was discarded and the supernatant was mixed with 1x SDS buffer and heated to 85°C for 10 min.

Proteins from seeds and leaves were used for separation by SDS-PAGE. The protein concentration was determined using the Bradford assay (BRADFORD, 1976) with Bradford dye reagent (Protein assay, Bio-Rad, Hercules, USA) and bovine serum albumin as a standard.

2.2.5.3 Protein separation by SDS-PAGE and Western blot analysis

The protein extracts were applied onto a 12% SDS-PAGE (LAEMMLI, 1970) and blotted on PVDF membrane (Millipore, Bedford, USA) by semi-dry electrotransfer. The solutions for the immunological reaction consisted of 50mM Tris-HCl (pH 7.5), 150mM NaCl, 5% (w/v) skim milk and 0.25% (v/v) Tween20. For primary antibody reaction, the protein gels were incubated for a minimum of 1 h with anti-DOG1 polyclonal antibody (1:10 000). As a secondary antibody a goat anti-rabbit IgG antibody conjugated to alkaline phosphatase (MP Biomedicals, Asse-Relegem, Belgium) (1:2000) was used. For visualization the blots were developed in a solution of 100 mM Tris-HCl (pH 9.6), 100mM NaCl, 0.1 mg/ml nitro blue tetrazolium, 0.05 mg/ml BCIP, 4mM $MgCl_2$ and 1% (v/v) DMF. (NAKABAYASHI ET AL., 1999)

3 Results

3.1 Identification of novel seed dormancy mutants by genetic screens

3.1.1 EMS mutagenesis experiments

The production and identification of mutants with altered physiological responses as well as other phenotypes represents the starting point of forward genetic approaches with the goal of identifying the respective genes and connecting the phenotype with the function of the protein encoded by the gene. Alkylating agents such as ethyl methanesulfonate (EMS) induce chemical modifications of nucleotides, which result in mispairing and base changes. Compared to irradiation mutagenesis, EMS induces relatively few strand breaks that lead to inversion or deletion mutations and was therefore chosen as the mutagenesis agent (KIM ET AL., 2006). In this study, three EMS mutagenesis populations of different Arabidopsis accessions were generated (Chapter 2.2.3) to screen for novel seed dormancy mutants: non-dormant mutants, after-ripening deficient mutants and stratification-insensitive mutants.

To identify non-dormant mutants, the near isogenic line NIL *DOG1* and Cvi were chosen as genetic backgrounds due to their strong dormancy (Figure 1.4). Cvi, whose strong dormancy can be broken by stratification after about 10 weeks of after-ripening (Figure 3.2, Figure 3.3), was additionally used for mutagenesis to screen for stratification-insensitive mutants. For the detection of after-ripening deficient mutants, the mutagenesis was performed with L*er*, because this accession has a low level of dormancy, which can be broken by a rather short period of after-ripening.

In all cases, M2 seeds arising from approximately 10 000 M1 plants were analyzed. M2 seeds were harvested in 330 seed batches, which included pooled seeds deriving from about 30 independent M1 plants. The numbering of the selected mutant lines corresponded to the numbering of the seed batch. Thus, mutants that were isolated from the same seed batch are very likely to originate from the same M1 plant, but it cannot be excluded that they might also be independent mutants deriving from different M1 plants present in the same pool.

3.1.2 Selection of non-dormant mutants

To date, mutant approaches to find non-dormant mutants were performed with relatively low dormant accessions and therefore revealed only a limited number of mutants (KOORNNEEF ET AL., 2000). In the present genetic screen non-dormant mutants were selected making use of the more dormant NIL *DOG1* and Cvi, with the expectation of finding additional mutants.

The selection strategy used a germination assay with mature seeds directly after harvest when wild type seeds were still dormant and non-germinating. Germinating seeds were selected after imbibing for three days at 25°C. Seeds were selected, if radicle protrusion could be observed under the microscope.

In total, 60 000 freshly harvested M2 seeds from EMS-mutagenized NIL *DOG1* plants were screened for germination. This screen resulted in 80 non-dormant mutant lines, of which five showed a confirmed non-dormant or reduced-dormant phenotype in the M3 generation with germination rates between 74-100% directly after harvest (Table 3.1). In the M4 generation these mutants were grown, along with two additional non-characterized, non-dormant mutants of former mutagenesis experiments with γ-irradiation performed by L. Bentsink and J. Jowett (2000, unpublished) in the same genetic background (mutant line E 36-2a) and by V. Raz (1999) in Columbia background (mutant line C 3-7, described in CLERKX ET AL., 2004).

After screening 60 000 seeds of the M2 population of mutagenized Cvi seeds, 30 non-dormant and reduced-dormant mutants with a germination percentage in M2 seeds between 61% and 100% were selected, of which six lines showed a confirmed phenotype in the M3 generation. The selected mutants were divided into phenotypic groups: Mutants with ABA-deficient or ABA-insensitive phenotypes, testa color mutants, and mutants without pleiotropic phenotypes at either the seedling or adult stages (Table 3.1, Table 3.5).

Table 3.1: M3 mutant lines with confirmed non-dormant or reduced-dormant phenotypes.
The germination phenotype was determined directly after harvest. The non-dormant γ-irradiation mutant line from L. Bentsink and J. Jowett (2000, unpublished) is indicated with *, the reduced dormant mutant line from V. Raz (CLERKX ET AL., 2004) is indicated with **.

Mutant line (#)	Genetic background	Phenotypic group	Germination (%)
6.2	NIL *DOG1*	ABA-deficient	100
20.1	NIL *DOG1*	ABA-deficient	100
31.1	NIL *DOG1*	ABA-deficient	100
29.1	Cvi	ABA-insensitive	97
252.1	Cvi	ABA-insensitive	100
252.2	Cvi	ABA-insensitive	61
47.1	Cvi	Testa mutant	80
17.1	NIL *DOG1*	WT, no pleiotropic phenotype	100
114.1	Cvi	WT, no pleiotropic phenotype	100
195.1	Cvi	WT, no pleiotropic phenotype	94
C3-7**	Col	WT, no pleiotropic phenotype	100
34.2	NIL *DOG1*	WT, no pleiotropic phenotype	74
E36-2a*	NIL *DOG1*	WT, no pleiotropic phenotype	98

The first group with ABA affected phenotypes contained two lines (mutant lines 20.1, 31.1) that showed an ABA-deficient phenotype with brownish yellow flower buds and leaves. These lines additionally showed a wilting phenotype, due to the inability of *aba* mutants to close their

stomata upon water stress. Complementation tests of these ABA-deficient mutant lines with various known *aba* mutants showed that they are all allelic to *aba1* (KOORNNEEF ET AL., 1982). Adult plants of mutant line 6.2 did not show a strong ABA-deficient phenotype; only during seedling establishment were the seedlings pale green, typical of ABA-deficient mutants. Sequencing of the *ABA1* gene in line 6.2 confirmed that there was a point mutation in the fourth exon of *ABA1* (At5g67030) resulting in a glycine to arginine change of amino acid 269 (R. Silady, pers. communication.). Three other mutants (mutant lines 29.1, 252.1, 252.2) were identified by characteristic green seeds as being ABA-insensitive 3 (*abi3*) mutants. This class of mutants was expected from this mutagenesis screen, because ABA has an inhibitory effect on seed germination and ABA-deficient mutants (*aba*) have reduced endogenous levels of ABA. The mutants consequently show absent or reduced dormancy. The second group contained one mutant line (47.1) with an altered seed color. Seeds of this mutant showed a yellow seed phenotype caused by a transparent testa, which in turn is caused by a delay in browning of the seed coat due to the oxidation of flavonoids. This suggested that this mutant was an allele of the *tt10* mutant (POURCEL ET AL., 2005), which was confirmed by an allelism test (I. Debeaujon, pers. communication). Unlike most known non-dormant mutants, which do have pleiotropic phenotypes, the mutants of the third group lacked obvious pleiotropic phenotypes (mutant lines 17.1, 114.1, 195.1, 34.2, E3-7, E36-2a), suggesting that these might represent new non-dormant mutants or might be new alleles of *dog1*, which is the only known non-dormant mutant without a pleiotropic phenotype. Complementation tests with *dog1-1* showed that two lines complemented *dog1* and are new putative non-dormant mutants (mutant line 34.2, E36-2a). These will be investigated in a future project and have been backcrossed with NIL *DOG1* to reduce background mutations. They were also crossed with Col to produce a mapping population (R. Silady, MPIZ). Four lines did not complement *dog1-1* and are possibly new mutant alleles of *dog1*. The *DOG1* gene of three out of four mutant lines was sequenced and revealed that mutant line C 3-7 carries two nucleotide changes from C to A in the first exon, which causes a stop-codon. The other mutant lines 114.1 and 195.1 showed a single basepair change from G to A at the same position in the first exon which also leads to a stop-codon (Appendix A 10). These two new mutant alleles of *dog1* were named *dog1-2* and *dog1-3*, respectively. The fourth non-complementing line (17.1) has not yet been sequenced and will be analyzed in the future. In addition, *DOG1* of the mutant line 34.2 was sequenced and contained as expected based on the complementation test no mutations in the *DOG1* gene.

3.1.3 Selection of after-ripening mutants

The dormancy of Arabidopsis seeds can be released by after-ripening. To gain further insights into the molecular mechanisms that regulate dormancy breaking by after-ripening, mutants with a reduced response to after-ripening were identified. Screens were performed using fully after-ripened M2 seeds from EMS treated L*er* and NIL *DOG1*. L*er* seeds only require a relatively short period of after-ripening to germinate completely. NIL *DOG1* seeds require about 12 weeks of after-ripening to germinate 100%. Non-germinating seeds of mutagenized L*er* and NIL *DOG1* seeds were selected after 12 weeks of after-ripening when a non mutagenized control seed batch germinated 100%. Non-germinating seeds were rescued by the removal of the seed coat.

In the NIL *DOG1* screen of 10 000 completely after-ripened M2 seeds, 50 mutants were isolated by embryo rescue after imbibing seven days at 25°C. After transferring the 50 seedlings to soil, 21 mutants survived, of which 10 mutant lines showed a confirmed non-germinating phenotype in the M3 generation after a storage period of 12 weeks at 25°C in the dark. The 10 mutant lines showed a broad range of pleiotropic phenotypes (Table 3.2, Figure 3.1). Two lines were GA-responsive dwarfs, which is the typical GA-deficient phenotype. The plant hormone gibberellin plays an important role in the promotion of germination. GA-deficient mutants are unable to germinate without exogenous gibberellin. Therefore mutants of this class were expected to be found in this screen.

The L*er* screen of 10 000 after-ripened M2 seeds yielded only four non-germinating mutants with very weak growth (Table 3.2). This low recovery can be partially explained by problems of seedling development after the embryo rescue and establishment in soil.

Table 3.2: Mutant lines with confirmed non-germinating phenotypes 12 weeks after harvest and with pleiotropic phenotypes.

Mutant line (#)	Genetic background	Germination (%)	Phenotypic description
45.1	NIL *DOG1*	0	non-germinating GA dwarfs, mutants require gibberellin for
106.1	NIL *DOG1*	0	germination and elongation growth, 106.1 semi-dwarf
52.3	NIL *DOG1*	0	shorter and deformed siliques
52.4	NIL *DOG1*	0	shorter and deformed siliques
111.2	NIL *DOG1*	0	shorter and deformed siliques
25.1	NIL *DOG1*	0	more siliques, bigger plant growth, flat-shaped siliques
90.2	NIL *DOG1*	0	slightly reduced plant growth, partially deformed and smaller siliques
47.2	NIL *DOG1*	0	normal plant growth, flat shaped siliques
90.1	NIL *DOG1*	25	small and weak plant growth, less shoot-branching
120.2	NIL *DOG1*	2	small and weak plant growth, less shoot-branching
114.1	L*er*	0	small and weak plant growth, less shoot-branching
22.1	L*er*	0	small and weak plant growth, less shoot-branching
37.2	L*er*	0	small and weak plant growth, less shoot-branching
133.1	L*er*	0	small and weak plant growth, less shoot-branching

When selecting for after-ripening mutants by isolating embryos of non-germinating seeds, a wide range of defects, causing this germination-deficient phenotype, is expected. Because the first attempts did not lead to any mutants without pleiotropic growth related phenotypes or mutants with an after-ripening specific phenotype, further analysis of the obtained mutants was not carried out.

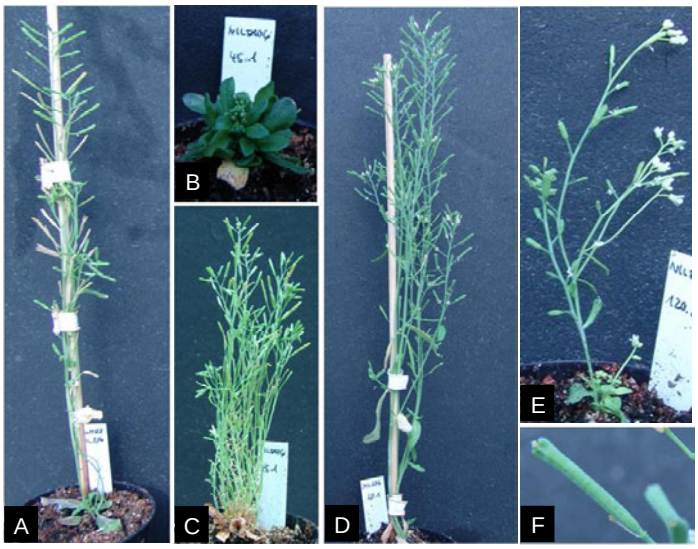

Figure 3.1: M2 after-ripening mutants with diverse pleiotropic phenotypes.
(A) Control plant NIL *DOG1*, (B) GA-deficient mutant line 45.1, (C) GA-deficient mutant line 45.1 that shows flowering and elongation growth after external application of GA_{4+7}, (D) mutant line 25.1 with a higher number of siliques, which are flat-shaped, (E) mutant line 120.1 with small and weak growth and less shoot-branching, (F) detail view of flat-shaped silique (mutant line 47.2).

3.1.4 Selection of stratification-insensitive mutants

Dormancy of Arabidopsis can be broken by a short period of 1-7 days at low temperature via stratification or moist-chilling. In order to study the molecular mechanisms that regulate stratification, the goal was to isolate mutants with an absent or reduced response to stratification. Therefore a two-step screen was designed, whereof the first step consisted of screening for non-germinating seeds after a dormancy-breaking stratification treatment. This allowed for the rapid identification of interesting mutants, with the disadvantage to possibly also select mutants with general germination defects. Therefore the mutants were further characterized in a second step using a more laborious assay to select stratification-insensitivity specific mutants.

To be able to develop an optimized selection strategy, it was important to understand the germination behavior of the accession Cvi, which was used for this screen. Seeds of this accession have a high dormancy level that can be broken completely by stratification after a limited time of after-ripening (ALI-RACHEDI ET AL., 2004). To characterize the germination behavior of Cvi in response to a combined treatment of after-ripening and stratification and to

find the most efficient stratification treatment, Cvi seeds at different stages of after-ripening were stratified for 0 to 7 days (Figure 3.2). Cvi seeds require a short period of after-ripening to be competent for the perception of the stratification signal. After this first period, the most efficient treatment was 7 days of cold. This was able to break dormancy leading to 80% germination 6 weeks after harvest and 100% germination 9 weeks after harvest. In this time window seeds that were not cold-treated (0 days of stratification) did not germinate, indicating that the release of dormancy was caused only by stratification. The application of 1 to 4 days of cold was not able to lead to the complete release of dormancy in such a stratification-specific time window. Repeating this characterization of germination with Cvi seeds grown at different maternal conditions confirmed that stratification for 7 days is the most efficient dormancy breaking treatment during the time period in which after-ripening itself does not yet release dormancy. However, the stratification-specific time-window varies. This variability in starting point an duration is likely due to evironmental factors during seed development and seed storage (compare Figure 3.2 with Figure 3.3).

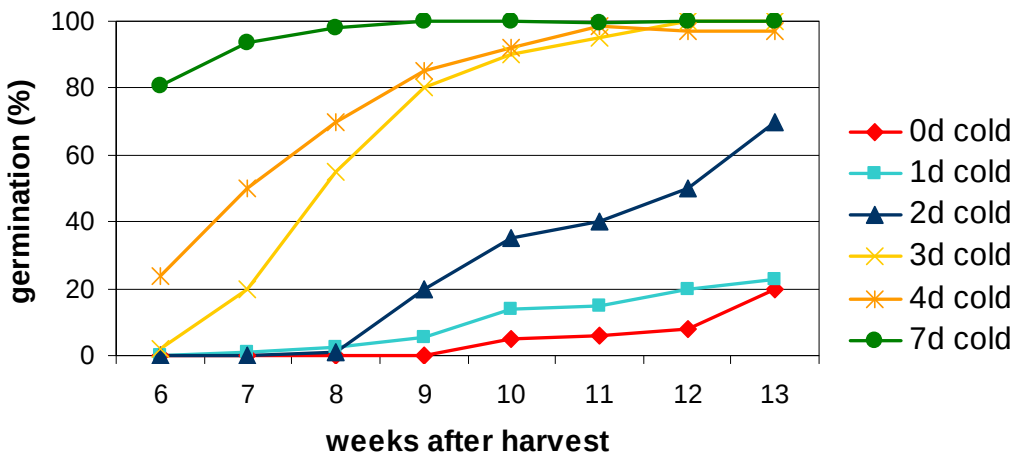

Figure 3.2: Germination response of Cvi seeds to stratification treatments at different stages of after-ripening.
Stratification treatments were performed for 0 to7 days at 4°C.

The first step of the two-step screening strategy was to identify mutants, which did not germinate after a combined after-ripening and 7 days stratification treatment. This same treatment resulted in 100% germination in a non mutagenized control seed batch. To choose the optimal time point for the screening of M2 seeds, continuous germination tests with control seeds and a random number of mutagenized seed batches were performed. At each defined stage of after-ripening, the germination of non-stratified and stratified seeds was compared to distinguish between the dormancy breaking effect of after-ripening and the cold treatment (Figure 3.3). The results of the germination assays of the random M2 seed batches and the control seeds could only be obtained

after the treatment time of 2 weeks (7 d, 4°C, 7d 25°C). Therefore the large scale first selection step was performed at 16 weeks after harvest, even though there was already a slight dormancy release effect of after-ripening itself.

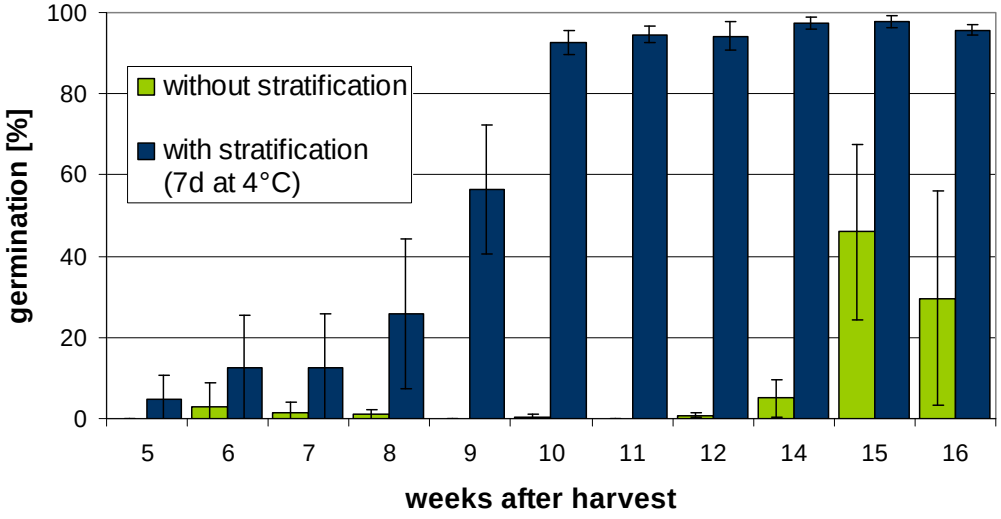

Figure 3.3: Germination response of Cvi control seeds and 5 random M2 seed batches at different time points after seed harvest with and without stratification.
The average of 6 seed batches with standard deviation is shown.

In the first selection step, 60 000 M2 seeds of Cvi were screened at 16 weeks after harvest after stratification for 7 days at 4°C. 620 mutants were selected by embryo rescue. To confirm the non-germinating phenotype of the first selection step, the M3 generation was re-screened at 6 weeks after harvest. At this point, the germination in the control was only promoted by the 7 days stratification treatment leading to 80% germination and 0% germination, when the seeds were not stratified. This difference was sufficient to distinguish cold-insensitive from cold-sensitive lines.

Of the 620 M2 lines, 125 lines with a confirmed phenotype in the M3 generation were selected. These lines germinated between 0 and 10% at 6 weeks after harvest (Table 3.5). 89 of these lines had either weak pleiotropic phenotypes or none at all. Similar to the screening of after-ripening mutants, GA-deficient mutants were expected to be found, because gibberellin promotes germination and GA-deficient mutants are not able to germinate without exogenous gibberellin. In total, 31 mutants with a GA-deficient phenotype were isolated. These mutants showed flowering and elongation growth only when GA was externally applied. Only three loci (*ga1*, *ga2*, *ga3*) lead to this 'non-germinating GA-dwarf' phenotype (KOORNNEEF ET AL., 1980), indicating that the 31 lines are likely all mutants with mutations in these three genes. In addition, five non-germinating dwarf-mutant lines did not show a response to GA application. This is typical for *sly* mutants in which the GA perception is disturbed. The large number of mutants

involved in GA-deficiency indicates that this screen was saturated and should allow for the identification of mutants that are not able to perceive dormancy breaking stratification signals.
The second selection step was used to select the lines with non-germinating phenotypes that were stratification-insensitivity specific and whose dormancy was released by after-ripening. This selection step eliminated the lines with a general germination defect and the lines in which after-ripening does not break dormancy. The lines were tested after 25 weeks of after-ripening when after-ripened non-mutagenized Cvi control seeds reached 100% germination. Several groups of mutants were found based on the degree of germination response to after-ripening 25 weeks after harvest. A group of 39 mutant lines germinated 0%. A second group of 27 mutant lines germinated 1-15%. A third group of 10 mutant lines germinated 30-40%. The fourth group of 13 mutant lines germinated 58-91% (Table 3.3). Groups 1-3 were eliminated, group 4 was further characterized.

Table 3.3: M3 mutant lines with stratification-insensitivity and dormancy release response to after-ripening.
A: Non-germinating phenotype at the first selection step (6 WAH, 7d 4°C) at a timepoint when wild type seeds germinated 80%. B: Germination phenotype at the second selection step (25 WAH) at a timepoint when wild type seeds germinated 100%. WAH: weeks after harvest.

Mutant line (#)	A germination (%)	B germination (%)
104.2	9	91
273.1	4	90
184.1	0	83
172.2	1	81
184.7	1	70
165.1	4	67
105.1	1	67
187.5	1	66
17.1	2	65
188.5	5	57
243.5	0	62
253.1	0	58
297.3	6	58

M4 seeds of the lines of the fourth group were retested by germination assays during a continuous time period to characterize their germination response to after-ripening in comparison to the combined treatment of after-ripening and stratification. Of the 13 lines, 10 lines showed a delayed dormancy release response to stratification. The germination graphs of four typical lines with delayed stratification response are shown in Figure 3.4. While these lines were initially selected at 6 weeks after harvest as being stratification-insensitive when given a sufficient period of time, they were able to germinate in response to stratification, indicating that they were only delayed. The mutant line 172.2 showed the earliest and fastest dormancy release by stratification, lines 253.1, 165.1 and 105.1 showed a slower stratification response. The response to stratification of line 105.1 was detected between 11 and 19 weeks after harvest, but especially slow and weak. In contrast to these lines with a delayed stratification reaction, three lines

(Figure 3.5) showed a stable stratification-insensitive phenotype. Despite this stratification-insensitivity, dormancy in these lines is released during after-ripening. However, they required a longer period of seed dry storage compared to non mutagenized Cvi seeds.

