Molecular and cytological analysis of seed dormancy

in *Arabidopsis thaliana*

Diplom-Biologin

Regina Geyer

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Zusammenfassung

Samendormanz oder Samenruhe ist definiert als das Ausbleiben der Keimung eines lebensfähigen Samens unter günstigen Bedingungen. Bei vielen höheren Pflanzen wird durch die Samenruhe der Zeitpunkt der Keimung in einer Samenpopulation optimiert. Samendormanz kann sowohl Austreiben vor der Ernte als auch verfrühte Keimung im Winter verhindern. Während der letzten Jahrzehnte wurden viele physiologische Untersuchungen zur Samendormanz durchgeführt. Aber obwohl die Rolle von Pflanzenhormonen bekannt ist wie auch die Auswirkungen einiger Chemikalien und der Umwelt, ist das Wissen über die zugrunde liegenden molekularen Mechanismen immer noch lückenhaft.

Diese Arbeit beschreibt zwei Ansätze um mehr Einblick in die molekularen Mechanismen zu gewinnen. Einerseits wurde die Chromatinstruktur während der Samenreifung und Dormanz untersucht. Während es nicht gelungen ist einen Unterschied in der Chromatinstruktur dormanter und nicht dormanter Samen zu messen, konnten Hinweise gefunden werden, dass das Volumen von Embryokernen während der Samenreifung abnimmt. Das könnte mit der Entwicklung der Trockentoleranz während der Samenreifung zusammen hängen. Außerdem wurde das Gen, das für den Dormanzphänotyp der *reduced dormancy2* (*rdo2*) Mutante verantwortlich ist, kloniert und als TFIIS Transkriptionselongationsfaktor identifiziert. Das kodierte Protein weist starke Konservierung mit dem Homolog aus Hefe auf, die Funktion ist aber pflanzenspezifisch, da es den Phänotyp der Hefemutante nicht komplementieren kann. Von Hefe ist bekannt, dass das TFIIS Protein direkt mit der RNA Polymerase II interagiert, um eine transkriptionelle Sperre zu lösen. *RDO2* ist in allen Geweben exprimiert, aber am höchsten im Samen. Die Menge des Transkripts steigt während der Samenreifung an, und erreicht im reifen Samen das Maximum. Stabile Transformanden, die ein RDO2-YFP Fusionsprotein exprimieren, zeigen Fluoreszenz im Zellkern, was mit der annotierten Funktion von RDO2 als TFIIS Protein übereinstimmt. Quantitative Real Time PCR hat gezeigt, dass *rdo2* Mutanten während der Samenreifung weniger *DOG1*, ein Dormanzschlüsselgen, exprimieren als L*er*. Das würde die geringere Dormanz der *rdo2* Mutante zumindest teilweise erklären. *RDO2* ist das zweite klonierte Gen nach *HUB1*, das eine Verbindung zwischen Transkriptionsregulation und Dormanz herstellt.

Es ist wahrscheinlich, dass während der Samenreifung, wenn sich der Wassergehalt der Samen verringert und die Kerne schrumpfen, die Effizienz der Transkriptionselongation abnimmt und deshalb Transkriptionselongationsfaktoren wie *RDO2* zunehmend benötigt werden.

Abstract

Seed dormancy is defined as the failure of an intact viable seed to complete germination under favorable conditions. Many higher plants express seed dormancy to optimize the distribution of germination over time in a population of seeds. Seed dormancy can prevent preharvest sprouting as well as the untimely germination during winter. During the last decades several physiological investigations were performed on seed dormancy. But even though the role of plant hormones is known as well as the effect of some chemicals and the environment, the knowledge about the underlying molecular mechanisms remains fragmentary.

This thesis describes two approaches to gain more insight in the molecular mechanisms. At the one hand, the chromatin structure during seed maturation and dormancy was investigated. While a difference of chromatin structure of dormant and non-dormant seeds was not measurable, indications were found that embryo nuclei reduce their volume during seed maturation. This could be related with the acquisition of desiccation tolerance during seed maturation.

In addition, the gene responsible for the dormancy phenotype of the *reduced dormancy 2* (*rdo2*) mutant was cloned and identified as a TFIIS transcription elongation factor. The encoded protein shows strong conservation with the yeast homologue, but has a plant specific function, as it cannot complement the phenotype of the yeast mutant. In yeast the TFIIS protein is known to interact physically with the RNA polymerase II complex to release it from transcriptional arrest. *RDO2* is expressed in all tissues, but highest in seeds. The transcript level increases during seed maturation and reaches a maximum in mature seeds. Stable transformants expressing an RDO2-YFP fusion protein express fluorescence in the nuclei, which is consistent with the annotated function of *RDO2* as a TFIIS protein. Quantitative Real Time PCR revealed, that the transcript level of *DOG1*, a key dormancy gene, is reduced in *rdo2* mutant seeds during maturation compared to L*er*. This would at least partially explain the reduced dormancy of the *rdo2* mutant.

RDO2 is the second cloned gene after *HUB1* that links transcription regulation to dormancy. It is probable, that during seed maturation, when the water content of the seeds is reduced and the nuclei shrink, transcription elongation becomes less efficient, therefore a transcription elongation factor like RDO2 is increasingly required.

Contents

1 Introduction

Seed dormancy, defined as the incapability of a viable seed to germinate under favorable conditions, is an important trait in nature and agriculture. To get access to the genomic mechanisms behind it the topic is studied in the model plant *Arabidopsis thaliana*.

1.1 *Arabidopsis thaliana*

The small dicot *Arabidopsis thaliana*, member of the *Brassicaceae*, started its career as a model organism in the 1940s, when Laibach recommended it for genetic and physiological investigation (Laibach, 1943). By now it is an intensively studied plant, being object to physiological, genetical, biochemical and molecular investigations (Meyerowitz, 1987). It is favored because of its short life cycle of six to eight weeks, the ease of self- or cross-fertilization at will, the numerous progeny, and the possibility to be grown on minimum space in greenhouses and climate chambers (Meyerowitz, 1987). The genome of *Arabidopsis thaliana* is distributed over five chromosomes and counts approximately 130 Mb encoding more than 27,000 genes. The genome is sequenced almost completely (The Arabidopsis Genome Initiative, 2000) and all information is publicly available (www.arabidopsis.org). Consequently mutant and knockout collections of practically every gene are available. Over the last decades, scientists of the Arabidopsis research community developed a variety of techniques including efficient transformation and ways to study its molecular genetics, biochemistry and physiology. Internet resources provide access to results of various large scale experiments, like microarray data, as well as *in silico* analysis tools. *Arabidopsis thaliana* is native to the northern hemisphere, even though its exact geographic origin is unknown. It has been collected from the arctic circle to the equator at various altitudes (Hoffmann, 2002). Most commonly used in laboratory work are the accessions Columbia (Col), which was chosen for sequencing, and Landsberg *erecta* (L*er*), background line of various mutants. Accessions collected from natural populations provide greater variation in genotype and phenotype and are utilized especially to analyze quantitative traits (Koornneef et al., 2004). Finally, variation in germination response and dormancy phenotypes of *Arabidopsis thaliana* is comparable to that of many higher plants, qualifying *Arabidopsis thaliana* as a model in seed research (Koornneef et al., 2002).

1.2 Seed development in *Arabidopsis thaliana*

Under greenhouse conditions *Arabidopsis thaliana* generates elongated siliques that contain 40 to 60 seeds each. The seed development takes about 20 days from pollination to release of the mature seed. Ripe L*er* seeds have an average length of 0.5 mm and a red-brown color. Embryogenesis

starts after pollination, when the haploid egg cell and the central cell of the embryo sac are fertilized in parallel by a sperm cell each. The fertilized egg cell forms a diploid zygote that generates the embryo. The two haploid nuclei of the central cell of the embryo sac fuse with one sperm cell and develop into the triploid endosperm. During the first ten days of development the embryo undergoes a phase of rapid cell growth and proliferation. From the zygote it develops into a heart-shaped then a torpedo-shaped structure. After bending of the cotyledons it looks like a walking stick before finally hypocotyl and cotyledons have similar volume (Figure 1.1). At the end of seed expansion, when the embryo has reached its final size and fills the seed coat completely, cell division in the embryo arrests (Raz et al., 2001). During the second half of seed development reserves are accumulated (reviewed by Meinke, 1994), primary dormancy is initiated (Bewley, 1997), chlorophyll is degraded and tolerance to desiccation is acquired. The induction of dormancy starts early during seed maturation and in the mature seed dormancy is at its highest level (Raz et al., 2001). During maturation drying 90 to 95 % of the original water content is removed. This results in a gradual reduction of metabolism and the embryo gets into a metabolically quiescent state. In the dehydrated state the seed can survive disadvantageous circumstances and the metabolism will be restarted when favorable conditions promote germination (Kermode and Finch-Savage, 2002).

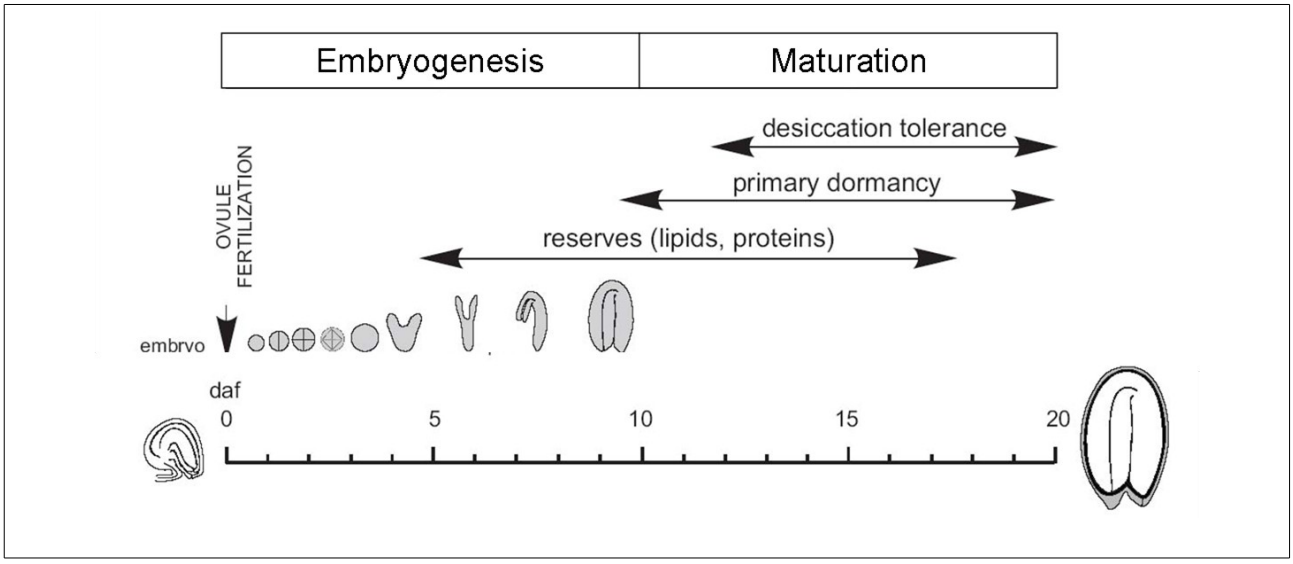

Figure 1.1: Seed development in *Arabidopsis thaliana.* During embryogenesis the seed grows from a zygote to a bend structure that fills the seed coat, before during the second half of seed development reserves are accumulated, chlorophyll is degraded, dormancy is initiated and dessication tolerance is acquired. daf, days after fertilization, equivalent to days after pollination (dap) (adapted from Debeaujon et al., 2007).

1.3 Seed dormancy in *Arabidopsis thaliana*

An intact viable mature seed is dormant, if it is not able to germinate under favorable conditions, which are presence of water, oxygen, light and appropriate temperature (Bewley, 1997). Dormancy can occur during time as primary dormancy, induced during seed maturation and determined by environmental and endogenous factors, or secondary dormancy, which might be induced by unfavorable conditions after release from the dormant state. Seed dormancy is an adaptive trait and assures that germination occurs during the most suitable time to establish seedlings and complete the plants life cycle. It is a survival strategy in which development is temporally adjourned and energy is saved (Bewley, 1997). The quality and duration of the dormant state is different and therefore characteristic among the accessions of *Arabidopsis thaliana* (Laibach, 1951; Evans and Ratcliffe, 1972; Ratcliffe, 1976).

There are two kinds of dormancy which are caused by different mechanisms. The coat-imposed or coat-enhanced dormancy is generated by the seed envelopes. When the physical barrier of the seed envelopes is removed, the isolated embryo is able to germinate. Here the growth potential of the embryo is important to overcome the constraints of the seed envelopes (Bentsink and Koornneef, 2002). Embryo dormancy, on the other hand, is intrinsic to the embryo and not promoted by some other structure (Debeaujon et al., 2007).

Dormancy is regulated by a combination of environmental and endogenous signals. Molecular studies of dormancy revealed changes in transcriptomes, proteomes, and hormone levels during different dormancy states (Finkelstein et al., 2008). The relation of abscisic acid (ABA) to gibberellin (GA) levels and sensitivity is a crucial regulator of dormancy status, but not the only one. ABA promotes dormancy induction and maintenance, whereas GA promotes progression from release through germination (Hilhorst, 1995; Debeaujon and Koornneef, 2000).

Dormancy release is a gradual process, a combination of changes in the constraints of the seed envelopes and the growth potential of the embryo. Besides changes in the hormonal state also exogenous factors can promote germination. Two possibilities are cold stratification, i.e. exposure to cold temperatures under moist conditions, and afterripening, which is dry storage of mature seeds. The requirement of these treatments to break dormancy are specific to *Arabidopsis thaliana* accessions. Winter annuals experience afterripening during summer, leading to germination in fall, while summer annuals rely on stratification during winter and are able to germinate in spring (Baskin and Baskin, 1998).

Even though some aspects of dormancy regulation have been explored, the knowledge about the underlying molecular mechanisms remains fragmentary (Finch-Savage and Leubner-Metzger, 2006).

Dormancy genes referred to in this thesis

One important regulator of dormancy, *DOG1*, was first identified as a quantitative trait locus (QTL) for dormancy in *Arabidopsis thaliana* (Alonso-Blanco et al., 2003). As described by Bentsink et al. (2006) the non-dormant mutant *dog1* was found in a mutagenesis screen for reduced seed dormancy. For this seeds of a near isogenic line containing a Cvi introgression in L*er* background at the position of the DELAY OF GERMINATION 1 QTL (NIL DOG1) were treated with γ-irradiation and mutants with no or strongly reduced dormancy were selected. The *dog1* mutant is completely non-dormant and expresses no pleiotropic phenotype except being less storable at room temperature than L*er*. The *DOG1* gene was cloned and turned out to be a member of a novel plant specific gene family with unknown function. Lately it was found that the DOG1 locus is also involved in the ABA-mediated sugar signalling pathway, since the DOG1 Cvi allele responds to addition of glucose (Teng et al., 2007).

Another gene involved in the dormancy process is *HISTONE MONOUBIQUITINATION1* (*HUB1*), which was recently cloned in our lab (Liu et al., 2007). Its mutant was identified in a mutagenesis screen for reduced dormancy in L*er*, and was originally named *rdo4* (Peeters et al., 2002). The *hub1* mutant is less dormant than L*er* and expresses several pleiotropic phenotypes. The *HUB1* gene was found to encode a C3HC4 RING finger protein responsible for ubiquitination of histone H2B. It was shown, that the expression of several dormancy-related genes in the *hub1* mutant is changed, suggesting a role for transcription control by histone ubiquitination in the dormancy mechanism.

1.4 The dormancy mutant *rdo2*

Four mutants with reduced dormancy, isolated in a dormancy screen after γ-irradiation of the L*er* accession, were described by Léon-Kloosterziel et al. (1996) and Peeters et al. (2002). *reduced dormancy 2* (*rdo2*) seeds are characterized by their ability to germinate directly after harvest in contrast to the wild-type L*er*, which needs two to six weeks of after-ripening. The recessive mutant also expresses a weak pleiotropic phenotype consisting of slightly darker green rosette leaves, less side shoots and a minor retardation of flowering time in comparison to L*er*. The normal sensitivity of the *rdo2* mutant to ABA suggests that *RDO2* functions in a different, yet unknown pathway than the typical non-dormant ABA mutant. A rough mapping localized the gene at the lower part of

chromosome 2 (Peeters et al., 2002) but did not show co-localization with known dormancy genes or QTLs.

1.5 Transcription elongation factor TFIIS

Transcription is the process of transcribing DNA sequence into RNA sequence. In eucaryots transcription of genes proceeds in three steps, which are initiation, elongation and termination. Initiation starts when the promoter sequence binds the RNA polymerase and all required additional factors. Consequently the DNA around the starting point unwinds, resulting in single-stranded DNA. After initiating the synthesis of the new RNA strand, the RNA polymerase gets into the elongation phase. During transcription, the polymerase complex also unwinds and reanneals the DNA in front and behind the point of transcription, and proofreads the new RNA strand. At the end of the transcribed sequence, the RNA and the enzyme complex dissociate from the DNA, which is called termination (Watson et al., 2008).

Transcription elongation by the RNA polymerase is not always continuous, but faces blocks of different nature. These include nucleosomes, DNA lesions, DNA binding proteins and specific DNA sequences itself (Uptain et al., 1997). One of the factors that enable the RNA polymerase to overcome these blocks is the TFIIS transcription elongation factor. At the beginning of the 1970s the TFIIS protein was identified in mouse (Natori et al., 1973), later in yeast (Sawadogo et al., 1980A), calf thymus (Rappaport et al., 1987), humans (Reinberg and Roeder, 1987) and Drosophila (Sluder et al., 1989). Most eucaryots have several copies with specific spatial or developmental expression (Uptain et al., 1997; Spencer and Groudine, 1990). TFIIS proteins have not yet been studied in plants, but the topic is deeply investigated in *Saccharomyces cerevisiae*. The protein contains three functional domains, of which the N-terminal TFIIS domain is composed of a four-helix bundle that is not required for the known biochemical and biological functions of TFIIS (Booth et al., 2000). Nevertheless a number of biochemical and genetical interactions between the TFIIS domain and other factors have been described (Pan et al., 1997; Wery et al., 2004; Malagon et al., 2004; Davie and Kane, 2000; Fish et al., 2006). The TFS2M domain forms a three-helix bundle and is connected to the zinc finger domain by a short linker (Kettenberger et al., 2003). The zinc finger domain is composed of three antiparallel β-sheets that form a zinc ribbon. The TFS2M domain and the linker are required for binding of the RNA polymerase II whereas the zinc finger domain is essential for stimulation of RNA cleavage (Awrey et al., 1998).

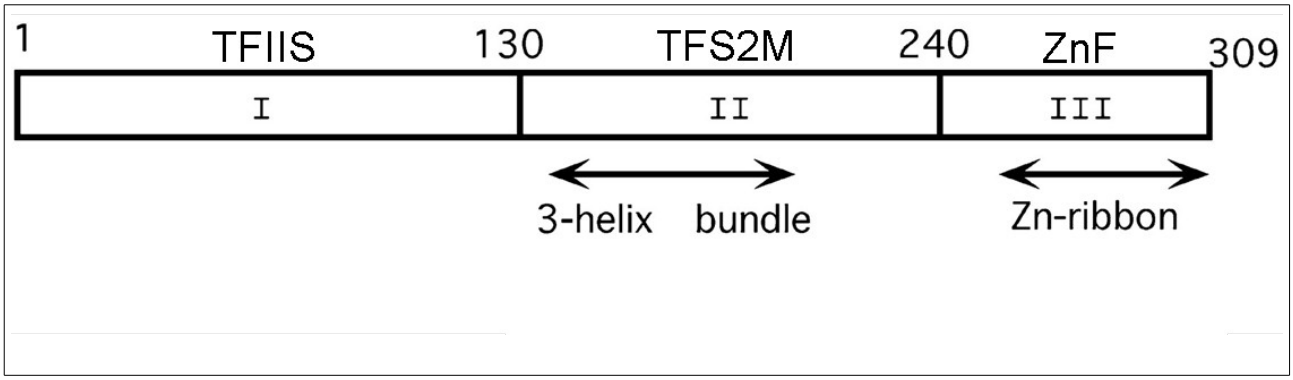

Figure 1.2: Structure of yeast TFIIS protein. The location of the three functional domains is depicted (adapted from Olmsted et al., 1998).

The function of the TFIIS protein which is best investigated is its ability to accelerate the overcome of transcriptional arrest of RNA polymerase II (Fish and Kane, 2002). Kettenberger et al., (2004) showed, that the C-terminus of the TFIIS protein inserts into the active center of the RNA polymerase II complex and induces a shift of the RNA strand. The TFIIS stimulated RNA cleavage creates a new RNA 3' end in the active center and facilitates read through of the block. Lately additional roles for TFIIS were found. It was shown to support the formation of the transcription preinitiation complex (Kim et al., 2007) and to act as a general RNA polymerase III transcription factor (Ghavi-Helm et al., 2008). In yeast knock-out of the single copy TFIIS gene leads to a phenotype only in presence of the NTP-depleting drugs 6-azauracil and mycophenolic acid, so the protein might not be essential under normal conditions or be functionally redundant with other transcription factors (Wery et al., 2004).

1.6 Chromatin structure

All eucaryots have chromosomes with more and less gene-rich regions. The different kinds of chromatin are structurally characterized by varying degrees of compaction and associated with specific proteins such as histones. In contrast to many other plants *Arabidopsis thaliana* has little highly condensed heterochromatin, which facilitates investigation of the chromatic structure. By staining with dyes that intercalate in the DNA helix (DAPI or propidium iodide) interphase nuclei display the highly aggregated chromatic areas as regions of strong fluorescence. These so called chromocenters contain the strongly condensed and repeat rich heterochromatin (Fransz et al., 2002). In yeast, *Drosophila melanogaster* and mammalian cells, a clear relationship has been established between epigenetic markers, chromatin organization and gene expression during cell differentiation (reviewed by Arney and Fisher, 2004). Grigoryev et al. (2004) found in mouse lymphocytes, that constitutive centromeric heterochromatin plays an active role in the transition from proliferation to

quiescence. At crucial points during the plant life cycle, when cells or tissues transform towards a new fate or function, the chromatin undergoes structural changes in organization. During floral transition pericentromeric heterochromatin as well as gene-rich chromatin decondensates, possibly to provide better access to the DNA for transcription (Tessadori et al., 2007B). Upon dedifferentiation of mesophyll cells into undifferentiated protoplasts the heterochromatin decondenses, and the repeat-rich areas are resorted (Tessadori et al., 2007A). Rearrangements can also be caused by low light conditions, biotic stress or infection by pseudomonas (Tessadori et al., 2007B, Paul Fransz, personal communication).

1.7 Objectives of the thesis

The work described in this thesis aimed to a better understanding of the molecular mechanisms of seed dormancy in *Arabidopsis thaliana*. The goal was to clone and characterize the dormancy gene *RDO2* and to investigate the chromatin structure of seeds during seed maturation and dormancy.

Chromatin structure during seed maturation and dormancy

The change from seed maturation to dormancy is an important transition during plant development, and is associated with large scale changes in the transcriptome. This could be reflected in alterations in the chromatin structure of embryonic nuclei. This thesis aimed to answer the question whether there is a measurable alteration of chromatin organization at the microscopic level during seed maturation and dormancy. Besides the comparison between dormant and non-dormant seeds as well as seeds during maturation another goal was to establish a technique to measure heterochromatin content.

Cloning and characterization of RDO2

To improve our understanding of the molecular mechanism of seed dormancy, the elucidation of gene functions and regulatory pathways involved in this process is an important goal. The aim of this thesis was to clone the gene that is responsible for the reduced dormancy of the *rdo2* mutant. Its molecular function and interactions with other dormancy genes or regulatory pathways would elucidate its role in dormancy.

2 Materials and methods

2.1 Materials

2.1.1 Chemicals and antibiotics

All chemicals used for the experiments were purchased from the following suppliers: AppliChem (Darmstadt, Germany), Aventis (Strasbourg, France), Becton, Dickinson & Co. (Le Pont de Claix, France), Bio-Budget (Krefeld, Germany), Biorad (Hercules, USA), Fermentas (St. Leon-Rot, Germany), Fluka (Buchs, Germany), Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), Promega (Mannheim, Germany), Roche (Mannheim, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany), Sigma (Deisenhofen, Germany).

All antibiotics for bacterial work were purchased from Duchefa (Haarlem, Netherlands). Stock solutions of dissolved antibiotics were stored at -20°C.

Antibiotic (Abbrevation)	**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
Kanamycin (Kan)	50	H_2O	50	25
Rifampicin (Rif)	50	DMSO	-	50
Tetracyclin (Tet)	10	H_2O	10	10

Table 2.1: Antibiotics

2.1.2 Buffers and culture media

General buffers and media were prepared as described by Sambrook and Russel (2001) and autoclaved for 20 min at 121°C. For some applications specific solutions were prepared.

Enzyme mix
- 0.3 % pectolyase,
- 0.3 % cytohelicase
- 0.3 % cellulase
- in citrate buffer

BVO Fix buffer (after Bauwens and Van Oostveldt)
- 1 % formaldehyde
- 10 % DMSO
- 2 mM EGTA
- 0.1 % Tween-20
- in PBS

PBT
0.1 % Tween-20
in PBS

Dellaporta DNA Extraction Buffer
50 mM Tris pH 8
10 mM EDTA pH 8
0,1 M NaCl
1 % SDS

Dellaporta 5M/3M potassium acetate
3 M potassium acetate
11,5 % glacial acetic acid

RNA High Salt Precipitation Solution
0.8 M sodium citrate
1.2 M sodium chloride

2.1.3 Enzymes and commercial kits

All restriction enzymes including their buffers were purchased from Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt a. M., Germany), or Roche (Mannheim, Germany).