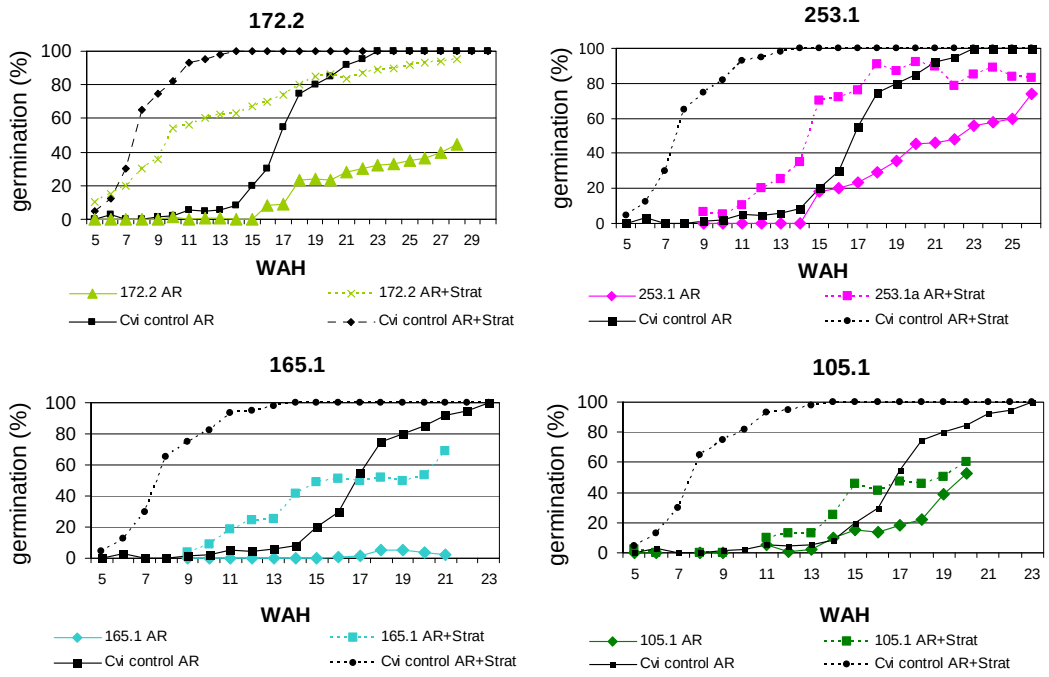

Figure 3.4: Germination response of M4 mutant lines with a delayed response to stratification.
WAH: weeks after harvest, AR: after-ripening period, AR+Strat: combined treatment of after-ripening and 7 d stratification at 4°C.

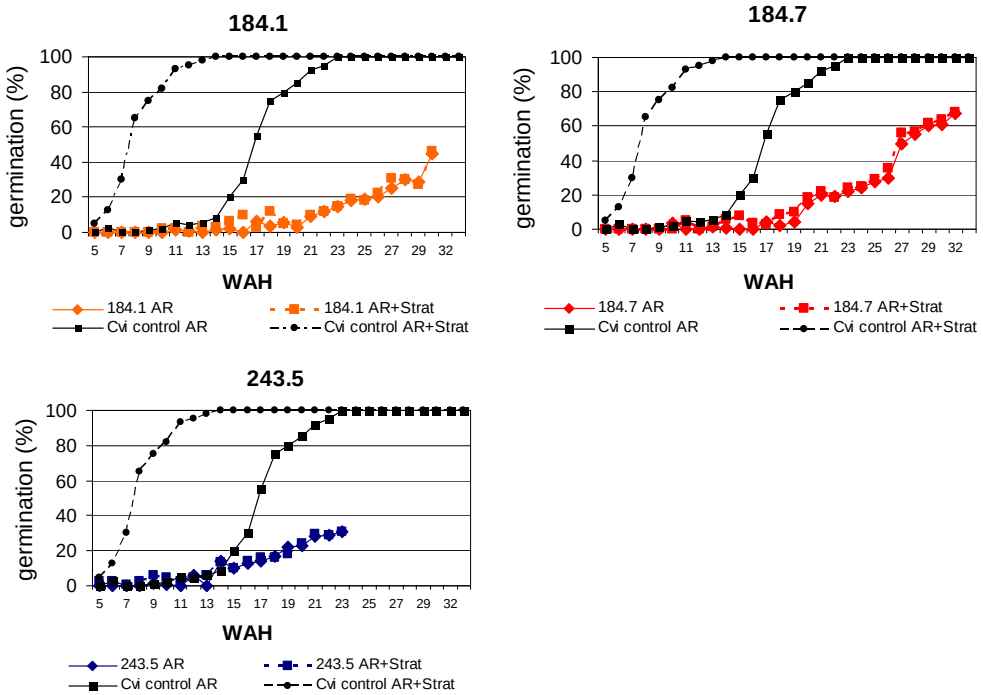

Figure 3.5: Germination response of M4 stratification-insensitive mutant lines.
Mutant lines with stable stratification-insensitive phenotype and prolonged after-ripening requirement. WAH: weeks after harvest, AR: after-ripening period, AR+Strat: combined treatment of after-ripening and 7 d stratification at 4°C.

The stratification-insensitive lines 184.1 and 184.7 showed an additional growth phenotype. The mutant plants grew more vigorously leading to taller plants with enlarged rosette leaves and a higher amount of siliques (Figure 3.6, A). Mutant lines 243.5 and 105.1 were both delayed in flowering compared to Cvi (Figure 3.6, B and C, respectively).

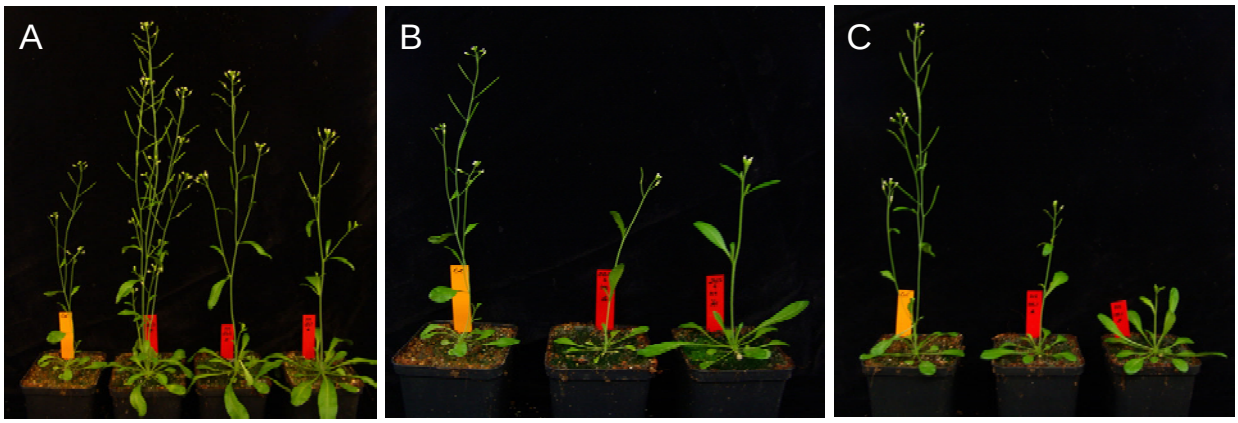

Figure 3.6: Growth phenotype of the mutants 184.1, 184.7, 243.5 and 105.1.
A: 184.1 and 184.7 show vigorous growth and enlarged rosette leaves, B: 243.5 shows delayed flowering, C: 105.1 shows delayed flowering. Plants with orange labels on the left of each picture are Cvi controls, plants with red labels are 2-3 independent M4 plants of the respective mutant.

To unravel whether the stratification-insensitive phenotypes of these three mutant lines and the weak stratification response of line 105.1 resulted from independent mutations in the same gene or in different genes, complementation tests were carried out. The four lines were crossed to each other and the F1 seeds arising from each cross were tested 13 weeks after harvest after 7 days of stratification. F1 seeds of crosses between the lines 184.1 and 184.7 did not germinate, indicating that they are allelic. The lines 243.5 and 105.1 were both able to complement the stratification insensitive-phenotypes of 184.1 and 184.7 (Table 3.4), indicating that line 243.5 and line 105.1 are not allelic to the lines 184.1 and 184.7. To determine if the lines 243.5 and 105.1 are independent mutations in different genes, crosses between the two lines were made and are a part of ongoing analysis.

Table 3.4: Complementation test among the stratification-insensitive mutant lines.
F1 seeds of crosses between different mutant lines were tested 13 weeks after harvest after 7 days of stratification.

Combination of mutant lines	Germination (%)
184.1 x 184.7	0
184.7 x 184.1	0
184.1 x 105.1	91
184.1 x 243.5	81
243.5 x 184.7	80

3.1.5 Overview of obtained dormancy mutants

In the low-dormancy screen, two new putative non-dormant mutants were found and three new alleles of *dog1* were identified (Appendix A 10).

The after-ripening screen in the genetic backgrounds of NIL *DOG1* and L*er* did not lead to after-ripening specific mutants.

The stratification screen required a complex screening strategy and produced three independent mutant lines, whose dormancy cannot be broken by stratification, but which are able to germinate after a prolonged period of after-ripening.

In addition, the obtained phenotypic classes of the stratification screen included a class of germination-deficient mutants, which did not show pleiotropic growth phenotypes. This class might also include after-ripening deficient mutants and provides an additional source of mutants for follow up work.

Table 3.5 gives an overview of all of the mutant phenotypes yielded from the different screenings.

Table 3.5: Overview of mutants from genetic screens for novel dormancy mutants.
Phenotypes indicated with * were confirmed by allelism test with the respective mutant. Mutants which were not tested by allelism test but which showed physiologically characteristic phenotypes corresponding to respective mutants were indicated with **. Examples of the general class of pleiotropic phenotypes *** resulting from the screen for after-ripening mutants are shown in Figure 3.1. The non-dormant mutant line that was isolated by Raz, 1999 (CLERKX ET AL., 2004) in Columbia background is indicated with ****.

Genetic background	Screen for	Phenotype	Number of mutant lines
NIL *DOG1*	non-dormant mutants	*aba1**	3
		*dog1**	1
		WT	2
Cvi	non-dormant mutants	*abi* -phenotype**	3
		*tt10**	1
		*dog1**	2
Col****	non-dormant	*dog1**	1
NIL *DOG1*	after-ripening mutants	GA-deficient phenotype**	2
		pleiotropic***	8
L*er*	after-ripening mutants	pleiotropic	4
Cvi	stratification mutants	GA-deficient phenotype, GA-responsive**	31
		sly1 , non-responsive to GA**	5
		germination-deficient	77
		delayed stratification response	9
		stratification-insensitive, dormancy release by prolonged after-ripening	3

3.2 Molecular and biochemical characterization of the seed dormancy gene *DOG1*

Although significant progress has been made in understanding seed dormancy, recent reviews still concluded that it is "one of the least understood phenomena in the field of seed biology" (BEWLEY, 1997a; FINCH-SAVAGE AND LEUBNER-METZGER, 2006; FINKELSTEIN ET AL., 2008). This emphasizes the importance of identifying novel genes with a role in seed dormancy. Functional analysis of these genes will contribute to a significant increase in the understanding of seed dormancy. The present study focused on the recently cloned key dormancy gene *DELAY OF GERMINATION 1* (*DOG1*). The expression pattern of *DOG1* is seed specific and loss of function mutants in several genetic backgrounds completely lack dormancy. *DOG1* encodes a protein with unknown biochemical function and does not contain domains of known function. Different splicing variants for the *DOG1* mRNA have been detected, however the significance of this alternative splicing has not yet been shown (BENTSINK ET AL., 2006). Furthermore, localization and the molecular function of the *DOG1* transcript and protein variants are unknown. The present study endeavored to find the initial answers to these questions by using molecular genetic, biochemical and transgenic approaches.

3.2.1 Localization studies

Previous expression studies showed that *DOG1* is only expressed during seed maturation and in dry seeds (BENTSINK ET AL., 2006; Figure 1.5). To obtain a more precise overview of the temporal and spatial *DOG1* expression pattern, the putative *DOG1* promoter was fused to two different reporter genes, endoplasmatic reticulum localized GFP (ER-GFP) and GUS. These constructs were transformed into *dog1-1* plants. Expression of the reporter genes was analyzed in different tissues and at different timepoints. In addition, RNA in-situ hybridization experiments were carried out to substantiate the results of the promoter fusion analysis.

3.2.1.1 *DOG1* promoter-reporter gene fusions

To gain knowledge about possible differences in the degree and localization of expression resulting from differences in the *DOG1* promoter activity between accessions, promoter sequences of the low dormant accession L*er* and the strong dormant accessions Cvi and Kashmir (Kas-2) were used (Figure 3.7). The main sequence difference in the putative *DOG1* promoter region between the three accessions was a 285 bp insertion present in L*er*.

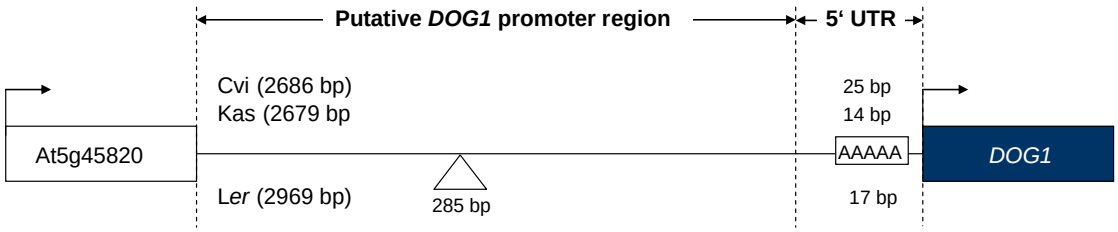

Figure 3.7: Putative *DOG1* promoter region of the accessions Cvi, Kas and L*er* used for the analysis of promoter::reporter gene fusions.
The triangle represents the 285 bp insertion present in L*er*. The box with AAAAA represents a polyA-stretch that is part of the 5' UTR which differs in length between the three accessions.

*DOG1*promoter-ER-GFP fusion constructs

For the first set of constructs, the *DOG1* promoters of each of the three accessions were fused with ER-GFP. Since GFP is able to move through the plasmodesmata from its initial site of transcription in young embryos (KIM ET AL., 2005), a fusion construct with ER-localized GFP (ER-GFP) was used to identify the original cells in which gene expression occurs. Fresh mature dry seeds from three independent T2 transformants for each promoter construct were used for the analysis of GFP fluorescence to exclude the influence of the transgene insertion position on promoter activity. GFP fluorescence was detected in the entire embryo, especially in the cotyledons and in the root tips of the isolated embryos of fresh mature seeds. Driven by the Kas and Cvi *DOG1* promoter, GFP showed a stronger fluorescence than under the control of the L*er* *DOG1* promoter (Figure 3.8). However, no significant GFP signal was observed in isolated embryos of the T3 generation at earlier timepoints during seed development when *DOG1* expression was expected to be highest (15 days after flowering). This might be caused by silencing of the transgene, but no proof for this was obtained by further experiments.

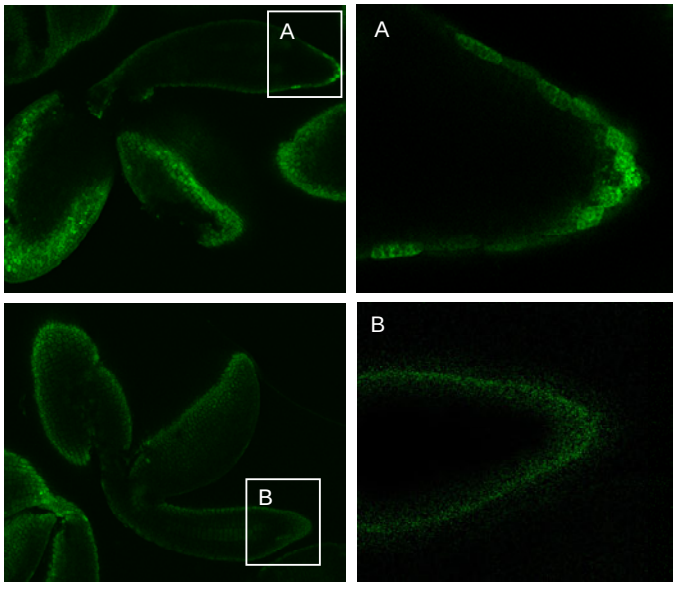

Figure 3.8: Expression pattern of ER-GFP driven by the native *DOG1* promoter from Cvi, Kas and L*er* in isolated embryos of fresh mature seeds from T2 transgenic plants.
Top row: isolated embryo from reporter lines with *pDOG1_Kas::ER-GFP*, A: magnified root tip. Bottom row: isolated embryo from reporter lines of *pDOG1_Ler::ER-GFP* construct, B: magnified root tip. Transgenic lines with *pDOG1_Cvi::ER-GFP* showed the same pattern and comparable signal intensity to *pDOG1_Kas::ER-GFP*.

*DOG1*promoter-GUS fusion constructs

In addition to the ER-GFP reporter constructs, promoter fusions with GUS were created. T2 plants of all three promoter constructs were analyzed by histochemical assay of GUS activity. The spatial expression pattern was determined by analysis of different plant tissues. The temporal seed-specific expression pattern was additionally analyzed by GUS staining of isolated embryos during a time course of 10 to 20 days after flowering (DAF). This time period covered the second half of seed development, which is the phase of seed maturation when seed dormancy is gradually induced (Figure 1.1, p. 2). Three independent lines per *DOG1* promoter-reporter gene construct were used for histochemical analysis (Figure 3.9). This revealed that GUS activity was present in T2 plants of all three promoter constructs in the entire embryo as well as in the endosperm (tested from 14 to 17 DAF). Although it was known that *DOG1* expression is seed-specific, and this was confirmed by the lack of GUS activity in leaves, seedlings, inflorescences and silique pods, this study gave new insights into the expression pattern of *DOG1* in the different compartments of the seed.

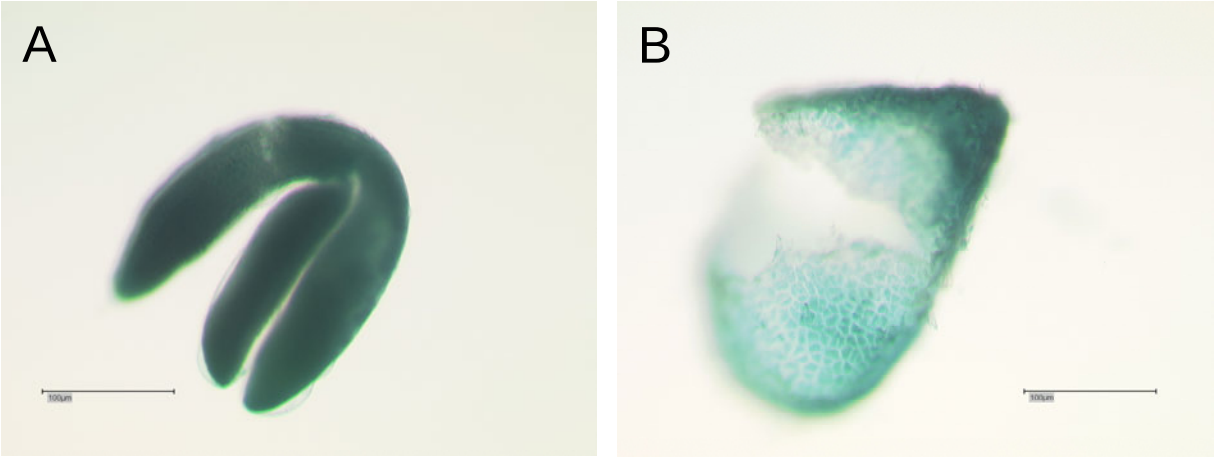

Figure 3.9: Histochemical localization of GUS activity of *dog1-1* plants transformed with the *pDOG1_Cvi::GUS* construct.
A: GUS activity detected in isolated embryos (15 DAF), B: GUS activity observed in the endosperm (15 DAF). DAF: days after flowering. Bar = 100 µm.

To follow the temporal expression pattern, 20 isolated embryos derived from three siliques of three independent T2 lines from all three promoter constructs were used, which allowed for a semi-quantitative analysis of GUS activity. The 20 isolated embryos were classified after GUS staining depending on the intensity of the GUS signal: white embryo, partially blue embryo and fully blue embryo (Figure 3.10, A). GUS activity was first detectable 12 DAF and increased to a maximum between 16 and 18 DAF, followed by a significant decrease at 20 DAF. This confirmed the results of the *DOG1* expression analysis (BENTSINK ET AL., 2006; Figure 1.5,

p. 12). Although solid quantitative conclusions cannot be drawn, there is a tendency that GUS expression driven by the L*er DOG1* promoter is weaker than expression driven by the Cvi and Kas *DOG1* promoters, which is in agreement with the results of the *DOG1* promoter-ER-GFP reporter lines (Figure 3.8). In addition, the GUS signal in transgenic lines with the Kas *DOG1* promoter seemed to be maintained longer at the end of seed maturation. However, these lines were only analyzed in the T2 generation in which the lines were still heterozygous. This data is only a preliminary description of the temporal expression pattern. To describe the temporal expression pattern quantitatively, a newly developed quantitative RT-PCR assay, which is described in chapter 3.2.2, was used. Therefore these lines were not further characterized.