The following nucleic acid modifying enzymes were used:

Accu Prime *Pfx* DNA Polymerase (Invitrogen, Karlsruhe, Germany)
Klenow fragment exo (Fermentas, St. Leon-Rot, Germany)
Lysozym (Roche, Mannheim, Germany)
Pfu DNA-Polymerase (Fermentas, St. Leon-Rot, Germany)
Ribonuclease Inhibitor (Roche, Mannheim, Germany)
RNase A (DNase-free) (Qiagen, Hilden, Germany)
RNase H (Promega, Mannheim, Germany)
Superscript™ II reverse transcriptase (Invitrogen, Karslruhe, Germany)
Taq DNA Polymerase (Invitrogen, Qiagen or Roche)
T4 DNA Ligase (Invitrogen, Karlsruhe, Germany)

The following commercial reagents and kits were used:

1kb DNA ladder (Invitrogen, Karlsruhe, Germany)
Gateway® BP/LR-Clonase™ II Enzyme Mix (Invitrogen, Karlsruhe, Germany)
MagAttract® 96 DNA Plant Kit (Qiagen, Hilden, Germany)
NucleoSpin® Plasmid (Macherey-Nagel, Düren, Germany)
NucleoSpin® RNA Plant (Macherey-Nagel, Düren, Germany)
Power SYBR® Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany)
QIAprep®Spin Miniprep Kit (Qiagen, Hilden, Germany)
QIAquick® Gel Extraction Kit (Qiagen, Hilden, Germany)
RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany)
RNAqueous™ Phenol-free total RNA Isolation (Ambion, Austin, USA)
RNeasy® Plant Mini Kit (Qiagen, Hilden, Germany)
RNA Isolation Aid (Ambion, Austin, USA)
Zero Blunt® TOPO PCR Cloning Kit (Invitrogen, Karlsruhe, Germany)

2.1.4 Oligonucleotides and plasmids

All synthetic oligonucleotides were purchased from Invitrogen (Karlsruhe, Germany) or Operon (Köln, Germany). The primers used for different applications, are listed below.

The differentiation of *wtRDO2* and mutant *rdo2* is possible with a primer that ends with the 4 bp deleted in the mutant. It does not anneal to the mutant sequence but amplifies the wt sequence selectively.

Name	Position (Mb)	Type	Forward Sequence 5' to 3'	Reverse Sequence 5' to 3'
T2N18	15,586	SSLP	TTTACGAATAGGATTGGGTTTCATC	ATGATTCTCTTTATGGCTCCTCAGC
F16M14	15,995	SSLP	TAGCTACAGTCACCACGAGCAC	CCTGGAACCTAAATCTAAGAATATGAC
T19C21	16,110	SSLP	AACAAGCCAGTCTTTCCAATGC	AAATTTTGGTGAATGCCTTTGC
T6A23-2	16,129	SSLP	CACAAAAAGCATCTCTTTCAGTCC	TGAATAATGCTTCATCTATCTTTCACG
T6A23-1	16,175	SSLP	TCACCTTTACATTGTTTGCTTTGG	CGTCTCAGATCTCTCACAGATGTTC
F12L6	16,487	SSLP	CGCAGCATTGCTATCACATCAG	GCCTGCATGGGAATAGTGACAG
T3K9	17,114	SSLP	AAATTGCTGTGATGGTGAG	GAAGGAGCATTATGGACATG
MHK10-2	17,694	SSLP	TTGTAAGATTTCCCGGAGTTTCG	CTAGCCACGGCCACGATTTC
T1O24	18,019	SSLP	TTAACAGAAACCCAAAGCTTTC	TGACCTCCTCTTCCATGGAG

Table 2.2: Primers for mapping of RDO2 and their physical position on chromosome 2

Name	Genotype	Forward Sequence 5' to 3'	Reverse Sequence 5' to 3'
RDO2-mut-R	RDO2 wt		GTCACAGTTAACACATGTAACAT
rdo2-mut-R	rdo2 mutant		GTCACAGTTAACACATGTAAAGT
nit1.2	NILD117	CGGAATTGATGTTTTGGACC	CCCTACATTCTACAACCATGTAGCC

Table 2.3: Primers for different genotypes

Name	Sequence 5' to 3'
35S-promotor	CAATCCCACTATCCTTCGC
BASTA_R	CAGTCGTAGGCGTTGCGTGCCTTC
KanR-F	CTATGGAACTGCCTCGGTGAG
KanR-R	CAATCGGTAGATTGTCGCACCTG
M13_F	GTAAAACGACGGCCAG
M13_R	CAGGAAACAGCTATGAC
pat_F	GCTTCAAGAGCGTGGTCGCTGTC
pat_R	GAAGTTGACCGTGCTTGTCTCG
RDO2-3UTR-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCAGTTGAGTCAGAATCAGTTTC
RDO2-3UTR-R	GCAGTTGAGTCAGAATCAGTTTC
RDO2-F	ATGGAGAGTGATTTGATTGATTTG
RDO2-F-SalI	GACATAGTCGACTTCATGGAGAGTGATTTGATTG
RDO2-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGAGTGATTTGATTGATTTG
RDO2-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAACAGAACTTCCAGTGGTTGTC
RDO2-proml-F	GAGTTGCGGTTGCTACTGTCTC
RDO2-proml-F-AscI	GGCAATCGGCGCGCCGAGAGAGTTGCGGTTGCTACTGTCTC
RDO2-proml-F-SfbI	GTACAAACCTGCAGGCTTCGAGTTGCGGTTG
RDO2-proml-GW-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAGTTGCGGTTGCTACTGTCTC
RDO2-proml-GW-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCCGTTCCGACAATCCCTAGCTC
RDO2-proml-R	CGTTCCGACAATCCCTAGCTC
RDO2-prom-R-XhoI	CAATCAACTCGAGCTCCAACGTTCCGACAATCCCTAGCTC
RDO2-proms-F	TTTGTGAAAAGCCCATCAAAC
RDO2-proms-F-AscI	CGAGGGCGCGCCTTTGTTTGTGAAAAGCCCATCAAAC
RDO2-R	TCAACAGAACTTCCAGTGGTTGTC
RDO2-R-EcoRI	CAAGAATTCTGGGTCTCAACAGAACTTCCAGTGG
RDO2-R-PstI	CAAGAAAGCTGCAGCTCAACAGAACTTCCAGTGG
SeLA	TCGCGTTAACGCTAGCATGGATCTC
SeLB	GTAACATCAGAGATTTTGAGACAC

Table 2.4: Primers for molecular cloning

Gene	Forward Sequence 5' to 3'	Reverse Sequence 5' to 3'
Actin 8	CTCAGGTATTGCAGACCGTATGAG	CTGGACCTGCTTCATCATACTCTG
DOG1	CGGCTACGAATCTTCAGGTGG	CTGCGTCTTCTTGTAGGCTTGAG
RDO2	CTCGTTGCGACTCAGGTGG	CAACCGTTAGTGCCTTCGG
wtRDO2	GCCCTGTTTGATTGTGAG	GTCACAGTTAACACATGTAACAT

Table 2.5: Primers for quantitative RT-PCR

Gene	Forward Sequence 5' to 3'	Reverse Sequence 5' to 3'
ACT2	GTATGGTGAAGGCTGGATTTGC	TGAGGTAATCAGTAAGGTCACGTCC
PER1	ATAAGAGAGGCGTGAAGCTCCTTGG	GTGGGAACATCTTTTTGGCTTCCTC
DOG1	GAGCTGATCTTGCTCACCGATGTAG	CTGCGTCTTCTTGTAGGCTTGAG
ABI4	GCTTCCCAACATCAACACAACCATC	GGAGACGGAGGAGGAAGAGGAAGAG
ATS2	CGTGGTGACTTGGATGACACACTTC	GCACCGCCTTATGGCTTCTTTAGAC
CYP707A2	TAAGCGGCTGGTCCAGTCTTCTTTC	GAGGAGATTGGGGTGGTCGTGTAAG
NCED9	AACCGCAGCGTTTAATCAAGAATCG	TTTCCACCGCGTCTAAAACCATAGC
SPT	GACTGTGAAAGCGAGGAAGGAGGAG	CGGGTGAAGTAAGGAGAGGGAAAGG
wtRDO2	GGAGATTTCACCAGAGAAACTC	GTCACAGTTAACACATGTAACAT
RDO2+rdo2	ATGGAGAGTGATTTGATTGATTTG	TCAACAGAACTTCCAGTGGTTGTC

Tabelle 2.6: Primers for semiquantitative PCR

All plasmids used for molecular cloning were provided by colleagues or purchased from Invitrogen (Karlsruhe, Germany).

Name	Resistance	Supplied/provided by
pCR-Blunt II-TOPO	Kanamycin	Invitrogen (Karlsruhe, Germany)
pDONR201	Kanamycin + Chloramphenicol	Invitrogen (Karlsruhe, Germany)
pDONR207	Gentamycin + Chloramphenicol	Invitrogen (Karlsruhe, Germany)
pENSG_YFP	Ampecilin/Carbenicilin + Chloramphenicol	AG Schnittger (MPIZ)
pEXSC_YFP	Ampecilin/Carbenicilin + Chloramphenicol	AG Schnittger (MPIZ)
pGreen_gw_MCS	Kanamycin + Chloramphenicol	AG Turck (MPIZ)
pLeela_GW	Ampecilin/Carbenicilin + Chloramphenicol	Melanie Schwab (MPIZ)
pSoup	Tetracyclin	Christina Philipp (MPIZ)

Table 2.7: Plasmids for molecular cloning

2.1.5 Bacterial strains

For molecular cloning chemically competent cells of *E. coli* strain DH5α were used (Hanahan, 1983). The *E. coli* strain DB3.1 was used for amplification of vectors carrying a Gateway cassette (Invitrogen) including the *CcdB* gene. For plant transformation, cells of the *A. tumefaciens* strain GV3101 were used, some of them carrying pSoup or pMP90RK, depending on the used plasmid.

2.1.6 Yeast strains

For the Yeast-two-hybrid screen the strains AH109 and Y187 were used. Furthermore the library HS-Ara in AH109, derived from complete Col plants (09.08.2004) was used.

For the growth test the strains CH1305 and CMKy3 were used, kindly provided by Caroline Kane (University of California, Berkley).

2.1.7 Plant material

Ecotypes of *Arabidopsis thaliana* used for the described experiments are Landsberg *erecta* (L*er*, Rédei, 1992), Cape Verde Islands (Cvi, Lobin, 1983) and Columbia (Col). Furthermore near isogenic lines (NILs), T-DNA insertion lines from SALK (Alonso et al., 2003) and GABI-Kat (Rosso et al., 2003) and mutants were used as listed below.

Name	Description	provided by
dog1	*dog1-1*, non-dormant allele of *dog1*, background NILD106	Melanie Schwab (MPIZ)
LCN2-18	Cvi introgression at position of *RDO2* (chromosome 2), background L*er*	Maarten Koornneef (MPIZ)
NILD106	Cvi introgression at position of DOG1 (chromosome 5), background L*er*	Maarten Koornneef (MPIZ)
NILD117	Cvi introgression at position of DOG6 (chromosome 3), background L*er*	Maarten Koornneef (MPIZ)
NILD73	Cvi introgression at position of DOG3 (chromosome 1), background L*er*	Maarten Koornneef (MPIZ)
rdo2-1	4 bp deletion in *RDO2*, background L*er*	Maarten Koornneef (MPIZ)
rdo2-2	GABI_817G07, T-DNA insertion 322 bp in front of ATG of *RDO2*	GABI-Kat
rdo2-3	SALK_056755, T-DNA insertion 876 bp behind ATG of *RDO2*	NASC
rdo2-4	GABI_273H04, T-DNA insertion 1059 bp behind ATG of *RDO2*	GABI-Kat
rdo2-5	SALK_027259, T-DNA insertion 1073 bp behind ATG of *RDO2*	NASC
rdo2-6	SALK_133631, T-DNA insertion 1195 bp behind ATG of *RDO2*	NASC

Table 2.8: *Arabidopsis thaliana* lines

2.2 Methods

2.2.1 Plant works

2.2.1.1 Germination, growth and harvest

Arabidopsis thaliana seeds were stratified on water-soaked filter paper (Macherey-Nagel, Düren, Germany) in plastic Petri dishes (Ø 6 cm) at 4°C in the dark for 4 d to break dormancy and synchronize germination. To induce germination, the Petri dishes were then placed in an incubator (MC785-VDB, van den Berg, Montfoort, Netherlands) under long day conditions (12 h 25°C light, 12 h 20°C dark) for ~ 24 h. The moist seeds were transfered one by one to soil, that contained a mixture of substrate and vermiculite. In general plants were grown in air conditioned growth chambers (Mobylux GroBanks, CLF Plant Climatics, Emersacker, Germany) under long day conditions (16 h 22°C light, 8 h 16°C dark) to reduce the influence of the seasons. Some plants (e.g. after transformation with *Agrobacterium tumefaciens*) grew in a greenhouse with ~16 h of natural and artifical light when needed. Plants were alowed to ripe without being covered by collection paper bags. Seeds were harvested from dry plants, sieved and filled in small, non sealed plastic bags. These bags were stored either in a closed cupboard at room temperature in the lab or for the

time of running germination tests in an incubator (Climacell, MMM Group, Planegg, Germany) at constantly 21°C and 50 % humidity in the dark.

2.2.1.2 Germination test

For dormancy measurement, the ability to germinate was determined in an periodic assay. About 100 seeds of each batch were evenly distributed in small Petri dishes with filter paper soaked with ~500 µl of demineralized water. The Petri dishes were placed in transparent plastic boxes with lids and wet filter paper. These moisture chambers were stored in an incubator under long day conditions (12 h 25°C light, 12 h 20°C dark). After seven days the total number of seeds and the number of germinated seeds were counted with the help of a dissecting microscope (MZ6 or MZ12.5, Leica Microsystems, Wetzlar, Germany) and a reflected light lamp (KL1500LCD, Schott, Mainz, Germany). The percentage of germinated seeds was calculated and represents the germination ability at the moment of sowing. The procedure was repeated weekly until the batch reached at least 95 % germination.

2.2.1.3 Stable transformation of *Arabidopsis thaliana* and selection of transgenics

A. thaliana plants were transformed following the Floral Dip method (Clough and Bent, 1998). For each experiment, 25 to 40 plants were grown in growth chambers under short day conditions (8 h 22°C light, 16 h 16°C dark) until 5-10 inflorescences opened. *Agrobacterium tumefaciens* carrying the plasmid of choice was streaked out onto YEB plates with the selective antibiotics and incubated for 2-3 d at 28°C. Bacteria were collected from the plates by scraping and resuspended in 30 ml YEB medium. Prior to dipping 120 ml of a solution containing 5 % sucrose and 0.03 % Silwet L-77 were added to the bacteria. The upper part of the plants with all inflorescences was dipped into the bacteria solution for 10-20 sec. The treated plants were kept horizontal in a moist chamber for one day before they were transferred to the greenhouse.

All plasmids used in this work carry the BASTA™ resistance gene as a reporter gene in *A. thaliana*. The T_1 seeds of the transformed (T_0) plants were sown on soil and when the first real leaves became visible, the plants were sprayed with a solution of the herbicide Glufosinat (200 mg/l in H_2O, BASTA™, Hoechst, Frankfurt a. M., Germany) three times with an interval of 2 d. The T_2 seeds of the surviving T_1 plants were treated the same way. After spraying, the ratio of survivors to non-survivors was determined and in case of a 3:1 splitting a single insertion of the T-DNA was assumed. The T_3 seeds of the T_2 plants again were treated with BASTA™. If all of the T_3 plants were resistant, the T_2 parent was identified as being homozygous for the insertion.

2.2.1.4 Transient transformation of *Allium ampeloprasum*

The transient transformation of leek epidermis was performed with the particle gun as described by Cole et al. (2006). 50 mg tungsten particles were kept on 60°C for 2 h in 1 ml of ethanol before they were chilled on ice for 10 min. After short centrifugation the supernatant was removed, and 1 ml of new ethanol was added prior to three periods of 5 min sonification of maximum 50 W with chilling on ice in between. The contents was centrifugated shortly and washed three times with water before the suspension was kept in 1 ml of water and stored at -20°C. The ingredients for coating the tungsten particles were all stored at -20°C prior to coating and were added to a tube while mixing with a vortex in the following order: 50 µl tungsten particles, 10-15 µg DNA in 20 µl TE buffer, 20 µl spermidine pH 7, and 60 µl sterile 2.2 M $CaCl_2$. The mixture was shaken for 10 min at room temperature and subsequently kept on ice until the DNA is on the microcarriers. 200 µl of ice-ethanol were added and the solution kept on ice for 10 min while the microcarriers were prepared. After short centrifugation and three times rinsing with ethanol, the material was taken up in 30 µl of ethanol, which is sufficient for two shots. 15 µl of the prepared tungsten suspension were pipetted on a microcarrier placed on a metalring and air dried. The lower white part of fresh leek was divided in halves and pieces of 5x5 cm cut out of the middle layers. For each shot one piece was placed on a plastik petri dish with wet filter paper and the surface was cut longitudinal to become plane. The bombardment was performed with a particle delivery system (PDS-100/He Biolistic®, Bio-Rad, München, Germany). The leech was kept dark and moist for some hours till over night and finally analysed with a stereo microscope (MZ16F, Leica Microsystems) with a YFP fluorescence filter (10447410, Leica Microsystems). Pictures were processed with Leica LAS software.

2.2.2 Bacteria works

Transformation of bacteria and selection of clones

E. coli chemically competent cells were transformed by heat shock (Hanahan, 1983). Aliquots of the bacteria were stored at -80°C in 26 % glycerol. After addition of ~200 ng of plasmid to the thawn cells, they were kept on ice for 30 min. The tubes were tranfered to a 42°C water bath for 90 sec before 950 µl LB medium was added. After 45 min of shaking at 37°C, the cells were plated on LB medium including the selective antibiotics and kept at 37°C over night.

A. tumefaciens chemically competent cells were treated similar (Dower et al., 1988). After addition of the plasmid, the cells are kept on ice for 30 min and then frozen in liquid nitrogen for 5 min.

After 5 min at 37°C 500 µl of YEB medium was added and the tubes shaked at 28°C for 2 h. After plating on YEB with the selective antibiotics, the cells were kept at 28°C for 2-3 d.
Some of the resistant colonies were grown to 10 ml liquid cultures. The plasmids were extracted with an appropriate kit and examined by restriction analyses and PCR.

2.2.3 Molecular Methods

Standard molecular procedures were performed following Sambrook and Russel (2001) and Ausubel (1994), if not indicated otherwise.

2.2.3.1 DNA extraction from plants tissue

DNA extraction of large numbers of samples was performed using the MagAttract® 96 DNA Plant Kit following the manufacturer's instructions.
Smaller numbers of samples were treated following the method described at Dellaporta et al. (1983). Some young leaves or inflorescences were collected in 2 ml tubes and frozen in liquid nitrogen. After addition of a tungsten-carbide bead (Qiagen, Hilden, Germany) the material was ground for 1 min at a frequency of 30/min using a Mixer Mill MM300 (Retsch, Haan, Germany). 750 µl extraction buffer was added and the tubes were shaken again before they were incubated at 65°C for 10 min. The addition of 200 µl 5M/3M potassium acetate solution was followed by 20 min incubation on ice and 10 min centrifugation at 13,000 rpm. The supernatant was transfered into a new tube and mixed with an equal volume of isopropanol followed by 10 min centrifugation at 13,000 rpm. The pellet was washed with 80 % ethanol and air dried. The DNA was eluted in 50 µl demineralized water and stored at -20°C.

2.2.3.2 Plasmid extraction from bacteria

Plasmid isolation from *E. coli* and *A. tumefaciens* was accomplished using the column based QIAprep®Spin Miniprep Kit or NucleoSpin® Plasmid following the manufacturer's protocol.

2.2.3.3 PCR conditions

All PCR reactions wer performed with a Thermocycler T3 (Biometra, Göttingen, Germany). For general application *Taq* DNA polymerase was used, whereas for cloning and sequencing Accu Prime *Pfx* DNA Polymerase or *Pfu* DNA Polymerase was used following the manufacturer's instructions. The composition and conditions of a standard reaction is listed below.

Reagent	Concentration	Volume (µl)
PCR buffer	10 x	1
dNTPs	10 mM	0.2
Primer F	10 µM	0.4
Primer R	10 µM	0.4
DNA template	50-200 ng/µl	1
DNA polymerase	1 U/µl	0.05
dH2O		6.95
Annealing temperature	55-65°C	
Elongation time	1 min/1 kb	
Number of cycles	35	

Table 2.9: Standard PCR conditions

2.2.3.4 Analyses of DNA fragments and purification

DNA fragments were generally analysed by agarose gel electrophoreses. The DNA as well as the 1kb DNA ladder was supplemented with loading buffer and filled into the slots of a prepared gel. The concentration of the agarose depended on the size of the expected fragments. Electrophoreses was performed at 5 V/cm using TAE buffer. Finally the separated fragments were visualized on a transilluminator with UV light (254 nm) and photographed with a gel documentation system (INTAS, Göttingen, Germany). The exposure of fragments which were subsequently purified from the gel was kept at minimum. Gel extraction and purification of DNA fragments was conducted using the QIAquick® Gel Extraction Kit following the manufacturer's protocol.

2.2.3.5 RNA extraction from plant tissue

RNA extraction from leaf material was done using the RNeasy® Plant Mini Kit or NucleoSpin® RNA Plant following the manufacturer's protocol.

RNA extraction from siliques and seeds was performed as established by Kushiro et al. (2004). 40 mg material was frozen in liquid nitrogen and growned with mortar and pestle without being thawn and subsequently processed with the RNAqueous™ Phenol-free total RNA Isolation Kit + RNA Isolation Aid following the manufacturer's instructions. The quality of the RNA was determined with the Nanodrop ND-1000 spoctrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). As further purification steps, high salt precipitation to remove polysaccharides and precipitation of high molecular weight RNA with lithium chloride was performed. The RNA was diluted with RNase free water to 1 ml and precipitated by addition of 250 µl isopropanol and 250 µl high salt precipitation solution. The mixed solution was kept on ice for 2 h before the RNA was recovered by 15 min cetrifugation at 14,000 rpm and 4°C. After rinsing the pelet with 70 % ice

cold ethanol air drying, it was dissolved with RNase free water to end up with a concentration of > 300 ng/µl. By addition of 0.5 volume of 5 M LiCl and subsequent storage on ice over night the RNA was precipitated. 20 min centrifugation at 13,000 rpm and 4°C was followed by washing with 70 % ethanol and air drying. The pellet was finally dissolved in 10 µl RNase free water and the quality was controlled. A ratio of absorbance A_{260nm}/A_{280nm} between 1.8 and 2.0 and A_{260nm}/A_{230nm} between 2.5 and 3.0 would show optimal low presence of peptides and polysaccharides. If needed the purification steps were repeated. Additionally 1 µl of the RNA solution was run on an agarose gel to test the integrity by visualization of the 18S and 28S ribosomal RNA. Total RNA was stored at -80°C.

2.2.3.6 cDNA synthesis

cDNA first strand synthesis was done with the RevertAid™ H Minus First Strand cDNA Synthesis Kit following the manufacturer's instructions. 3 µg of total RNA and oligo$(dT)_{16-18}$ primers were used.

2.2.3.7 Nucleic acid quantification

For precise validation DNA and RNA was quantified using the Nanodrop ND-1000 Spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

2.2.3.8 Sequencing

DNA sequencing was carried out by the MPIZ DNA core facility ADIS on Abi Prism 377, 3100 and 3730 sequencers (Applied Biosystems, Darmstadt, Germany) using BigDye-terminator v.3.1 chemistry and reagents from Applied Biosystems. The output data was analyzed with DNAstar Lasergene SeqMan Software (GATC Biotech AG, Konstanz, Germany).

2.2.3.9 Molecular cloning

All constructs were created using the Gateway® technology (Invitrogen, Karlsruhe, Germany). PCR products amplified with specific primer extensions (see table 2.6) were pasted into an entryvector (pDONR201 or 207) by BP reaction. The resulting plasmid was checked by PCR, restriction digest and sequencing of the inserted fragment. By LR reaction with a Gateway compatible expression vector the final construct was built and subsequently checked by PCR and restriction analyses.

2.2.3.10 Expression analyses

RNA expression was measured by semiquantitative PCR or quantitative Real Time PCR. To detect putative contamination with genomic DNA the primers were either located in different exons or across exon-exon boundaries.

For semiquantitative PCR the reaction mix was prepared as described above, but the PCR was run for 15, 20, and 25 cycles. The result of the reaction that did not yet lead to maximal amplification was chosen for analysis.

Quantitative real time PCR was performed with SYBR Green (RealMasterMix SYBR ROX, 5Prime, Hamburg, Germany) and a Mastercyler ep *realplex* (Eppendorf, Hamburg, Germany). The PCR reaction was prepared and performed following the distributors instructions. To monitor appearance of undesired PCR fragments or primer dimers subsequent to every PCR run a melting curve analysis was performed (60°C to 95°C with a heating rate of 0,5°C/s and continuous fluorescence measurement). Quantification of the normalization gene Actin8 was calculated based on a 10-fold dilution series of a reference sample. Each data point represents the mean of three technical replicates on the same PCR plate. Plant material was grown under controlled conditions (22°C/16 °C, long day) and for each time point siliques from five to ten different plants were collected.

2.2.4 Yeast-two-hybrid and growth test with yeast

The yeast-two-hybrid screen was performed following the method described by Soellick and Uhrig (2001). Transformation of yeast cells and growth assay were performed as described in the Yeast Protocols Handbook (2001).

2.2.5 Cytological methods

2.2.5.1 Spreading and staining of embryo nuclei on slides

Seeds were imbibed on wet filter paper for 2 h, embryos were isolated and directly transfered to ice cold ethanol/acetic acid 3:1; then stored in a tube at -20°C over night. The fixed embryos were washed in the tube with Aqua dest for 2 x 5 min, then washed with 10 mM NaCitrate pH 4,5 for 2 x 5 min. The embryos were digested with enzyme mix in a closed tube at 37°C for at least 3 h. The enzyme mix was replaced in the tube by Aqua dest. The embryos were placed on a piece of parafilm, tapped with a blunt needle under the binocular until they became a homogeneous suspension. The suspension was transferred to a tube and placed on ice. A drop (3-5 µl) of the cell suspension was placed on a slide, 20 µl of 45 % acetic acid added with the help of a blunt needle,

the slide placed on a heat block at 45°C, and the drop gently stirred with a blunt needle for 30-45 s. The acetic acid was removed with drops of ice cold 3:1, and the slide tilt to remove liquid and air dry. Addition of 8-10 µl of 2 µg/ml DAPI or PI in Vectashield at the center of the slide and of a 22 x 22 mm coverslip.

2.2.5.2 Whole mount staining of embryos

Seeds were imbibed on wet filter paper for 2 h, embryos were isolated and fixed with BVO for 45 min. The embryos were washed with PBT for 2x5 min, with methanol for 2x5 min, with ethanol for 5 min, and finally fixed with xylene:ethanol 1:1 for 30 min. After washing with ethanol for 2x5 min the material was rehydrated with 90%, 70%, 50%, 30% ethanol for 2 min each and washed with PBT for 5 min. Incubation with RNase (100µg/ml) + 1% Tween in PBS for 90 min at 37°C was followed by washing with PBT for 2x5 min and postfixation with 1% formaldehyde in PBT for 30 min. After washing with PBT for 2x5 min the embryos were dehydrated with 70%, 90%, 100% ethanol 2 min each and transfered to slides. After air drying it was mounted in Vectashield + DAPI or PI (2µg/ml) and kept at 4°C in the dark. Analysis by confocal microscope was performed 3 days later.

2.2.5.3 Microscopy

For fluorescence light microscopy an Axioshot microscope with Differential Interference Contrast (Normarsky)-Optics (Zeiss, Heidelberg, Germany) was used. Pictures were taken with DISKUS version 4.10.19 (Carl H. Hilgers Technisches Büro, Königswinter, Germany). Confocal-laser-scanning microscopy was performed with TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany).

2.2.6 Amino acid sequence alignment (*in silico*)

Alignment of amino acid sequences was performed with MAFFT Version 6 (Katoh et al., 2008, http://www.ebi.ac.uk/Tools/mafft/index.html), sequences were transfered to ClustalW2 (Larkin et al., 2007, http://www.ebi.ac.uk/Tools/clustalw2/index.html), and visualization with Jalview (Clamp et al., 2004).

3 Results

3.1 Chromatin

Before the start of this project initial observations in the lab showed differences in the chromocenter appearance between dormant and non-dormant embryos. While immature L*er* seeds as well as ripe seeds of the non-dormant mutant *dog1* show strong and distinct chromocenters, the nuclei of Cvi seeds after harvest appeared evenly stained without spots.

In active cells during the interphase the transcriptionally active euchromatin can be distinguished from the highly condensed inactive heterochromatin (Sadoni et al., 1999). Staining of the DNA reveals weakly stained euchromatin consisting of less compacted DNA and brightly stained heterochromatic spots called chromocenters.

It has been shown that the chromocenter structure can be disrupted during developmental changes (Tessadori et al., 2007 A+B). The transition from an immature seed to a dormant dry seed and finally a non-dormant seed could also be associated with changes in chromatin structure. It is known that at certain points during the plant life cycle, when gene transcription adapts new fate or function, the chromatin undergoes structural changes in organization (Tessadori et al., 2007B). Also dormancy could be determined by specific chromatin organization.

To determine whether there is a relationship between dormancy and chromatin structure, the distribution of heterochromatin in non-dormant and dormant seeds was compared. We analyzed the non-dormant mutant *dog1* and its background line NIL DOG1 and seeds early in seed maturation compared to ripe seeds. For visualization of the chromatic structure specific fluorescent dyes were applied. 4',6-Diamidino-2-phenylindol (DAPI) and propidium iodide (PI) incorporate in the heteroduplex structure of the DNA and lead to stronger signals at positions of DNA condensation, i.e. chromocenters. The relative intensity of the fluorescence can be quantified at every position of a digital picture and this data can be processed by appropriate software. High resolution pictures of the chromatin stained nuclei were made with the confocal microscope. Subsequently the pictures were analyzed with two specific software programs. The DISKUS CROMO software requires a manual definition of the area of interest (nucleus) and counts the number of pixels above (chromocenters) and below (nuclear background) a given threshold of fluorescence intensity. The ACAPELLA software is able to recognize a closed area (nucleus) on a picture and can identify and measure spots with given characteristics (chromocenters) within the nucleus.

3.1.1 Chromatin structure of non-dormant *dog1* and dormant NIL DOG1 embryos

To compare the chromatin structure of dormant and non-dormant embryos we made use of the *dog1* mutant.

For the described experiments we used mature non-dormant *dog1* mutant, the highly dormant NIL DOG1 and moderately dormant L*er* seeds. Whole mount staining and analysis of the seeds allows the distinction of the cells by tissues. The embryos were isolated from the seed coat, fixed, treated with RNase and finally stained with DAPI or PI. Whole mount confocal microscopy was used to take pictures from cells of the outer cell layer which usually showed the best staining. The compact and dense embryo tissue is not easily permeated by the solutions as well as by the confocal laser, which leads to less sharp pictures of inner tissue layers.

Several whole mount stainings were performed with different material and a number of changes in the protocol were made to improve the quality of the pictures. The results showed large variation, possibly caused by environmental conditions during the growth of the plants. One example of a staining displaying more defined and stronger stained chromocenters in the non-dormant *dog1* than in the dormant NIL DOG1 cells is shown in Figure 3.1.

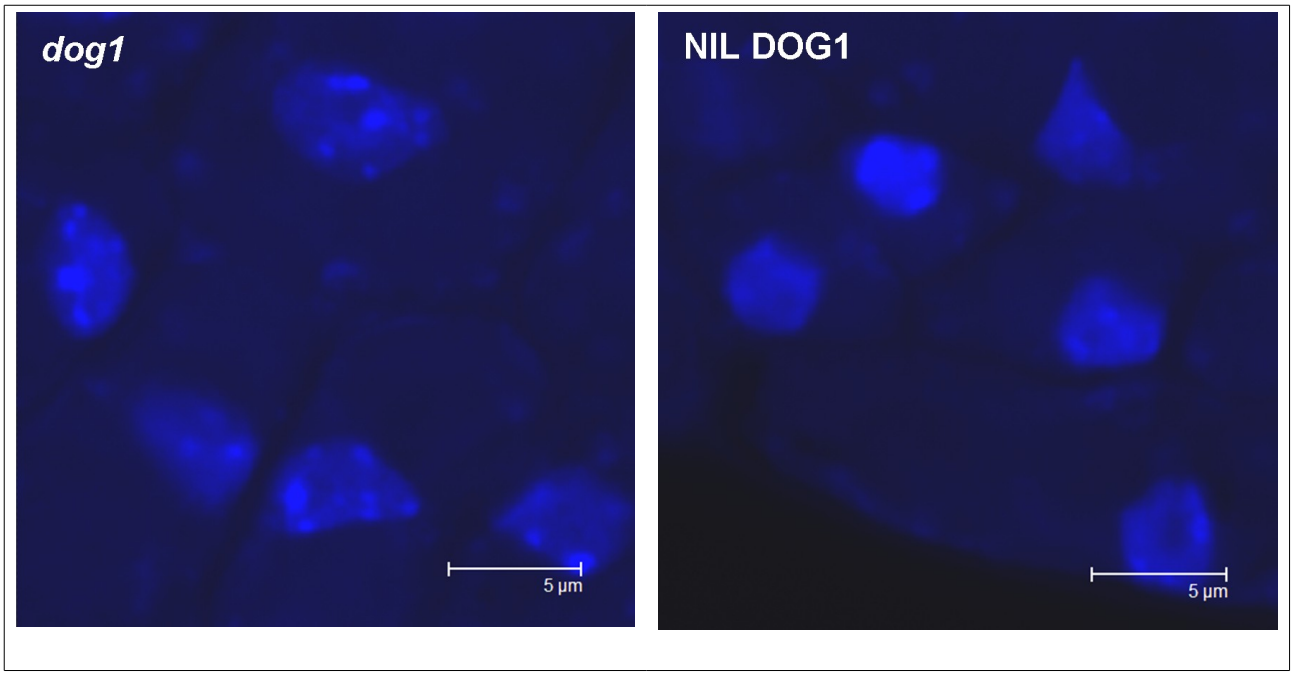

Figure 3.1: Chromatin structure of non-dormant *dog1* and dormant NIL DOG1. Confocal pictures of whole mount DAPI stained *dog1* (left) and NIL DOG1 (right) mature embryos. Scale bars are 5µm.

The results had to be quantified to measure their significance. For every measurement, 50 nuclei of cotyledon and hypocotyl from five to ten different embryos per line were photographed in high

resolution by confocal microscopy. These pictures were analyzed with the DISKUS CROMO software (Figure 3.2) by manual selection of the area of the nucleus in the picture and a part of the background. Subsequently the pixels of the picture are displayed as black or white depending on the value of fluorescence intensity of each pixel. The threshold was set and defined the areas set above the limit (chromocenters) or below the limit (nuclear background).

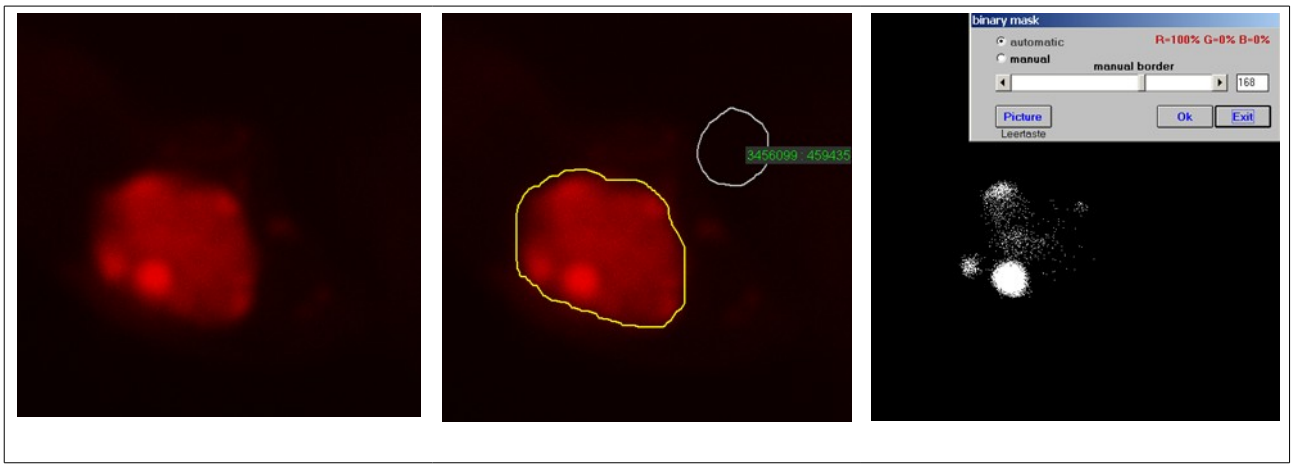

Figure 3.2: Principle of DISKUS CROMO measurement. Left: Confocal picture of a PI stained nucleus. Middle: Result of the manual selection of the nucleus (yellow line) and a marked area of background (white line). Right: Screen shot of the black-and-white mask to set the intensity threshold.

A prerequisite for the measurement of the heterochromatin fraction is an appropriate staining of the nucleus. The pictures of three experiments were chosen for measurement (Table 3.1). The results are not consistent. Although the fraction of heterochromatin is always higher in *dog1* seeds than in the dormant NIL DOG1 or L*er* seeds, it varies between two to ten times. Therefore the results are not reliable due to the big differences between the experiments. The variation could be caused by a low quality of the analyzed pictures or the principle of the measurement with DISKUS CROMO. Most chromocenters contain strongly stained and lower stained parts. Sometimes chromocenters of lower intensity are located in less stained parts of the nuclei. By setting a threshold of intensity, these less strongly stained parts of the chromocenters fall below the limit and are counted as "nucleus". But the main reason for the difficulties of the measurement is the compactness of the nuclei. The nuclei in embryos are much smaller and more dense than for example in leaves, where this method has proven to be successful (Soppe et al., 2002). Due to the density of the nuclei, the software cannot identify and measure chromocenters properly.

staining	sample	area (fraction of chromocenter to nucleus)	intensity (fraction of chromocenter to nucleus)
DAPI	*dog1*	0,28 % ± 0,07	0,55 % ± 0,15
	NIL DOG1	0,02 % ± 0,01	0,06 % ± 0,02
DAPI	*dog1*	3,43 % ± 0,43	5,86 % ± 0,66
	L*er*	2,60 % ± 0,54	4,29 % ±0,79
PI	*dog1*	0,86 % ± 0,08	2,11 % ± 0,19
	NIL DOG1	0,51 % ± 0,08	1,23 % ± 0,19

Table 3.1: DISKUS CROMO measurement of heterochromatin in nuclei of non-dormant *dog1* and dormant NIL DOG1 or L*er* embryos. Mature *dog1*, NIL DOG1 and L*er* embryos were whole mount stained with DAPI or PI. Pictures of longitudinal section with highest fluorescence of 50 single nuclei from cotyledon and hypocotyl were taken with the confocal microscope. Percentage of area and intensity of chromocenters compared to nucleus was measured with the DISKUS CROMO software. Values give means and standard errors.

To improve the measurement, we used the ACAPELLA software which is an algorithm specialized to identify areas like cells and to identify dots within these areas. In this case the dots are the chromocenters in the area of the nucleus.

To optimize the settings for our purpose, some example pictures with discrete chromocenters and unstructured heterochromatin with DAPI and PI staining were chosen from all experiments. The DAPI stained nuclei were found to be inappropriate for the ACAPELLA software. The PI pictures were converted into 8 bit gray scale by Adobe Photoshop. Dr. Kurt Stüber (MPIZ) wrote a program to automate the identification of the nuclei and the measurement of the chromocenters (see appendix). Figure 3.3 shows an example of a recognized nucleus (middle) and the selected chromocenters (right). The criteria for the identification of the chromocenters include the size, intensity and distance of the spots (see appendix). The number and intensity of the pixels within the spots and the intensity of the pixels of the nuclear area without the spots are summed up and all data transfered to a table.

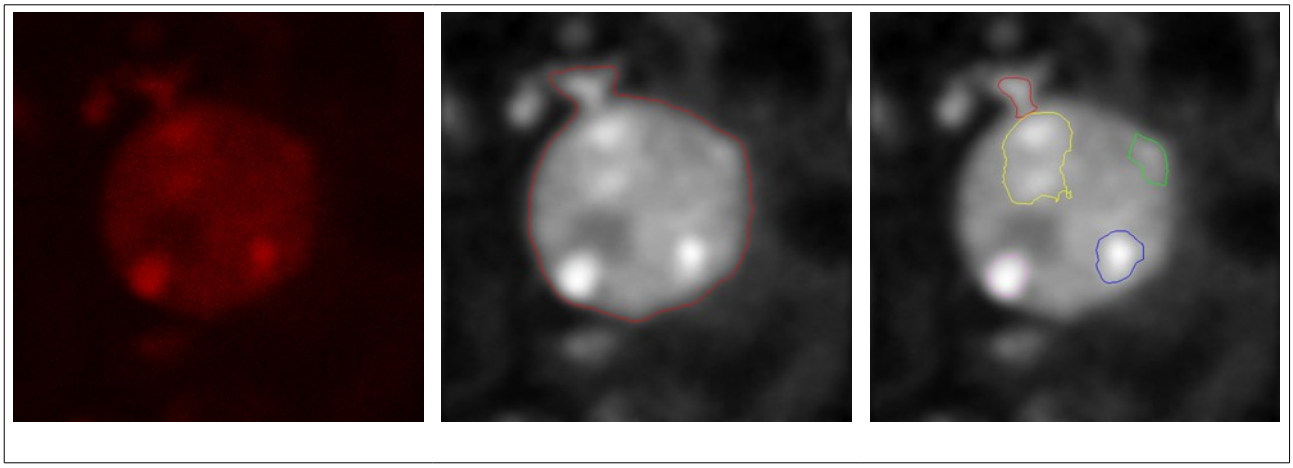

Figure 3.3: Principle of ACAPELLA measurement. Left: Confocal picture of a PI stained nucleus. Middle: Result of the automatic identification of the nucleus (red line). Right: Result of automatic selection of five chromocenters (red, yellow, green, blue and purple lines).

Three data sets of non-dormant *dog1* and dormant NIL DOG1 embryos stained with PI were analyzed (Table 3.2). One set of pictures could not be processed by the software. The other two data sets show that the ACAPELLA software identifies larger areas of chromocenters in the NIL DOG1 than in the *dog1* nuclei. Even though the mean pixel intensity is lower in the non-dormant nuclei, no significant difference is measured in the percentage of chromocenter intensity to intensity of the total nucleus between the *dog1* and NIL DOG1 samples.

sample	mean area of chromocenters	mean pixel intensity of chromocenters	chromocenter to nucleus intensity
dog1 NIL DOG1	4092 6412	31,1 28,9	94,8 % ± 0,8 97,4 % ± 0,7
dog1 NIL DOG1	3394 no data	28,5 no data	92,3 % ± 2,2 no data
dog1 NIL DOG1	6385 6709	43,7 42,6	96,4 % ± 0,9 96,0 % ± 0,9

Table 3.2: ACAPELLA measurement of heterochromatin in nuclei of non-dormant *dog1* and dormant NIL DOG1 embryos. Mature *dog1* and NIL DOG1 embryos were whole mount stained with PI. Pictures of the longitudinal section of highest fluorescence of 50 single nuclei from cotyledon and hypocotyl were taken with the confocal microscope. Area and intensity of the nuclear parts identified as chromocenters in relative values and percentage of chromocenter intensity to intensity of total nucleus measured with the ACAPELLA software. Values give means and standard errors in the third column.

In comparison to the results from the measurement with DISKUS the ACAPELLA values are strikingly different in general. While the fraction of chromocenters to the complete nucleus are in the DISKUS measurement below 4 % for all samples, all results from the ACAPELLA measurement are above 90 %. The only possible explanation for this huge discrepancy is a fundamentally different principle of measurement.

A structural difference of non-dormant and dormant nuclei is not measurable with the available methods. The reason is the difficulty to define the less distinct chromocenters in the small and dense nuclei of the dormant embryos.

During the course of the experiments data from Paul Fransz (personal communication) showed that the heterochromatin fraction in the Cvi accession is lower than in the L*er* accession. This explains the difference that was originally found between the dormant Cvi seeds and the non-dormant seeds in L*er* background.

3.1.2 Dynamics in nuclear size during seed maturation

During the second half of seed development the embryo matures and desiccates for a possibly long duration in the soil until germination. This structural change could become visible in a change of nuclear appearance.

Cvi seeds were dissected from siliques at different time points during seed maturation. Embryos were digested, the material was spread on slides and stained with DAPI. Pictures of 15 nuclei were taken with a fluorescence microscope at 10 days after pollination (dap), 15 dap and 21 dap. They show a strong decrease of nuclear size as visible on the representative pictures of Figure 3.4.

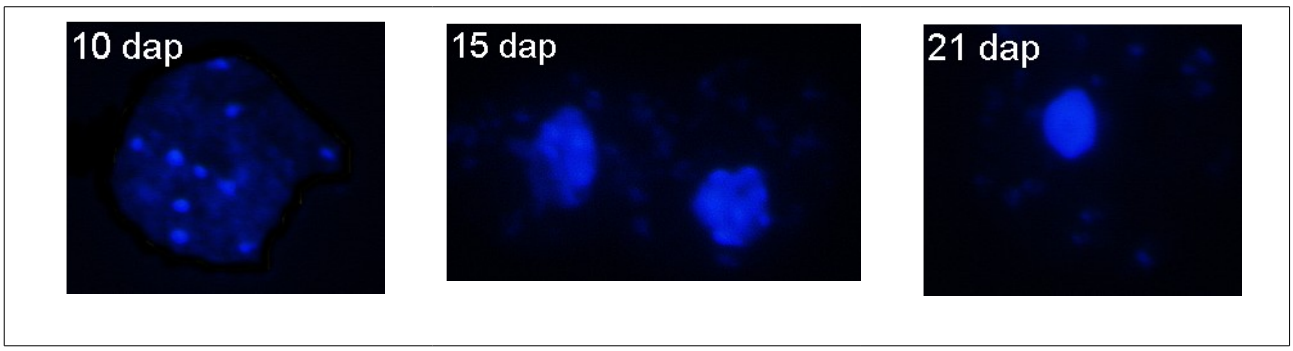

Figure 3.4: Nuclei of Cvi during seed maturation. Cvi seeds were harvested 10 dap, 15 dap and 21 dap, embryos digested and the nuclei spread on slides. After DAPI staining pictures were taken by light microscopy. Microscope magnification 100x.

Measurement of nuclear areas and intensities was performed with the DISKUS CROMO software. The mean values are summarized in Table 3.3. The data reflects the strong reduction in area of the nuclei between 10 dap and 15 dap and a slight decrease between 15 dap and 21 dap as shown in Figure 3.4.

days after pollination	area nucleus	intensity nucleus
10	13362 ± 1221	778679 ± 30430
15	2827 ± 151	332230 ± 13696
21	2646 ± 200	337273 ± 22687

Table 3.3: Size of spread Cvi nuclei during seed maturation. Cvi seeds were harvested 10 dap, 15 dap and 21 dap, embryos digested and the nuclei spread on slides. After DAPI staining pictures were taken by light microscopy. Percentage of area and fluorescence intensity of nuclei and chromocenters were measured with the DISKUS CROMO software. Values give means and standard errors of 15 nuclei.

Due to the spreading of the nuclei it is possible that the size difference appears stronger than it is in three dimensions. Furthermore, it is possible that the more condensed looking nuclei do not spread that broadly as the nuclei from earlier stages but remain more three-dimensional. To exclude this factor, in a second experiment whole mount stained embryos were analyzed.

L*er* seeds were harvested at 10 dap, 13 dap, 16 dap, 18 dap and when mature. The embryos were isolated from the seed coat and whole mount staining with PI was performed. Confocal microscope pictures of single nuclei were taken. Figure 3.5 shows three examples for each time point.

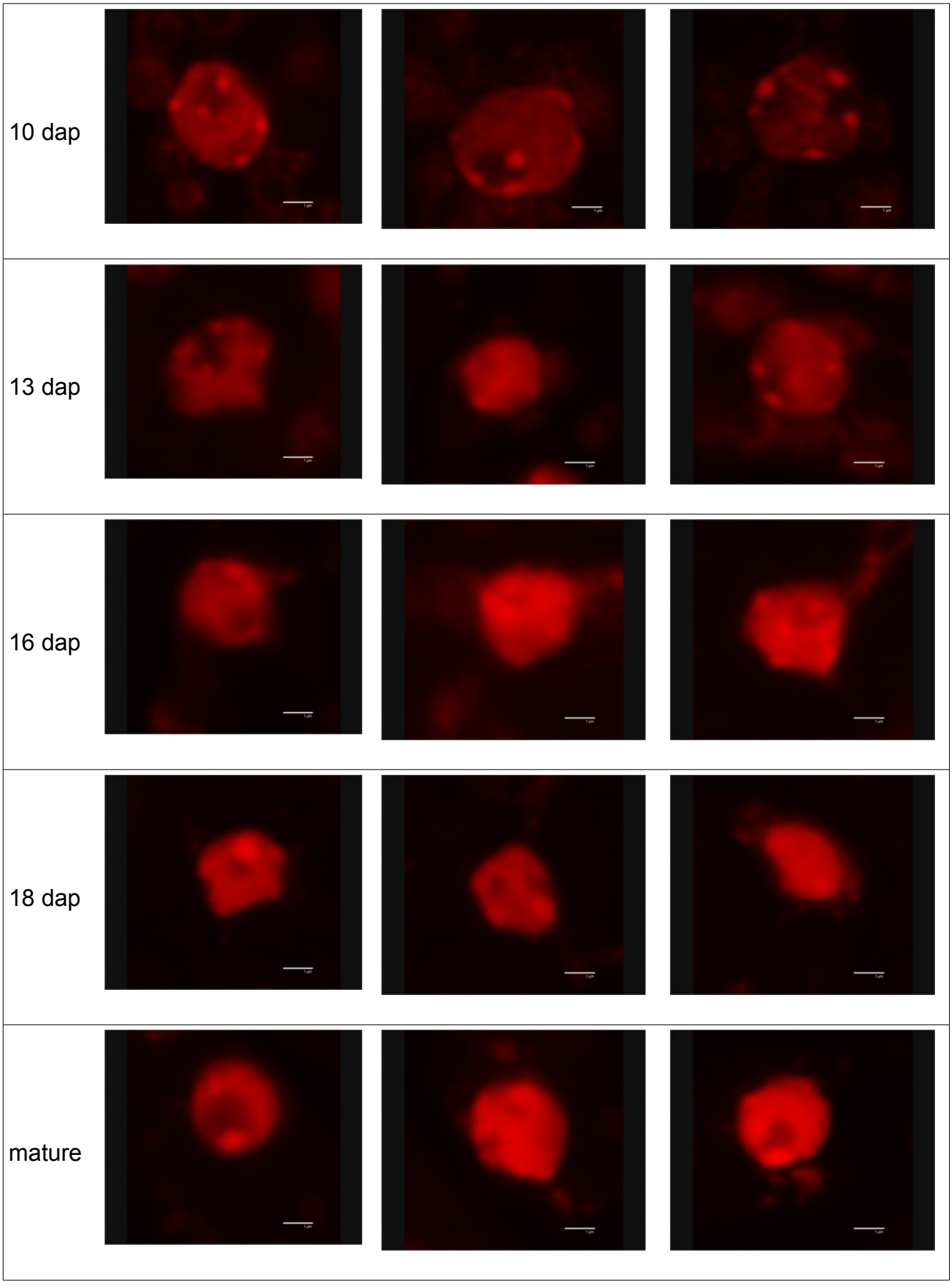
10 dap
13 dap
16 dap
18 dap
mature

Figure 3.5: Whole mount L*er* nuclei during seed maturation. L*er* seeds were harvested 10 dap, 13 dap, 16 dap, 18 dap and mature, embryos were isolated from the seed coat and whole mount stained with PI. Shown are three confocal pictures of single nuclei of each time point. Scale bars are 1 µm.