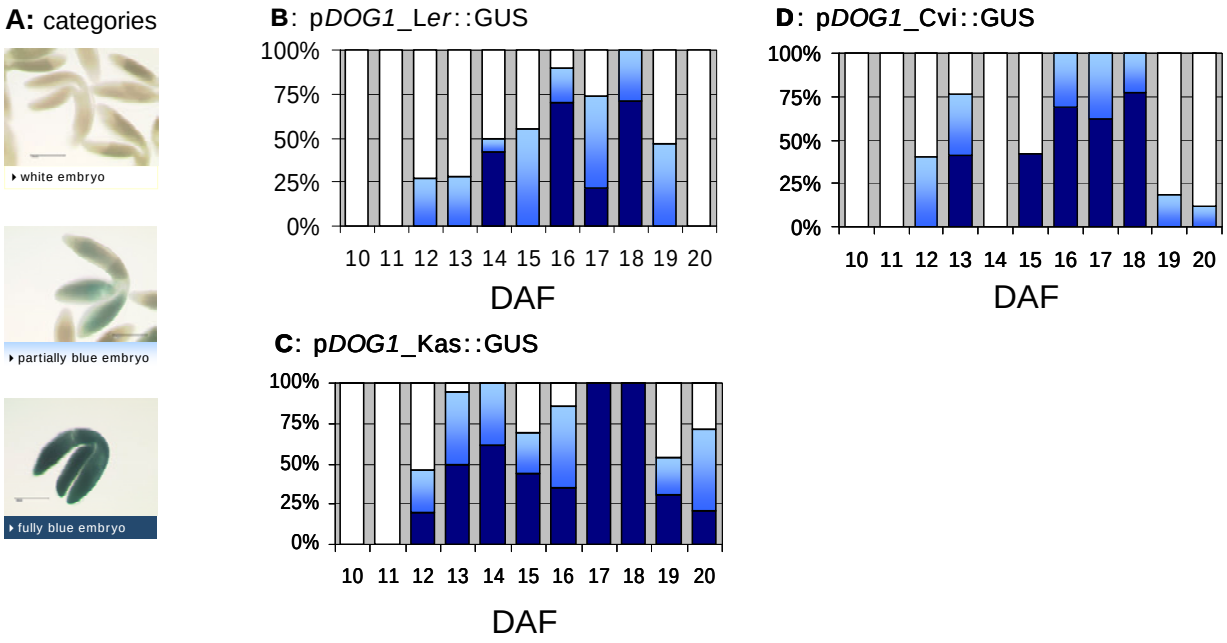

Figure 3.10: Temporal expression pattern of the reporter gene GUS driven by the *DOG1* promoter from L*er* (B), Kas (C) and Cvi (D).
GUS activity is represented semi-quantitatively showing the percentage of embryos that were classified in one of three groups (A) no GUS activity (white embryo), medium intensity of GUS activity (partially blue embryo) or strong GUS activity (fully blue embryo). There was no GUS activity detected in siliques of transgenic plants with the p*DOG1*_Cvi::GUS construct at 14 DAF. This timepoint was judged as an outlier. At every timepoint 20 embryos were analysed for GUS activity.

3.2.1.2 RNA in-situ hybridization with *DOG1* probe

The analysis of promoter-reporter gene fusions is one of the most widely used techniques for identifying the temporal and spacial expression patterns of cloned genes. However, promoter fusions are an insufficient measure of gene expression because they can be prone to artificial expression that does not necessarily reflect the in vivo regulation of the gene (TAYLOR, 1997). To study the localization pattern of the *DOG1* transcripts, RNA in-situ hybridization with an antisense *DOG1* probe containing nucleotides 197-575 relative to the ATG of the cDNA was

performed in collaboration with S. Schulze (MPIZ). Fixed siliques of the strong dormant accession Cvi at 14 DAF were used. *DOG1* transcripts were highly accumulated in the shoot apical meristem of the embryo as well as in the vascular system extending into the cotyledons and the hypocotyl (Figure 3.11).

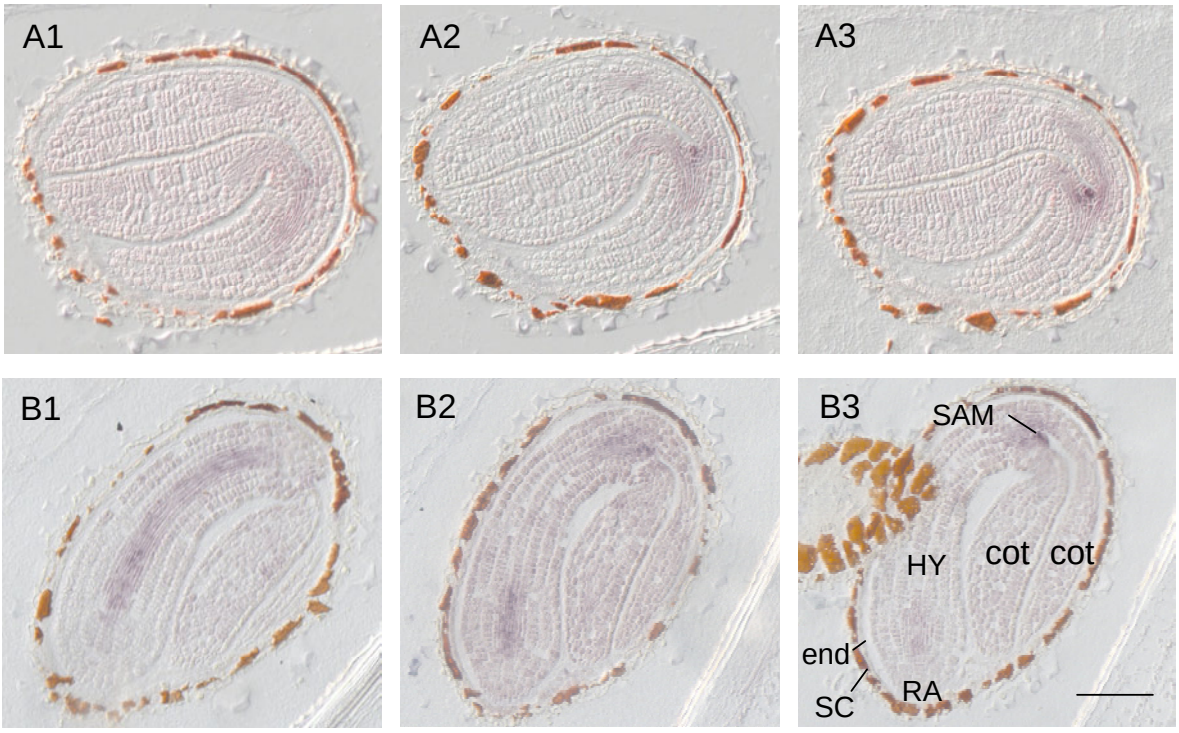

Figure 3.11: In-situ hybridization analysis of *DOG1* mRNA in Cvi seeds at 14 DAF.
A and B show serial cuttings with longitudinal sections of two independent Cvi seeds at 14 DAF hybridized with antisense *DOG1* RNA probe. In-situ hybridizations were performed by S. Schulze (MPIZ). DAF, days after flowering. end, endosperm layer; cot, cotyledon; HY, hypocotyl; RA, radicle; SAM, shoot apical meristem; SC, seed coat. Bar = 100 µm.

3.2.1.3 Subcellular localization of the DOG1 protein variants

Localization of DOG1 protein variants in transgenic Arabidopsis plants

To examine the subcellular localization of the different DOG1 protein variants, translational fusions with soluble-modified green-fluorescent protein (smGFP, DAVIS AND VIERSTRA, 1998) were produced. SmGFP was fused to the 3' end of the alpha and delta DOG1 protein variants and the resulting DOG1::smGFP fusion was expressed under the control of the endogenous *DOG1* promoter from Cvi. The constructs were transformed into *dog1-1* plants. The transformation efficiency with these constructs was very low. Only 16 T1 transformants with the *pDOG1::alphaDOG1::smGFP* and 3 T1 transformants with the *pDOG1::deltaDOG1::smGFP* construct were obtained. These lines showed a non-dormant phenotype in the T2 generation. The presence of the transgene was confirmed by PCR. However, GFP could not be detected in

isolated embryos and endosperm of siliques during seed maturation and in fresh mature seeds. This might be caused by instability of the fusion protein in planta, leading to degradation. It is also possible that the C-terminus of the DOG1 protein has a crucial role in the function or regulation of the protein, which is interfered with by the fused smGFP. Generating additional translational fusions with an N-terminal smGFP fusion would adress the problem of possible interference with the C-terminus. Furthermore, fusion protein levels could be increased by using a constitutive promoter such as the 35S promoter.

Transient expression of DOG1 protein variants in *N. benthamiana* leaves

Because the subcellular localization of the DOG1 protein variants was not determined in stable transformants, DOG1 protein variants C-terminally fused to smGFP under the control of the 35S promoter were expressed in *Nicotiana benthamiana* leaves using an *Agrobacterium*-mediated transient expression assay. All three DOG1 protein variants (α, β, δ DOG1) showed the same subcellular distribution pattern and were detected mainly in the nucleus, but also to a lesser degree in cytoplasmatic strands (Figure 3.12 A, B). Confocal images additionally indicated that the protein variants might also localize in the plasma membrane or the tonoplast, the vacuolar membrane (Figure 3.12 C, D). Localization either at the tonoplast or the plasma membrane is depicted by a visible cell-wall space between two neighboring cells. Organelle markers (NELSON ET AL., 2007) for the plasma membrane and the tonoplast are required to distinguish between these two possibilities.

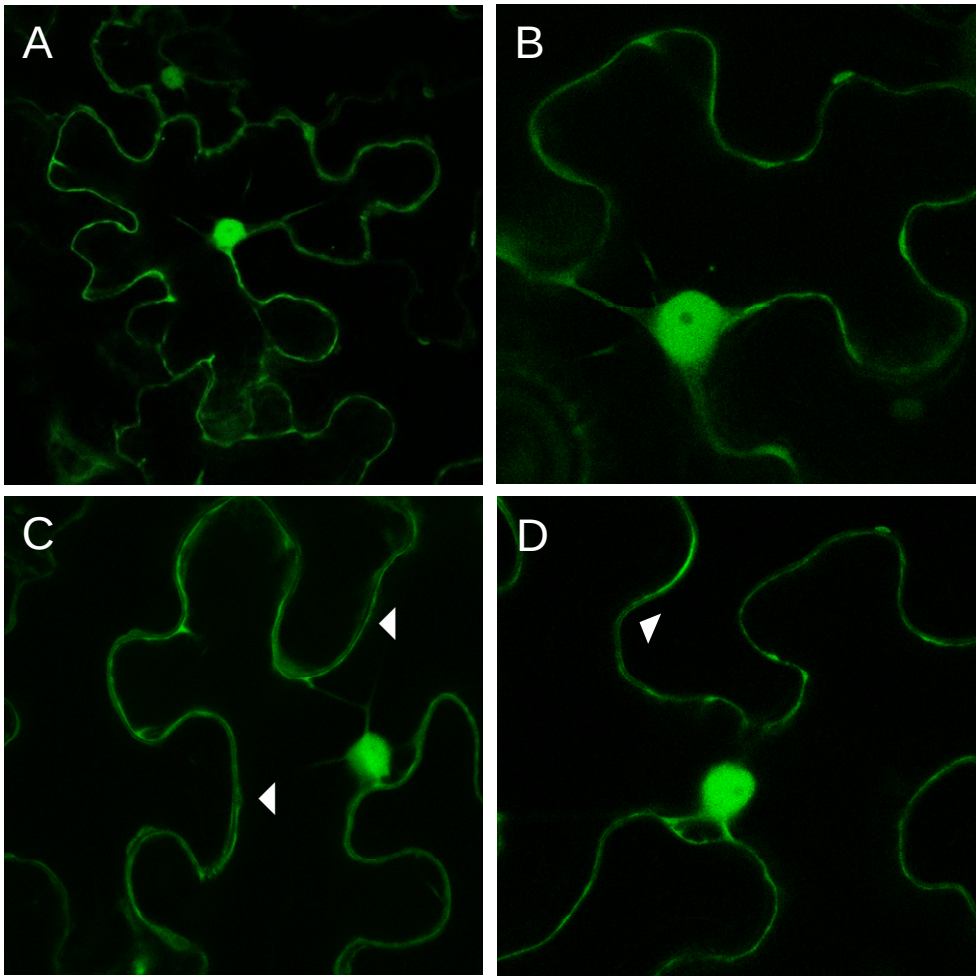

Figure 3.12: Subcellular distribution of α-, β/γ- and δ-DOG1 proteins.
Confocal images of *N. benthamiana* leaves transiently overexpressing *α-DOG1::smG*FP, *β/γ-DOG1::smGFP* and *δ-DOG1::smGFP*. A shows a complete cell with a strong GFP signal in the nucleus and a weaker signal in the cytoplasm, B shows a magnified cell compartment with cytoplasmatic strands. C and D suggest that the protein variants might also be localized at the plasma membrane or the tonoplast (indicated with arrows). Pictures represent the distribution of all three DOG1 protein variants.

3.2.2 Differential expression analysis of *DOG1* splicing variants during seed development

Alternative splicing is an important posttranscriptional regulatory mechanism that can increase protein diversity and may affect mRNA stability. Recent reviews emphasized that alternative splicing in plants occurs on a much wider scale than previously thought. To date, only a few alternative splicing events have been identified experimentally in plants. These include genes involved in splicing, transcription, flowering regulation, disease resistance and enzyme activities (Chapter 1.5) (MODREK AND LEE, 2002; JORDAN ET AL., 2003; KAZAN, 2003). *DOG1* is alternatively spliced, however it is not clear if alternative splicing is important for the *DOG1* function.

Pyrosequencing assays to quantify the relative abundance of the different transcripts in mature dry seeds revealed that there are different relative levels of the four splicing variants of *DOG1*. However, these relative levels did not differ significantly between several accessions (BENTSINK ET AL., 2006). This raised the question of whether alternative splicing of *DOG1* functions as a regulatory mechanism during dormancy induction and release. Therefore, the transcript levels of the different splicing variants were analyzed during seed development.

3.2.2.1 Experimental setup of the qRT-PCR assay

A set of transcript-specific primers has been designed, of which each reverse primer anneals in a overlapping region of two specifically combined alternatively spliced parts (Figure 3.13). PCR conditions were optimized to maximize stringency. In PCR reactions with plasmid DNA, which included mixtures of alpha, beta and delta *DOG1* in different ratios, these primer sets only amplified the specific transcript for which they were designed (data not shown). cDNA synthesis and real-time PCR conditions were optimized to perform quantitative RT-PCR based on SYBR® green detection. Melting curve analysis confirmed that the primers did not produce primer dimers. Further details of the procedure are described in chapter 2.2.4.9.

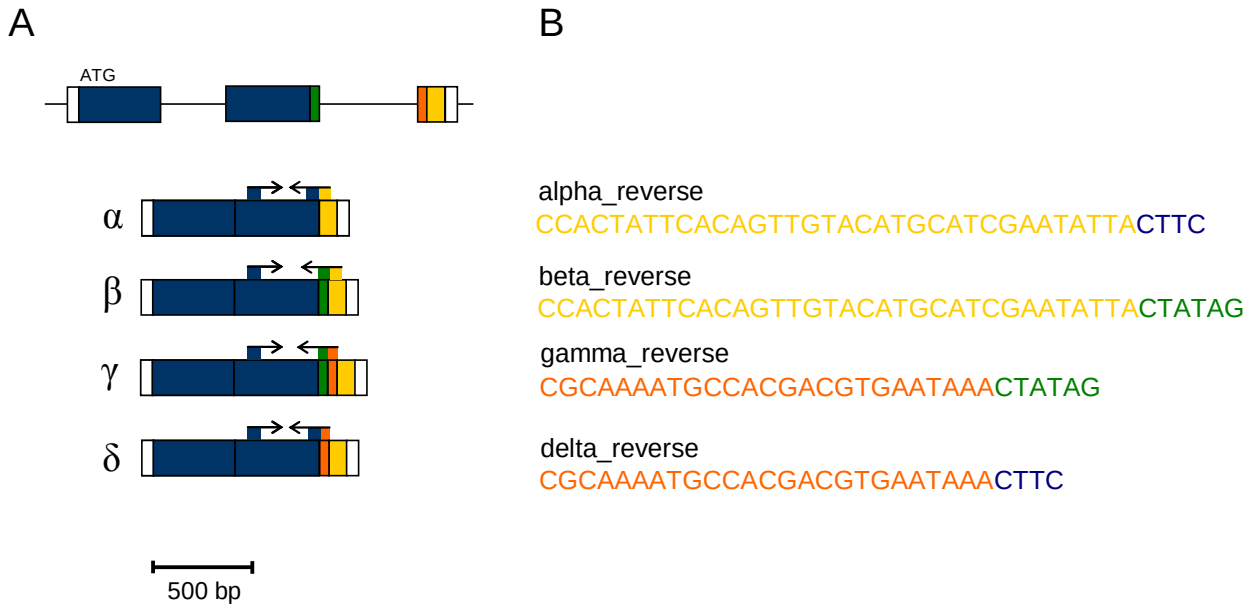

Figure 3.13: Schematic representation of the four different *DOG1* splicing forms with annealing positions of the primer pairs used for qRT-PCR (A) and sequences of the transcript-specific reverse primers (B).
The white boxes represent the 5' and 3' UTR. The colored boxes represent the different exons. Primer sequences are shown in 5' to 3' direction.

The genotypes L*er* and NIL *DOG1* were used as plant material for quantitative RT-PCR. Total RNA was extracted from siliques harvested in a time course from 8 to 19 DAF, covering the final days of embryogenesis and the complete seed maturation phase, during which seed

dormancy is induced (Figure 3.14). Seed development of the two genotypes proceeded simultaneously and required 19 days from pollination to the end of seed maturation, at which time the seeds were desiccated and completely mature. In addition to these time points, fresh mature dry seeds directly after harvest and fresh mature seeds that were imbibed for 24 hours in light were used.

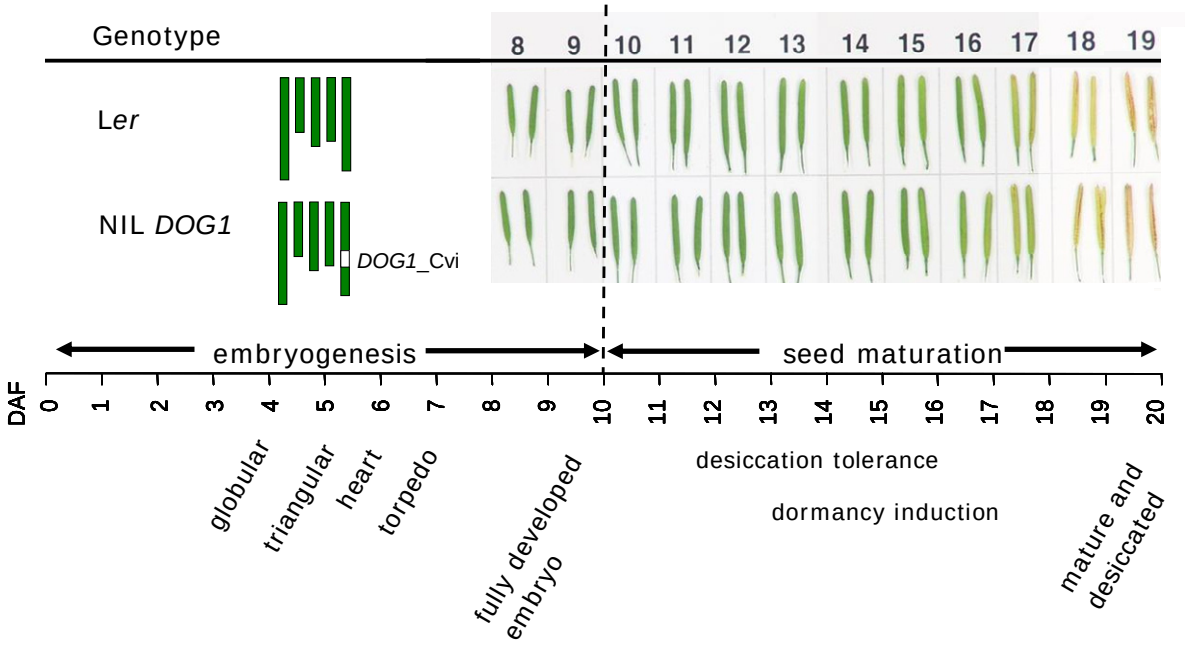

Figure 3.14: Siliques harvested from L*er* and NIL *DOG1* throughout seed development and used for qRT-PCR.
The schematic time course shows the entire phase of seed development which lasts for 20 days from pollination to the end of seed maturation under standard conditions. The genotypes L*er* and NIL *DOG1* grown in climate chambers at 22°C day temperature/16°C night temperature with 16 h light completed seed development in 19 days.

3.2.2.2 Expression levels of *DOG1* splicing variants during seed maturation and imbibition

Quantitative RT-PCR using L*er* and NIL *DOG1* in two biological replicates demonstrated that the four splicing forms of *DOG1* reached maximum levels of expression in the mid-stage of seed maturation between 14 and 17 days after flowering. The expression levels decreased toward the end of seed maturation and completely disappeared during imbibition (data shown for one representative biological replicate of NIL *DOG1*, Figure 3.15). This confirmed the expression pattern that was obtained by Northern blot analysis (BENTSINK ET AL., 2006, Figure 1.5). Low levels of *DOG1* expression could first be detected just before the beginning of the seed maturation phase. This indicated that *DOG1* is likely to play a role during seed maturation. The alpha splicing form was the major variant throughout this phase representing about 85% of the total expression level.

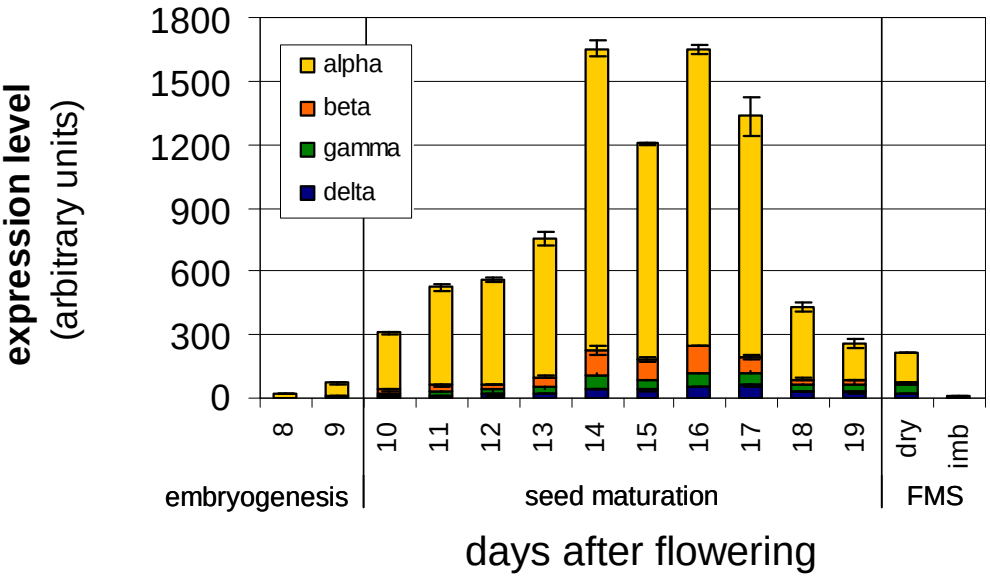

Figure 3.15: Expression pattern of *DOG1* splicing variants during seed maturation and imbibition.
Transcript levels of each splicing variant during seed maturation, and of dry and 24h imbibed fresh mature seeds in one representative transcription experiment of NIL *DOG1* are shown. The values were normalized by the housekeeping gene *ACTIN8*. Error bars indicate the standard deviation of duplicated measurements. FMS, fresh mature seeds directly after harvest; imb, 24h imbibed seeds.

Analyzing the relative abundance of each splicing form at each time point showed that the relative amount of each splicing form stayed stable between 8 and 17 days after flowering, but changed during the latest stages of seed maturation, when seeds became desiccated and mature. At 19 days after flowering and in dry seeds the gamma and delta *DOG1* splicing forms were relatively higher.