Confocal pictures of the of 40 to 50 single nuclei were taken from the cotyledons and the hypocotyl of at least five different embryos at each stage. The nuclear area was defined manually and measured with the DISKUS CROMO software (Figure 3.6). The pictures in Figure 3.5 as well as the measurement show a decrease of nuclear size at the start of seed maturation between 10 dap and 13 dap.

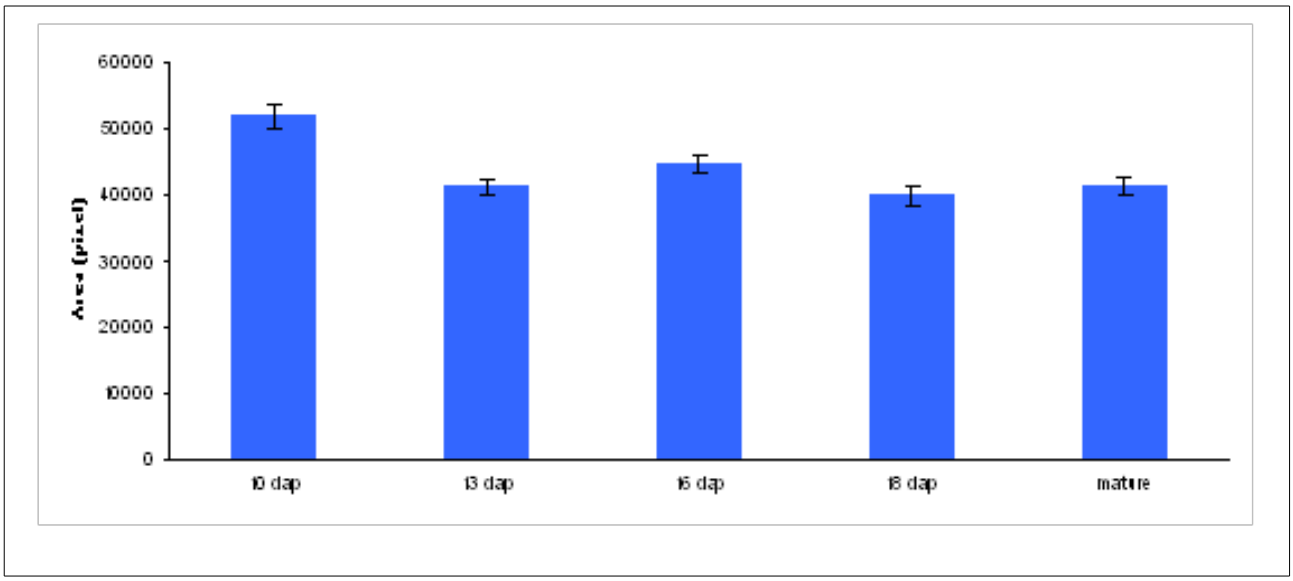

Figure 3.6: Size of whole mount L*er* nuclei during seed maturation. L*er* seeds were harvested 10 dap, 13 dap, 16 dap, 18 dap and mature, whole mount stained with PI and the longitudinal section with the maximal area of single nuclei of cotyledons and hypocotyl photographed by confocal microscopy. The pictures were analyzed with DISKUS CROMO software. Values are means of pixels and error bars represent standard errors.

Therefore there are indications for a reduction in nucleus size during seed maturation. However, the data is not yet confirmed, which should be done by a further improved method of measuring.

3.2 *RDO2*

A mutagenesis screen for reduced dormancy after γ-irradiation was performed by Karen Léon-Kloosterziel in the lab of Maarten Koornneef and resulted in four mutants named *reduced dormancy (rdo) 1* to *4*. All of them display higher germination after harvest than L*er* and they have been roughly mapped and characterized (Léon-Kloosterziel et al., 1996 and Peeters et al., 2002). *RDO4* (*HUB1*) has recently been cloned and characterized (Liu et al., 2007). It encodes a C_3HC_4 RING finger protein responsible for histone H2B monoubiquitination and consequently influences transcription activity. The expression level of some dormancy-related genes is changed in the *rdo4* mutant.

reduced dormancy 2 (*rdo2*) seeds are characterized by a reduced dormancy and mild pleiotropic phenotypes. The mutant plants are somewhat smaller and take one to two days longer till flowering than L*er*. The arrangement of the single flowers in the inflorescence is disturbed and the rosette leaves look slightly darker green than the wild type. Because of the germination phenotype of the mutant *RDO2* could play a role in the dormancy network and the pleiotropic phenotypes facilitate the identification of the mutant in the greenhouse. Therefore, *RDO2* was selected for fine-mapping and cloning in a forward genetic approach.

3.2.1 Mapping of *RDO2*

Previously *RDO2* was roughly mapped to the bottom of chromosome 2, south of nga168 (Peeters et al., 2002). Map based cloning of *RDO2* was generally performed as described by Jander et al. (2002). Fine mapping of a gene that influences a quantitative trait such as dormancy is difficult because the genetic variation between the two parents of the mapping population can interfere with the phenotype of the mutant. To reduce this variation the *rdo2* mutant in L*er* background was crossed to the Near Isogenic Line (NIL) LCN2-18 (Keurentjes et al., 2007) which contains an introgression of Cvi only at the estimated position of *RDO2* in L*er* background. The precise location of the Cvi introgression in LCN2-18 is the very bottom of chromosome 2 starting between marker M323 (14.9 Mb) and marker T2N18 (15.6 Mb).

F2 plants showing pleiotropic phenotypes of the *rdo2* mutant were selected in the greenhouse and reduced dormancy was confirmed by a germination test. The position of *RDO2* was narrowed down by analyzing the genotype of the F2 plants at different molecular markers in the region. The *rdo2* mutant is homozygous L*er* and recombinants in the region between *RDO2* and the marker should have a Cvi allele for the tested marker. In this way the area of interest could be narrowed down from

both sides. The location of *RDO2* was previously mapped to a region of 3 Mb by Dr. Yongxiu Liu. Further fine mapping was performed with molecular markers; about 1100 F2 plants were screened with 10 simple sequence length polymorphism (SSLP) markers based on the Monsanto Polymorphism Collection (http://www.arabidopsis.org/Cereon/) (Table 2.2 in Materials and methods). Finally the position of *RDO2* was narrowed down to an area of 45 kb (Figure 3.7) which contains 15 annotated genes.

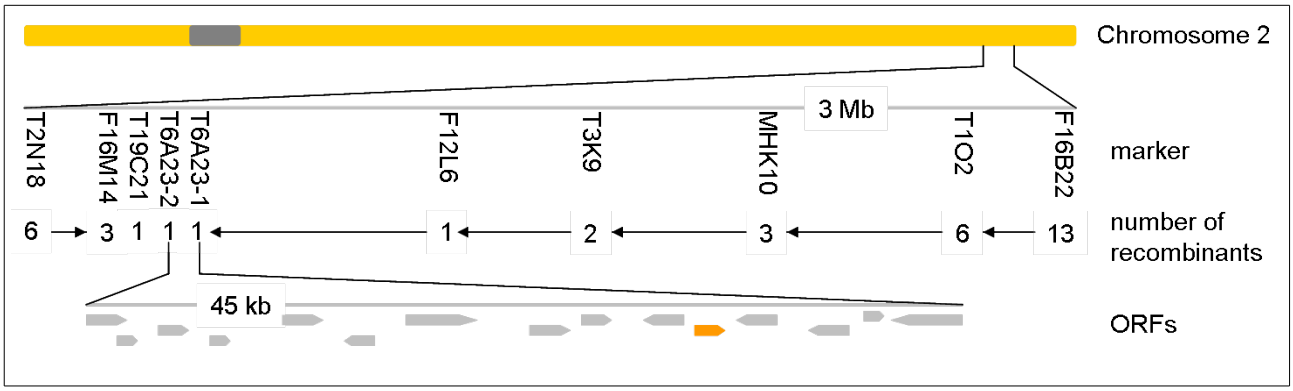

Figure 3.7: Fine mapping of *RDO2*. From top to bottom are depicted chromosome 2, the area of fine mapping with the approximate location of the molecular markers, the number of plants found to be recombinant between the molecular marker and *RDO2*, and the final 45 kb area with a scheme of 15 open reading frames (ORFs) annotated in this area, *RDO2* is shown in orange.

Since the main phenotype of the *rdo2* mutant is observed in seeds, candidate genes are likely to be expressed in seeds. Publicly available microarray data was analyzed using Genevestigator software (http://www.genevestigator.etzh.ch; Zimmermann et al., 2004). It revealed a few genes which show high expression in seeds. After evaluating the predicted functions of these genes based on the TAIR (http://www.arabidopsis.org) annotation, one gene was chosen for sequencing as the most promising candidate. This was At2g38560, which is expressed constitutively in the plant and highest in mature siliques, i.e. ripe seeds, and germinating seeds (Figure 3.8). It is annotated as a putative TFIIS transcription elongation factor based on sequence similarity to a yeast protein.

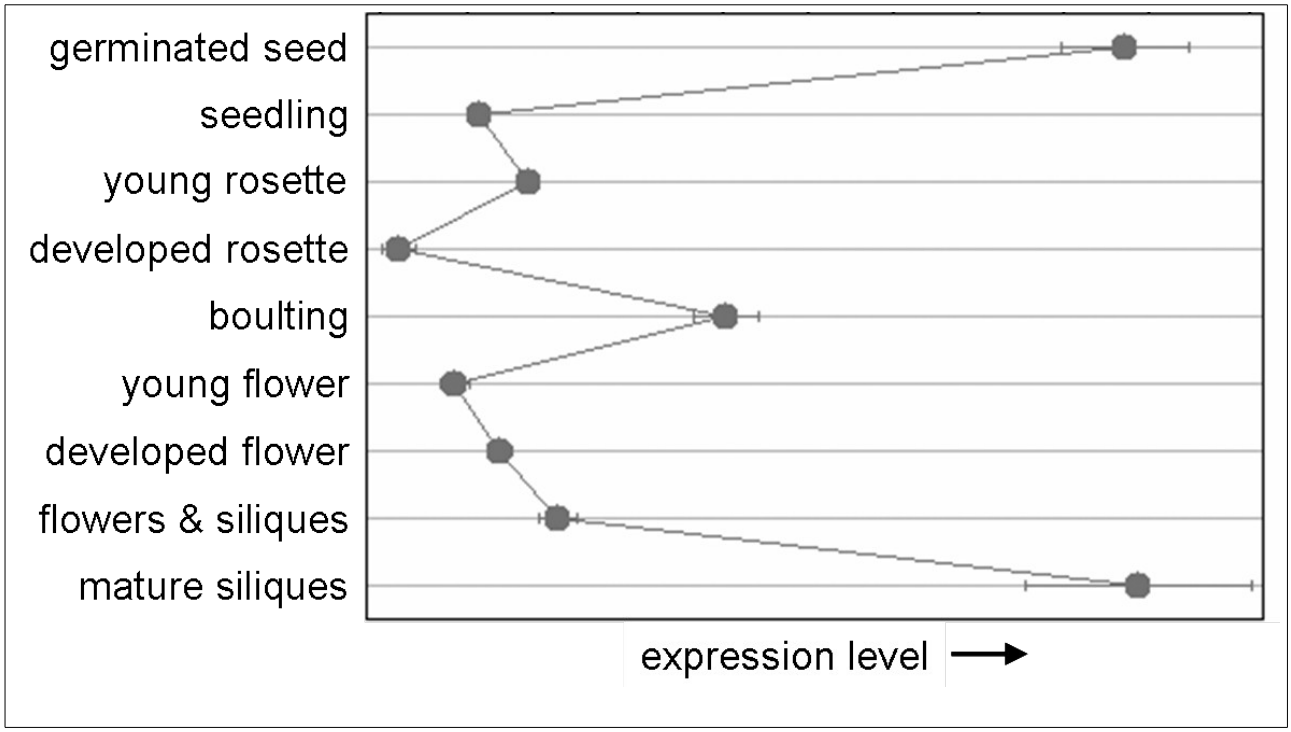

Figure 3.8: Expression of At2g38560 in different organs. X-axes gives relative expression level increasing from left to right. Graph taken from Genevestigator.

Sequencing of At2g38560 in L*er* and *rdo2* revealed a 4 bp deletion in the *rdo2* mutant at the end of the coding sequence. This deletion causes a frameshift that results in an elongated nucleic acid sequence coding for a protein with a longer but different C-terminal domain. In the *rdo2* mutant the zinc finger domain is destroyed. The TAIR database (http://www.arabidopsis.org) displays the genomic sequence of At2g38560 as depicted in the appendix. Figure 3.9 and Figure 3.10 show the sequence schematically. It consists of two exons of 165 bp and 972 bp, respectively, and an intron of 381 bp (Figure 3.9).

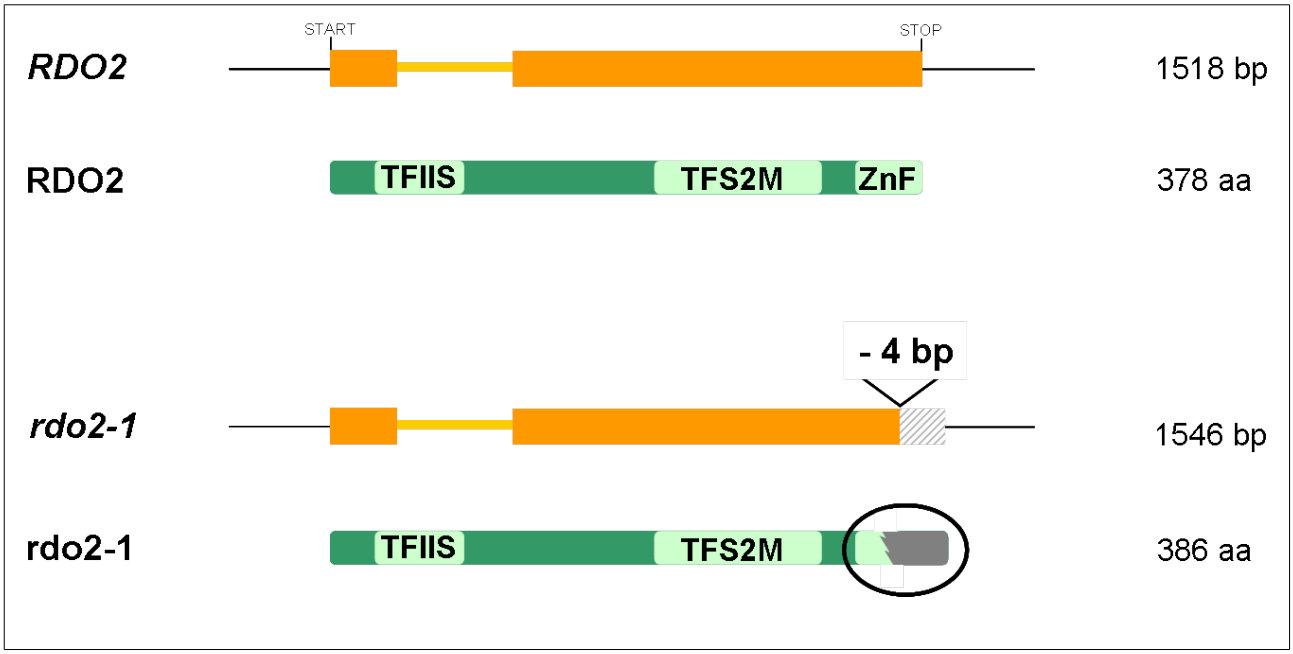

Figure 3.9: Genomic and protein structure of At2g38560 in L*er* and *rdo2*. Scheme of genomic structure of At2g38560 in L*er* (orange, top) and position of the 4 bp deletion in the *rdo2* mutant (orange, bottom). Scheme of the corresponding protein and position of the functional domains annotated for At2g38560 in L*er* (green, top) and *rdo2* mutant (green, bottom).

RDO2 is located in a gene-rich region. Additional to the 5'UTR of 163 bp the distance to the previous gene is only 223 bp (Figure 3.10). The 3'UTR comprises of 376 bp, followed by 158 intergenic basepairs in front of the next gene. The complete genomic sequence of At2g38560 from its ATG to the stop codon contains 1518 bp; the cDNA is 1137 bp long. The analogous protein consists of 378 aa. The predicted protein includes a N-terminal TFIIS domain, a TFS2M domain and a zinc finger C2C2 domain at the C-terminal end (Figure 3.9).

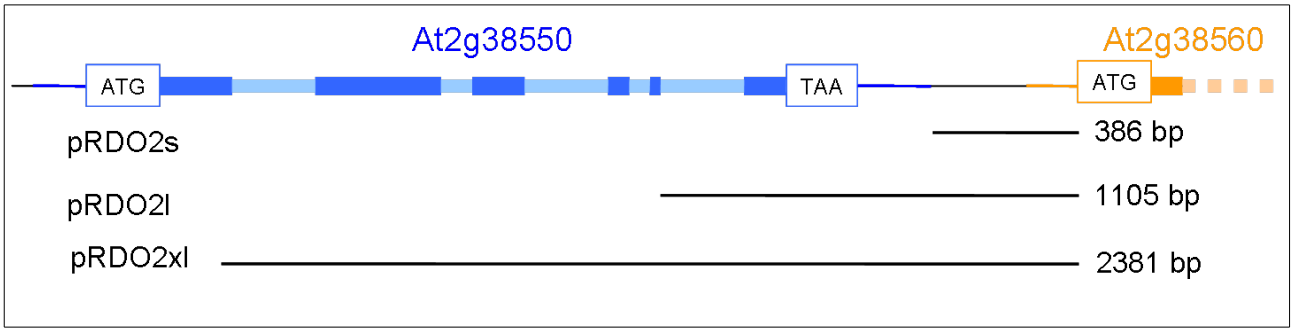

Figure 3.10: Region in front of At2g38560. Scheme of At2g38550 and the intergenic region of At2g38550 and At2g38560 including the position of three promoter sequences (pRDO2s, pRDO2l, pRDO2xl) used for complementation.

The TFIIS domain is characteristic for transcription elongation factor S. The TFS2M domain assembles the protein to the RNA polymerase II. Responsible for the binding to the DNA is the zinc

finger domain (Awrey et al., 1998). TFIIS proteins are conserved through all eucaryots, many organisms have several genes whereas the *Arabidopsis thaliana* and yeast genome contain only one copy. The TFIIS protein is thoroughly investigated in yeast, where it is responsible for activation of RNA polymerase cleaving function for restarting after transcriptional arrest (Kettenberger et al., 2004). The mutation in *rdo2* destroys the zinc finger domain and probably causes a non-functional RDO2 protein.

To confirm the identity of At2g38560 as *RDO2*, a complementation assay of the *rdo2* mutant with the L*er RDO2* sequence was performed. The insertion of the wild type *RDO2* sequence into the genome of the mutant should revert the mutant phenotypes if this gene is responsible for the observed phenotypes.

Two genomic fragments of 2.6 kb and 1.9 kb L*er* genomic sequence were used for complementation. Both contain the genomic sequence of At2g38560 and a shorter or longer putative promoter fragment of 0.4 kb and 1.1 kb, respectively (Figure 3.10). They were named pRDO2s:gRDO2 and pRDO2l:gRDO2. The fragments were transformed into *rdo2* plants. T4 seeds of five homozygous lines with independent single insertions of the short promoter construct (Figure 3.11A) and three homozygous lines with independent single insertions of the long promoter construct were analyzed (Figure 3.11B). Twelve plants per line were grown next to *rdo2* and L*er* plants under controlled conditions and germination tests were performed on individual plants. All lines show a germination phenotype intermediate between L*er* and *rdo2,* regardless the length of the putative *RDO2* promoter that was included in the construct.

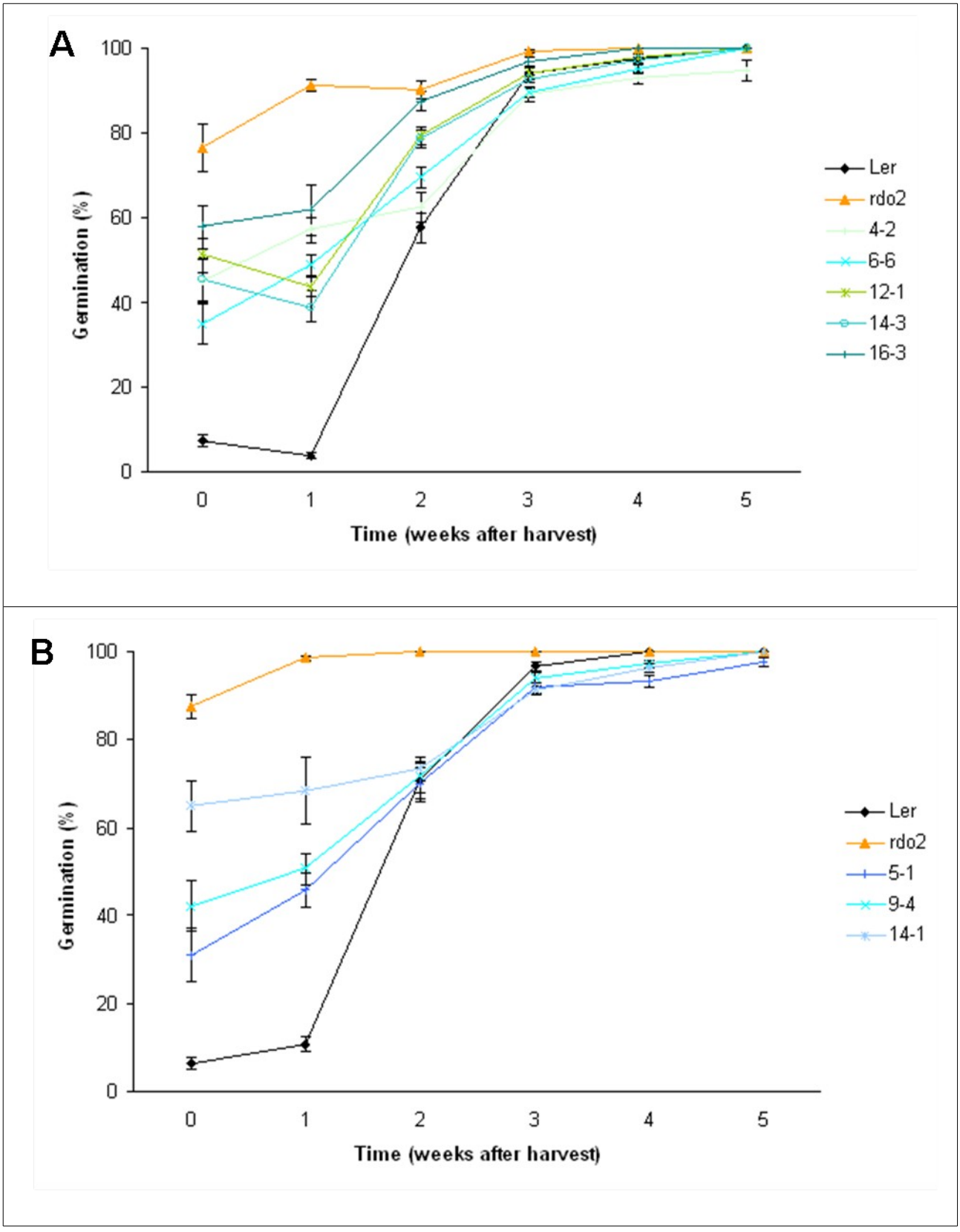

Fig. 3.11: Germination of *rdo2* mutant plants transformed with pRDO2s:gRDO2 (A) or pRDO2l:gRDO2 (B). The percentage of germinated T4 seeds of independent homozygous lines, *rdo2* and L*er* is shown. Twelve plants per line were analyzed individually for germination during after-ripening, standard errors are depicted as bars.

Therefore both constructs partially complement the *rdo2* mutation. However, they probably lack some unknown regulatory part that is necessary for full complementation of the mutant phenotype.

A second confirmation for the identity of At2g38560 as *RDO2* was obtained by the study of additional mutant alleles of this gene. For *Arabidopsis thaliana* a large collection of lines with T-DNA insertions in specific genes is available (http://arabidopsis.info/). The long T-DNA insertions can interrupt the open reading frame of a gene causing a disruption of transcription and an absence of full-length mRNA.

Five lines with T-DNA insertions in At2g38560 were obtained from the Salk insertion mutant collection (Alonso et al., 2003) and the GABI-Kat collection (Rosso et al., 2003). All these lines are in Columbia (Col) background. They were named as alleles of *rdo2* in the order of their insertions along the genomic sequence. The original *rdo2* mutant will further be referred to as *rdo2-1*. The position of the insertions were verified by PCR and the seeds were analyzed for their germination phenotype. The location of the insertions in the genomic sequence of *RDO2* is depicted in figure 3.12. Line *rdo2-2* contains an insertion in the promoter region while the four other T-DNA insertions are all located within 320 bp at the beginning of the second exon.

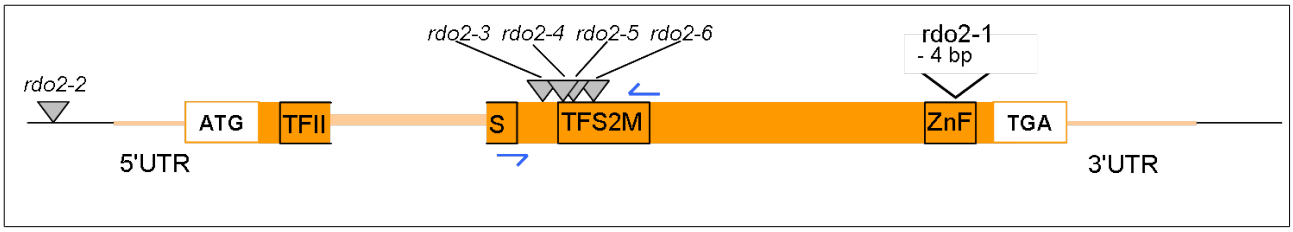

Figure 3.12: Location of the insertions of the T-DNA insertion lines in At2g38560. The scheme depicts the structure of the genomic sequence, the parts that code for the three annotated functional domains and the position of the 4 bp deletion in *rdo2-1*. The position of each T-DNA insertion is shown by a triangle on top of the sequence. Blue arrows mark the position of the primers used for expression analyses.

A semiquantitative RNA expression analyses of total RNA from leaves was performed to analyze whether the production of full-length mRNA is disturbed in the insertion lines (Figure 3.13). Total RNA was extracted from leaves of the plants and cDNA synthesis was performed. The position of the primers used for the PCR are marked by blue arrows in Figure 3.12. The result shows that the expression of full-length *RDO2* RNA is strongly reduced in the four lines that carry insertions at the beginning of the second exon but not in line *rdo2-2* that contains an insertion in front of the gene.

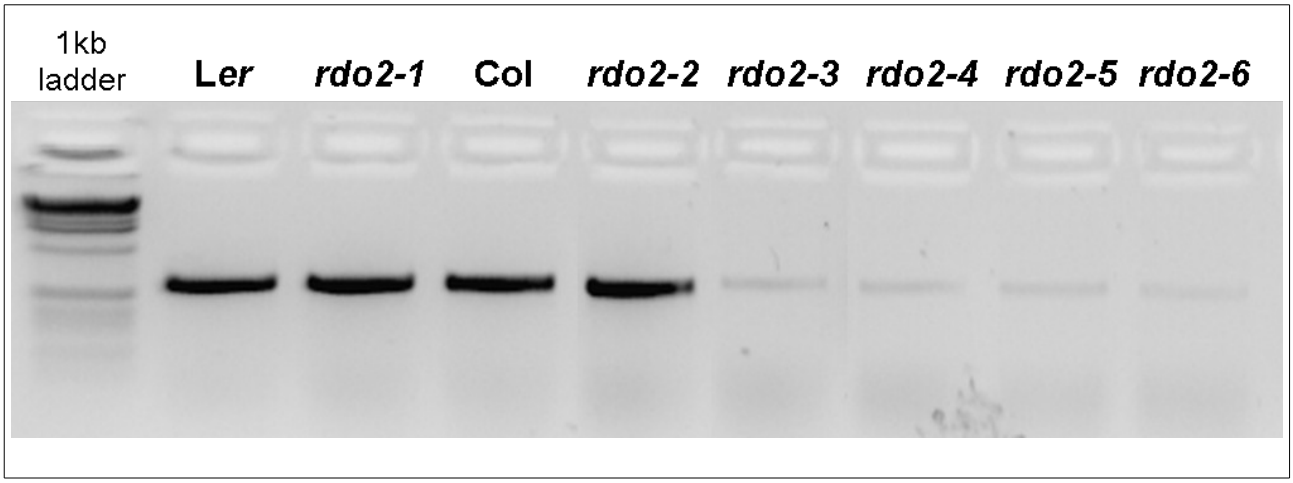

Figure 3.13: RDO2 mRNA expression of T-DNA insertion lines of At2g38560. Gel picture of semiquantitative PCR with cDNA synthesized from total RNA extracted from leaf material.

The dormancy phenotype of the five T-DNA insertion lines was obtained in a germination test (Figure 3.14). Two of the four lines with T-DNA insertions in the second exon show a germination phenotype very similar to the *rdo2-1* mutant while the other two express a phenotype intermediate to *rdo2-1* and L*er*. Line *rdo2-2* with the T-DNA insertion in front of the coding sequence of At2g38560 behaves like its background Col, which is less dormant than L*er*.

Therefore the reduced dormancy phenotype of *rdo2-1* is confirmed in the insertion mutants.

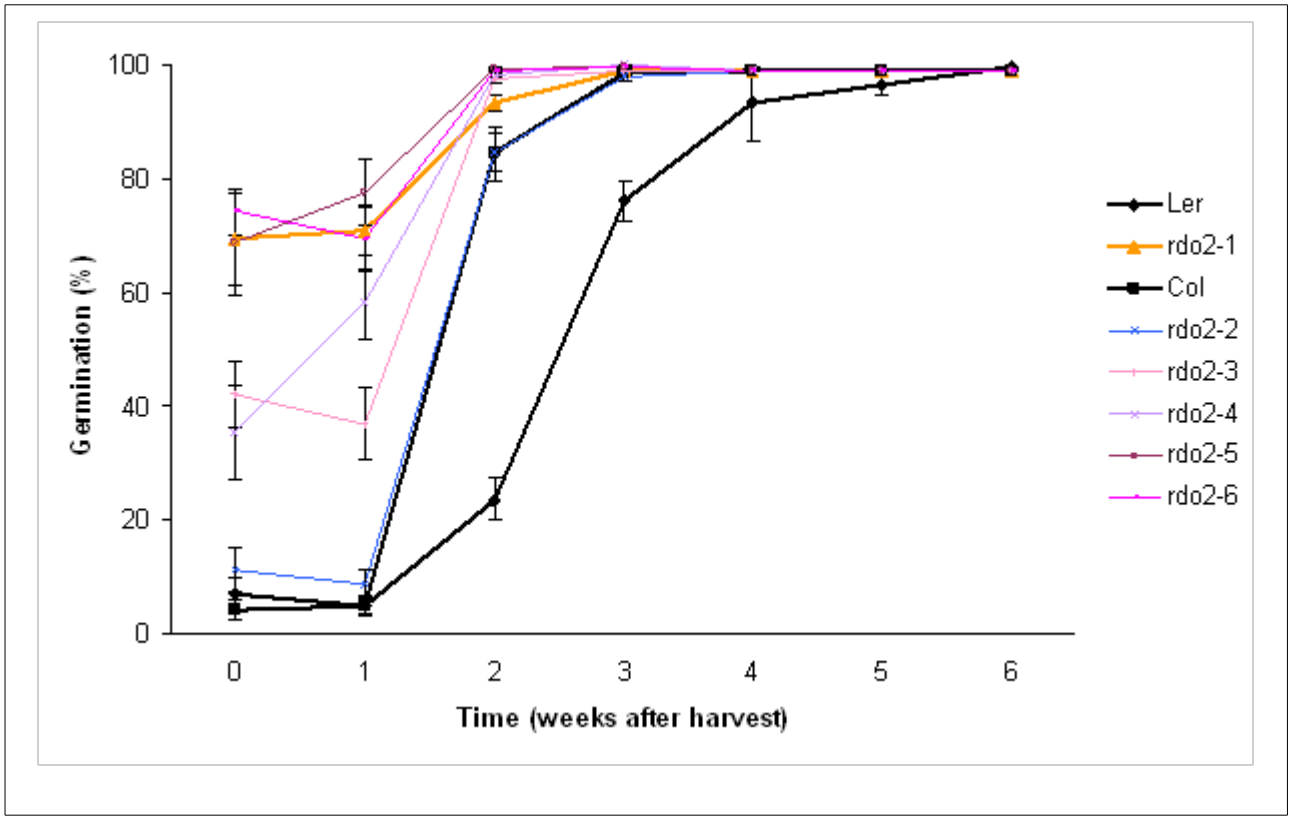

Figure 3.14: Germination of T-DNA insertion lines of At2g38560. Percentage of germinated seeds of the five T-DNA insertion lines, L*er*, Col and *rdo*2. Twelve plants per line were analyzed separately during after-ripening, standard errors are depicted as bars.

In summary we found three confirmations for the identity of At2g38560 as the *RDO2* gene. There is a 4 bp deletion in the coding sequence of At2g38560 in the *rdo2-1* mutant compared to L*er*. The mutation destroys the C-terminal zinc finger domain of the encoded protein. The transformation of *rdo2-1* mutant plants with the L*er* genomic sequence of At2g38560 leads to a partial complementation of the mutant phenotype. Finally two of the four T-DNA insertion lines of At2g38560 display a germination phenotype very similar to that of the *rdo2-1* mutant.

3.2.2 Characterization of *RDO2*

As described in the previous chapter, the gene *RDO2* was found to encode a TFIIS transcription elongation factor. This chapter describes the characterization of the gene and its role in seed dormancy control.

RDO2 is annotated as a TFIIS transcription elongation factor in the TAIR database (http://www.arabidopsis.org). While little is known about this protein in plants, TFIIS is well studied in *Saccharomyces cerevisiae*. It was found to accelerate the overcoming of trancriptional arrest of RNA polymerase II (Fish and Kane, 2002), to support the formation of the transcription preinitiation complex (Kim et al., 2007) and to act as a general RNA polymerase III transcription factor (Ghavi-Helm et al., 2008). Furthermore, in yeast knocking out the TFIIS gene leads to a phenotype only in presence of the NTP-depleting drugs 6-azauracil and mycophenolic acid, so the protein might be functionally redundant with other factors (Wery et al., 2004). A sequence search revealed that homologues of the *Arabidopsis thaliana* TFIIS protein can be found in many eucaryotic organisms. In *Arabidopsis thaliana* it is a unique gene, since no matches for a second gene with a similar sequence or the same set of domains were found. PCR with specific primers showed that *RDO2* and the putative promoter sequence of 1 kb upstream of its transcription start can also be found in the close relatives *Arabidopsis lyrata* and *Arabidopsis halleri* (Marilyne Debieu, personal communication). Altogether these findings suggest a fundamental and conserved function of RDO2.

The TFIIS protein is involved in the general process of transcription but the *rdo2* mutant in *Arabidopsis thaliana* has been identified by its seed dormancy phenotype. The relation between transcription elongation and seed dormancy can be revealed by study of the *RDO2* gene and protein.

3.2.2.1 *RDO2* expression during seed maturation

Dormancy is established during seed maturation, i.e. the second half of seed development (Raz et al., 2001). The expression of the *RDO2* gene during seed development was analyzed. Since *RDO2* influences the dormancy phenotype of *Arabidopsis thaliana*, it is important to determine the expression of the gene during seed development.

L*er* plants were grown under controlled conditions, which lead to ripe seeds within 20 days after pollination (dap). Total RNA was extracted from siliques at 10 dap, 12 dap, 14 dap, 16 dap, 18 dap and 20 dap and the expression of *RDO2* was assessed by quantitative real time PCR. Three biological replicates of the experiment showed the same tendency. The amount of *RDO2* mRNA is

increasing during the second half of seed maturation and is highest in mature seeds. A representative experiment is shown in Figure 3.15.

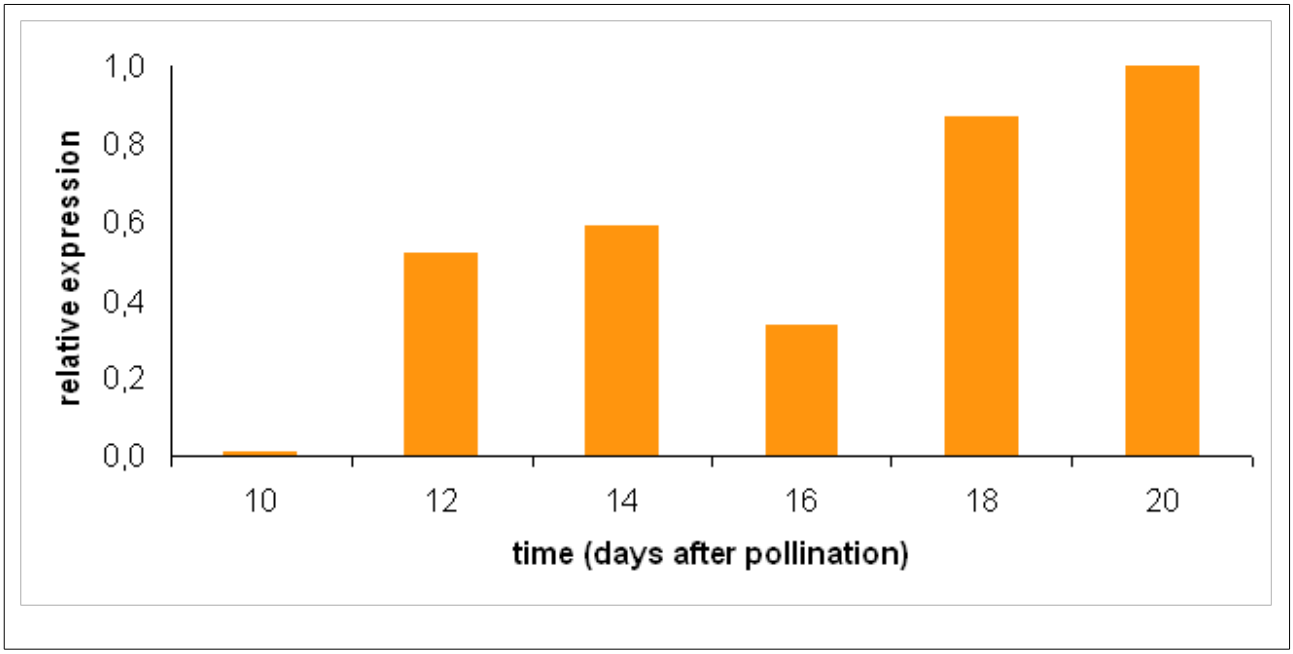

Figure 3.15: Transcript levels of *RDO2* in L*er* seeds during seed maturation. Transcript levels of *RDO2* were determined by quantitative RT-PCR. cDNA was generated from mRNA from 40 mg L*er* siliques harvested 10 dap, 12 dap, 14 dap, 16 dap, 18 dap and 20 dap. The expression values of *RDO2* were normalized using the expression level of *ACT8* as internal standard.

3.2.2.2 Overexpression of *RDO2*

The *rdo2* mutant shows that loss of RDO2 function leads to reduced dormancy. Increased RDO2 expression could lead to a different effect on dormancy. The high abundance of a protein can lead to enhanced or additional phenotypic effects (Lloyd, 2003). For overexpression of a gene in *Arabidopsis thaliana*, the double 35S promoter (Jakoby et al., 2004) can be used, which is highly expressed in all tissues.

rdo2 plants were transformed with cDNA of *RDO2* expressed from the p2x35S promoter. Four independent homozygous lines with single insertions were selected in the T2 generation on the basis of 3:1 ratio of BASTA resistant to BASTA sensitive plants. The germination phenotype of the homozygous lines was compared to L*er* and *rdo2* (Figure 3.16). One of the transformant lines (14-1) showed the same germination pattern as the *rdo2* mutant, which could indicate that *RDO2* is not expressed. The other three lines show intermediate phenotypes which are very similar to each other. The germination of the lines 5-1, 7-4 and 8-3 is in between the phenotype of L*er* and *rdo2*.

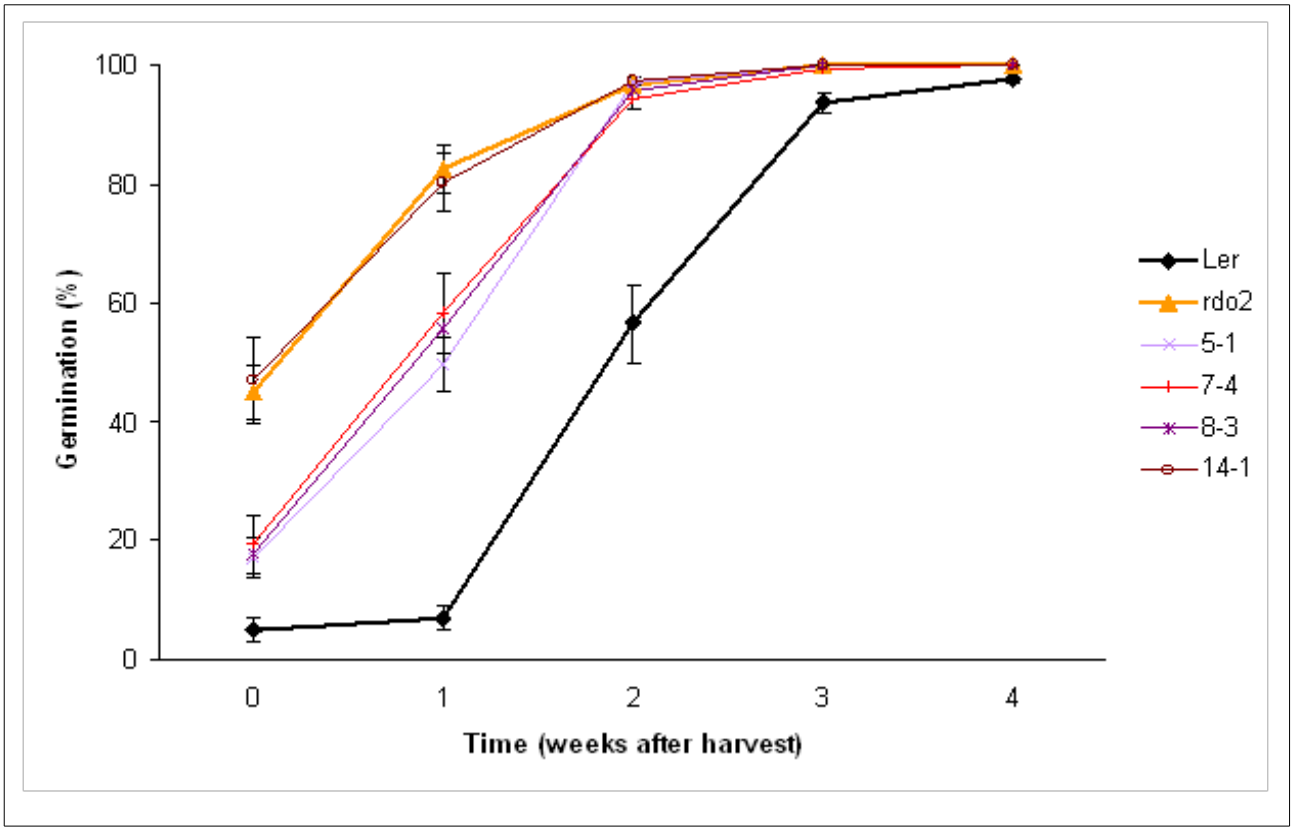

Figure 3.16: Germination of *rdo2* plants transformed with p2x35S:cRDO2. Percentage of germinated seeds on water in the light after different periods of dry storage is shown for L*er*, *rdo2* and four independent homozygous transformants of *rdo2* with p2x35S:cRDO2. Values are means of ten plants, the bars represent standard errors.

The dormancy level of the overexpression lines is similar to that of the complementation lines (Chapter 3.2.1). The reason could be that an increased level of *RDO2* does not lead to a change in dormancy or that the expression of *RDO2* is not increased in the transformed lines.

One disadvantage of overexpression constructs is the possibility of silencing. Gene silencing can be induced by multiple tandem insertions of the transgene (Assaad et al., 1993). Silencing results in the degradation of the mRNA of the gene. However, tandem repeats of the insert which could induce a silencing process were excluded by PCR analysis. Furthermore, silencing of a gene should lead to a phenotype similar to the knock out mutant. This is not the case for the *RDO2* overexpression transformants.

The expression level of *RDO2* was examined in seeds of L*er*, *rdo2* and the transformants at 17 dap. mRNA was extracted from seeds 17 dap and primers amplifying specifically *wtRDO2* were used for quantitative RT-PCR (Figure 3.17).

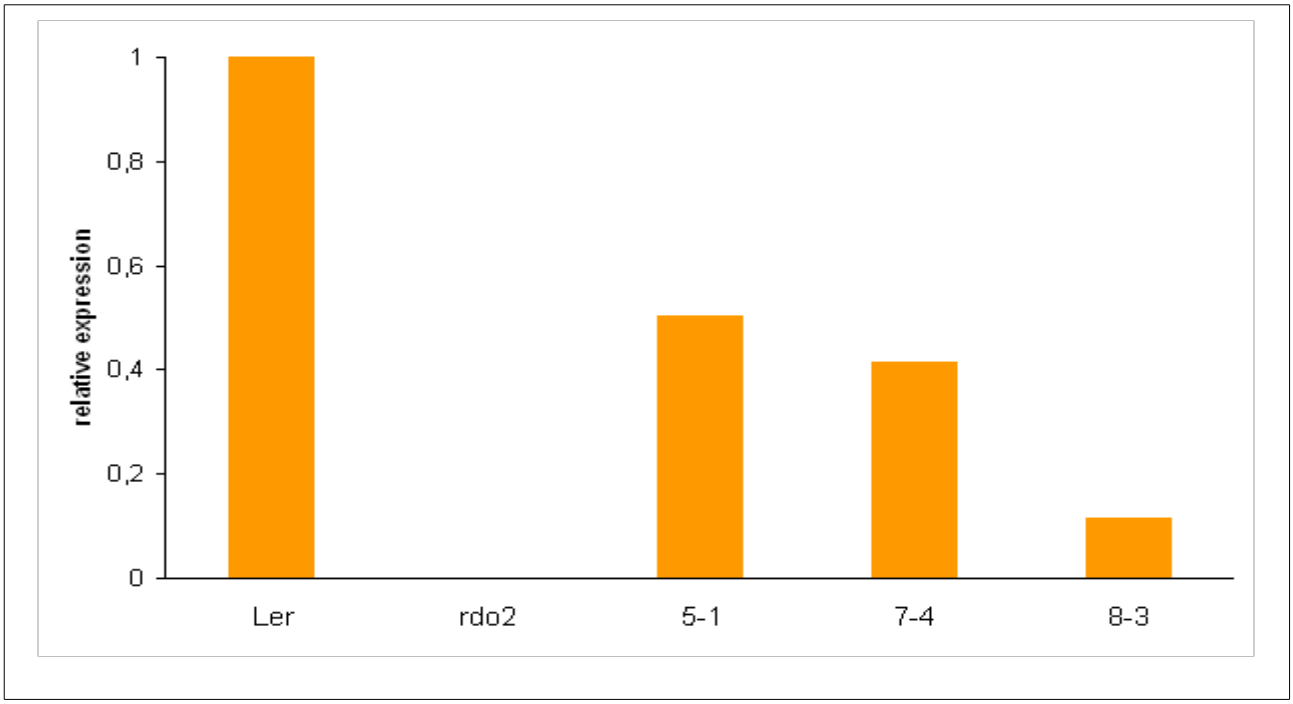

Figure 3.17: Transcript levels of *wtRDO2* in *rdo2* transformed with p2x35S:cRDO2. Transcript levels of *wtRDO2* were determined by quantitative RT-PCR. cDNA was generated from mRNA from 40 mg seeds 17 dap of L*er*, *rdo2* and three independent homozygous transformants of *rdo2* with p2x35S:cRDO2. The expression values of *wtRDO2* were normalized using the expression level of *ACT8* as internal standard.

As expected no *wtRDO2* can be detected in *rdo2* seeds. The three investigated transformants show between 0,1x and half of the expression of *RDO2* in L*er*. This is consistent with the observed dormancy phenotype of the lines.

To exclude the possibility that lines of high dormancy were missed by the mode of selection of transformants, all 50 T2 populations were screened again for high dormancy. However, no highly dormant transformants were found.

In summary, we could not obtain a line that overexpresses *RDO2*. After transformation of *rdo2* plants with *RDO2* under the double 35S promoter all obtained lines show an expression level of *RDO2* similar to the endogenous promoter, corresponding with the phenotypes.

The transformants contain the mutated *rdo2* gene as well as the wt *RDO2* gene, and both genes are presumably expressed. It is possible that the total amount of transcript is regulated. Transcript regulation can be mediated by a gene specific micro RNA (Brodersen and Voinnet, 2006). However, evidence for regulation of *RDO2* by this mechanism was not found. No miRNA is annotated for *RDO2* (http://mpss.udel.edu/at/).

3.2.2.3 Intracellular localization of the RDO2 protein

The annotation of RDO2 as a TFIIS factor is based on *in silico* data (http://www.arabidopsis.org). A transcription elongation factor should localize in the nucleus, therefore I tested the intracellular localization of RDO2. The localization of a protein inside the cell, i.e. its confinement to a certain compartment, could hint to the process the protein is working in. To visualize a protein inside an organism it can be tagged with a fluorescent marker and when the fusion protein is expressed its precise position in the cell can be determined with the help of a fluorescence microscope (Leffel et al., 1997).

To gain knowledge of the intracellular localization of the RDO2 protein, the L*er* cDNA of the gene was fused with YFP at the N- or C-terminus and expressed by a 2x35S promoter. In an initial experiment the constructs were expressed in leek epidermal cells by micro particle bombardment. Some cells incorporate the sequence and express the encoded protein when bombarded with particles that are coated with a plasmid. Leek leaves were used because they consist of large cells and fluorescence can be detected easily in the lower chlorophyll-less parts of the leaves.

After bombardment of white parts of leek leaves with one of the plasmids the fluorescent signal became visible after a few hours of incubation. Both the C-terminal and the N-terminal fusion protein lead to strong fluorescence exclusively in the nuclei (Figure 3.18). The strong expression was possibly due to the overexpression promoter. We concluded that the RDO2 protein is localized in the nucleus and the attached YFP does not hinder its nuclear import. This agrees with the role of RDO2 as TFIIS factor.