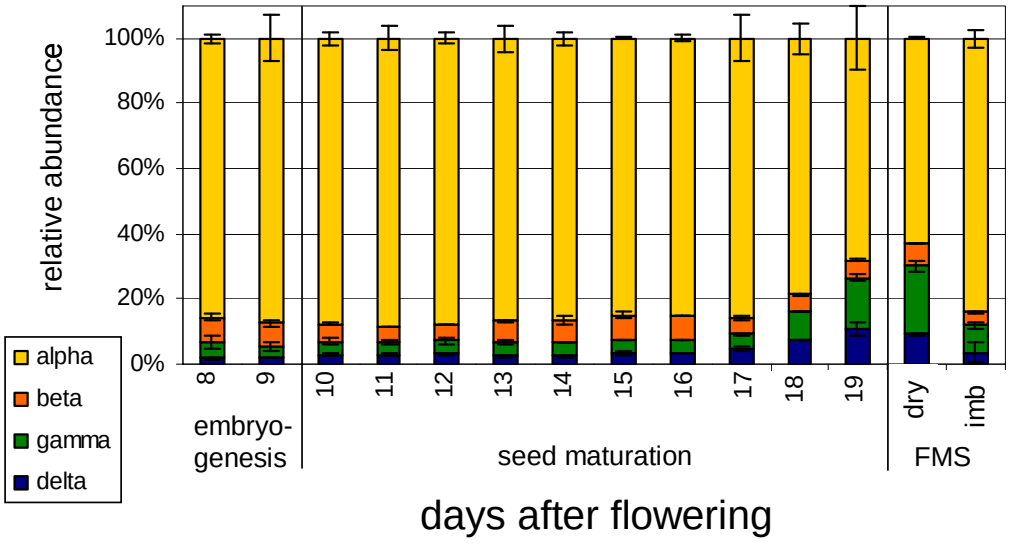

Figure 3.16: Relative abundance of *DOG1* splicing variants during seed maturation and imbibition.
Transcript level of each *DOG1* splicing variant is shown as relative amount at each sampling point. Values represent the average and standard deviation of a duplicated qRT-PCR assay of one representative biological replicate of NIL *DOG1*. Values were normalized by *ACTIN8*. FMS, fresh mature seeds directly after harvest; imb, 24h imbibed seeds.

3.2.2.3 Comparison of *DOG1* transcript levels between L*er* and NIL *DOG1*

Comparing the transcript levels of the four *DOG1* splicing variants between L*er* and NIL *DOG1* revealed that expression levels in both genotypes were similar until the early-stage of seed maturation (13 DAF). However, higher expression was observed in NIL *DOG1* compared to L*er* in the mid-stage of seed maturation, and this difference remained apparent until the last stage of maturation (19 DAF). The difference of *DOG1* expression levels between L*er* and NIL *DOG1* correlated with the different dormancy levels of the two genotypes (Figure 3.17) and are in agreement with the GUS patterns observed in transgenic promoter-reporter lines with the *DOG1* promoters of L*er* and Cvi.

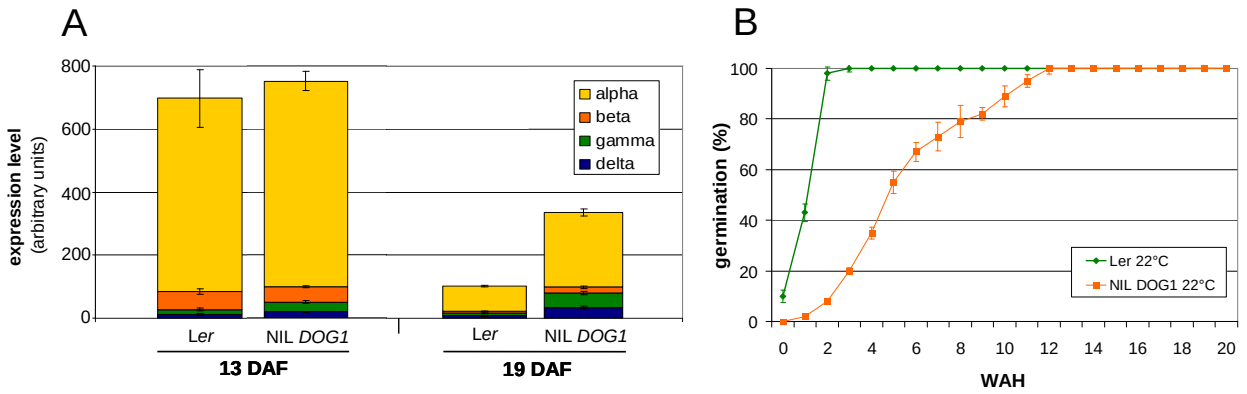

Figure 3.17: Comparison of *DOG1* transcript levels and dormancy between L*er* and NIL *DOG1*.
A: Transcript levels of each splicing variant in L*er* and NIL *DOG1* at 13 and 19 DAF. Values were normalized by *ACTIN8*. DAF, days after flowering. B: Germination phenotype of the low dormant accession L*er* and the strong dormant NIL *DOG1* at different timepoints after seed harvest (average of 10 plants with standard deviation). WAH, weeks after harvest.

3.2.2.4 Effect of temperature on *DOG1* expression

To study the effect of temperature on *DOG1* expression, the genotypes L*er* and NIL *DOG*1 were grown at 22°C and 15°C. Seed development at 22°C was completed after 19 days (Figure 3.14); at 15°C seed development was not completed until 32 days. To circumvent this developmental variation, siliques at the last stage of seed maturation were used as comparable samples (19 DAF, Figure 3.14). The accumulation of *DOG1* transcripts in L*er* was higher when plants were grown at 15°C (Figure 3.18, A). Similar results were obtained for NIL *DOG1*. The ratios of the different splicing forms did not change in the different growth environments. This implied that *DOG1* expression can be regulated by temperature. In addition, temperature had an effect on the dormancy level. Seeds developed at lower temperatures showed an increased dormancy level (Figure 3.18, B). Thus, higher *DOG1* expression correlated with a higher dormancy level, both when comparing between genotypes and when comparing between growth temperatures.

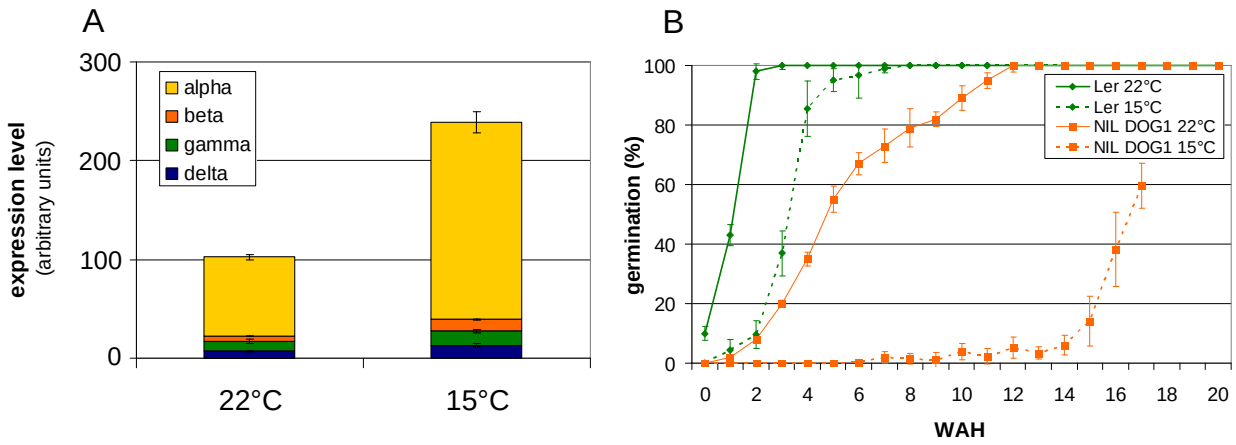

Figure 3.18: Effect of temperature on *DOG1* expression and dormancy level of L*er* and NIL *DOG1*.
A: Transcript levels of each splicing variant at the last stage of seed maturation from L*er* plants grown under standard (22°C) and low (15°C) temperature conditions. Values were normalized by *ACTIN8*. Similar results were obtained for NIL *DOG1*. B: Germination phenotype of L*er* and NIL *DOG1* at different timepoints after harvest, plants were grown at 22°C or 15°C (average of 10 plants with standard deviation). WAH, weeks after harvest. Germination assay of NIL *DOG1* at 15°C is only shown until submission of this thesis and will be continued until germination reaches 100%.

3.2.3 Analysis of DOG1 protein

A polyclonal antibody was raised against a unique region of the DOG1 protein comprising the first 151 amino acids (Chapter 2.2.5.1). It was determined in a first antiserum check that a 1:10 000 dilution of the antibody was able to detect about 10 ng of recombinant antigen protein using alkaline-phosphatase detection. Analysis of DOG1 protein was performed on Western blots (in collaboration with K. Nakabayashi, MPIZ) using soluble proteins from fresh mature seeds of L*er*, NIL *DOG1* and *dog1-1*. DOG1 accumulation was higher in NIL *DOG1* compared to L*er*, which was consistent with the transcript level (Figure 3.17). No protein, not even a truncated protein, was detected in the *dog1-1* mutant, which has a premature stop-codon. This confirmed the specificity of the antiserum to DOG1.

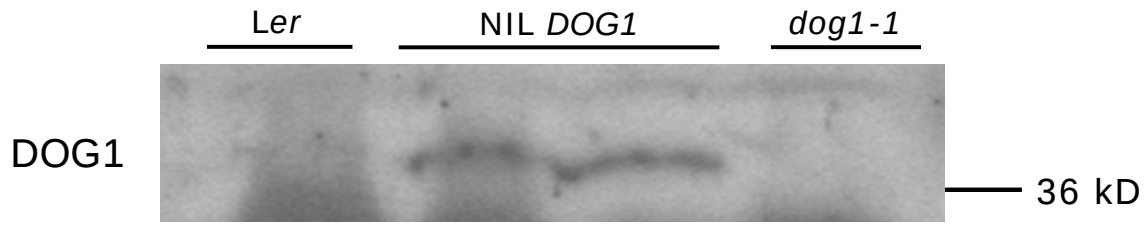

Figure 3.19: DOG1 protein accumulation in fresh mature dry seeds in the genotypes L*er*, NIL *DOG1* and *dog1-1*.
Immunoblot of soluble proteins from fresh mature seeds probed with anti-DOG1 antibody. The two lanes of NIL *DOG1* represent duplicates.

The presence and amount of DOG1 protein will be analyzed in future experiments by immunoblot analysis at different timepoints during seed development and storage and under different environmental conditions.

To enable protein detection using alternative methods, constructs were made that contained fusions of the DOG1 protein variants with a strepII tag driven by a double 35S promoter or by the native *DOG1* promoter from Cvi (Table 2.12). However, the generation of stable transformants with these constructs failed. No primary transformants of constructs with the double 35S promoter were obtained in four independent transformation experiments, but a number of primary transformants with strepII tagged alpha and delta DOG1 protein variants under the control of the native *DOG1* promoter were selected. Genomic DNA was tested and the presence of the transgene was confirmed by PCR. However, these transformants were not resistant to Basta when screened in the T2 generation. This could be caused by silencing of the transgene. Although these plants were not investigated further, the lack of resistance to Basta indicates that C-terminal fusions of the DOG1 protein variants might lead to instability and degradation of the protein or to a higher susceptibility to silencing. These results are similar to those of the C-terminal GFP fusion constructs (Chapter 3.2.1.3),

3.2.4 Functional analysis of *DOG1* splicing isoforms

The functional relevance of the different splicing forms was studied using a transgenic approach. A set of constructs was made with the alpha, beta and delta forms of *DOG1* (Table 2.12). The gamma form encodes the same protein as the beta form of *DOG1*, therefore this transcript was not used for cloning. Sequence analysis of the coding region of *DOG1* from different accessions showed that several polymorphisms exist, caused by indels and SNPs, of which only a few lead to amino acid changes (Figure 3.20). However, a correlation between the sequence and the dormancy level of the different accessions was not observed. To confirm that the L*er* and Cvi alleles of *DOG1* have the same function, both alleles were used for each splicing form to generate constructs.

```
alpha DOG1_Col    1 mgsssknieqaqds-ylewmslqsqripelkqllaqrrshgdedndnklrkltgkiigdfknyaakradlahrcssnyyaptwnsplenaliwmggcrps
alpha DOG1_Ler    1 mgsssknieqaqeccylewmslqsqripelkqllaqrrshgdedndnklreltgkiigdfknyaakradlahrcssnyyaptwnsplenaliwmggcrps
alpha DOG1_Cvi    1 mgsssknteqaqeccylewmslqsqripelkqllaqrrshgdedndnklreltgkiigdfknyaakradlahrcssnyyaptwnsplenaliwmggcrps

alpha DOG1_Col  100 sffrlvyalcgsqteirvtqflrnidgyessgggggaslsdlsaeqlakinvlhvkiideeekmtkkvsslqedaadipiatvayemenvgepnvvvdqa
alpha DOG1_Ler  101 sffrlvyalcgsqteirvtqflrnidgyessgggggaslsdlsaeqlakinvlhvkiideeekmtkkvsslqedaadipiatvayemenvgesnvvvdqa
alpha DOG1_Cvi  101 sffrlvyalcgsqteirvtqflrnidgyess-gggggaslsdlsaeqlakinvlhvkiideeekmtkkvsslqedaadipiatvayemenvgepnvvvdqa

alpha DOG1_Col  200 ldkqeeamarllveadnlrvdtlakilgilspvqggadfllagkklhlsmhewgtmrdrrrrdcmvdtevifdacttvnsgprptettnnern&
alpha DOG1_Ler  201 ldkqeeamarllveadnlrvdtlakilgilspvqggadfllagkklhlsmhewgtmrdrrrrdcmvdtevifdacttvnsgprptettnnern&
alpha DOG1_Cvi  200 ldkqeeamarllveadnlrvdtlakilgilspvqggadfllagkklhlsmhewgtmrdrrrrdcmvdtevifdacttvnsgqrptettnnern&
```

Figure 3.20: Amino acid sequence alignment of Col, L*er* and Cvi alleles of alpha DOG1.

3.2.4.1 Inducible expression of *DOG1* splicing variants

In order to study when *DOG1* expression is required during seed development for dormancy induction, a ß-estradiol inducible *DOG1* system was developed for alpha, beta and delta *DOG1*. A ß-estradiol inducible GFP construct was shown to induce GFP expression in seedlings that were grown on inductive medium. The response to induction was first detectable 30 min after transfer onto inducible medium (ZUO ET AL., 2000). However, it was not yet shown, if this inducible system is also functional in adult plants and during seed development. A modified pER8 vector, which carries the Basta-resistance gene and inducible GUS expression, was used for *DOG1*. The GUS expression allows for visualization of any leakyness of gene expression, which could occur without induction. In addition, a similar inducible system with *ABA1* was designed to examine the effects of inducible *ABA1* expression during different stages of seed development. The constructs were transformed into *dog1-1* and *aba1-1* plants, respectively, and non-leaky homozygous single-insertion lines were selected.

A pilot study was performed to test the functionality of the inducible system in adult plants. T3 plants were grown under controlled conditions at 22°C and 16 h day light. Seed development under these conditions required 20 days from pollination to the end of seed maturation. Expression was induced by daily spraying of a 2 µM ß-estradiol solution at all parts of the plant, especially the siliques. To confirm that the induction worked, GUS activity was assayed in various tissues at different time points during seed development. Preliminary results showed that it was possible to induce GUS expression in different tissues of ß-estradiol treated plants including leaves, pollen, petals, silique velves and embryos (Figure 3.21). However, GUS activity was not detectable in all samples of each tissue tested, indicating that the induction did not work consistently or throughout the whole plant. Germination assays of harvested transgenic seeds confirmed that the induction did not work properly. Even when ß-estradiol was supplied continuously during seed development, neither *DOG1* nor *ABA1* were expressed sufficiently to complement the *dog1* or *aba1* mutations respectively.

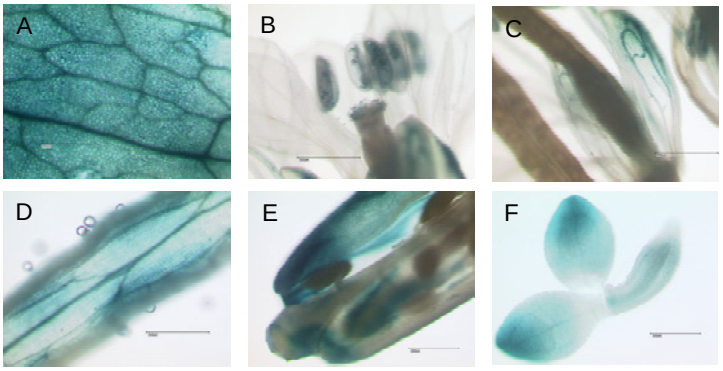

Figure 3.21: Induction of GUS expression in leaves (A), anthers/pollen (B), petals (C), silique velve (D, E) and embryos (F) of T3 ß-estradiol inducible *DOG1* lines.

Technical problems could have caused the high variability of induced GUS expression in adult plants, thus limiting the uptake of ß-estradiol. GUS staining was diffuse in the tissues tested and only in a few cases was the vascular tissue stained. To improve the experimental setup and to enhance the uptake of the inducer through the vascular system, plants were grown on rockwool and watered with nutrient solutions that contained the inducer. The functionality of the induction was analyzed as described before by testing the presence of GUS activity in different tissues. The application of the inducer through a hydroponic system, which should lead to the uptake of the hormone by the roots and transport through the vascular system, was not successful in inducing GUS expression homogenously throughout the plant. GUS signal was detectable in similar tissues as shown in Figure 3.21, but only in a few of the tested samples. This led to the conclusion that ß-estradiol might not be able to be transported in adult plants and that it seems especially difficult for the inducer to reach the developing siliques and the embryo. The transportation characteristics of the hormone might also be limited by the size of the molecule. The application of the ß-estradiol inducible system in adult plants and during seed development has not been reported before and was shown in this study to be not functional. Therefore it will be a future task to establish inducible expression by the use of alternative inducible systems.

3.2.4.2 Complementation of *dog1-1* by a single splicing isoform

A 5.6-kb genomic fragment containing the Cvi allele of *DOG1*, including the putative *DOG1* promoter region and the 5' and 3' UTR, was able to induce a strong dormant phenotype when transformed into plants of the low dormant accession L*er* (BENTSINK ET AL., 2006). This confirmed that the Cvi allele of *DOG1* is responsible for the strong dormancy of NIL *DOG1*. To determine the functionality of each protein isoform, complementation tests of the non-dormant *dog1-1* with each isoform were performed. Each cDNA from the alpha-, beta- and delta-type transcripts was cloned under the native promoter of *DOG1* derived from Cvi and *dog1-1* plants were transformed by floral dipping. Two to three independent transgenic lines per construct were selected and confirmed by segregation analysis to be homozygous and to contain a single insertion (following the same selection strategy as described for the selection of transgenic overexpression lines, chapter 3.2.4.3, p. 63). The germination phenotype of T3 plants was analyzed directly after harvest. All of the transformants showed 100% germination indicating that none of the single splicing isoforms was able to complement the non-dormant phenotype of *dog1-1*, although the genomic construct complemented the low dormancy of L*er* plants. This suggested that more than one isoform might be required for the *DOG1* function. To examine whether expression of two splicing forms together in a single plant is sufficient to confer

complementation, transgenic T3 plants expressing alpha, beta or delta *DOG1* were combined in all possible combinations by crosses. F1 seeds derived from the crosses were predicted to express both splicing forms in a similar ratio. The germination percentage of F1 seeds was tested directly after harvest. All the combinations (alpha/beta, alpha/delta, and beta/delta) showed 100% germination indicating that the expression of two forms together was not sufficient for functional complementation. The double transformants will be used for the generation of triple transformants.

Expression analysis of complementation lines

To characterize the expression level of the transgene in the complementation lines, qRT-PCR, with the same transcript-specific primers as for the natural expression analysis, was performed using siliques at 15 days after flowering. At that timepoint, *DOG1* was shown to be highly expressed (Figure 3.15). The beta transgene contained only the coding region ending at the stop-codon in the green exon (Figure 3.22). Thus, the transcript-specific beta primer for the detection of beta cDNA does not anneal in the expressed transgene. Therefore a different primer ($ß_{TR}$) was used for detection that annealed in the overlapping region of the blue and the green exon, amplifying the beta-transgene as well as the endogenous beta and gamma cDNA. The expression values of the beta-transgene presented in the following graphs were calculated by subtracting the endogenous beta and gamma-specific expression values from the total expression level detected by the $ß_{TR}$ primer.

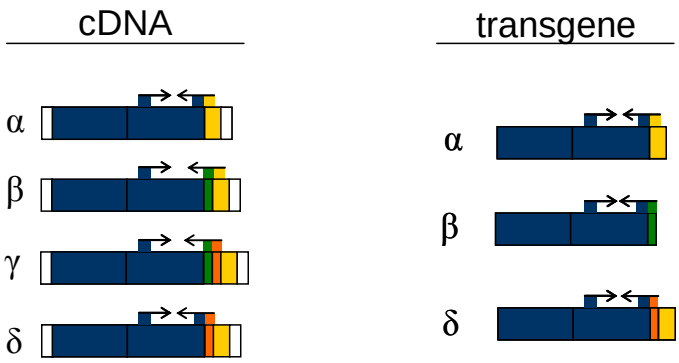

Figure 3.22: Schematic representation of the primer sets used for qRT-PCR detection of the endogenous splicing variants (cDNA) and the expressed transgenes in T3 complementation lines and overexpression lines.

The expression level of the transgene in T3 complementation lines was adjusted by subtracting the non-functional background expression of the endogenous splicing forms. The expression pattern of the splicing forms in L*er*, *dog1-1* and NIL *DOG1* provided a control. Expression levels of the respective cloned cDNA driven by the native *DOG1* promoter in the transgenic lines were higher than the background expression levels. T3 lines expressing the alpha transgene

showed on average 20 times more alpha transcript than the *dog1-1* mutant. Transgenic lines expressing the beta transgene showed an average of 140 times higher expression levels of the beta transcript than the *dog1-1* mutant. Transgenic lines expressing the delta *DOG1* transgene showed 90 times higher expression levels of delta *DOG1*. All of the transgenic lines had a non-dormant phenotype similar to *dog1-1*, indicating that absence of expression did not cause low dormancy.

In addition, the L*er* and the Cvi alleles of the cloned alpha transcript led to comparable expression levels. This confirmed the hypothesis that the sequence polymorphisms in the coding region are not responsible for differences in *DOG1* expression and dormancy levels.

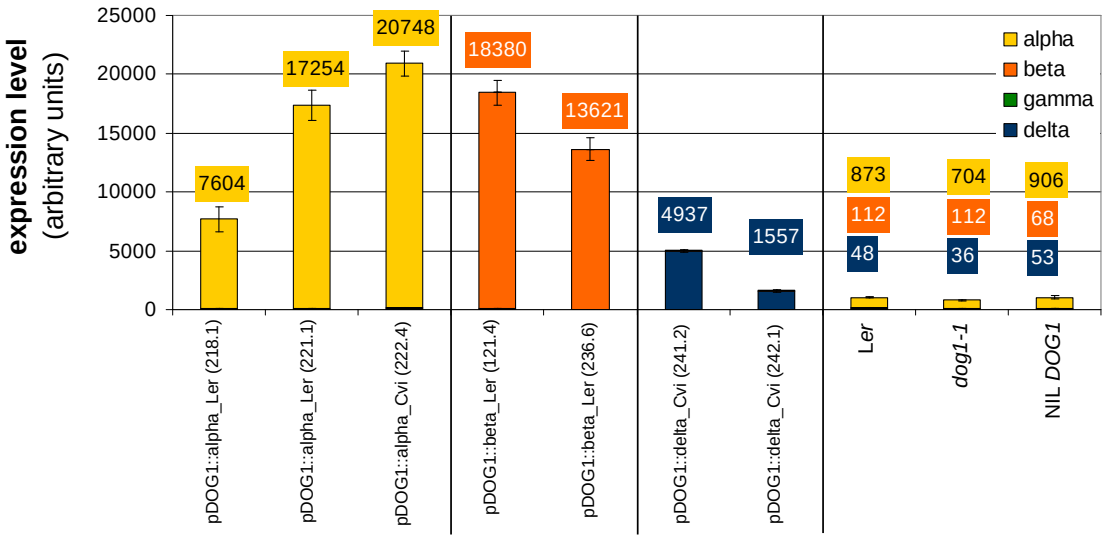

Figure 3.23: Expression levels of the *DOG1* splicing forms in the T3 complementation lines in comparison to L*er*, *dog1-1* and NIL *DOG1* controls.
qRT-PCR was performed using siliques at 15 days after flowering, sampled from 5-6 individual plants per T3 line. The absolute transcript level of the transgene at 15 days after flowering is shown and indicated in the boxes, colored with the color code of the respective splicing form. Values are shown as the mean of duplicate measurements and error bars represent the standard deviation.Values were normalized by *ACTIN8*.