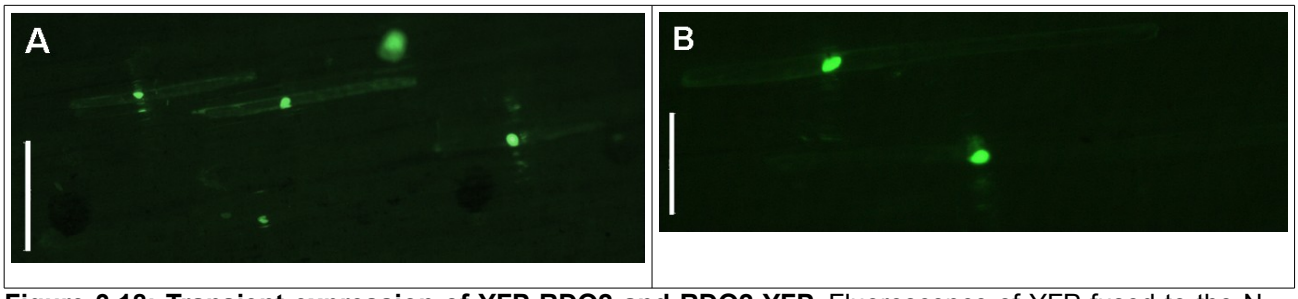

Figure 3.18: Transient expression of YFP-RDO2 and RDO2-YFP. Fluorescence of YFP fused to the N-terminus (A) or C-terminus (B) of RDO2 in leek epidermis cells after bombardment is detected in the nucleus. The cell walls reflect the fluorescent shine from the nuclei. Scale bar is 1 mm.

To gain insight into the natural location of RDO2 in *Arabidopsis thaliana* cells the fusion protein was stably transformed ito *rdo2* mutant plants. For stable expression of the RDO2-YFP fusion protein, *rdo2* mutant plants were transformed with one of the constructs. Independent homozygous lines with single insertions were tested for the intracellular localization of the fusion protein by

investigation of embryos and seed coats. Additional staining of the material with propidium iodide (PI), which stains the DNA, was performed.

Figure 3.19 shows an embryo with its seed coat in overview and a part of the cotyledon in higher magnification. The microscope analysis shows that plants transformed with the overexpressed RDO2 fused to YFP have strong fluorescence signals that co-locate with the PI stained nuclei. This is consistent with the result of the transient expression of the constructs in leek. Therefore the RDO2-YFP fusion protein is located in the nucleus.

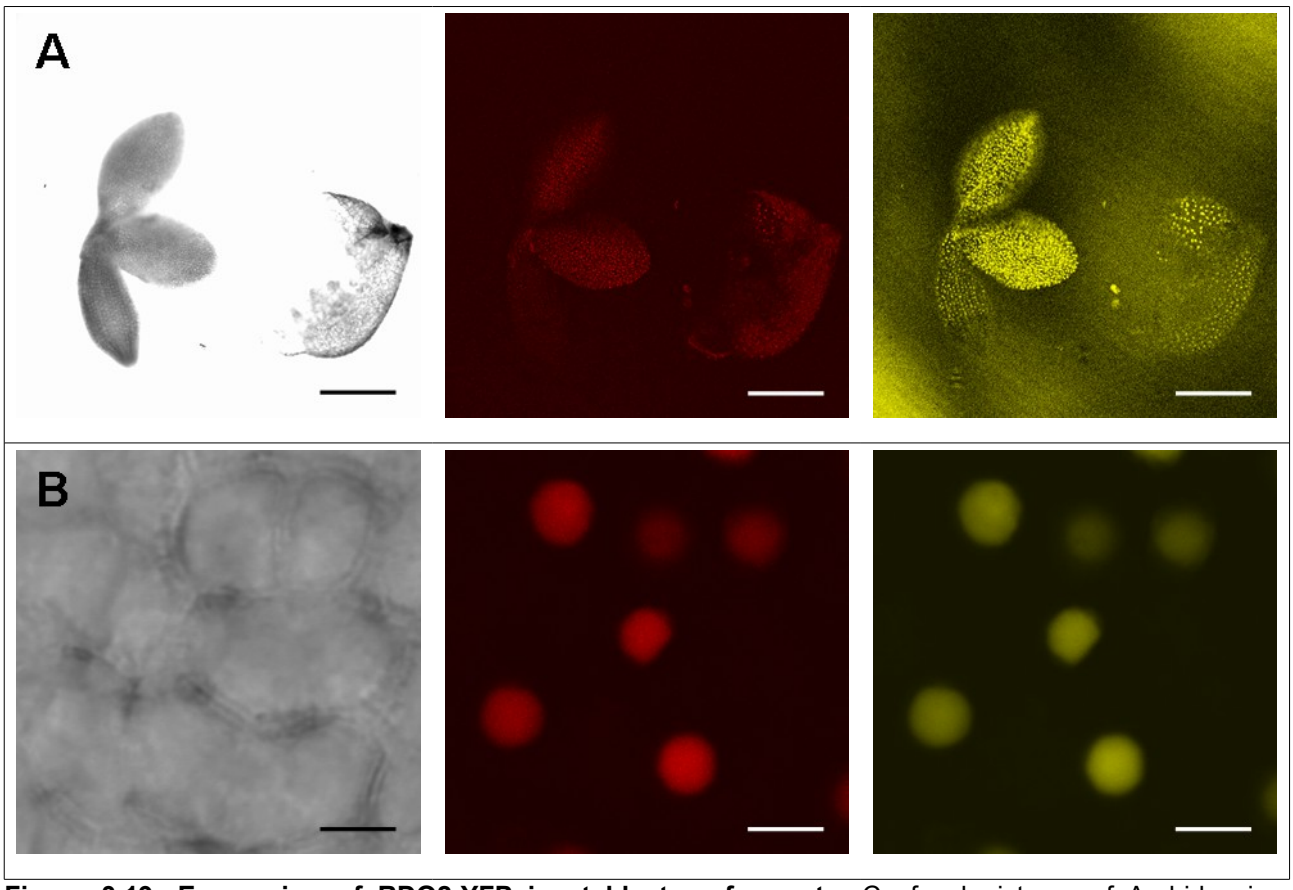

Figure 3.19: Expression of RDO2-YFP in stable transformants. Confocal pictures of Arabidopsis embryo and seed coat (A) and embryo nuclei (B) expressing RDO2 protein with C-terminal fusion of YFP. Left: transmission, middle: PI staining, right: YFP fluorescence. Scale bars are 75 µm (A) and 3 µm (B).

The functionality of the RDO2-YFP fusion proteins was tested by analysis of the dormancy phenotype of some of the lines (Figure 3.20). Surprisingly, the plants with the overexpressed RDO2-YFP protein germinated faster than the *rdo2* mutant. The protein does not delay germination as in L*er*. It is possible that the attachment of the YFP tag blocks the function of RDO2 and could even act as a dominant-negative mutation as indicated by the stronger phenotype. It could inhibit the function of the protein complex that is involved in transcription elongation.

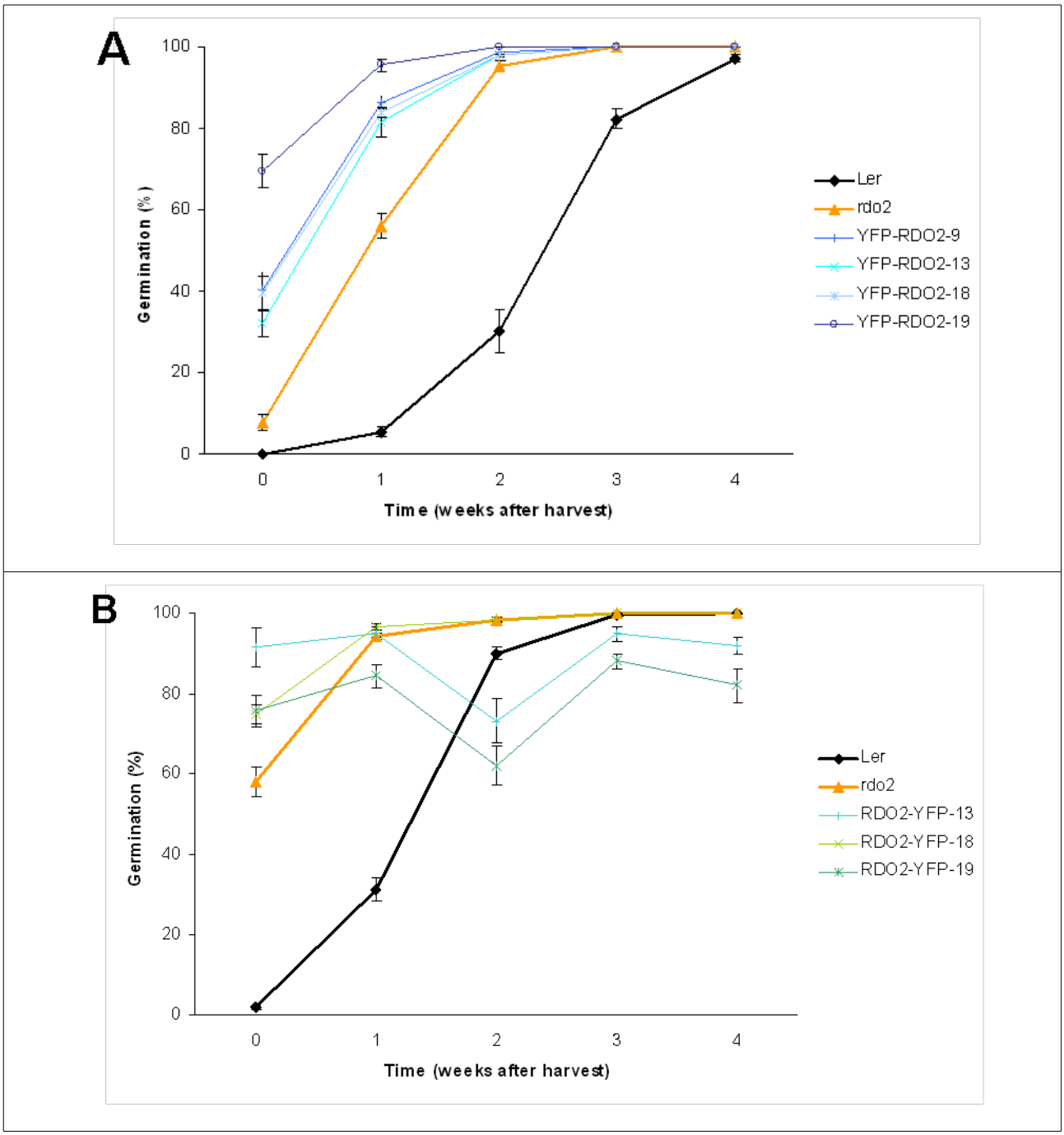

Figure 3.20: Germination of *rdo2* plants transformed with p2x35S:YFP-RDO2 (A) and p2x35S:RDO2-YFP (B). Percentage of germinated seeds on water in the light after different periods of dry storage is shown for L*er*, *rdo2* and four independent homozygous transformants of *rdo2* with p2x35S:YFP-RDO2 (A) and three independent homozygous transformants of *rdo2* with p2x35S:RDO2-YFP(B). Values are means of ten plants, the bars represent standard errors.

To analyze the expression level of *RDO2* in the transformed lines semiquantitative RT-PCR of *YFP-RDO2* and *RDO2-YFP* mRNA in leaves and seeds was performed (Figure 3.21). Total RNA was extracted from leaves of three and two independent lines, respectively. cDNA was prepared and

used for PCR with primer pairs amplifying parts of *Actin 2* (*ACT2*), *wtRDO2* and both *wtRDO2* and mutant *rdo2*.

The signal of *ACT2* of all lines was similar in strength indicating comparable amounts of mRNA in these samples. The two lines with C-terminally fused YFP showed a strongly increased expression of *RDO2* compared to L*er* and *rdo2*, while the lines with N-terminal fused YFP did not.

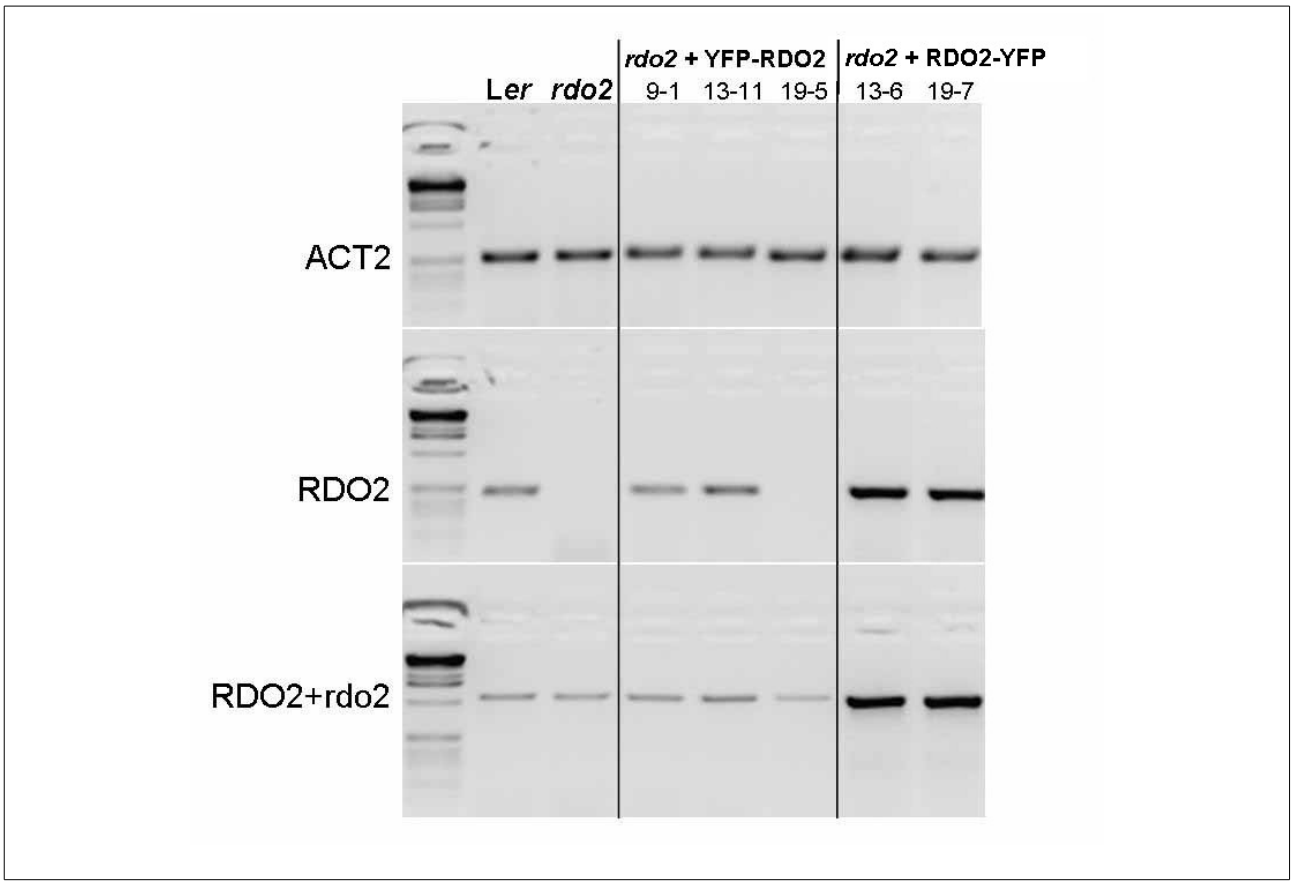

Figure 3.21: Expression of *RDO2* RNA in leaves of RDO2+YFP fusion lines. Semiquantitative RT-PCR of cDNA synthesized from total RNA of leaves.

Transgenic plants with a fusion of RDO2 with YFP visualized its location in the nucleus both in transiently and stably transformed plant cells. Even though the transgene transcript is expressed from the 2x35S promoter, *RDO2* mRNA is highly abundant solely when fused with YFP at the C-terminus. Furthermore, the germination phenotype of the mutant could not be rescued by the transgene indicating that the tagged RDO2 protein is not functional. Therefore the result of the localization study has to be considered with care.

3.2.2.4 Functional conservation of *Arabidopsis thaliana* and yeast TFIIS function

The TFIIS transcription elongation factor is well investigated in the yeast *Saccharomyces cerevisiae*. The yeast TFIIS gene, named *DST1*, is a single copy gene. It encodes a protein of 309 amino acids and is ubiquitously expressed; the knockout leads to minor defects under specific conditions (Wery et al., 2004). *DST1* was found to be responsible for the restart of RNA polymerase II complexes after transcriptional arrest (Fish & Kane, 2002) as well as for the assembly of the transcription preinitiation complex assembly (Kim et al., 2007) and it was shown to function as a general transcription factor for RNA polymerase III (Ghavi-Helm et al., 2008). The molecular structure of the yeast TFIIS protein and its interaction with the arrested polymerase II was resolved by crystallization studies (Kettenberger et al., 2004).

The phenotypes of the mutated TFIIS gene in yeast and *Arabidopsis thaliana* are similar. Both mutants are viable and show only minor defects under specific conditions or in a specific developmental phase (Wery et al., 2004, Léon-Kloosterziel et al., 1996). Alignment of the amino acid sequences of the two proteins reveals conservation especially in the functional domains (Figure 3.22). Considering the similarity between DST1 and RDO2, it is possible, that they can complement each others function.

The yeast TFIIS mutant was transformed with two different plasmids containing the cDNA of *RDO2* which are expressed as single copy in each cell or as multiple copies. In parallel, the yeast genomic sequence of *DST1* expressed from the p2x35S or the short *RDO2* putative promoter was stably transformed in *rdo2* mutant plants.

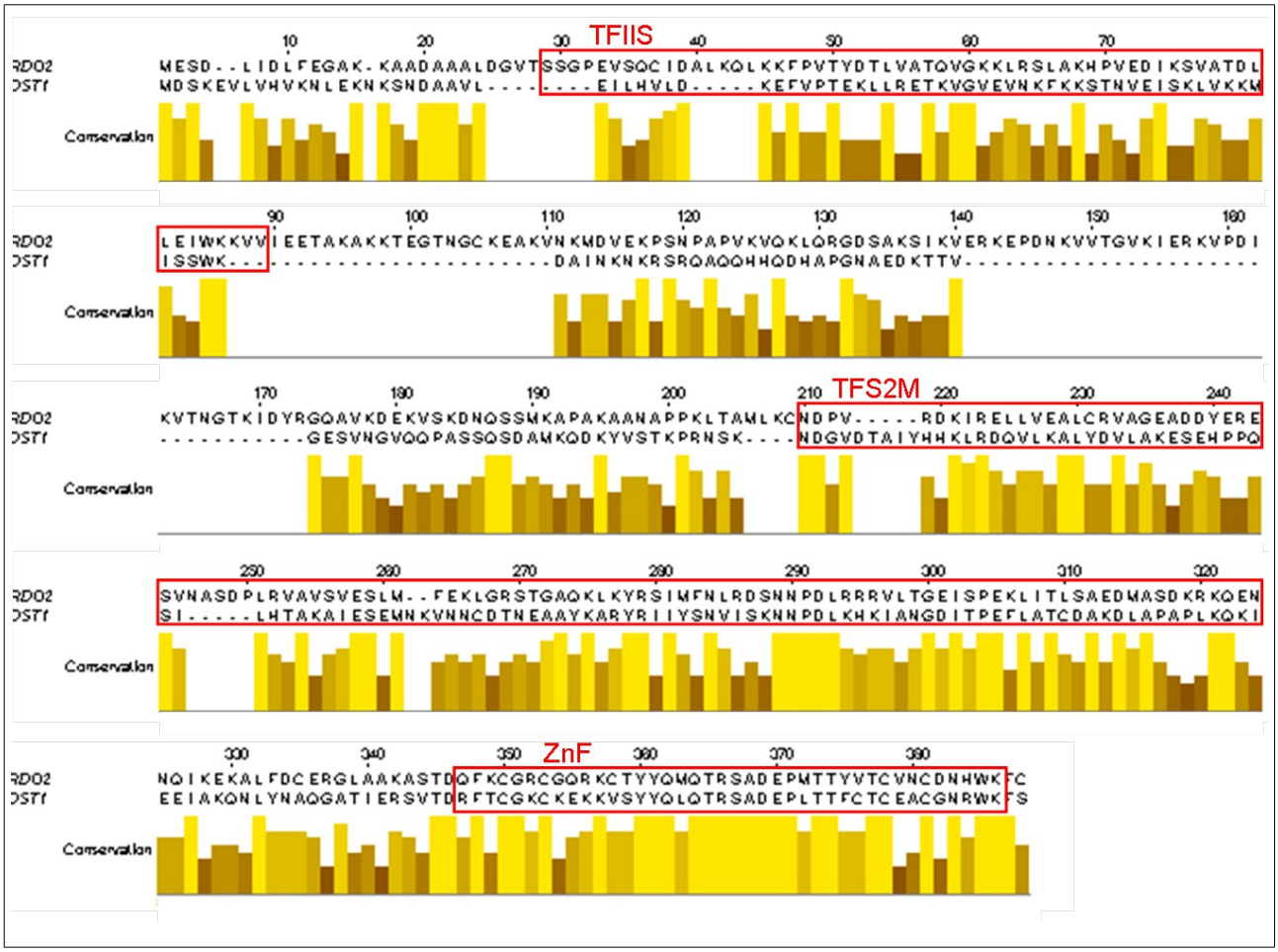

Figure 3.22: Conservation of RDO2 and DST1. Shown are the amino acid sequences of *RDO2* and *DST1* and the position of the functional domains. The degree of conservation is displayed as yellow to brown columns; the taller and lighter the column, the higher is the degree of conservation of the amino acid.

Complementation of the yeast TFIIS mutant with RDO2

The yeast TFIIS mutant named *Δdst1* shows reduced growth on medium with the transcription inhibiting chemical 6-azauracil (6-AU) (Exinger and Lacroute, 1992). For the complementation experiment the *Δdst1* mutant CMKy3 and its background strain CH1305 (Davie and Kane, 2000) were used. In the mutant yeast strain a large part of the *DST1* sequence is replaced by the open reading frame of the *URA3* gene, orientated in the opposite direction. *URA3* is coding for orotidine 5-phosphate decarboxylase, involved in the synthesis of pyrimidine ribonucleotides, and is used as a reporter gene (http://db.yeastgenome.org/). The reduced growth phenotype of the mutant was visible on 100 µg/ml 6-AU and the substitution of the *DST1* gene by the *URA3* gene was confirmed by PCR.

To test the ability of *RDO2* to rescue the yeast mutant, *Δdst1* was transformed with the cDNA of *RDO2* controlled by an *ADH* promoter that leads to constitutive expression. The construct was

cloned in a CEN plasmid and a 2µ plasmid, respectively. The CEN plasmid is present in one to two copies in each cell and is inherited like a chromosome during cell division. The 2µ plasmid creates 20 to 100 copies per cell and will lead to high transcript levels. As a control *Δdst1* was transformed with plasmids containing *DST1* or no insert.

On medium without 6-AU all strains grew with similar density. The growth phenotype of the strains was screened on selective medium with concentrations of 6-AU ranging from 1 µg/ml to 200 µg/ml. Concentrations up to 25 µg/ml did not lead to a visible difference in growth of the strains. Concentrations of 6-AU between 50 µg/ml and 200 µg/ml did show a differential growth pattern. As an example the growth of eight strains on 100 µg/ml 6-AU is shown in Figure 3.23. Transformation with an empty plasmid did not change the decreased growth of the *Δdst1* mutant compared to its background. Transformation of the mutant with a plasmid containing *DST1* recovered the growth phenotype, while *RDO2* did not. The number of plasmid copies does not influence the growth phenotype.

Therefore we conclude that *RDO2* is not able to complement the yeast TFIIS mutant.

Cloning of these constructs and all work concerning yeast was performed by Christina Philipp.

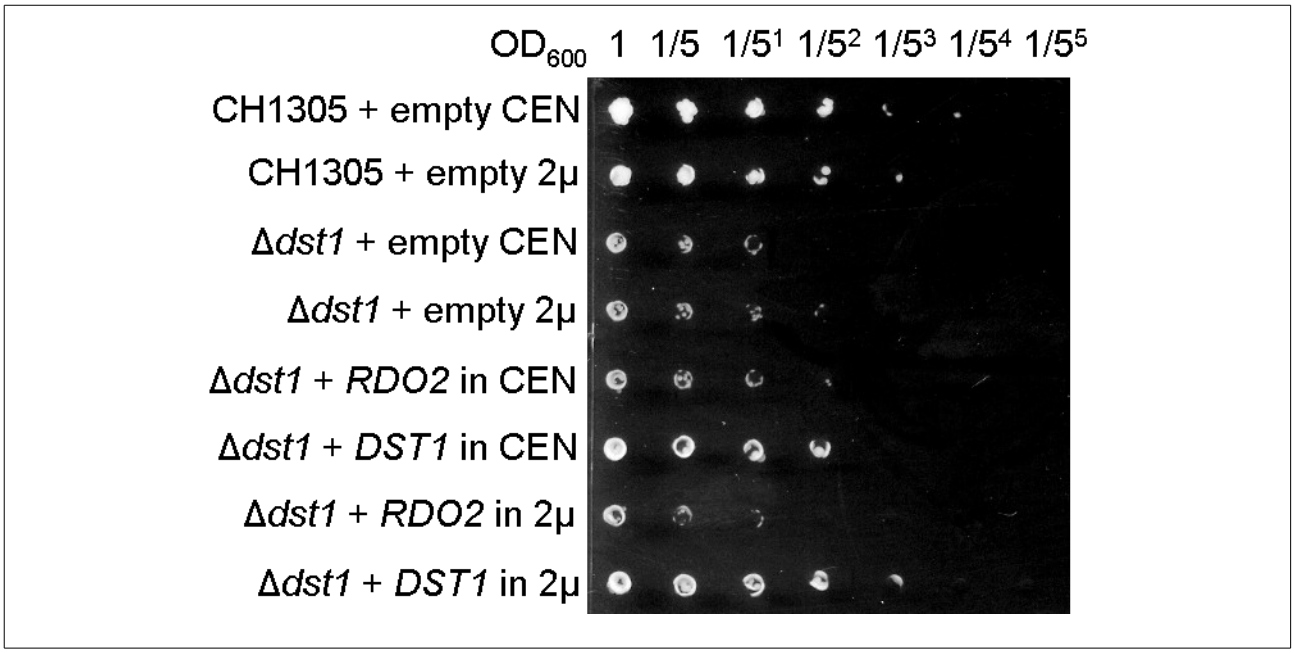

Figure 3.23: Growth phenotype of *Δdst1* mutant transformed with RDO2 and DST1. Dilution series of eight different yeast strains grown on medium containing 100 µm 6-AU.