3.2.4.3 Overexpression of single splicing forms and a genomic *DOG1* fragment

To study the effect of ectopic and constitutive *DOG1* expression, the single splicing variants alpha, beta and delta *DOG1* were overexpressed using a double 35S promoter. These constructs were transformed into non-dormant *dog1-1* and L*er* plants. The complementation lines revealed that a single splicing form driven by the native promoter does not complement the *dog1-1* mutant. Therefore, it was determined, whether overexpression of a single splicing variant in L*er* plants, which have a natural composition of all splicing variants, could lead to a phenotypic difference.

In addition to the overexpressed single splicing variants, a 2.8 kb genomic *DOG1* fragment that contained the partial 5' UTR, the entire gene and the partial 3' UTR (GF_*DOG1*) was cloned under the double 35S promoter and transformed into L*er* plants (Figure 3.24). All of the splicing forms were expected to be generated from this sequence. Therefore, this construct represented a control that allowed for the comparison of the overexpression phenotypes of a single splicing variant with the phenotypes that are caused by the overexpression of all the splicing variants at the same time.

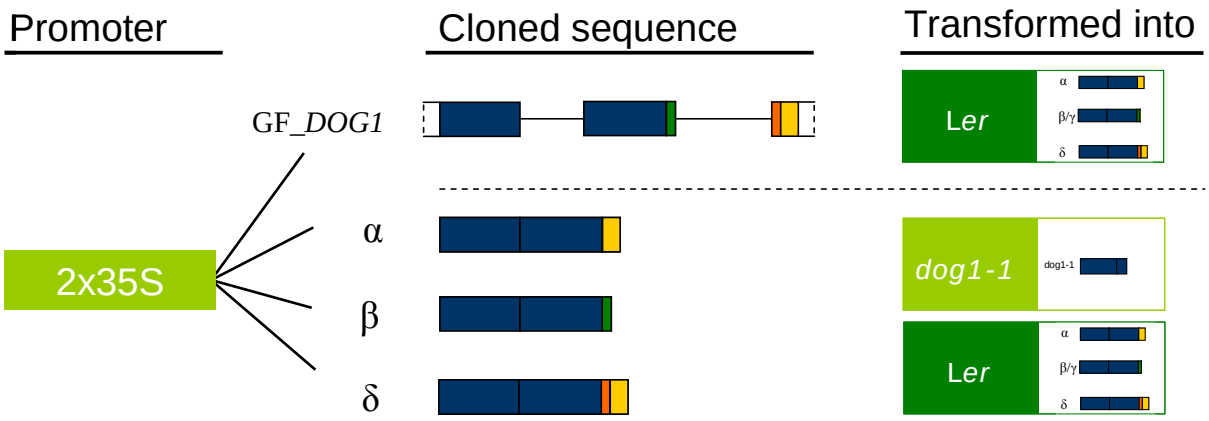

Figure 3.24: Schematic illustration of the different overexpression constructs and the genotypes into which they were transformed.
The single splicing variants were transformed into L*er* and *dog1-1* plants and the genomic fragment (GF) containing the region between the start- and stop-codon (GF_*DOG1*) was transformed into L*er* plants. The white boxes of the GF_*DOG1* contruct indicate the partial 5' and 3' UTRs. The two genotypes are characterized by a different composition of endogenous *DOG1* splicing forms and protein variants, indicated by the schematic representation of the potentially present DOG1 protein variants.

Selection strategy of the transgenic lines and dormancy classification

DOG1 expression in fresh mature seeds was shown to correlate with the dormancy level (Figure 3.17). Therefore, overexpression of *DOG1* or a specific *DOG1* splicing variant could lead to increased dormancy. In order not to omit strongly dormant T1 transformants caused by overexpression, all T1 seeds were selected after 8 weeks of after-ripening and 7 days of stratification, which was shown to break dormancy in the stronger dormant accession Cvi (Figure 3.2).

Dormancy phenotypes were determined for segregating T2 seeds from all T1 plants. Transgenic T2 lines in the non-dormant *dog1-1* background were analyzed directly after harvest; transgenic T2 lines in the L*er* background were tested two weeks after harvest, when L*er* control seeds germinated 90-100%. The dormancy phenotypes of the transgenic lines were clustered in two phenotypic groups: non-dormant lines, which showed 100% germination and dormant lines, which segregated for dormancy (Table 3.6). The dormant lines showed about 25% germination,

indicating that the homozygous and hemizygous transgenic seeds were dormant and segregating 3:1 (dormant:non-dormant). To test whether the dormancy phenotype correlated with Basta-resistance, seedlings derived from the isolated embryos of the dormant fraction and seedlings of the germinating non-dormant fraction were tested for Basta resistance. Dormant lines were selected, whose dormancy phenotype followed the 3:1 segregation, had isolated embryos that were all Basta resistant, but whose seedlings of the 25% non-dormant fraction did not show Basta resistance. In addition, non-dormant T2 single insertion lines, segregating 3:1 for Basta resistance, were selected.

In the T3 generation, homozygous lines were selected by screening for lines with 100% Basta resistance in the non-dormant group and lines with 0% germination in the dormant group. The dormant lines were first tested directly after harvest or two weeks after harvest, depending on the genetic background. A second confirmation of the dormancy phenotype was obtained after an 8 weeks after-ripening period and 7 days stratification treatment. At that point, the dormant lines did not show any release of dormancy. This indicated that the transgene induced a high level of dormancy in the dormant lines that could not be broken with a dormancy breaking treatment that is able to break the dormancy of the strong dormant accession Cvi (Figure 3.2). During the selection process, two to three independent homozygous lines with a single insertion were selected per construct and per dormancy phenotypic group. T3 plants and siliques harboring T4 seeds of these selected lines were used for further characterization.

For all constructs, dormant lines represented a minority of only 11% to 41% of total transformants (Table 3.6). The percentage of dormant lines per construct was the same in both genetic backgrounds. This suggested that the background did not influence the induction of dormancy by the transgene. Therefore, further phenotypic characterization and expression analysis were only performed with overexpression lines which were transformed into non-dormant *dog1-1* plants, except for the overexpressed genomic fragment of *DOG1* (GF_*DOG1*) for which transformants in L*er* background were analyzed.

Table 3.6: Dormancy phenotypes of T2 overexpression lines.
Dormancy phenotypes were classified as non-dormant or dormant. T2 lines in *dog1-1* background were tested for dormancy directly after harvest and classified as being dormant when they showed a 3:1 dormancy segregation with 25% germinating T2 seeds. T2 lines in L*er* background were tested similarly, but at two weeks after harvest when L*er* seeds germinated 90-100%.

			Phenotypic group	
Transformed into	**Construct**	**T2 lines**	**non-dormant**	**dormant**
dog1-1	***p2x35S::alpha DOG1***	**# of lines**	**59**	**11**
		(%) of lines	84%	16%
dog1-1	***p2x35S::beta DOG1***	**# of T2 lines**	**19**	**13**
		(%) of lines	59%	41%
dog1-1	***p2x35S::delta DOG1***	**# of T2 lines**	**16**	**2**
		(%) of lines	89%	11%
L*er*	***p2x35S::alpha DOG1***	**# of T2 lines**	**11**	**6**
		(%) of lines	65%	35%
L*er*	***p2x35S::beta DOG1***	**# of T2 lines**	**24**	**10**
		(%) of lines	71%	29%
L*er*	***p2x35S::delta DOG1***	**# of T2 lines**	**30**	**9**
		(%) of lines	77%	23%
L*er*	***p2x35S::GF_DOG1***	**# of T2 lines**	**8**	**2**
		(%) of lines	80%	20%

Expression analysis of overexpression lines

To characterize the expression level of the transgene in the overexpression lines, qRT-PCR with transcript-specific primers was performed using siliques at 15 days after flowering (Figure 3.22). The quantification of the expression of the beta transgene was calculated as described previously for the complementation lines (Chapter 3.2.4.2, p. 61). It was determined, whether the dormancy phenotypes of the transgenic overexpression lines could be explained by their *DOG1* expression levels. The expression level of the transgene in T3 overexpression lines was adjusted by subtracting the non-functional background expression of the endogenous splicing forms. In addition, the expression levels of the low dormant accession L*er*, *dog1-1* and the stronger dormant NIL *DOG1* were analyzed as controls.

The background expression of the different splicing variants in *dog1-1* was not affected by the overexpression of the transgene and was comparable to the expression levels in the controls. The level of overexpression of the transgene varied considerably among the transgenic lines overexpressing alpha, beta or delta *DOG1*. Some of the non-dormant lines showed no overexpression or only weak overexpression (e.g. transgenic lines overexpressing alpha 97.3, 114.3, 69.2). Other non-dormant lines showed increased levels of the respective transgene. However, the comparison of expression levels of the different transgenes between the two

phenotypic groups, revealed a strong correlation between expression levels of the transgenes and the dormancy phenotype of the transgenic lines (Figure 3.25).
Non-dormant lines overexpressing alpha *DOG1* had between 33 and 53 times higher expression levels of alpha compared to *dog1-1*, whereas dormant lines with the same transgene showed a 270 times higher expression level.
Out of the non-dormant beta *DOG1* overexpression lines, line 389.4 showed a 313 times increased level of beta *DOG1*, whereas a 2 366 times increased level of beta *DOG1* expression was detected in line 400.1. Nevertheless dormant lines with the overexpressed beta transgene showed much higher expression levels of beta *DOG1*, with an average of 12 164 times more beta transcript than in *dog1-1* background.
Similarly, two non-dormant delta *DOG1* overexpression lines (48.1, 423.3) showed a 133 to 158 times increase in delta *DOG1* expression levels. In the non-dormant line 428.3, there was even a 2 134 times higher delta *DOG1* expression. However, an average of 6 039 times increased expression level of the delta transcript was detected in the dormant lines.
The non-dormant overexpression lines showed similar expression levels of the respective transgene than in the non-dormant complementation lines (Figure 3.23, Figure 3.25). Although the expression level of the respective transgene was increased in these non-dormant lines and higher than in the strongly dormant NIL *DOG1*, it was not sufficient to induce dormancy. This indicated that a single splicing form was only able to induce dormancy when it was highly overexpressed.

The L*er* and Cvi alleles of the alpha transgene in the overexpression lines led to comparable expression levels (e.g. compare non-dormant alpha lines 102.7 (L*er*) and 77.3 (Cvi), dormant alpha lines 109.4 (L*er*) and 352.9 (Cvi), similarly as shown before for the complementation lines.

The dormant lines overexpressing the genomic *DOG1* fragment showed an increased level of all four splicing variants, but they were not generated in the same ratio as in the controls. There was an average of 42 times more alpha, 1 021 times more beta, 465 times more gamma and 125 more delta transcript. Although each splicing form was not as highly overexpressed as in the dormant lines overexpressing a single splicing form, expression levels were high enough to induce strong dormancy.

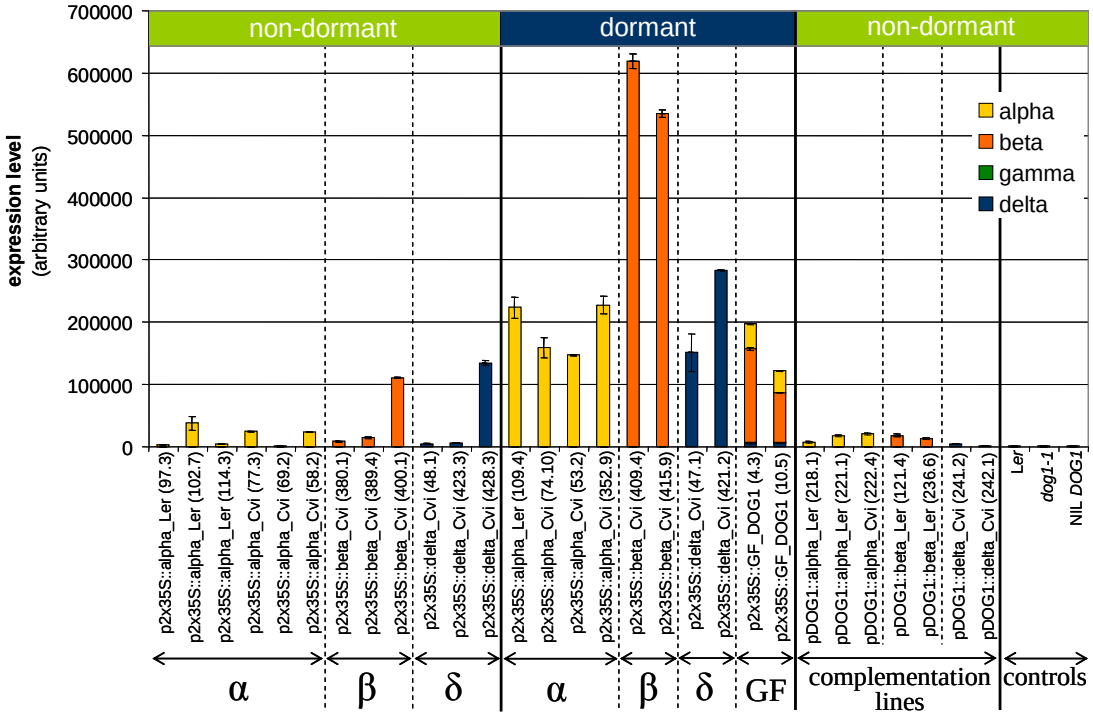

Figure 3.25: Expression levels of transgenes in non-dormant and dormant T3 lines overexpressing a single *DOG1* splicing variant or a genomic fragment of *DOG1* (GF_*DOG1*) in comparison to the non-dormant complementation lines with a single splicing form and L*er*, *dog1-1* and NIL *DOG1* as controls.
QRT-PCR was performed using siliques at 15 days after flowering, sampled from 5-6 individual plants per T3 line. Absolute transcript levels are shown as the mean of duplicate measurements and error bars represent the standard deviation. Values were normalized by *ACTIN8*.

Protein detection in leaves of T3 overexpression lines

The overexpression lines were not only tested for their *DOG1* transcript level, but also for their DOG1 protein abundance. Due to the absence of DOG1 protein produced by the endogenous gene, this analysis was performed on leaf material.

Immunoblot analysis, using DOG1 specific antibody, demonstrated the absence of protein in leaves of non-dormant lines overexpressing a single *DOG1* splicing variant, and a high protein accumulation in leaves of dormant lines overexpressing a single splicing variant or a genomic fragment of *DOG1* (Figure 3.26, K. Nakabayashi). This indicated that the protein was probably degraded and only visible when protein production was sufficiently high. Protein accumulation correlated with the dormant phenotype in 18 of 20 transgenic plants. The two exceptions are the dormant overexpression lines p2x35S::alpha_Cvi (352.9) and p2x35S::delta_Cvi (47.1) which did not show clear protein accumulation. It is possible that these two outliers might still show a higher protein level in seeds, which has to be tested. The two dormant transgenic lines overexpressing a genomic fragment of *DOG1* (p2x35S::GF_DOG1 (4.3 and 10.5) differed in their degree of protein accumulation.

The predicted protein size of the alpha DOG1 protein variant is 32.6 kD, beta and gamma DOG1 is 30.8 kD and delta DOG1 is 33.9 kD. These size differences were visible on the immunoblot (Figure 3.26). Based on these differences, it was observed that the protein size in the lines overexpressing a genomic fragment of *DOG1* was similar to the migration size of the beta DOG1 variant. This was consistent with the expression data, which revealed that the beta transcript was the major *DOG1* variant generated from the genomic fragment.

Further confirmation of the degree of protein accumulation in fresh mature seeds of these overexpression lines is necessary and is part of the research of K. Nakabayashi (MPIZ).

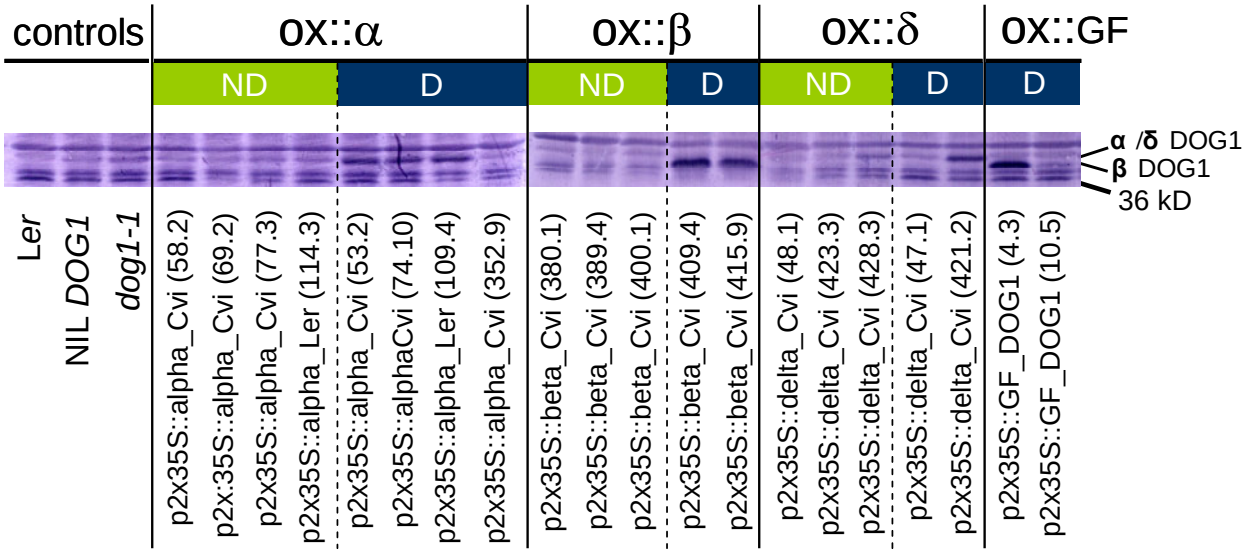

Figure 3.26: Immunoblot of leaf proteins from non-dormant and dormant T3 lines overexpressing a single *DOG1* splicing variant or a genomic fragment of *DOG1*.
Protein accumulation of DOG1 protein derived from the transgene of non-dormant (ND) and dormant (D) overexpression lines in leaf tissue using anti-DOG1 antibody. The DOG1 protein variants differ in size, which is indicated by the arrows. OX: overexpression construct with a double 35S promoter. Immunoblots were performed by K. Nakabayashi (MPIZ).

Growth retarded phenotype of the dormant T3 lines overexpressing a single splicing variant

In addition to the previously described dormancy phenotypes of the overexpression lines, the ectopic expression of *DOG1* caused a growth related phenotype. T3 plants of the dormant lines overexpressing a single splicing variant showed growth retardation resulting in a significant reduction of rosette leaf size, a thinner stem and a reduced number of siliques, whereas non-dormant lines overexpressing a single splicing form showed the same growth phenotype as *dog1-1* plants (Figure 3.27). Thus, this phenotype correlated with *DOG1* induced dormancy, and with the expression level of the transgene and the protein level in the transgenic lines. There are only two exceptions of dormant lines overexpressing a single splicing form with no detected

protein in leaves but with growth retardation. However, the dormant lines, overexpressing the genomic fragment of *DOG1* (GF_*DOG1*) did not show a growth retarded phenotype. This suggested that the disturbed presence of a high amount of a single splicing variant might lead to the observed aberrant growth, whereas an increased level of all splicing forms did not.

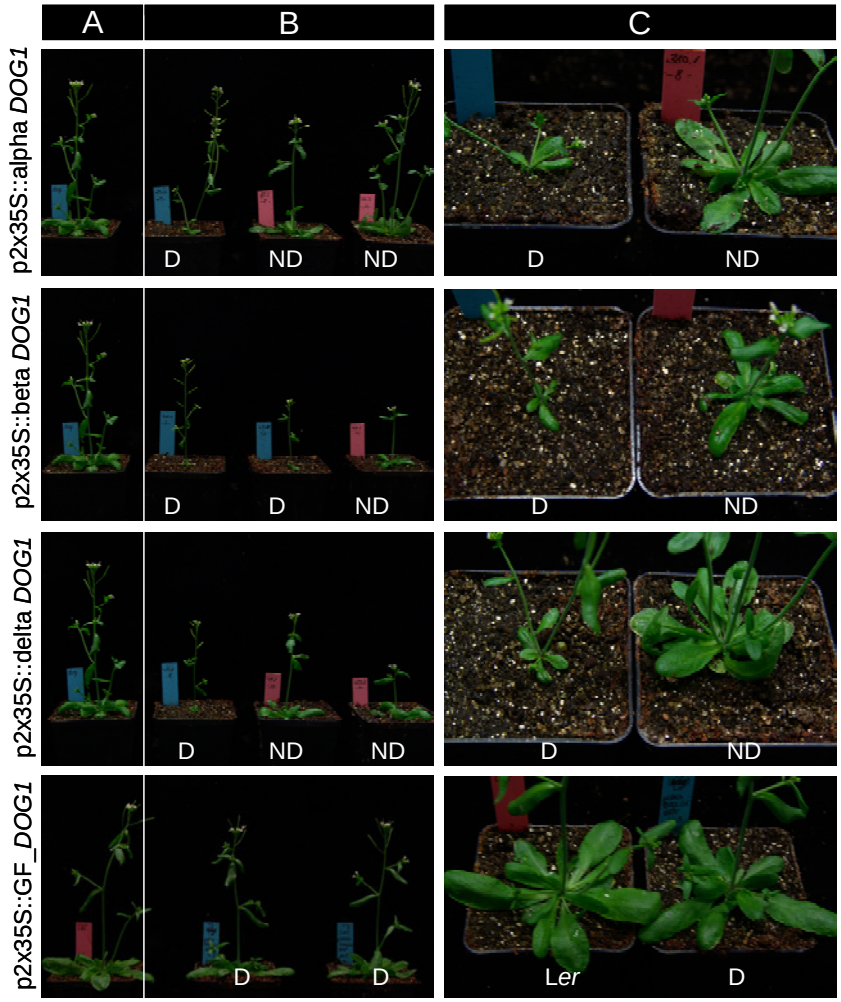

Figure 3.27: Growth phenotype of dormant and non-dormant T3 lines overexpressing a single *DOG1* splicing form or a genomic fragment of *DOG1*.
A: *dog1-1* control plants, and L*er* plants as control for *p2x35S::GF_DOG1*. B: growth phenotype of dormant (D) and non-dormant (ND) T3 lines with indicated transgene. C: close-up view of the rosette of dormant and non-dormant overexpression lines.