Complementation of the* rdo2 *mutant with the yeast TFIIS gene

To test whether *DST1* is able to complement the *rdo2* mutant phenotype two plasmids for expression *in planta* were designed to express *DST1* at different levels. The genomic sequence of *DST1* was cloned into a plasmid containing the p2x35S promoter for overexpression. To investigate expression of *DST1* at endogenous levels the genomic sequence of *DST1* was expressed under the short putative *RDO2* promoter. Assuming *DST1* can complement the *rdo*2 mutant, the transformants should show a similar change in dormancy as the complementation of the mutant with *RDO2* itself. Seeds from the transformed plants will be obtained soon.

3.2.2.5 The relation between *RDO2* and dormancy QTLs

To gain insight into the genetic interaction between dormancy loci, plants containing these loci can be crossed and the phenotype of the double lines analyzed.

The *rdo2* mutant was crossed with three different near isogenic lines (NILs) containing Cvi introgressions in L*er* background at locations of the dormancy QTLs DOG1, DOG3 and DOG6. The double lines were created in Wageningen based on the pleiotropic phenotypes of the *rdo2* mutant. The single NILs display a higher dormancy level than L*er*. Putative relations between the *rdo2* mutant and the QTLs would result in a change of the germination phenotype of the double lines. Therefore the dormancy phenotype of the three double lines, the single lines (NILD73, NILD106 and NILD117) and controls (L*er* and *rdo2*) was analyzed.

All double lines containing the *rdo2* mutation in the background of NILD73 (DOG3) and NILD106 (DOG1) or NILD117 (DOG6) did show reduced dormancy compared to the single NILs (Figure 3.24a). Since the tested plants turned out to be not homozygous for the rdo2 mutation, the analysis was repeated with homozygous plants of the double line with NILD117 (Figure 3.24b), which showed the strongest effect. Also the homozygous lines show a similar reduction of dormancy as the segregating lines.

All NILs are additive to the *rdo2* mutation, the genes seem to function in different pathways than *RDO2*.

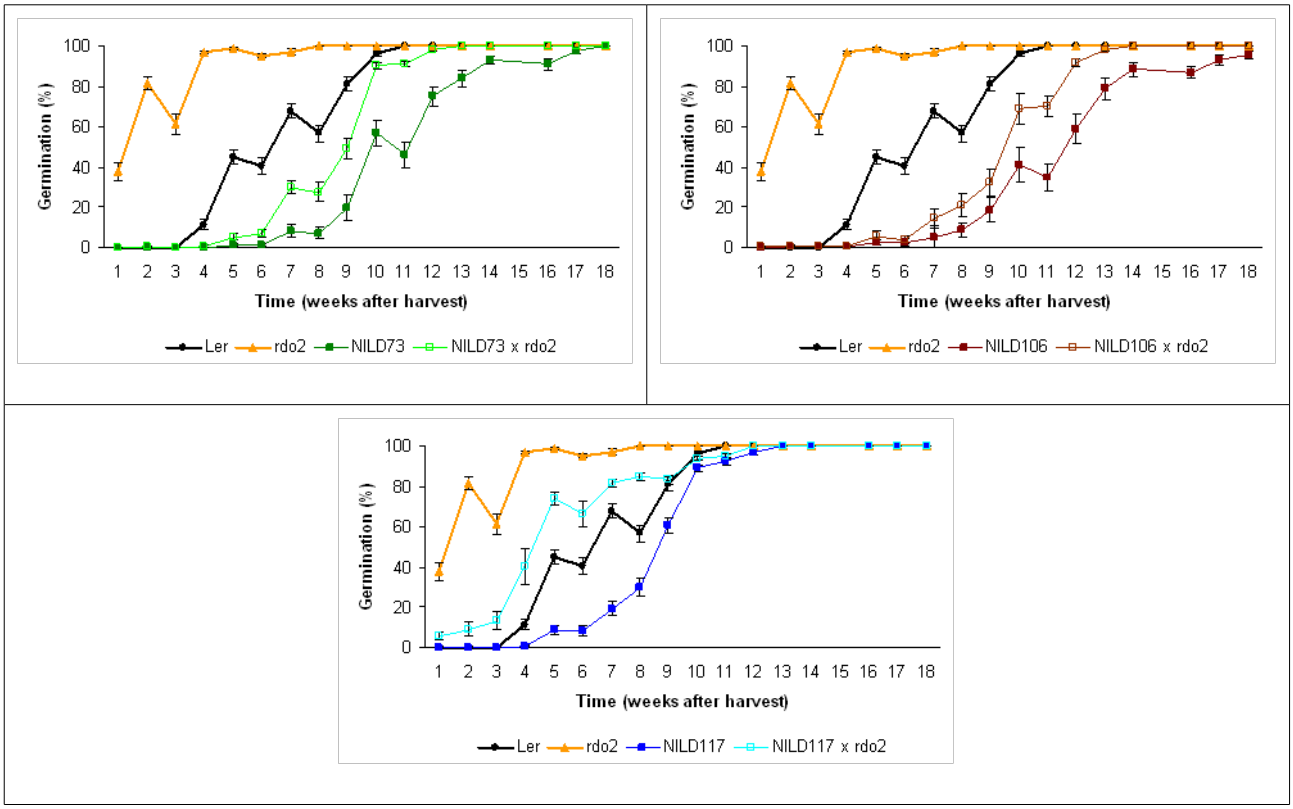

Figure 3.24a: Germination of NILD73*rdo2,* NILD106*rdo2* and NILD117*rdo2* double lines. Percentage of germinating seeds on water in the light after different periods of dry storage is shown for L*er*, *rdo2*, NILD73 and NILD73*rdo2*, NILD106 and NILD106*rdo2,* and NILD117 and NILD117*rdo2*. Values are means of twelve plants, the bars represent standard errors.

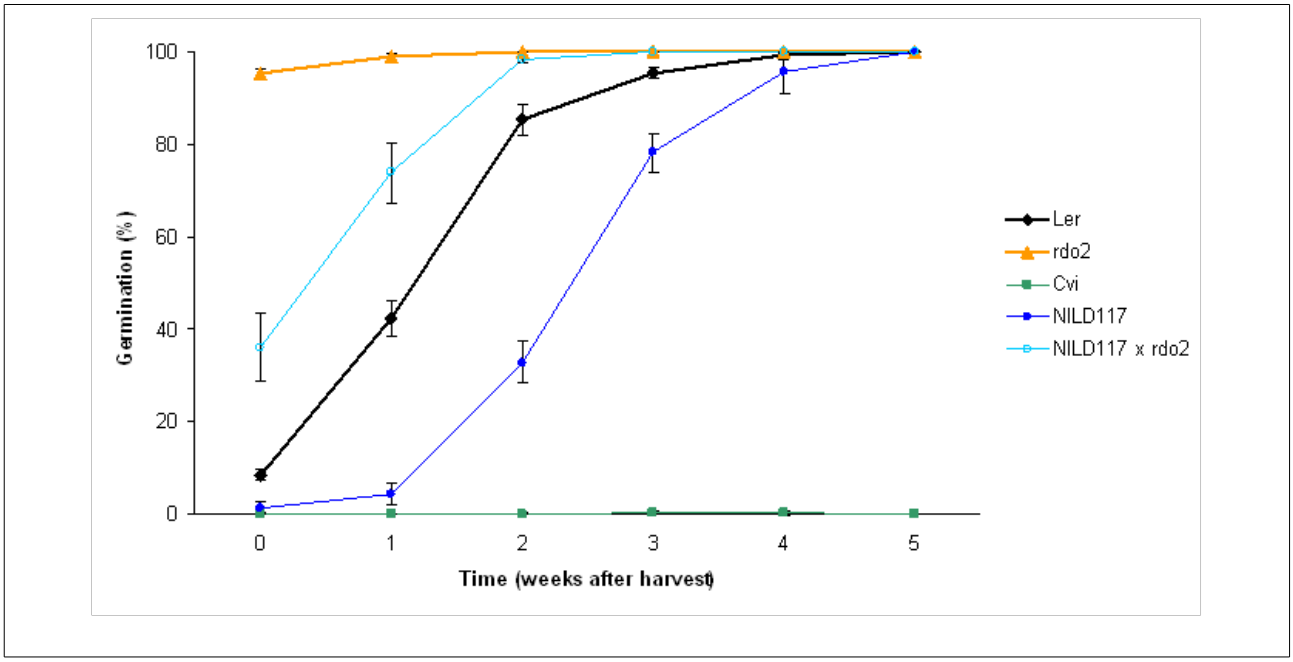

Figure 3.24b: Germination of the homozygous NILD117*rdo2* double line. Percentage of germinating seeds on water in the light after different periods of dry storage is shown for L*er*, *rdo2*, Cvi, NILD117 and NILD117*rdo2*. Values are means of twelve plants, the bars represent standard errors.

3.2.2.6 The analysis of *rdo2* transcriptome data and qRT-PCR for downstream genes

If RDO2 is acting as a transcription elongation factor, it is likely to affect the expression of genes.

Analysis of transcriptome data

An *in silico* analysis of the *rdo2* transcriptome was performed, using microarray data from the group of Mike Holdsworth, available via NASCArray (http://arabidopsis.info/). Data provided by Mike Holdsworth listed the genes down- or upregulated in ripe seeds for a certain mutant in alphabetical order. The data of *rdo2* was compared with those of two other mutants that completely lack dormancy, the ABA deficient mutant *aba1-1* and the ABA insensitive mutant *abi1-1*.

The set of genes which is down- or upregulated in the *rdo2* mutant did not show an enrichment of genes related to dormancy or germination or any other specific process. To compare the gene expression in *rdo2* to other dormancy mutants, we searched the transcription data of *aba1-1* and *abi1-1* for similarities. Figure 3.25 shows the number of genes which are down- or upregulated in mature fresh seeds of *rdo2*, *aba1-1* and *abi1-1* plants compared to L*er*. It also indicates how many of these differentially expressed genes are shared by the different mutants. We find 2/9 genes down-/upregulated in all three dormancy mutants compared to L*er*. Furthermore 105/131 genes were down-/upregulated only in *rdo2*, 4/19 regulated genes shares *rdo2* with *abi1-1,* 16/18 genes with *aba1-1*. The results show that the three dormancy genes participate in different pathways, because the majority of the differentially expressed genes is specific for each of the mutants.

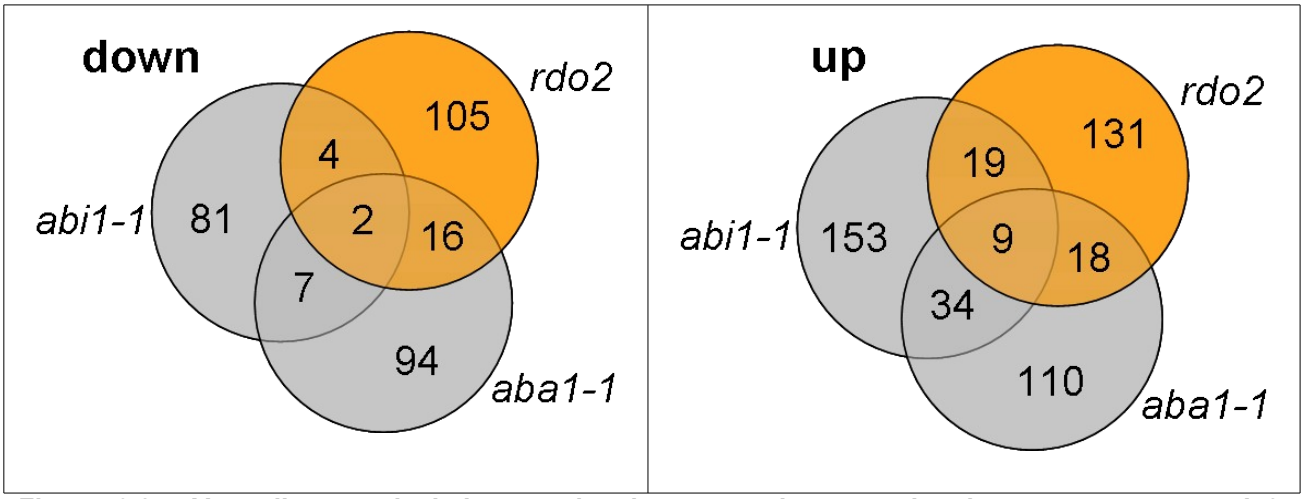

Figure 3.25: Venn-diagram depicting overlapping genes between the dormancy mutants *rdo2*, *aba1-1* and *abi1-1*. Number of genes downregulated (left) and upregulated (right) compared to L*er* in one, two and three of the mutants, respectively.

We analyzed the identity of the 9/2 genes up-/downregulated in all of the three mutants because they could be involved in a general dormancy process. However, none of them is related to

dormancy or germination. *RDO2* is active during seed maturation, but probably not in ripe seeds because they are transcriptionally inactive. The arrays were performed with mature seeds so no conclusion can be drawn for differential gene expression during seed maturation. Instead these results might represent genes far downstream of *RDO2*.

The hypothesis is that *RDO2* is needed at the end of seed maturation to support RNA transcription. This support could be especially important for long genes (Grasser, 2005). In the *rdo2* mutant, this support is absent. According to this hypothesis it would lead to a down regulation of long transcripts in the *rdo2* mutant seeds. We analyzed the length of the genes differentially regulated in *rdo2* and the other mutants based on the genomic sequence given in the TAIR database (http://www.arabidopsis.org). There is only a slight difference in the average length between the genes up-/downregulated in the three mutants (Fig. 3.26). While the genes downregulated in *aba1-1* and *abi1-1* tend to be shorter than the upregulated ones, in *rdo2* the downregulated genes are slightly longer than the upregulated genes. This fits to the theory.

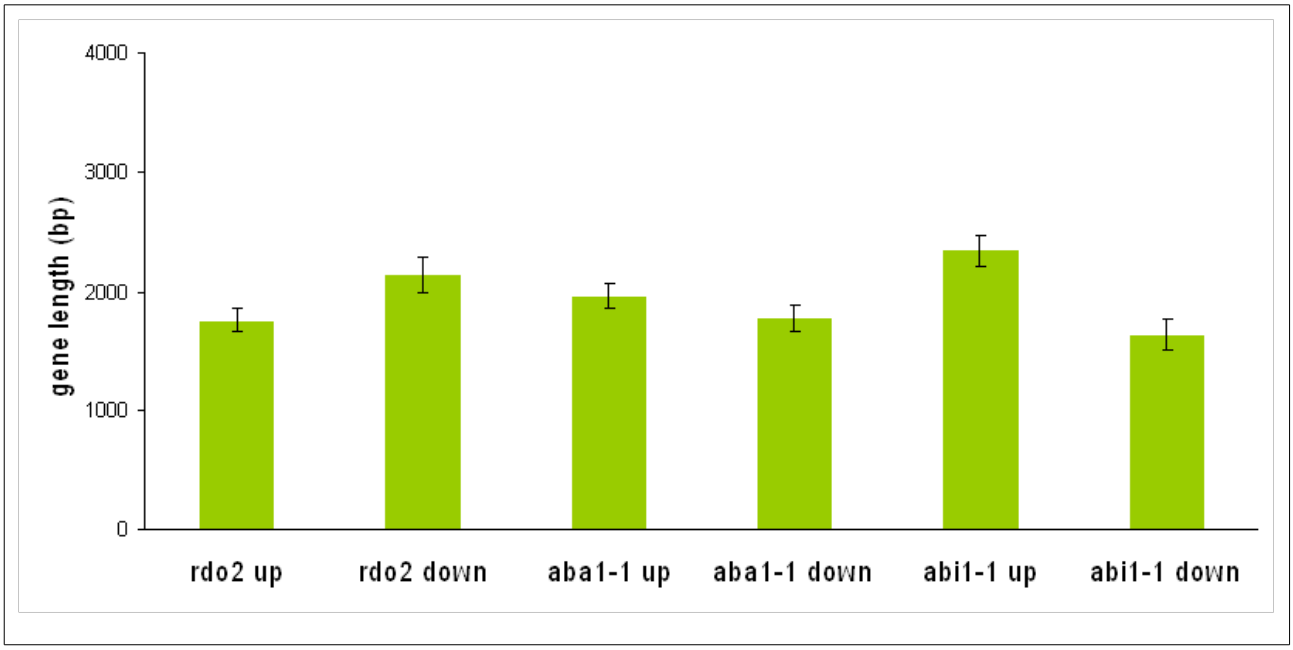

Figure 3.26: Length of genes differentially expressed in three dormancy mutants. Average length of the genomic sequence of the genes up- or downregulated in *rdo2*, *aba1-1* and *abi1-1* mature seeds according to the transcriptome data and standard errors.

Expression analysis by quantitative real time PCR

A different approach to identify downstream genes of *RDO2* is to compare the mRNA expression of a gene between L*er* and *rdo2* mutant. The disadvantage of this analysis is, that candidate genes have to be selected beforehand. I decided to analyze the expression of a number of dormancy and germination related genes.

Initially a semiquantitative reverse transcriptase assay was carried out. Total RNA was extracted from L*er* and *rdo2* ripe seeds and siliques harvested 8 dap, 10 dap, 15 dap, after which cDNA synthesis was performed. A PCR was run with primers for *Actin 2* (*ACT2*), *RDO2, DOG1, SUPRESSOR OF ABI3 4 (SUA4), NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 9 (NCED9), 1-CYS PEROXIREDOXIN (PER1), CYP707A2, ABA INSENSITIVE 4* (ABI4), *ATS2* and SPATULA (*SPT)* (Figure 3.27).

The similar intensity of bands of *ACT2* indicate a comparable amount of mRNA in all probes. Most genes show a similar expression in L*er* and *rdo2* at the same time point during seed maturation. Only for *DOG1* different levels of mRNA could be detected in L*er* and *rdo2* probes during seed maturation.

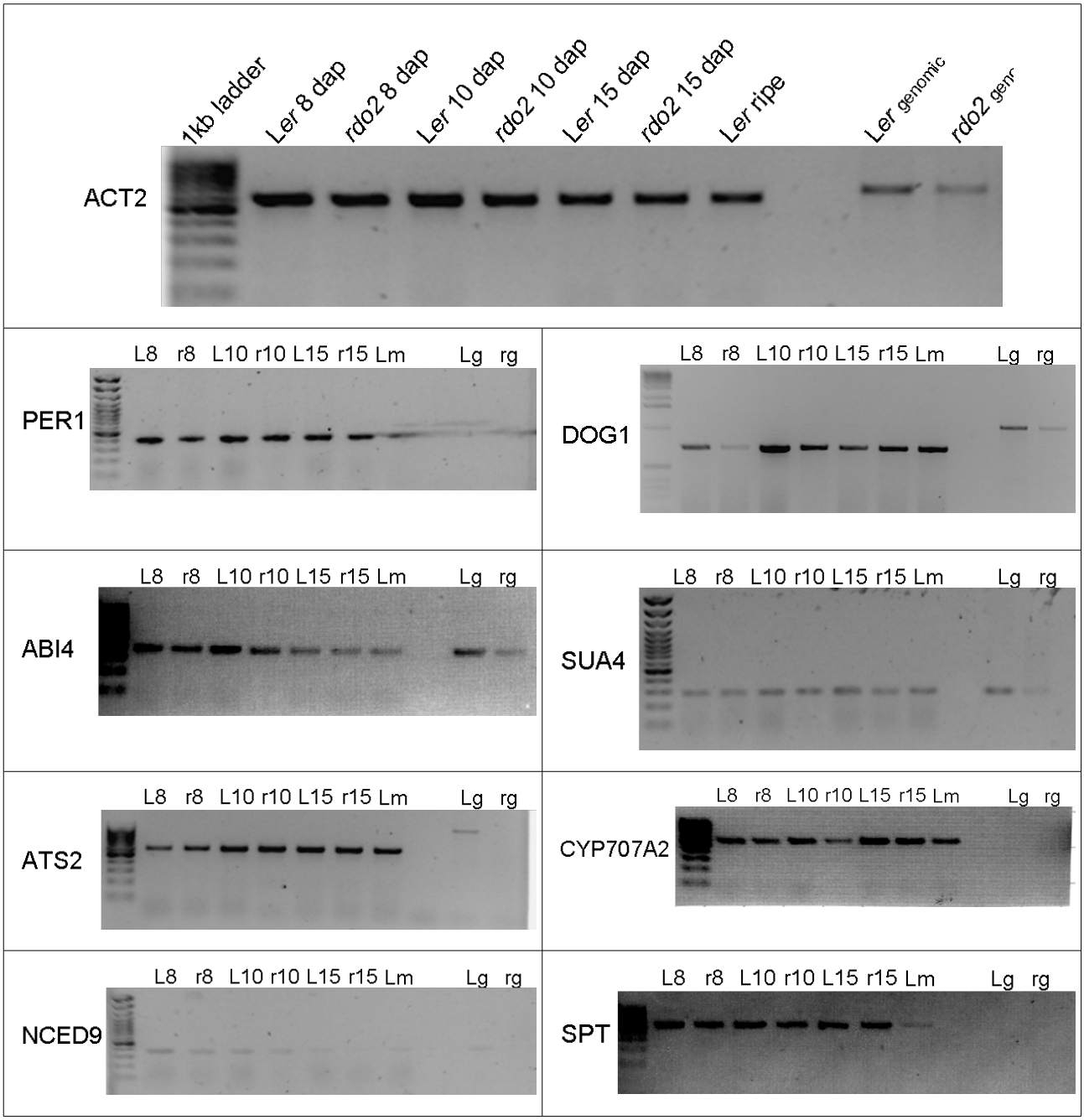

Figure 3.27: Transcript levels of dormancy genes in L*er* and *rdo2* during seed maturation. Transcript levels were determined by semiquantitative RT-PCR. cDNA was generated from mRNA from 40 mg seeds.

To investigate the mRNA expression of *DOG1* in L*er* and *rdo2* during seed maturation more in detail, a quantitative real time PCR assay was performed. Siliques were harvested 10 dap, 12 dap, 14 dap, 16 dap, 18 dap and 20 dap. Total RNA was extracted and cDNA syntheses performed. qRT-PCR was carried out with appropriate primers for *Actin 8* as internal control and *DOG1* (Figure 3.28). Similar results from three experiments results reveal that *DOG1* mRNA is expressed in *rdo2* to a lower level than in L*er* at every tested time point during seed maturation.

Therefore *DOG1* is regulated by *RDO2* - either directly or indirectly.

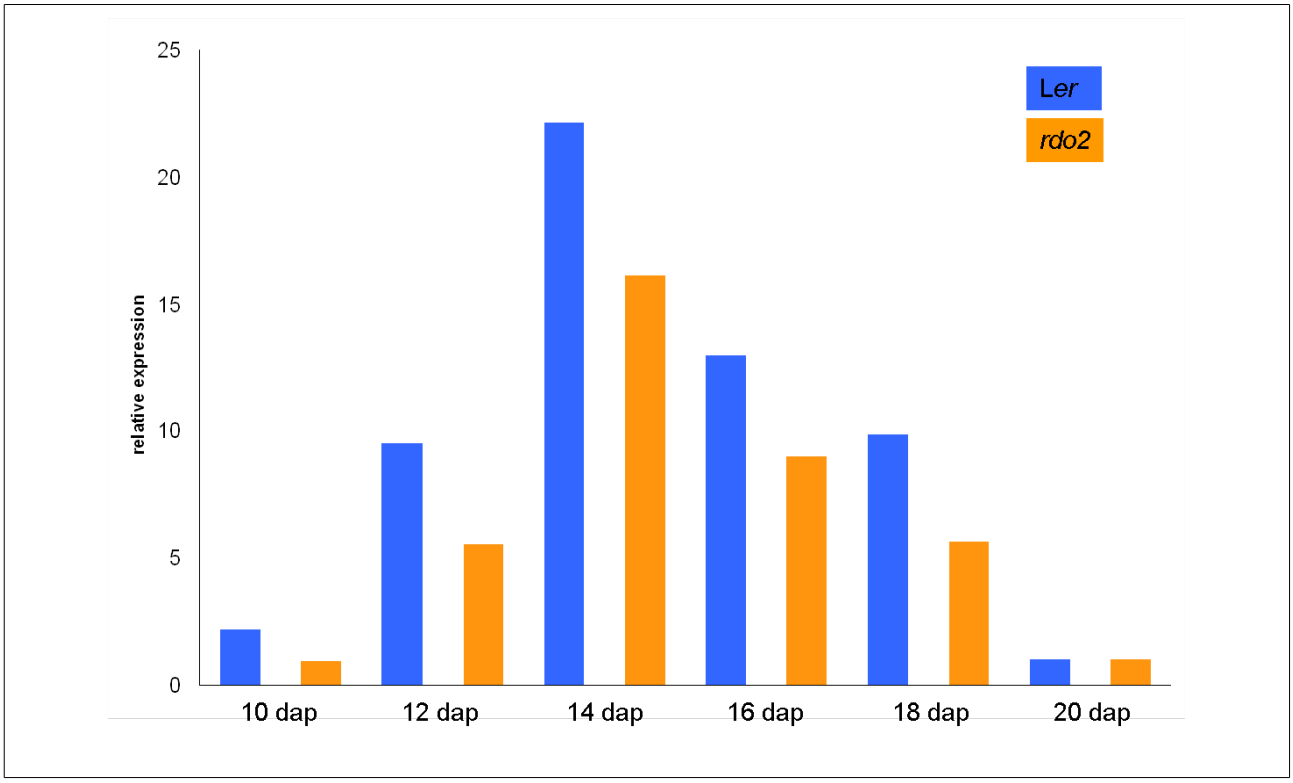

Figure 3.28: Transcript levels of *DOG1* in L*er* and *rdo2* during seed maturation. Transcript levels of *DOG1* were determined by quantitative RT-PCR. cDNA was generated from mRNA from 40 mg seeds. The expression values of *DOG1* were normalized using the expression level of *ACT8* as internal standard.