Mucilage deficient phenotype

Embryo rescue from the non-germinating seeds of dormant T3 overexpression lines was necessary in order to grow homozygous plants. During this procedure it was observed that T3 seeds had a defect in the production of mucilage. The secretion of mucilage, a pectinaceous hydrogel layer, upon imbibition is a process that supports seed germination by retaining water around the seed. In mature seeds mucilage is present in a dehydrated form within each epidermal cell of the seed coat. Upon contact with water, the mucilage expands leading to rupture of the primary cell wall and finally extrusion of mucilage from the seed coat. Mucilage forms a thick

capsule around the seed that can be visualized by the pectate stain ruthenium red (WILLATS ET AL., 2001; PENFIELD ET AL., 2001; WESTERN, 2006).

The presence of a mucilage layer in imbibed T3 seeds of the overexpression lines was detected by ruthenium red staining and compared to *dog1-1*, L*er* and NIL *DOG1* as controls. A thick mucilage layer was detectable in all of the controls, even *dog1-1* mutant seeds in which all *DOG1* transcripts are present, but which have no functional DOG1 proteins (Figure 3.28, B). This indicated that the DOG1 protein was not required for mucilage function. However, seeds which were overexpressing only a single splicing form in the *dog1-1* background showed a clear reduction in mucilage extrusion. This mucilage deficiency was independent from the dormancy phenotype because it was observed in both dormant and non-dormant seeds overexpressing the same transgene. The same mucilage deficient phenotype was detected in T3 seeds of the non-dormant complementation lines which are expressing a single splicing form driven by the native DOG1 promoter. This suggested that the deficiency was not caused by the artificial and ectopic expression of the 35S promoter. Dormant lines overexpressing the genomic fragment of *DOG1* had a wild type pattern of mucilage staining. In both cases, the growth and the mucilage phenotype, overexpression of a single splicing variant caused an altered phenotype, whereas overexpression of all of the splicing variants, generated from the genomic fragment, did not cause a phenotypic alteration. This suggests that the presence of a single splicing variant represents an artificial situation that is able to cause developmental aberrations. However, in contrast to the growth retardation, which correlatd with dormancy, the absence of mucilage did not correlate with dormancy.

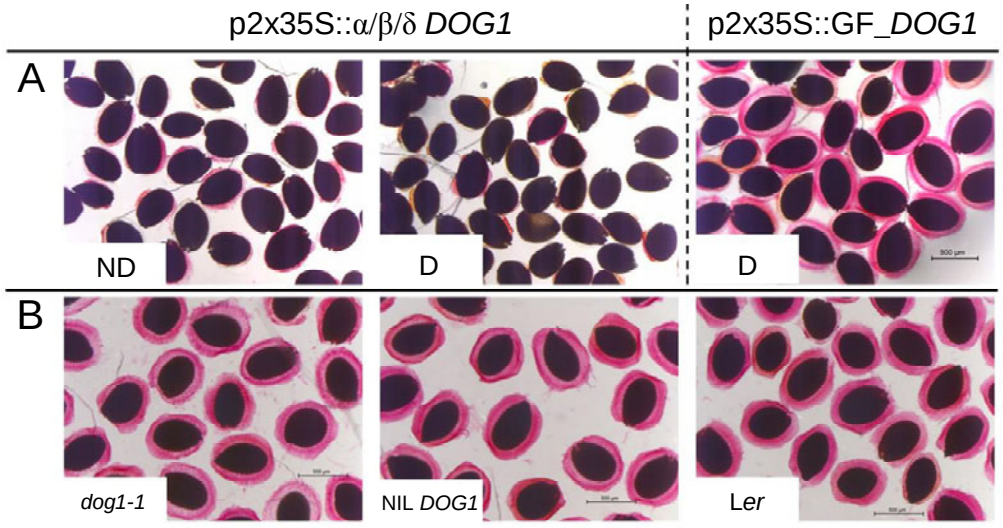

Figure 3.28: Mucilage detection by ruthenium red staining in imbibed T3 seeds of lines overexpressing a single *DOG1* splicing variant or a genomic *DOG1* fragment.
Mucilage phenotype of T3 overexpression lines (A) and of *dog1-1*, NIL *DOG1* and L*er* as controls (B). D: dormant, ND: non-dormant.

4 Discussion and conclusions

4.1 Identification of new seed dormancy mutants by using novel selection strategies and strong dormant genotypes

Seed dormancy is an adaptive trait, based on a complex network of interactions between different genes that integrate environmental signals and regulate endogenous developmental processes such as dormancy induction during seed maturation and dormancy release during after-ripening and stratification. However, the number of known genes that influence the regulation of seed dormancy is still limited (HOLDSWORTH ET AL., 2008; FINKELSTEIN ET AL. 2008). The present study made use of novel selection strategies to identify new non-dormant mutants and mutants with absent or reduced responses to dormancy releasing processes such as after-ripening and stratification.

4.1.1 A screen for non-dormancy yielded mutants affected in known dormancy genes and two putative novel mutants

In previous decades most dormancy related genes were identified by mutant screens using the low dormant lab strains L*er* or Col. During this work, a mutant approach was applied, using the strong dormant accession Cvi and the dormant near isogenic line NIL *DOG1* to screen for non-dormant mutants. Germination assays were performed directly after harvest, when non-mutagenized seeds of the two background genotypes were still deeply dormant. This strong selection identified mutants, which were affected in key dormancy genes: ABA deficient and ABA-insensitive mutants, a testa mutant and *dog1* mutants.

ABA is one of the main regulators of dormancy, promoting dormancy induction and maintenance (FINKELSTEIN ET AL., 2008). The identification of ABA mutants confirmed that the selection successfully identified mutants affected in essential pathways for seed dormancy.

This was also confirmed by the identification of three novel mutant alleles of *DOG1*. *DOG1* was previously identified as the strongest QTL in a QTL analysis of genetic differences in seed dormancy between the strong dormant accession Cvi and the low dormant accession L*er*. The γ-irradiation mutant allele of *DOG1*, in NIL *DOG1* background, is completely non-dormant and lacks pleiotropic phenotypes (BENTSINK ET AL., 2006), indicating that *DOG1* is essential for seed dormancy. The new *dog1* alleles in highly dormant backgrounds confirmed the important and central role of *DOG1* as a key player involved in seed dormancy. Sequencing of the mutant *dog1* alleles revealed that they carried single basepair changes in the first exon, which led to early

stop-codons and thus to a potentially non-functional truncated *DOG1* protein (Appendix A 10). However, it is possible that weaker mutations, causing merely a reduction in *DOG1* function, were not identified due to the strong selection strategy. To identify the importance of specific amino acids, mutants in which particular amino acids are changed could be characterized. Such mutants can be obtained by a TILLING approach (Targeting induced local lesions in genomes), which generates an allelic series of mutations for functional studies (TILL ET AL, 2003; HENIKOFF ET AL, 2004; TILL ET AL., 2006).

The aim of this screen was to identify novel non-dormant mutants. Two such mutants were obtained, which did not show pleiotropic phenotypes and were confirmed to not be allelic to ABA-deficient or *dog1* mutants. Therefore, they will be further investigated in a map-based cloning approach to identify the mutated genes.

4.1.2 Selection for after-ripening deficiency yielded mutants with pleiotropic phenotypes and general germination defects

Seed dormancy increases during seed maturation and gradually decreases during after-ripening until the seeds are able to complete germination when imbibed under favorable conditions (HOLDWORTH ET AL. 2008). The duration of the after-ripening phase until dormancy is completely released is variable and depends on environmental conditions during seed maturation, seed storage and germination (DONOHUE ET AL., 2005). However, the molecular mechanisms that regulate the dormancy release during after-ripening are not understood.

A screen was performed to identify genes that are involved in after-ripening. Low dormant L*er* seeds were mutagenized and mutants were selected, which were not able to germinate after a period of after-ripening, which was sufficient to completely release dormancy in non-mutagenized control seeds. Non-germinating mutants were then isolated by embryo rescue.

A number of GA-deficient mutants were identified. These mutants were expected because GA is required for the promotion of germination. However, finding these mutants was not the focus of this screen. Only a limited number of non GA-deficient mutants were isolated, all of which showed various pleiotropic growth phenotypes.

However, an additional group of mutants obtained in the stratification-insensitivity screen could constitute a group of mutants from which after-ripening-deficient or after-ripening delayed mutants can be selected. In the screen for stratification-insensitive mutants in the strong dormant Cvi background, mutants were selected, whose dormancy was only released by after-ripening and not by stratification (Chapter 4.1.3). In addition, this stratification screen also yielded 77 mutant lines with general germination defects without severe growth phenotypes. These 77 lines

were not able to germinate after a combined after-ripening and stratification treatment. This failure to germinate could be caused by defects that are not specific for after-ripening or the combination of after-ripening and stratification, for example genes acting downstream of these processes). However, out of these 77 lines, mutants that are able to germinate after a prolonged period of after-ripening could be identified through an additional selection step, for example more than 50 weeks after-harvest. This would identify lines whose reaction to after-ripening is strongly delayed, indicating that they have an increased capacity for seed dormancy, but are generally able to germinate. It would also be necessary to test their responsiveness to stratification, to be able to distinguish between the two dormancy releasing processes. The link between after-ripening and stratification is not well understood.

However, the characterization of the dormancy release of the strong dormant accession Cvi in response to after-ripening and the combination of after-ripening and stratification showed that a stratification response requires an earlier reduction in dormancy by after-ripening. In this stratification screen, this level might not have been reached yet, when the germination tests were performed (Figure 3.2; Figure 3.3). Because Cvi seeds required an initial period of after-ripening before they responded to stratification, it is possible that Cvi mutants with absent, reduced or delayed after-ripening responses may not be responsive to stratification.

4.1.3 A novel class of stratification-insensitive mutants was identified by a two-step selection strategy

Plants are sessile organisms for which adaptation to the environment is crucial for survival. Temperature is one of the major environmental factors to which plants must adapt. They have developed three different responses to low temperatures: cold acclimation to protect the plant from freezing damage, vernalization to induce flowering, and stratification to release dormancy (WILSON AND DEAN, 1996; Sung et al., SUNG AND AMASINO, 2005). Some characteristics of stratification resemble vernalization, but others are more similar to acclimation. Stratification and vernalization determine the timing of the major developmental transitions germination and flowering induction. However, in Arabidopsis stratification requires a much shorter period of low temperatures than vernalization. Similar to acclimation, a few days are sufficient to saturate the stratification response. The specific molecular mechanisms for acclimation and vernalization have each been characterized. Cold acclimation initiates a transcriptional cascade that induces downstream genes that encode proteins involved in cold protection (YANG ET AL., 2005). In contrast, vernalization results in covalent histone modifications in the chromatin of flowering repressors resulting in downregulation (AMASINO, 2005; SUNG AND AMASINO, 2005; SCHMITZ

AND AMASINO, 2007). However, the molecular mechanisms for seed stratification are still largely unknown. Therefore, identification of mutants specifically affected in stratification is important for gaining new insights into the stratification response at the molecular level.

A mutagenesis screen of the strongly dormant accession Cvi was performed, which identified a novel class of mutants that do not respond to stratification, but can germinate after prolonged seed storage. The capacity of after-ripening and the combination of after-ripening and stratification to release dormancy in the Cvi accession was characterized, revealing that stratification can induce 100% germination in a stratification-specific time-window, in which after-ripening alone does not lead to dormancy release. This time-window was shown to be influenced by environmental conditions during seed maturation and seed storage. Therefore, it was crucial to select non-germinating mutants in the confirmed stratification-specific time-window. Due to this complexity, non-germinating mutants were identified in a first selection step and confirmed to be able to germinate by after-ripening in a second selection step. This led to the identification of three independent mutant lines, whose dormancy was not released by stratification, but only by a prolonged period of after-ripening. These mutants were backcrossed and a first mapping population was produced. The current goal is to clone and characterize the genes underlying this stratification-insensitivity (C. Boehme, MPIZ).
As dicussed in the context of the after-ripening-deficiency screen, the possible link between after-ripening and stratification should also be considered. It is possible that after-ripening influences the ability of seeds to respond to stratification and that therefore after-ripening is a prerequisite for responsiveness to stratification. In less dormant accessions such as L*er*, the dormancy is level is so low that the after-ripening requirement is already fulfilled and seeds are able to directly respond to stratification, whereas in strong dormant accessions the time window in which stratification is effective is delayed.

These stratification-insensitive mutants must be further investigated and integrated with recent studies, which have highlighted connections between hormonal pathways and external environmental signals in the stratification process. Low temperature and exposure to light are the main environmental factors that lead to the release of seed dormancy during imbibition and they were shown to influence ABA and GA biosynthesis and catabolism pathways (HOLDWORTH ET AL, 2008). GA biosynthesis genes *AtGA3ox1* and *AtGA3ox2* are upregulated by stratification at 4°C in light-imbibed seeds (YAMAUCHI ET AL., 2004). In addition, large scale expression analysis revealed that 25% of cold-responsive genes were GA-regulated genes, indicating that stratification promotes germination by increasing the potential for bioactive GA accumulation. In

the *ga3ox1* mutant stratification was not able to promote germination and no increase of bioactive GAs was observed, indicating that this gene is responsible for mediating the temperature signal (YAMAGUCHI ET AL., 2007). In addition, the basic helix-loop-helix (bHLH) transcription factor *SPATULA* (*SPT*) inhibits expression of *AtGA3ox1* and *AtGA3ox2* during seed imbibition in the cold, thus mediating germination in response to temperature (PENFIELD ET AL., 2005). Expression levels of *AtGA3ox1* and *AtGA3ox2* are being analyzed in the stratification-insensitive mutant lines to determine if these mutants function before or after the *GA3ox* genes.

4.2 *DOG1* is a novel key dormancy gene with complex regulation and unknown function

The present study made a major contribution in gaining initial insights into the molecular and biochemical characteristics of *DOG1*, a key gene in seed dormancy. *DOG1* is a 'pioneer gene' because it is not homologous to any known genes nor does it contain any known domains. Therefore, the implementation of unbiased and broad approaches was crucial. The results described here provide the basis for more focused follow-up research strategies.

4.2.1 Promoter activity studies show that the *DOG1* promoter is able to drive expression in the embryo and the endosperm

Expression profiling showed that *DOG1* transcription is seed-specific (BENTSINK ET AL., 2006), but the tissue- and cell-specific expression pattern was unknown. In this study it was shown through a transgenic approach with *DOG1* promoter-reporter gene fusions, that the putative *DOG1* promoter is active in the embryo and the endosperm.

The capacity for seed dormancy is determined by three major components of the seed: the embryo, the endosperm and the seed coat. A complex cross-talk between the three tissues is necessary for the regulation of dormancy. The pressure of the radicle is opposed by the resistance of the endosperm and the testa. The endosperm exerts control over germination by secreting cell wall loosening enzymes. This weakens the mechanical resistance of the micropylar endosperm and enables radicle protrusion (BEWLEY, 1997b; NONOGAKI ET AL., 2007). Recent microarray expression profiling of isolated embryos and endosperm from germinating seeds demonstrated that the endosperm plays a specific role in the control of seed germination by ABA. *ABI4*, the crucial determinant of ABA-sensitivity for lipid mobilization in the seed, was shown to be specifically expressed in the embryo. *ABI5* was shown to be expressed in the embryo as well as in a restricted region of the micropylar endosperm (PENFIELD ET AL., 2006). This study highlighted the importance of tissue- and cell-specific responses in germination control.

In the future, constructs with endosperm specific or embryo specific promoters fused to the genomic sequence of *DOG1* could be generated and used to complement non-dormant *dog1-1* plants, to study whether *DOG1* expression in the endosperm and the embryo are both necessary for dormancy induction. Two options for an endosperm-specific promoter are the *AtEPR1* (*EXTENSIN PROLINE-RICH1*) promoter, which drives expression in the endosperm during seed germination (DUBREUCQ ET AL., 2000), and the *Catalase1* (*Cat1*) promoter, which leads to

expression during seed maturation and in germinating seeds during imbibition (SUZUKI ET AL., 1994; LEUBNER-METZGER AND MEINS, 2001; LEUBNER-METZGER 2005; G. Leubner-Metzger, pers. communication). The *Catalase1* promoter has only been used in castor bean and tobacco, functionality in *Arabidopsis thaliana* has not yet been shown.

Two options for an embryo-specific promoter are the *ABI4* and the *ABI5* promoters. As described previously, *ABI4* is highly expresed in the embryo and *ABI5* is expressed in the embryo and the micropylar endosperm (PENFIELD ET AL., 2006).

The temporal expression of *EPR1*, *ABI4* and *ABI5* during seed maturation is not known. However, while the temporal expression pattern of *DOG1* is known, the timepoint when *DOG1* expression is actually required for dormancy induction is not known. Therefore the use of endosperm and embryo specific promoters with various temporal expression patterns during seed maturation could adress this question.

4.2.2 Localization studies demonstrate that *DOG1* transcripts are present in the vascular system and highly accumulated in the shoot apical meristem of the embryo

Although promoter-reporter gene fusions are widely used to analyze the temporal and spacial regulation of genes, they are an insufficient measure of gene expression. Several cases have been documented in which promoter-reporter gene fusions illustrated artifactual expression that did not reflect the accurate in vivo regulation of the gene of interest (TAYLOR, 1997).

Therefore, RNA in-situ hybridization experiments were performed, which demonstrated that *DOG1* mRNA is highly accumulated in the shoot apical meristem of the embryo and also present in the vascular system extending into the cotyledons and the hypocotyl. Although transgenic plants expressing the GUS reporter gene under the control of the putative *DOG1* promoter showed expression in the entire embryo, this expression could result from diffusion, which has also been seen in previous studies (MASCARENHAS AND HAMILTON, 1992). Localization of *DOG1* transcripts in the endosperm was not observed in in-situ hybridizations. While in-situ hybridizations detect actual expression of the gene, promoter-reporter gene fusions illustrate only the potential promoter activity. A correct expression pattern is restricted by the putative promoter region and often additional sequences outside of the promoter region contain important regulatory elements. The half-life of GUS in plants is relatively long; therefore rapid changes in transcription of the reporter gene are not reflected by correspondingly rapid changes on the protein levels (TAYLOR, 1997). In contrast to the histochemical analysis of the *DOG1* promoter-GUS fusions, localization studies by in-situ hybridization directly detect the presence of *DOG1*

transcripts, which are possibly influenced by highly dynamic RNA turnover-processes and RNA stability. Therefore the detected GUS activity in the endosperm could reflect transient *DOG1* expression that was no longer occurring in the endosperm, when the in-situ hybridization was performed.

4.2.3 *DOG1* expression level correlates with dormancy and is influenced by temperature

The expression of the single *DOG1* splicing variants during seed development was quantified with qRT-PCR. This expression time-course showed that *DOG1* was highly expressed during seed maturation, when dormancy induction occurs. Comparing *DOG1* expression levels between the low dormant accession L*er* and the strong dormant NIL *DOG1* revealed a strong correlation between *DOG1* expression levels and dormancy. In addition, low temperatures during seed maturation (15°C compared to 22°C) led to a proportional increase of *DOG1* expression and dormancy in both genotypes.

Temperature has a profound influence on the dormancy status of seeds (HILHORST, 2007; DONOHUE ET AL., 2007). Phytochrome is one of the mediators of germination responses to seasonal changes of light and temperature (DONOHUE ET AL., 2007). In the present study it was shown that cold temperatures during seed maturation result in higher *DOG1* expression levels as well as higher dormancy levels. Sensing seasonal temperature changes during seed maturation is crucial for the propper timing of seed germination. The temperature response of *DOG1* transcription strongly suggests that its expression level is important in defining the degree of dormancy. The temperature of the natural habitat of Arabidopsis is closer to 15°C than to 22°C, suggesting that dormancy is stronger under natural conditions than under typical greenhouse conditions.

A role for membranes in dormancy regulation has been postulated. Membranes were suggested to be the primary target of temperature perception at the cellular level (MURATA AND LOS, 1997). The actual model proposes that temperature alters the membrane fluidity, which results in a conformational change of membrane proteins, in particular membrane phospholipids. These conformational changes could affect signal transduction cascades and thus gene expression leading to changes in dormancy (YANGA ET AL., 2005; HILHORST, 2007). In cyanobacteria *Synechocystis* changes in membrane fluidity result in altered gene expression of cold-induced genes (MIKAMI AND MURATA, 2003). How and if changes in membrane-fluidity lead to changes in expression of cold-induced genes and then to changes in dormancy is still unknown. However, it is possible that *DOG1* could be involved in such a cold-responsive cascade.

4.2.4 Understanding the scale and characteristics of alternative splicing in *DOG1*

A major challenge in the study of alternatively spliced genes is to identify the complete set of possible transcripts. Although splicing sites are based on conserved sequences (AG|GU), many more signals and consensus sequences are important for the splicing of introns. These are still not well characterized for plants (LORKOVIC ET AL., 2000). Therefore, *in silico* splicing site prediction is not sufficient to identify possible splicing forms in a genomic sequence.

The four splicing forms of *DOG1* were originally identified by sequence analysis of cDNA pools obtained by the use of a reverse primer annealing at the end of the last exon of the predicted ORF (in the yellow part, Figure 1.6, p. 13; BENTSINK ET AL., 2006). Based on this sequence data, transcript-specific primers were used to quantify the amounts of each splicing form during seed development. However very recently, it was determined that additional *DOG1* splicing forms are present. This was concluded by comparing the total expression level of the four known transcripts with the detected expression level using primers annealing in a common region of all four transcripts in the first and second exons. The detected *DOG1* overall expression was higher than the sum of the four known splicing forms. However, the ratio between the four known splicing forms and the additional unknown transcripts was consistent in the two tested genotypes and the two environmental conditions. Currently, these new *DOG1* splicing forms are being identified by a 5' and 3' RACE approach (K. Nakabayashi, MPIZ). If these new splicing forms also result in new protein variants, additional experiments will be required to adress the role of the new protein variants in the function of *DOG1*.

4.2.5 Variation in dormancy is potentially caused by variation in cis-regulatory regions

A correlation between dormancy levels and sequence differences in the *DOG1* coding region of various accessions was not observed (BENTSINK ET AL., 2006). This was confirmed by the transgenic approach in this study, which demonstrated that L*er* and Cvi alleles of the *DOG1* coding region conferred the same phenotypes. Although the present study did not focus on the analysis and dissection of the *DOG1* promoter, the promoter-reporter gene fusions using promoter sequences from the low dormant accession L*er* and the strong dormant accessions Cvi and Kas indicated that promoter differences do cause variation in *DOG1* expression levels, which correlate with dormancy levels. This suggests that the variation in dormancy phenotypes is mainly caused by functional variation of the cis-regulatory region. Promoter dissection studies could identify putative regulatory elements and provide insight into the molecular basis of *DOG1* regulation. Known motifs in the putative *DOG1* promoter region have already been identified.