3.2.2.7 Search for interactors of RDO2 using a yeast-two-hybrid screen

In yeast the proteins Med13 (Srb9) and Spt8 were isolated as two-hybrid partners of the conserved TFIIS N-terminal domain; they are part of the RNA polymerase II complex (Wery et al., 2004). Based on sequence comparison, no similar proteins were found in *Arabidopsis thaliana*. Therefore a yeast two hybrid cDNA library screen was performed.

To test the interaction of RDO2 with other proteins we used the GAL4 yeast two hybrid (Y2H) technique in *Saccharomyces cerevisiae.* RDO2 was fused to a domain of the transcription factor GAL4 which is the bait protein while the cDNA library was combined with the prey domain. If RDO2 interacts physically with any of the proteins in the library, the two halves of the transcription factor GAL4 couple and become functional. Subsequently, the reporter gene responsible for replication of the yeast in selective medium is expressed. This is a fast and low cost technique to identify protein-protein interactions. The disadvantage of a Y2H is that the conditions in yeast do not always reflect the conditions in *Arabidopsis thaliana*. Furthermore, the method solely reveals direct interactions but cannot detect interactions that require additional factors or bigger complexes.

Christina Philipp performed two Y2H screens with *RDO2* cDNA and the all-*Arabidopsis thaliana* cDNA library HS-Ara. Only one of the screens revealed a weak interaction with LOS4 (At3g53110), a putative DEAD-box RNA helicase. The protein might be involved in temperature sensing and the export of polyA RNA (Gong et al., 2002, 2005). However, the interaction could not be confirmed by exchanging bait and prey in the Y2H screen and a T-DNA insertion line of *LOS4* did not show a dormancy phenotype compared to the background Col. Therefore the analysis of this gene was not further continued.

4 Discussion and conclusions

4.1 Cytological analysis of seed dormancy

A dependency of chromatin organization and gene expression during cell differentiation is known in yeast, *Drosophila* and mammalian cells (reviewed by Arney and Fisher, 2004). Major changes in gene expression are likely to be associated with changes in chromatin organization, which are even visible at the microscopic level (Tessadori et al., 2007A). Also in *Arabidopsis thaliana* the chromatin structure is modified during developmental changes throughout the plant life cycle. For example Tessadori et al. (2007B) reported a dramatic decondensation of pericentromeric and gene-rich chromatin in leaf mesophyll nuclei during floral transition. They propose, that decondensation of chromatin in flowering-induced plants may enhance the accessibility of DNA for the transcription machinery on a genome-wide scale. From expression analysis it is known that during floral transition hundreds of genes are up or down regulated (Schmid et al., 2003).

A similar change in gene expression and chromatin organization could take place during seed maturation, when development and growth of the embryo has been completed and the seed prepares for duration in the soil until favorable conditions promote germination.

The aim of this project was to analyze the phase of seed maturation and the dormant state for possible changes in nuclear appearance by cytological methods.

4.1.1 No measurable difference in chromatin structure of *dog1* and NIL DOG1

An initial comparison of dormant Cvi seeds and non-dormant *dog1* mutant seeds gave some indication for a structural difference of the chromatin of dormant an non-dormant embryo nuclei. The heterochromatic structure of mature non-dormant *dog1* and dormant NIL DOG1 seeds was compared. Whole mount staining of embryos with DNA intercalating dyes was followed by confocal analysis. Confocal pictures were analyzed with two different software programs, DISKUS CROMO and ACAPELLA, but the heterochromatin could not be measured reliably.

The quantification of the initially observed difference in chromatic structure of non-dormant and dormant seeds was not possible. It turned out that the difference in heterochromatic appearance between non-dormant seeds in L*er* background and dormant seeds in Cvi background is caused rather by the general difference of the heterochromatic fraction of L*er* and Cvi (Paul Fransz, personal communication) than by the difference in dormancy level.

There are examples where measurement of the amount of heterochromatin was performed successfully (Soppe et al., 2002, Tessadori et al., 2007A+B). However, the described experiments

were not performed in mature seeds, but protoplasts or leaf cells of *Arabidopsis thaliana.* Baroux et al. (2007) investigate endosperm nuclei during early seed development, stating that these nuclei are even bigger than the ones from leaf cells. We found that the cells and nuclei of mature embryos are much smaller and the tissue and chromatin is more dense than for example in leaves. This could be an effect of dessication. It is known that in dessication-tolerant seeds chromatin is highly condensed (reviewed by Pammenter and Berjak, 1999). When the density of the chromatin is high it is more difficult to distinguish between heterochromatin and euchromatin.

4.1.2 Nuclei shrink during seed maturation

During investigation of mature seeds, we noticed that the embryo nuclei were much smaller than reported for embryos during early seed development (Baroux et al., 2007). Therefore this phenomenon was further analyzes in the embryos during maturation. Seeds were harvested at different points during seed maturation The size of spread nuclei isolated from embryos as well as pictures of three-dimensional whole mount preparations were measured with the DISKUS CROMO software. The data display a decrease of nuclear size at the start of seed maturation between 10 dap and 13 dap but not during further development.

Even though the decrease of nuclear size during seed development is not significant in this data set, the research was continued. It was noticed that during the confocal analyses of the embryos the nuclei differ slightly in size in different tissues of the same embryo. Additionally some embryos show variation also between neighboring cells, possibly caused by incomplete fixation of the tissue. The main disadvantage of the used method, the reduction of a nucleus to a two-dimensional picture, can be avoided by measuring the volume of the nuclei. Dr. Vittoria Brambilla analyzed three-dimensional data and excluded embryos with obvious defects. The preliminary results show a convincing decrease of nuclear volume during seed maturation.

Even though decrease of nuclear volume during seed maturation was never reported, it is known since years, that the chromatin gets more condensed during desiccation. It is possible that during maturation drying the conformation of the DNA changes from the B-helix to the more dense Z-form (Osborne and Boubriak, 1994). Loss of water increases the intracellular concentration of ions, modificates the histone H1 content as well as phosphorylation and changes the activity of the poly(ADP-ribose)polymerase (reviewed by Deltour, 1985). It is likely that during drying also the nuclear membrane shrinks, thereby reducing the nuclear volume. These structural changes complicate all processes taking place in the cells during late embryo maturation.

4.2 Mapping and characterization of *RDO2*

The *rdo2* mutant described by Léon-Kloosterziel et al. (1996) displays reduced seed dormancy and is possibly involved in the network of dormancy genetical factors. The aim was to identify the gene and characterize it in the means of dormancy mechanism.

4.2.1 *RDO2* is a TFIIS transcription elongation factor

RDO2 was fine mapped and a 4 bp deletion at the end of the gene At2g38560 was found to be responsible for the reduced dormancy phenotype of *rdo2* seeds. *RDO2* is annotated as a TFIIS transcription elongation factor that in yeast is catalyzing the release of the RNA polymerase II from transcriptional arrest (Izban and Luse, 1992). The protein contains three functional domains, the C-terminal zinc finger is destroyed in the mutant *rdo2* protein.

Transformation of the *rdo2* mutant with the wild type *RDO2* gene led to partial complementation, suggesting that some essential element is missing in the construct. One possibility is, that the stability of the *RDO2* mRNA is reduced because the construct misses the 3'UTR part. It is known, that this area increases the stability of the mRNA (Brodersen and Voinnet, 2006). Nevertheless often cDNA constructs which lack the UTRs are successfully used for transformation experiments. Transcription factors and other trans-acting elements should be present in the mutant background. Therefore the absent regulatory element could be something which has to be located in proximity of the ORF of *RDO2* and is not present in the selected genomic sequence used for transformation. It is known, that for the correct expression of a gene several *cis*-regulatory elements are needed, which can be located thousands of bp from the promoter both upstream and downstream (Watson et al., 2008). An example for an essential regulatory element located far downstream of the ORF, is the promoter of *LATERAL SUPPRESSOR* (*LAS*). Complementation experiments with deletion constructs demonstrated that at least 820 bp upstream of the ATG and 3547 bp downstream of the ORF are necessary to restore the wild type phenotype (Eicker, 2006). An additional attempt of complementation was started recently with a construct including the 3'UTR of *RDO2* and the 2381 bp upstream of the ATG, including the previous gene without its first exon. *rdo2* mutant plants were transformed, the T1 seeds will be harvested soon. If a larger part of the genomic area including *RDO2* is needed for complementation, it would include more ORFs from this gene-rich area. These genes would have to be inactivated, for example by excluding the ATGs from the sequence, to avoid additional effects of their expression. Finally one could think of conformational reasons. The three dimensional structure of the DNA where the *RDO2* gene is located could be important to get regulatory elements in close proximity to the activation site. This is known from the promoters of

higher eucaryots (Watson et al., 2008). Since the transformed sequence is inserted in the plant DNA randomly, the three dimensional structure would not be the same as at the endogenous location.
Four alleles of *rdo2* in Col background with T-DNA insertions at the beginning of the second exon did not express the *RDO2* mRNA and show dormancy phenotypes very similar to the original mutant. One line containing a T-DNA introgression in the putative promoter region of *RDO2* does express *RDO2* mRNA at the same level as L*er* and *rdo2-1* and expresses dormancy like its background Col. This confirms the identity of *RDO2* as At2g38560 which is annotated as a TFIIS transcription elongation factor.
Even though little is known about TFIIS proteins in plants, the topic is deeply investigated in other eucaryots. Studies in *Saccharomyces cerevisiae* showed, that the TFIIS protein contains three independently folding domains, of which the first is not required for the known biochemical and biological functions of the protein (Booth et al., 2000). A short linker connects the second to the third domain, which forms a zinc ribbon (Kettenberger et al., 2003). Initially the TFIIS protein was found to release the RNA polymerase II from transcriptional arrest (Fish and Kane, 2002). Later it was reported to support also the formation of the transcription preinitiation complex (Kim et al., 2007) and to act as a general RNA polymerase III transcription factor (Ghavi-Helm et al., 2008). The three functional domains found in yeast are also annotated for the RDO2 protein. A sequence search showed, that there is no second protein in *Arabidopsis thaliana* with the same combination of functional domains. Even though the TFIIS protein seems to play a role in the ubiquitous process of transcription, the *rdo2* mutant is viable without severe defects. Also in yeast knock-out of the TFIIS gene leads to a non-severe phenotype, so the protein might be functionally redundant with other transcription factors (Wery et al., 2004).

4.2.2 *RDO2* Characterization

Our main interest is to uncover the regulation of dormancy on the molecular level. The *rdo2* mutant displays a dormancy phenotype thus RDO2 seems to be part of such a network. The characterization of *RDO2* might lead us to additional members of the dormancy regulation and reveal information about the nature of the network.

4.2.2.1 *RDO2* expression increases during seed maturation

Genevestigator data shows, that *RDO2* is expressed in all tissues of *Arabidopsis thaliana*. The transcript level of *RDO2* during seed maturation was analyzed by quantitative real time PCR. The

amount of *RDO2* mRNA is increasing during the second half of seed development and is highest in mature seeds.

Transcript abundance is not necessarily related to protein abundance but it is a commonly used approximation. It is possible that the RDO2 protein is needed towards the end of seed maturation in a high concentration, because the condition for transcription is getting more difficult in condensed and dry nuclei. Another possibility is that the mRNA is not translated directly but stored for use during the dormant phase or at the beginning of germination. It was argued, that even though genes remain transcriptionally competent in dry seeds, they might not be expressed (Comai and Harada, 1990). However, increasing transcript levels during after-ripening were reported from *Nicotiana tabacum* (Leubner-Metzger, 2005), *Nicotiana plumbaginifolia* (Bove et al., 2005) and barley (Leymarie et al., 2007). Because of the low water content of quiescent seeds, transcription promoting factors like TFIIS could be of special need.

4.2.2.2 Transformation with overexpressing construct of *RDO2* did not lead to plants with increased transcript level

I could not establish a line that overexpresses *RDO2*. After transformation of *rdo2* plants with *RDO2* under the double 35S promoter all obtained lines show an expression level of *RDO2* similar to the endogenous promoter, in agreement with the phenotype, which is intermediate to L*er* and the *rdo2* mutant.

It is possible that highly dormant lines got lost in the T1 because the seeds were stratified for only four days. However in that case I would still expect some moderately dormant lines to be found with this approach. Another possibility is that overexpression of *RDO2* is lethal. This is not reported from yeast, and when we performed the tranformation of the yeast mutant with the yeast TFIIS gene in an overexpressing vector the growth phenotype did not differ from the lower expressing line (Chapter 3.2.2.4). The cDNA of *RDO2* used for transformation does not include 5' and 3' UTR, which might affect stability of the mRNA (Brodersen and Voinnet, 2006). But even if the in excess transcribed mRNA is partially degraded, more than the amount of mRNA in L*er* should remain. No indication for silencing could be found, since tandem insertions, known to lead to silencing (Assaad et al., 1993), were excluded by PCR and a miRNA (Brodersen and Voinnet, 2006) is not annotated for *RDO2*. Finally, the total amount of *RDO2* mRNA transcripts could be limited by an unknown mechanism. In that case the expression level of the mutant *rdo2* mRNA and the wild type *RDO2* mRNA in the transformed lines would sum up to a total amount not higher than the expression of

RDO2 in L*er*. A qRT-PCR analysis of the mutant *rdo2* transcript level to test this theory failed, but will be repeated.

4.2.2.3 RDO2-YFP fusion protein is localized in the nucleus, but not functional

Transient expression of *RDO2* fused with YFP in leek as well as stable expression of the construct in *Arabidopsis thaliana* revealed a nuclear localization of the fusion protein. This is consistent with the annotated function in the transcription process taking place in the nucleus.

However, the transformed lines express a lower dormancy than the *rdo2* mutant which suggests that the fusion protein has a dominant negative function. Obviously the fused YFP inhibits the RDO2 function completely and possibly even disturbs the activity of interaction partners. Maybe this is caused by sterically hindered folding of RDO2 or blocking of the functional domains. These are common reasons for non-functionality of fluorescent fusion proteins (Snapp, 2005). In yeast it was shown that the TFIIS protein inserts in the active center of the polymerase complex (Kettenberger et al., 2003). It is possible that the size or the conformation of the fusion protein is inappropriate to fulfill this function. The fusion protein is expressed under the p2x35S overexpressing promoter. A semiquantitative expression analysis of some lines shows that lines transformed with the N-terminal fusion construct have transcript levels similar to L*er* and *rdo2* mutant, while lines with the C-terminal fusion construct show increased transcript levels. As discussed in 4.2.2.2 an unknown mechanism could limit the transcript level of *RDO2*, which would explain no increase in *RDO2* expression level under the overexpressing promoter. Maybe the C-terminal YFP tag prevents regulation or degradation of the *RDO2* mRNA.

4.2.2.4 Functional conservation does not lead to complementation of the yeast TFIIS mutant with the *Arabidopsis thaliana RDO2* gene

In silico comparison of the amino acid sequence of *RDO2* and the yeast TFIIS protein reveals conservation, especially in the functional domains. There are several examples for successful complementation of yeast mutants with a homologous gene from a different species (Boocock et al., 2006; Bassett et al., 1996; Dotan et al., 2001), also for *Arabidopsis thaliana* (Klutstein et al., 2008).

However, the complementation of the yeast TFIIS mutant with the *RDO2* cDNA was not successful. It is reported, that the TFIIS genes from different eucaryots, even though highly conserved, do not function in exchange (Shimoaraiso et al., 1997; Sawadogo et al., 1980B). Probably the interaction with species specific interactors is disturbed. In yeast the SAGA component Spt8 and the Mediator component Med13 were identified as interactors of the TFIIS protein in a yeast-two-hybrid screen

(Wery et al., 2004). Sequence search of the Arabidopsis genome did not identify homologous proteins in *Arabidopsis thaliana.* Maybe their function is taken over by proteins with different structure.

4.2.2.5 *RDO2* is additive with three dormancy QTLs

Lines in which the *rdo2* mutant was combined with three different NILs were analyzed for their dormancy phenotype. All "double lines" show increased germination compared to the NILs. Therefore, the three genes underlying the QTLs work in different pathways than *RDO2* explaining their additive effect on seed dormancy.

Results from double mutant approaches are not always easy to interpret, as Koornneef et al. (1998) discussed for a number of late-flowering mutants in *Arabidopsis thaliana*. Like flowering time, dormancy is a quantitative trait and likely to be regulated by a network of genes, potentially interacting in a complex way instead of linear pathways. Therefore more information and detailed investigation would be needed to elucidate the interplay of the loci analyzed in this experiment. However, at present further analysis of the interaction of *DOG3* and *DOG6* with *RDO2* is not possible since the genes responsible for the QTLs DOG3 and DOG6 are not identified. The influence of the mutated *rdo2* on the effect of the strong *DOG1* allele of Cvi is consistent with the finding, that the level of DOG1 transcript is reduced in the *rdo2* mutant (Chapter 3.2.2.6). A lower expression of *DOG1* reduces dormancy also in the *rdo2* NILDOG1 double line.

4.2.2.6 The analysis of *rdo2* transcriptome data for downstream targets

Mature dry seeds consist of dense tissue with condensed nuclei (Chapter 3.1.2) and show a highly decreased metabolism (Holdsworth et al., 2008). Our hypothesis is that the RNA polymerase II faces more difficulties during transcription in maturing seeds than in active tissues due to low water content. These complications might require a high concentration of transcription elongation factor TFIIS. Therefore the loss of proper RDO2 function might lead to a misexpression of specific genes in the *rdo2* mutant.

Transcriptome data from ripe seeds of different dormancy mutants provided by Mike Holdsworth was analyzed to identify downstream effectors of *RDO2*. No candidate associated with dormancy or germination could be identified. As discussed above it is possible, that the ripe seed is not the developmental phase when *RDO2* function is crucial, because ripe seeds contain mainly stored mRNA that has been transcribed during seed maturation. It could also be before the end of maturation, when dormancy is established. To address that phase, seeds harvested about 17 dap

should be used and the transcriptome searched for genes associated with dormancy and germination. Dr. Yongxiu Liu is working on this experiment at the moment.

The genes less expressed in the *rdo2* mutant compared to L*er* are slightly longer than the ones in *aba1-1* and *abi1-1*. This is consistent with our theory that genes with longer cDNAs might need the support of RDO2 more than shorter genes, since they are more often object of transcriptional arrest. Indeed it is discussed whether the length of a gene could be a reason for transcriptional arrest (Grasser, 2005).

4.2.2.7 qRT-PCR reveals decreased *DOG1* transcript level in *rdo2* during seed maturation

Transcript levels of *DOG1* in L*er* and *rdo2* siliques during maturation were analyzed by quantitative real time PCR. The data shows that the amount of *DOG1* mRNA is lower in *rdo2* than in L*er* at all timepoints. Therefore, *DOG1* is a downstream target of *RDO2*. The lower expression of *DOG1* in the *rdo2* mutant does explain the reduced dormancy phenotype of the mutant. Dr. Melanie Schwab (2008) found, that the amount of *DOG1* transcript correlates with the dormancy level.

DOG1 did not show up during the analysis of the transcriptome data as being down regulated by *RDO2*. The reason could be, that the change in expression is not strong enough to be reported, or it was not detected in all three replicates of the microarray. In general, the analysis of the transcript level of a gene by quantitative real time PCR is more sensitive than the results of a microarray experiment.

4.3 Further analyses to elucidate the connection between transcription elongation and seed dormancy

To confirm the impression that embryo nuclei shrink during seed maturation, the volume of nuclei should be measured by a more advanced technique. Initial experiments were performed by Dr. Vittoria Brambilla and the results are more convincing showing a similar tendency as the ones described above. To identify more downstream targets of *RDO2*, further quantitative real time PCR assays should be performed to investigate the transcript level of other dormancy or germination related genes in L*er* and *rdo2* seeds. To address the phase of seed maturation, seeds harvested about 17 dap should be used and the transcriptome searched for genes associated with dormancy and germination.

Although transcription elongation does not seem to be connected to seed dormancy, a second gene which was cloned in our lab recently, *HUB1*, was found to be involved in transcriptional regulation,

too (Liu et al. 2007). Dr. Yongxiu Liu performed a Chromatin Immunoprecipitation (ChIP) assay, testing the success of transcription of some genes. After cross-linking the proteins to the DNA, transcribed regions are identified by antibodies against the RNA polymerase II. After reversing the cross-linking quantitative PCR with primers for 3' and 5' regions of selected genes was performed. The ratio of the amount of transcript tells about the achievement of the transcription process. Preliminary results show that transcription of *DOG1* is reduced in the *hub1* mutant compared to L*er*. The effect on the transcription process should also be tested for *RDO2* in such an assay.

Altogether, it seems that nuclei of embryos shrink during seed maturation, which is the developmental phase when the TFIIS transcription elongation factor *RDO2* is highly expressed and *RDO2* influences the expression of the dormancy regulating gene *DOG1*.
These findings suggest that the factors regulating seed dormancy include proteins that influence transcription during seed maturation.

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Appendix

ACAPELLA script

written by Dr. Kurt Stüber

```
singlewell( )
convolutionmask("Disk",6)
convolution(image=image1, faster=yes)
set(image1=image)
nuclei_Detection_A(image1)
cytoplasm_Detection_A(image1)
//spot_Detection_D(image1)
```

Genomic sequence of *RDO2* and *rdo2*

The following sequence is the genomic sequence of the coding region of *RDO2*. CAPITAL LETTERS show exons, gray lower case letters show the intron, the yellow box marks the four bp that are deleted in the *rdo2* mutant.

ATGGAGAGTGATTTGATTGATTTGTTCGAGGGAGCTAAGAAGGCAGCCGATGCGGCGGCTCTTGAC
GGTGTTACCTCTTCAGGTCCTGAGGTTTCTCAATGTATCGATGCCCTTAAACAGCTCAAGAAGTTT
CCTGTCACATACGATACCCTCGTTGCGACTCAGgttcgtttctgattatcttctcgtaaccctaaa
aaagattgatttattttgcatttaggaaaaggtttatatatgttgcatttaggagggtttttctag
gtgttgattgcttaatctcgttgcattgacttgtgttgttgttaccttagattttttaattagggtt
atttaatttcatctgaaaaagtttatagaagtttaaaaagagtaactttaattcaatagatgaaaga
agcttgagctaactgatttgtataattgactaattgtttgtacgtccccgttgatattgttggaac
aaaccaatttggacattatcctctggaatgttgatagtgtgtgtatttgcttttgagaaagttata
ttctttttcttgcttccagGTGGGAAAGAAGCTGAGGTCTCTTGCAAAACATCCTGTTGAAGATATC
AAAAGCGTAGCTACTGATCTGCTTGAGATATGGAAGAAAGTTGTCATTGAAGAGACAGCCAAAGCT
AAGAAAACCGAAGGCACTAACGGTTGTAAAGAGGCTAAGGTGAATAAGATGGATGTTGAGAAGCCT
TCAAATCCTGCTCCTGTTAAAGTTCAGAAACTTCAGAGGGGTGATTCGGCTAAGAGTATCAAGGTT
GAGAGAAAGGAACCTGACAATAAAGTTGTTACCGGTGTCAAGATAGAGAGAAAGGTACCTGACATC
AAAGTCACCAATGGAACCAAGATAGATTATCGTGGTCAGGCTGTGAAAGATGAAAAGGTCTCAAAG
GACAACCAATCAAGTATGAAAGCTCCAGCTAAAGCAGCTAATGCTCCTCCAAAACTAACTGCAATG
CTCAAATGCAATGATCCTGTGCGTGACAAAATCCGTGAGTTGCTTGTGGAGGCGTTGTGCAGGGTT
GCTGGAGAAGCTGATGACTATGAGAGAGAGTCAGTAAATGCTAGTGATCCTTTACGTGTTGCTGTC
TCAGTGGAATCACTGATGTTTGAGAAATTGGGTCGCTCAACTGGAGCTCAGAAGCTTAAGTACAGA
TCTATAATGTTCAACCTGAGGGATAGTAACAACCCGGACTTAAGAAGGAGGGTTCTCACTGGGGAG
ATTTCACCAGAGAAACTCATAACATTGTCTGCCGAAGATATGGCAAGTGACAAGAGGAAACAAGAG
AACAACCAGATCAAAGAGAAAGCCCTGTTTGATTGTGAGCGTGGTCTTGCTGCAAAAGCATCTACC
GACCAGTTCAAGTGCGGGCGGTGTGGTCAGCGCAAATGCACCTACTATCAGATGCAAACAAGAAGT
GCTGATGAGCCAATGACGACTTATGTTACATGTGTTAACTGTGACAACCACTGGAAGTTCTGTTGA

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Erklärung

Köln, 2008

Ich versichere, dass die von mir vorgelegte Dissertation selbstständig 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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07/2007	2nd International Max Planck PhDnet Workshop: Communication, Frankfurt a.M. (Poster)
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