These include an RY repeat (CATGCA), which is required for seed-specific expression and two abscisic acid-responsive elements (ABRE; TACGTGTC), which are known to be involved in the ABA response (NAKABAYASHI ET AL., 2005). Additionally, the *DOG1* promoter region from the low dormant accession L*er* contains a 285 bp insertion, in which a pyrimidine box element (TTTTTTCC) is present that is required for GA induction. The regulatory relevance of this element could be tested by applying GA and analyzing the effect on the expression of *DOG1*. This could determine if *DOG1* expression in L*er* is downregulated by GA more than in other accessions due to the additional presence of the GA-inductive cis-regulatory element. In addition, the existing natural variation among accessions can be explored using promoter swap experiments. The *DOG1* promoter regions from various accessions with different degrees of dormancy could be fused with a genomic fragment of *DOG1* and be used for complementation of non-dormant *dog1-1* plants. This approach is expected to highlight subtle differences. Therefore, position effects of transgene insertion should be taken into account. Cis-regulatory regions could play a major role in environmental adaptation because their functional architecture is thought to allow for faster evolution than coding regions (DE MEAUX ET AL., 2005; DE MEAUX ET AL., 2006). However, the extent to which variation in cis-regulatory regions significantly affects the phenotype is not known. Researchers in J. de Meaux's group (MPIZ) are studying the influence of adaptive evolution on cis-regulatory and coding variation of *DOG1*.

4.2.6 Transgenic approaches indicate a regulatory role of alternative splicing in the function of *DOG1*

A single splicing form is not sufficient to restore dormancy induction

A 5.6 kb genomic fragment containing the Cvi allele of *DOG1*, including the complete upstream intragenic region and the 5' and 3' UTR was able to induce strong dormancy when transformed into L*er* plants (BENTSINK ET AL., 2006). Because this construct included intron sequences, all of the *DOG1* splicing variants are generated.

To study the functional relevance of alternative splicing of *DOG1*, transgenic approaches using the single splicing variants were used. In a complementation approach, the single splicing forms were under the control of the putative *DOG1* promoter and were transformed into non-dormant *dog1-1* plants. This revealed that a single splicing form driven by the native promoter was not able to complement and therefore was not sufficient to induce dormancy. In addition, double transgenics of all possible combinations of alpha, beta and delta *DOG1* also did not complement. It remains to be tested, if the combination of all three splicing forms in triple transgenics is able to restore a dormant phenotype. Expression analysis of the transgene in the complementation lines demonstrated that the transgenes were expressed at a higher expression level than in wild

type. This was surprising because the putative native promoter was used, which should have led to comparable expression levels. It is possible that important regions of the promoter that contain cis-regulatory elements were missing in these constructs. The putative *DOG1* promoter used for the complementation construtcs of single *DOG1* splicing forms did neither contain intron sequences nor the 5' and 3' UTR. It was shown that 5' and 3' UTRs (*TT10*; I. Debeaujon, pers. communication), large intragenic regions (SIEBURTH AND MEYEROWITZ, 1997), and even sequences far up- or downstream of the gene of interest (EICKER, 2005) can all be necessary for proper transcriptional regulation. Such regions can contain target sites for positive and negative regulators of gene expression (TAYLOR, 1997). Therefore, it is possible that the putative *DOG1* promoter sequence from Cvi used in this study was missing regions that contain target sites for such negative regulators that would decrease the *DOG1* expression level.

Overexpression lines give indirect evidence that *DOG1* function is tightly regulated by its expression of different splicing variants and their ratio

In addition to the complementation approach with the putative native promoter, transgenic plants were generated, which overexpressed a single splicing form or a 2.8 kb genomic fragment of *DOG1*. The selection strategy for identifying transformants accounted for the possibility that overexpression of a dormancy gene might induce a high degree of dormancy. However, it is possible that very dormant T1 seeds were missed in the selection process and that dormant transgenics would represent a larger class of lines than the present data suggests. In the selection process, no transgenics with multiple insertions were obtained, which is an indirect hint, that those might have been missed due to their strong dormancy phenotype. Two phenotypic classes of transformants were identified: non-dormant lines, which constituted the major group and a minor group of deeply dormant lines. The homozygous dormant lines, resulting from a single insertion event, were not able to germinate after 8 weeks of after-ripening and 7 days of stratification. However, these dormant lines showed a reduced level of dormancy in the next generation, indicating that their dormancy level is also influenced by environmental conditions during seed maturation and seed storage.

The selection problem of dormant transgenic seeds can be avoided in the future by the use of alternative selection markers that allow for selection before germination. Fluorescent proteins have been used as visual selection markers to identify transgenic Arabidopsis seeds. These markers contain the promoters of the seed storage protein genes napin or cruciferin and were used to drive fluorescent proteins like DsRed (Red fluorescent protein from *Discosoma* sp.), EGFP (Enhanced green fluorescent protein), EYFP (Enhanced yellow fluorescent protein) or ECFP (Enhanced cyan fluorescent protein). Transformation of these constructs allowed the

identification of mature transformed seeds in a large background of untransformed seeds by fluorescence microscopy (STUITJE ET AL., 2003). The use of visual seed-specific selection markers will provide an unbiased and useful tool, especially for transgenic approaches with dormancy genes.

Expression analysis of the overexpression lines showed that overexpression of a single *DOG1* splicing form or of the 2.8 kb genomic fragment only resulted in dormancy induction when the expression level of the transgene exceeded a certain threshold. Once expression was above this threshold neither the particular splicing form nor the ratio of the transcripts were important for the function. Overexpression of a single splicing form below the threshold did not restore the dormant phenotype in either these overexpression construtcs or the complementation lines. Although the expression levels in these overexpression and complementation lines were higher than in the control genotypes L*er* and NIL *DOG1*, it seems that when expression was below this critical threshold, the presence of all of the different splicing forms was necessary to induce dormancy. This can be concluded from the expression analysis of the endogenous *DOG1* gene in the two genotypes L*er* and NIL *DOG1*, which demonstrated that there was a two to three fold higher *DOG1* expression in the strongly dormant NIL *DOG1*. This suggests that small differences in the expression level influence dormancy when all splicing forms are present in the natural ratio. This indicates that there could be a very fine and sensitive regulation of *DOG1* in wild type in which alternative splicing plays an important role.

The expression difference between the genotypes L*er* and NIL *DOG1* correlated with the difference in protein accumulation. However, a different correlation was observed in the overexpression lines. Western blot analysis of the overexpression lines demonstrated a high DOG1 protein accumulation in leaves of the dormant, but no accumulation in the non-dormant lines. In addition, there were two outliers of dormant overexpression lines, in which no protein was detected. However, these lines might still have high protein level in seeds.
Although the non-dormant lines showed a 20 to 140 times increase of expression of the respective splicing form compared to the dormant NIL *DOG1*, there was no corresponding increase in protein levels. This suggests that the single overexpressed transgene might produce an instable protein that is quickly degraded, whereas in wild type, the different protein variants might form a stable protein. In the dormant overexpression lines increased levels of a single DOG1 protein variant was detectable. It is possible that protein degradation also occurs in these lines, but that the effect is mitigated by the extremely high levels of transcripts and protein.

In addition to these overexpression constructs, it would be interesting to analyze the expression and dormancy levels of transgenic complementation lines which include a genomic sequence of *DOG1* (including 5' and 3' UTR) and the *DOG1* promoter. Such a construct was used for complementation of low dormant L*er* plants with a Cvi allele of *DOG1*. Two independent T2 transformants were shown to have a strong dormant phenotype, but there is no information available about the expression levels of the different *DOG1* splicing variants. However, the dormancy phenotypes were characterized and they showed 60-80% germination 13 weeks after harvest (BENTSINK ET AL., 2006). Although these lines were analyzed in the T2 generation and the germination characterization only studied the dormancy release by after-ripening, it seems that the dormant T3 overexpression lines obtained in this study were more dormant. Currently the dormant T4 overexpression lines are being analyzed in a continuous germination assay to characterize dormancy release in response to after-ripening and a combined treatment of after-ripening and stratification. Preliminary results indicate that dormancy is reduced in comparison to the T3 seeds, perhaps caused by environmental variation during seed maturation and seed storage. In addition, these lines show a reduced sensitivity to stratification, which might indicate that strong dormancy, induced by high *DOG1* expression and DOG1 protein accumulation, counteracts the dormancy breaking effect of stratification. However, further experiments are needed to confirm this.

It must be concluded that the transgenic approaches in this study were not able to mimic wild type *DOG1* function. To simulate this, it might be necessary to use the upstream intragenic putative *DOG1* promoter region, the 5' and 3' UTR for an appropriate regulation of gene expression and a genomic sequence of the *DOG1* gene including intron sequences that are necessary for proper alternative splicing. However, it seems likely that the function of *DOG1* is tightly regulated by its expression and possibly also by the ratio between different transcripts, which is required to sustain protein stability.

Extensive analysis of alternative splicing is rare in plants. However, one example with a similar functional relevance of alternative splicing is the alternatively spliced *N* resistance gene in tobacco. The *N* gene was shown to encode two splicing variants: a short transcript encoding the putative full-length N protein (*Ns*) and a long transcript (*Nl*) which included an additional exon leading to a reading frame shift and premature stop-codon. The two transcripts were shown to be present in a ratio of 25:1 (*Ns*:*Nl*) before infection with tobacco mosaic virus (TMV) and in a completely reversed ratio of 1:20 (*Ns*:*Nl*) 7 h after infection. TMV was assumed to trigger alternative splicing of the *N* gene, which then induced the defense mechanism. The functional

significance of the alternative splicing was determined by using a set of *N* gene deletion constructs for functional complementation. The only construct that allowed for the induction of the resistance reaction included a coding sequence that allowed for the generation of both proteins and a 3' genomic sequence, in which important regulatory elements were present. This indicated that both transcripts and presumably the resulting protein variants are necessary to confer complete resistance to TMV (DINESH-KUMAR AND BAKER, 2000).

Strong dormancy inducing overexpression of single *DOG1* splicing variants caused an additional growth retarded phenotype

A growth retarded phenotype was observed in T3 plants of dormant lines overexpressing a single splicing form, which resulted in a significant reduction of rosette leaf size. This phenotype was not observed in the non-dormant lines, indicating that the high expression level of the transgene in leaves caused this aberration. However, the dormant lines overexpressing the 2.8 kb genomic fragment of *DOG1* in which all of the splicing forms were overexpressed showed a wild type growth phenotype. Although the expression level was only tested in siliques and not in leaves it is likely that a similar expression level was present in both tissues. This is an additional indication that alternative splicing is important for the proper function and that the unnatural presence of a single splicing form causes side-effects leading to aberrant growth when ectopically expressed. Although this is an unnatural artifact caused by overexpression, further analysis of the retarded rosette leaf phenotype might provide further information about the function of *DOG1*. It is known that organ size is regulated through cell growth and proliferation processes (DE JAGER ET AL., 2005; HORVATH ET AL., 2006). Similar processes are also involved in radicle protrusion during germination (DE CASTRO ET AL, 2000; MASUBELELE ET AL., 2005). Therefore understanding the role of *DOG1* in leaf size could provide further insight into a parallel role in germination.

Self-binding of *DOG1* might lead to the formation of a stable functional heterocomplex of *DOG1* splicing variants that is necessary for the function

A yeast two hybrid screen performed by researchers in W. Soppe's group (MPIZ) only yielded one potential DOG1 protein interactor. However, further work of K. Nakabayashi (MPIZ) revealed that DOG1 is able to bind to itself in yeast. This was demonstrated for all DOG1 protein variants and in all combinations. A set of truncated proteins was produced and tested for their binding in yeast. This approach identified a region of ten amino acids at the beginning of the first exon that is responsible for binding. This region is present in all of the protein variants. Within this region substitution mutants were created and the single amino acid responsible for binding

was determined by alanine scanning. Currently, the role of self-binding of DOG1 is being studied in planta. Unlike several other examples of alternatively spliced genes (KOO ET AL., 2007; CARVALHO AND DUQUE, 2007), the different protein variants of DOG1 co-locate in the same cellular compartments, indicating that self-binding could be relevant in planta. A substitution construct of the binding site was generated. This construct contains the genomic sequence including the promoter sequence and 5' and 3' UTR. Complementation of *dog1-1* plants with this construct is being tested. Additionally, the interaction of the splicing variants will be confirmed by using the Split-YFP system. This will reveal whether the self-binding of DOG1 occurs in planta and if it is necessary for the function of *DOG1* (K. Nakabayashi, pers. communication).

Results of the self-binding in planta could confirm that several *DOG1* splicing forms are required for dormancy induction, which was observed in the transgenic overexpression and complementation approaches. It is possible that the different splicing variants form a stable functional heterocomplex. This would explain why a single splicing form, below a certain threshold, led to protein degradation and was not sufficient to induce dormancy. Combinations of two splicing forms, which might build a dimer was also not sufficient, however it remains to be tested if the combination of three splicing forms with the possibility of a terameric conformation is able to restore dormancy induction.

This hypothesis, that the presence of all splicing variants is required for the formation of a functional complex, was tested by transforming L*er* plants, in which a wild type composition of all splicing forms is present, with overexpression constructs of a single splicing form. Although the *DOG1* expression and protein levels have not been tested yet, this did not lead to a phenotypic difference compared to transformation of *dog1-1* plants. This suggests that the function is not only determined by the presence of all splicing forms, but that a specific ratio is necessary for the formation of a functional complex. A similar example has not yet been reported in the literature and *DOG1* represents the first example of the influence of alternative splicing on protein stability.

4.2.7 Future directions to unravel the function of *DOG1*

The results obtained in this thesis revealed important aspects about the regulation of *DOG1* and the functional relevance of alternative splicing, but the function of this key dormancy gene still remains unknown. Because the DOG1 protein does not contain any domains with a known function nor is it homologous to any annotated genes, the design of research strategies for studying the function lacks a clear starting point. Additional approaches to those taken in the

present study are necessary to unravel the function of the DOG1 protein. Three possible approaches are a biochemical approach, a microarray approach and a genetic approach.

A biochemical approach would be to identify proteins that interact with DOG1 by immunoprecipitation using the antibody against DOG1, which was created in this study. The nature of the proteins that immuniprecipitate together with DOG1 could be determined by MALDI-TOF. The analysis of their role in dormancy should reveal insights into the function of DOG1. If the interactor is a transcription factor for example, this would suggest that DOG1 is part of a transcription factor complex. In addition, the localization of the interators could give important information about the function, for example if DOG1 interacts with membrane proteins or nuclear localized proteins.

Another approach would be the identification of the primary downstream target genes of *DOG1*. These could be detected by using microarrays in combination with the inducible expression of *DOG1*. In the present study, it was demonstrated that a chemical inducible system based on ß-estradiol was not able to induce expression during seed development. Therefore, it will be necessary to establish an alternative inducible system, which is able to induce expression in the developing seeds during seed maturation. It was shown that the small molecule ethanol can quickly penetrate within the plant tissues and is able to induce expression during early embryogenesis (SAKVARELIDZE ET AL., 2007). However, it is possible that ethanol promotes germination (VREUGDENHIL ET AL., 2006). This should be considered in the implementation of the system. Complementation of non-dormant *dog1-1* plants with ethanol-inducible *DOG1* expression, using a genomic *DOG1* construct, could determine at which timepoint during seed development *DOG1* is required for dormancy induction. Subsequent microarray analysis could reveal the early target genes of *DOG1* by the identification of genes that show altered expression levels directly after the induction of *DOG1* expression. Studying the influence of these genes on dormancy and their relationship to *DOG1* will provide information about the function of *DOG1* as well. This approach was successfully used to identify the target genes of *CONSTANS*, which promotes flowering in response to day length (SAMACH ET AL., 2000).

Another promising approach would be a *dog1* suppressor mutagenesis screen to identify mutants that can suppress the non-dormant phenotype of *dog1*, leading to increased dormancy. Cloning and functional analysis of the underlying genes could provide additional information about the DOG1 function.

The genes and proteins identified with these additional approaches could reveal the molecular mechanism and biochemical function of DOG1.

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Appendix

Appendix A 1: Table of mutants involved in the regulation of seed dormancy and germination.
Germination phenotype of the mutant compared to wild type is indicated, reduced dormancy/increased germination (+) and reduced/no germination (-). Proteins are classified as follows: transcription factor (TF), regulatory protein (RP), enzyme (E), photoreceptor (P) and transporter protein (TP). (updated tables, based on BENTSINK ET AL., 2002)

Seed maturation mutants

Mutant	Gene	Germination phenotype	Encoded protein	Protein class	Accession number
abi3	*ABI3*	+	B3 domain protein with B1 and B2 domain	TF	AJ002473
fus3	*FUS3*	+	B3 domain protein with B2 domain	TF	AF016264
lec1	*LEC1*	+	HAP3 subunit of CCAAT box binding protein	TF	AF036684
lec2	*LEC2*	+	B3 domain transcription factor	TF	AF400124

Mutants with a role in biosynthesis or signaling pathways of plant hormones

Mutant	Gene	Germination phenotype	Encoded protein	Protein class	Accession number
Abscisic acid biosynthesis and signaling mutants					
abi1	*ABI1*	+	serine/threonine phosphatase 2C	RP	X78886
abi2	*ABI2*	+	serine/threonine phosphatase 2C	RP	Y11840
abi4	*ABI4*	+*	APETELA2 domain protein	TF	AF040959
abi5	*ABI5*	+*	basic leicine zipper transcription factor	TF	AC006921.5
aba1	*ABA1*	+	zeaxanthin epoxidase	E	AF283761
aba2	*ABA2*	+	xanthoxin oxidase	E	
aba3	*ABA3*	+	molybdenum cofactor sulfurase	E	AF325457
cyp707a1	*CYP707A1*	-	protein with ABA 8'-hydroxylase activity	E	NM101819
cyp707a2	*CYP707A2*	-	protein with ABA 8'-hydroxylase activity	E	NM128466
cyp707a3	*CYP707A3*		protein with ABA 8'-hydroxylase activity	E	NM180805
cyp707a4	*CYP707A4*		protein with ABA 8'-hydroxylase activity	E	NM112814
era1	*ERA1*	-	farnesyl transferase	RP	AF214206
* germination on ABA concentrations that inhibit wild type germination					
Gibberellin biosynthesis and signalling mutants					
ga1	*GA1*	-	copalyl diphosphate synthase (CPS)	E	U11034
ga2	*GA2*	-	ent-kaurene synthase (EKS)	E	AF034774
ga3	*GA3*	-	ent-kaurene oxidase	E	AF047719
gai	*GAI*	-	DELLA protein	TF	NM101361
rga	*RGA1*		DELLA protein	TF	NM126218
rgl2	*RGL2*	+	DELLA protein	TF	NM111216
sly1	*SLY1*	-	Putative F-box subunit of an SCF E3 ubiquitin ligase	RP	NM118554
Ethylene and Brassinosteroid mutants					
ein2	*EIN2*	-	bifunctional transducer	RP	AF141202
etr1	*ETR1*	-	ethylene receptor with histidine kinase activity	RP	L24119
det2	*DET2*	-	steroid 5 -reductase	E	U53860
bri1	*BRI1*	-	transmembrane receptor kinase	RP	AF017056

Seed coat or testa mutants

Mutant	Gene	Germination phenotype	Encoded protein	Protein class	Accession number
tt1	*TT1*	+	zinc finger protein	TF	NM103201
tt2	*TT2*	+	R2R3 MYB domain protein	TF	AJ299452
tt3	*DFR*	+	dihydroflavonol-4-reductase	E	AB033294
tt4	*CHS*	+	chalcone synthase	E	AF112086
tt5	*CHI*	+	chalcone isomerase	E	M86358
tt6	*F3H*	+	flavonol 3-hydroxylase	E	U33932
tt7	*F3'H*	+	flavonol 3'-hydroxylase	E	AF155171
tt8	*TT8*	+	basic helix-loop-helix domain protein	TF	AJ277509
tt9		+	unknown		
tt10	*TT10*	+	protein similar to laccase-like polyphenol oxidases		NM124184
tt11		+	unknown		
tt12	*TT12*	+	MATE family protein, proton anitporter	T	AJ294464
tt13		+	unknown		
tt14		+	unknown		
tt15		+	unknown		
ttg1		+	WD40-repeat protein	RP	AJ133743

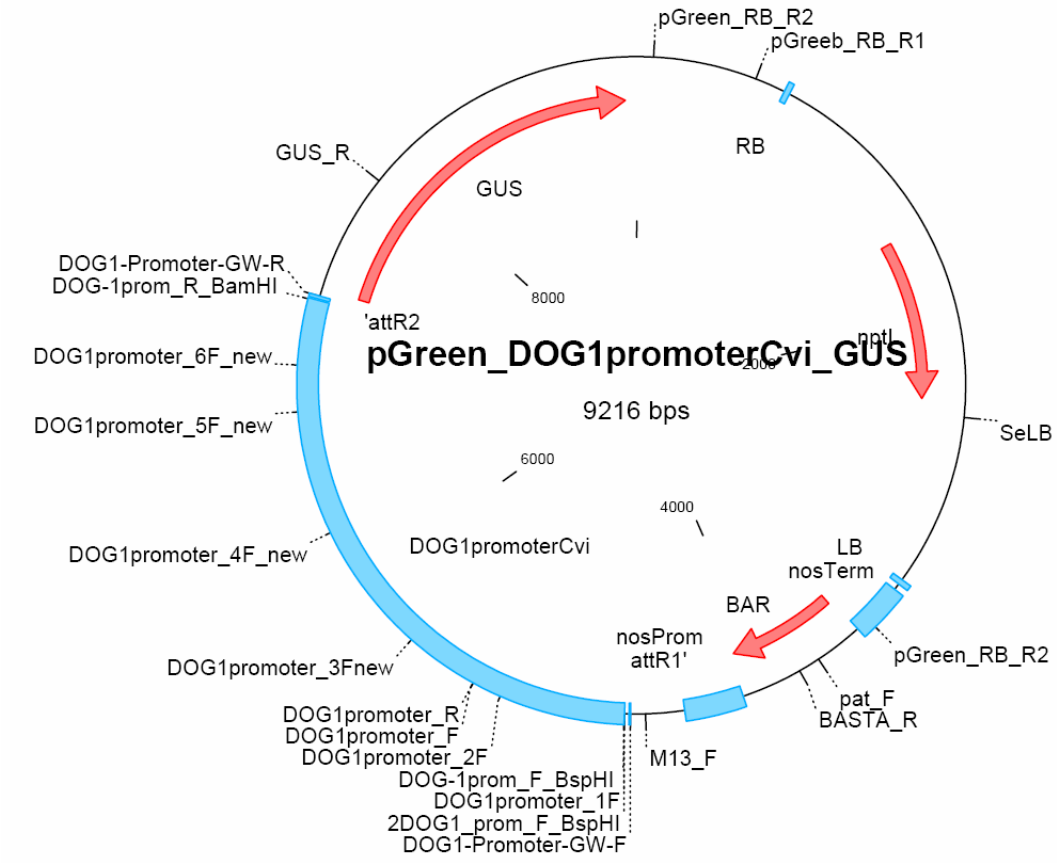
Mutants affected in light response

Mutant	Gene	Germination phenotype	Encoded protein	Protein class	Accession number
hy1	*HY1*	-	ferredoxin-dependent heme oxygenase	E	AB021858
hy2	*HY2*	-	phytochromobilin synthase	E	AB045112
phyA	*PHYA*		phytochrome A apoprotein	P	X17341
phyB	*PHYB*	-	phytochrome B apoprotein	P	X17342
pil5	*PIL5*	+	basic helix-loop-helix domain protein	TF	NM179665

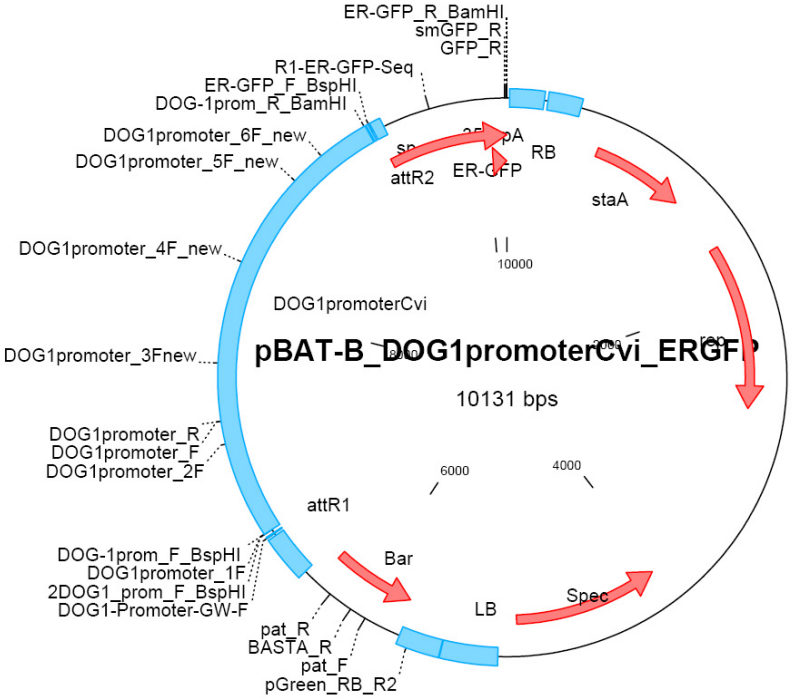
Miscellaneous dormancy mutants

Mutant	Gene	Germination phenotype	Encoded protein	Protein class	Accession number
dag1	*DAG1*	+	DOF transcription factor	TF	AJ224122
dag2	*DAG2*	-	DOF transcription factor	TF	AJ237810
rdo1		+	unknown		
rdo2	*RDO2*	+	TF2S transcription elongation factor	RP	Geyer, unpublished
rdo3		+	unknown		
rdo4/hub1	*HUB1*	+	E3 ligases responsible for monoubiquitination of H2B	RP	AAL91211
hub2	*HUB2*	+	E3 ligases responsible for monoubiquitination of H2B	RP	AAG51572
cts	*CTS*	-	peroxisomal ABC transporter	T	NM120148

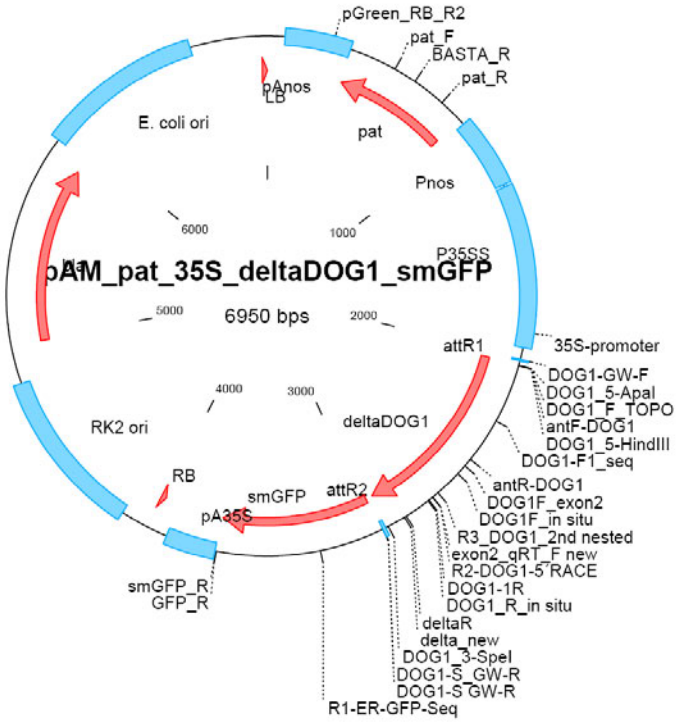
Appendix A 2: Vector map of a representative construct of native promoter GUS fusions for localization studies (*pGreen_DOG1promoterCvi_GUS*)



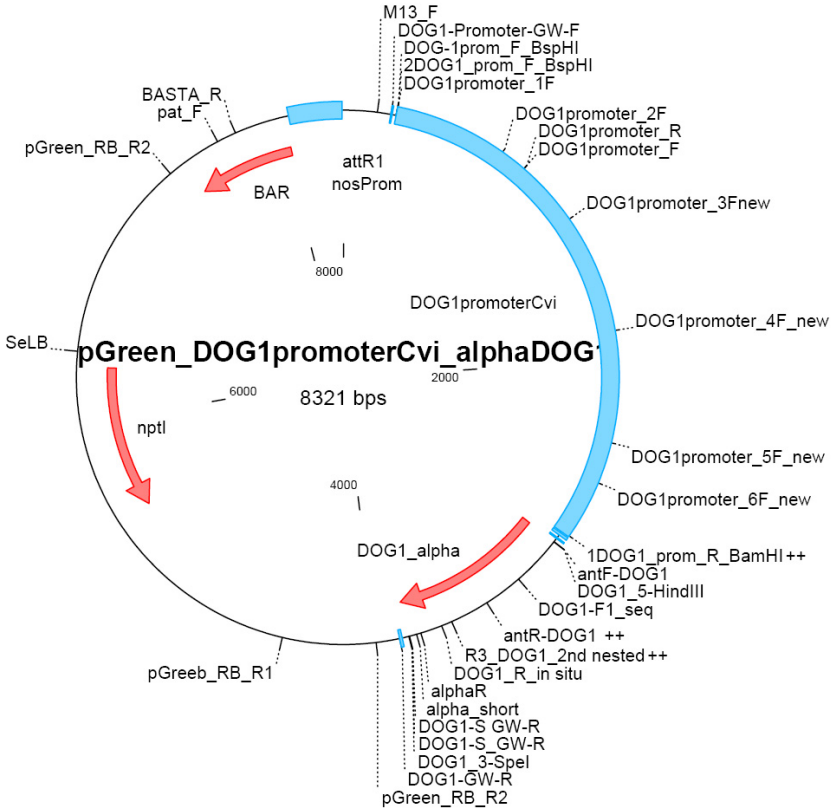
Appendix A 3: Vector map of a representative construct of native promoter ER-GFP fusions for localization studies (*pBAT-B_DOG1promoterCvi_ERGFP*)



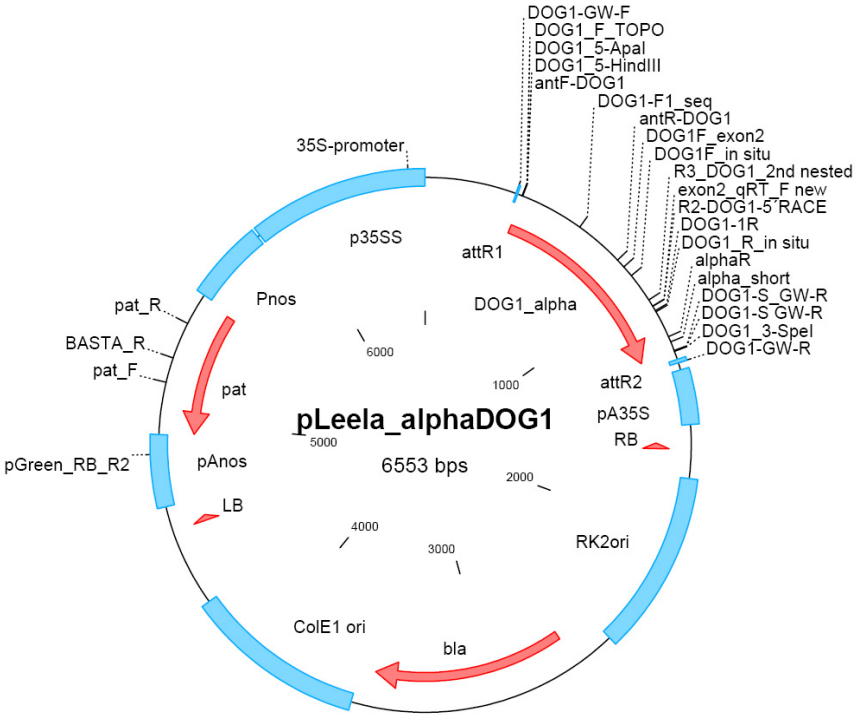
Appendix A 4: Vector map of a representative construct for protein localization (*pAM_pat_35S_deltaDOG1_smGFP*)



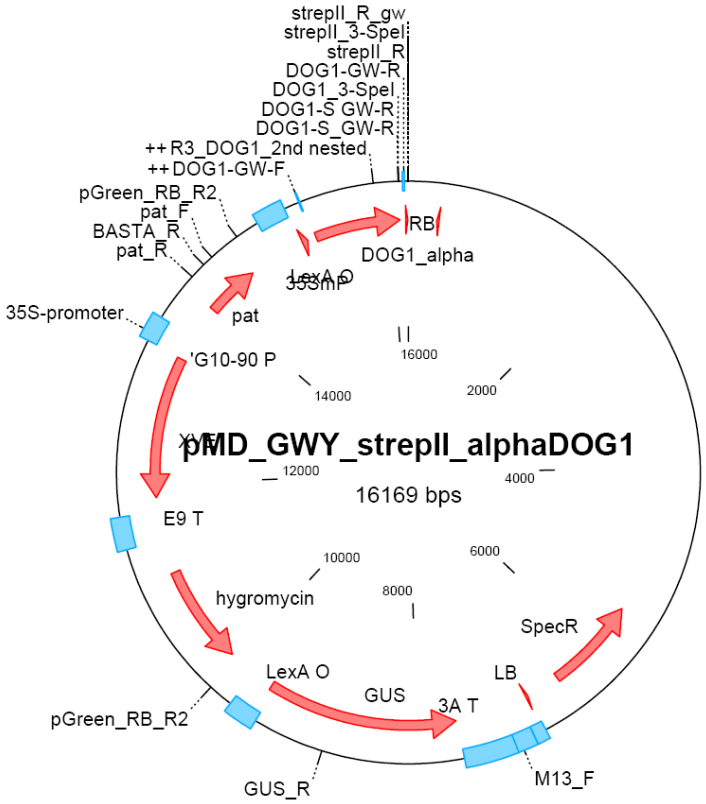
Appendix A 5: Vector map of a representative construct for complementation with a single splicing variant driven by the native promoter from Cvi (*pGreen_DOG1promoterCvi_alphaDOG1)*



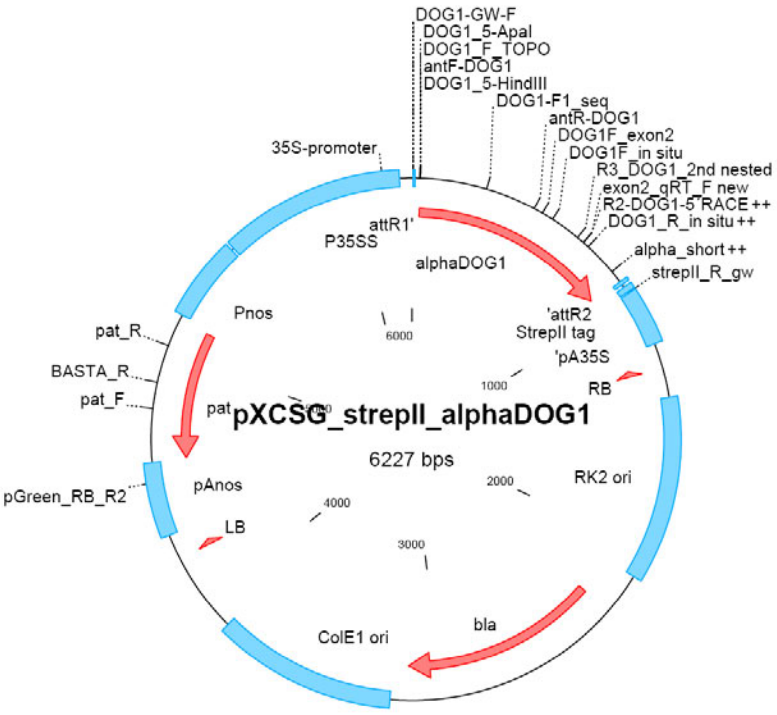
Appendix A 6: Vector map of a representative construct for overexpression of a single splicing variant (*pLeela_alphaDOG1*)



Appendix A 7: Vector map of a representative construct for ß-estradiol inducible expression of a single splicing variant (*pMD_GWY_strepII_alphaDOG1*)



Appendix A 8: Vector map of a representative construct for protein analysis of a strepII-tagged DOG1 protein variant (*pXCSG_strepII_alphaDOG1*)



Appendix A 9: Generated constructs and selection characteristics.

Transgenic designation	Name of construct	Selection in *E. coli*	*A. tumefaciens* strain	Selection in *A. tumefaciens*	Selection in plants	Transformed into
Sequences in *pDONR* or *pENTR*						
pENTR clones for Gateway cloning						
	pDONR201_DOG1promoter_Cvi	50 Kan				
	pDONR201_DOG1promoter_Ler	50 Kan				
	pDONR201_DOG1promoter_Kas	50 Kan				
	pDONR201_ABA1_COL	50 Kan				
	pDONR201_alphaDOG1_Col	50 Kan				
	pENTR_alphaDOG1_Cvi**	50 Kan				
	pENTR_alphaDOG1_Ler **	50 Kan				
	pDONR201_alphaDOG1 (no STOP)	50 Kan				
	pENTR_alphaDOG1_Cvi (no STOP)**	50 Kan				
	pENTR_alphaDOG1_Ler (no STOP)**	50 Kan				
	pDONR201_betaDOG1_Cvi	50 Kan				
	pENTR_betaDOG1_Ler**	50 Kan				
	pDONR201_betaDOG1_Cvi (no STOP)	50 Kan				
	pENTR_deltaDOG1_Cvi**	50 Kan				
	pDONR201_deltaDOG1_Ler	50 Kan				
	pENTR_deltaDOG1_Cvi (no STOP)**	50 Kan				
	pDONR201_deltaDOG1_Ler (no STOP)	50 Kan				
Localization studies						
native promoter GUS fusions						
pDOG1_Cvi::GUS	pGWB3_DOG1promoterCvi_GUS	50 Hyg, 50 Kan	GV3101	50 Rif, 50 Gent, 50 Kan, 50 Hyg	25 Hyg, 50 Kan	*dog1-1*
pDOG1_Kas::GUS	pGWB3_DOG1promoterKas_GUS	50 Hyg, 50 Kan	GV3101	50 Rif, 50 Gent, 50 Kan, 50 Hyg	25 Hyg, 50 Kan	
pDOG1_Ler::GUS	pGWB3_DOG1promoterLer_GUS	50 Hyg, 50 Kan	GV3101	50 Rif, 50 Gent, 50 Kan, 50 Hyg	25 Hyg, 50 Kan	
pDOG1_Cvi::GUS	pGreen_DOG1promoterKas_GUS	50 Kan	GV3101 + psoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	L*er* , Cvi
pDOG1_Kas::GUS	pGreen_DOG1promoterCvi_GUS	50 Kan	GV3101 + psoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	
pDOG1_Ler::GUS	pGreen_DOG1promoterLer_GUS	50 Kan	GV3101 + psoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	
native promoter ER-GFP fusions						
pDOG1_Cvi::ER-GFP	pBAT-B_DOG1promoterCvi_ER-GFP	100 Spec	GV3101	50 Rif, 50 Kan, 100 Spec	BASTA	*dog1-1*
pDOG1_Kas::ER-GFP	pBAT-B_DOG1promoterKas_ER-GFP	100 Spec	GV3101	50 Rif, 50 Kan, 100 Spec	BASTA	
pDOG1_Ler::ER-GFP	pBAT-B_DOG1promoterLer_ER-GFP	100 Spec	GV3101	50 Rif, 50 Kan, 100 Spec	BASTA	
localization of DOG1 protein						
pDOG1_Cvi::alphaDOG1::smGFP*	pGreen_DOG1promoterCvi_deltaDOG1*_smGFP	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	*dog1-1*
pDOG1_Cvi::deltaDOG1::smGFP*	pGreen_DOG1promoterCvi_alphaDOG1*_smGFP	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	
p35S::alphaDOG1::smGFP-Term*	pAM-PAT-35S_alphaDOG1*_smGFP-Term	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	*N. benthamiana*
p35S::betaDOG1::smGFP-Term*	pAM-PAT-35S_betaDOG1*_smGFP-Term	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	
p35S::deltaDOG1::smGFP-Term*	pAM-PAT-35S_deltaDOG1*_smGFP-Term	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	
Analysis of *DOG1* splicing variants						
Complementation lines						
*pDOG1_Cvi::alphaDOG1**	pGreen_DOG1promoterCvi_alphaDOG1*	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	*dog1-1*
*pDOG1_Cvi::betaDOG1**	pGreen_DOG1promoterCvi_betaDOG1*	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	
*pDOG1_Cvi::deltaDOG1**	pGreen_DOG1promoterCvi_deltaDOG1*	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	
Overexpression lines						
*p2x35S::alphaDOG1**	pLeela_alphaDOG1*	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	*dog1-1* , L*er*
*p2x35S::betaDOG1**	pLeela_betaDOG1*	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	
*p2x35S::deltaDOG1**	pLeela_deltaDOG1*	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	
*p2x35S::GF_DOG1***	pLeela_DOG1Cvi_ORF	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	L*er*
Inducible expression						
*pMD::alphaDOG1**	pMD_GWY_strepII_alphaDOG1*	100 Spec	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Spec	BASTA	*dog1-1*
*pMD::betaDOG1**	pMD_GWY_strepII_betaDOG1*	100 Spec	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Spec	BASTA	
*pMD::deltaDOG1**	pMD_GWY_strepII_deltaDOG1*	100 Spec	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Spec	BASTA	
pMD::ABA1_Col	pMD_GWY_strepII_ABA1_Col	100 Spec	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Spec	BASTA	*aba1-1, aba1-3, aba 1-5*
Analysis of DOG1 protein						
StrepII-tagged DOG1 protein						
p35S::alphaDOG1::strepII*	pXCSG_strepII_alphaDOG1*	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	*dog1-1*
p35S::deltaDOG1::strepII*	pXCSG_strepII_deltaDOG1*	100 Amp	GV3101 pMP90RK	50 Rif, 10 Gent, 25 Kan, 50 Carb	BASTA	
pDOG1_Cvi::alphaDOG1::strepII*	pGreen_DOG1promoterCvi_alphaDOG1*_strepII	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	*dog1-1*
pDOG1_Cvi::deltaDOG1::strepII*	pGreen_DOG1promoterCvi_deltaDOG1*_strepII	50 Kan	GV3101 + pSoup (Tet)	50 Rif, 25 Kan, 10 Gent, 10 Tet	BASTA	

* Cvi and L*er* alleles were used for the constructs of each *DOG1* splicing variant.
** Made by Kazumi Nakayashi (MPIZ, Cologne, Germany).
*** Made by Yongxiu Liu (MPIZ, Cologne, Germany).

Appendix A 10: Genomic sequence of *DOG1* with indicated nucleotide changes of the three different *dog1* mutant alleles.

Yellow blocks indicate the positions of base-pair changes in the first exon. The mutant allele *dog1-2* (mutant line C 3-7 in Col background) has two base-pair changes from C to A, whereof the first basepair change results in an early stop-codon indicated by **. The mutant allele *dog1-3* (mutant lines 114.1 and 195.1, in Cvi background) carries a single point-mutation with a change from G to A, which leads to an early stop-codon indicated by ***. The red circled nucleotide in the second exon indicates the 1bp deletion in the *dog1-1* mutant allele (in NIL *DOG1* background), * indicates the resulting stop-codon. Capital letters: exons, lower case letters: introns. The green, orange and yellow sequences show the parts that are alternatively spliced leading to four different transcripts.

ATGGGATCTTCATCAAAGAACATCGAACAAGCTCAAGATTCTTATCTCGAGTGGATGAGT
TTGCAATCTCAACGCATCCCTGAGCTCAAACAACTCTTAGCTCAACGACGATCTCACGGT
GATGAAGATAATGATAACAAGCTTCGTAAGTTAACGGGAAAAATCATCGGTGATTTCAAAA
ATTACGCCGCAAAAAGAGCTGATCTTGCTCACCGATGTAGCTCGAACTATTATGCACCCA
CGTGGAACAGTCCTTTAGAGAACGCTCTAATTTGG***ATGGGTGGTTGTCGACCATCTTC
TTTCTTTAGGCTCGTTTATGCTTTGTGTGGGTCA**CAAACTGAGATCCGTGTGACTCAGT
TTCTCCGCAACATCGACGGCTACGAATCTTCAGgtaagggtttggacgttttcggttatttcggttttgaggaata
aaaaaatagactaaatttaatttttggttcggtagtcagtacggtgcggcaaaaaattttattgtcttccttccgatttctaaaatttatttatt
ctgttcattttgcttatgacaaaaataatagattcttaggttttatattaagttgggttcggtttgaatttggaatttgttcagttctatatattatac
aaatatattattatttttaaataaaattaatcataaaaaatgattccgaaccatagatctataccaaaattctaatggtttcggttcggttgg
ttcaatatggtttggtttgacagGTGGTGGCGGCGGTGCATCACTTAGCGACTTAAGTGCGGAGCAG
CTAGCTAAAATCAATGTGTTGCATGTAAAAATTATAGACGAAGAAGAGAAGATGACCAAG
AAAGTCTCAAGCCTACAAGAAGACGCAGCGGATATTCCCATCGCCACTGTGGCTTACGA
GATCTAGAATGTCGGAGAGCCTAA*CGTAGTGGTGGATCAAGCTCTCGACAAGCAAGAA
GAAGCTATGGCTCGTTTATTGGTCGAGGCCGATAATCTAAGGGTTGATACTTTAGCGAAG
ATCCTCGGGATTCTATCTCCGGTACAAGGAGCGGATTTCTTGCTCGCTGGGAAAAAGCTT
CATCTTTCGATGCATGAGTGGGGAACTATGAGAGATCGTCGCCGTCGTGACTGTATGGT
TGACACCGAAGGTAATGCCGGAGGAGAGGAAGGAAAGTAGTCGTTATTATATTAGATATG
ATACTATAGgtacgtacgtgtcatatttaaaattgcataataacaaggttttcaatcttgatattataaagttatatatttggcatatatt
ctagggatatataacccgtatgtgtgttacttttatttttctgaagacttatggtagcaaggtgcaatgcgttatgttagtttatattataaataa
ttaagattttgctttgtataagatggatcttttgttttttattaaatgcagaaatatagttttatatataatcttatataataattattgtttcgttataa
gattgtagtttgtaaggataaaaaataacctttttttttggttaatggataagaaatcatttataagttacattttgatgttttaaattttatcactt
atcgaagatatatttgaattattctatttttttatagTTTATTCACGTCGTGGCATTTTGCGAAATACAGTAATAT
TCGATGCATGTACAACTGTGAATAGTGGCCCACGCCCCACGGAGACGACAAATAATGAG
AGAAATTGA

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Erklärung

Köln, 2008

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit – einschließlich Tabellen, Karten und Abbildungen –, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht worden ist sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Herrn Prof. Dr. Maarten Koornneef betreut worden.

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Sprachen und weitere Fähigkeiten

Sprachkenntnisse	Englisch:	sehr gute Kenntnisse
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