Analysis of Natural Allelic Variation at Seed Dormancy Loci of *Arabidopsis thaliana*

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ABSTRACT

Arabidopsis accessions differ largely in their seed dormancy behavior. To understand the genetic basis of this intraspecific variation we analyzed two accessions: the laboratory strain Landsberg *erecta* (L*er*) with low dormancy and the strong-dormancy accession Cape Verde Islands (Cvi). We used a quantitative trait loci (QTL) mapping approach to identify loci affecting the after-ripening requirement measured as the number of days of seed dry storage required to reach 50% germination. Thus, seven QTL were identified and named delay of germination (*DOG*) 1–7. To confirm and characterize these loci, we developed 12 near-isogenic lines carrying single and double Cvi introgression fragments in a L*er* genetic background. The analysis of these lines for germination in water confirmed four QTL (*DOG1*, *DOG2*, *DOG3*, and *DOG6*) as showing large additive effects in L*er* background. In addition, it was found that *DOG1* and *DOG3* genetically interact, the strong dormancy determined by *DOG1*-Cvi alleles depending on *DOG3*-L*er* alleles. These genotypes were further characterized for seed dormancy/germination behavior in five other test conditions, including seed coat removal, gibberellins, and an abscisic acid biosynthesis inhibitor. The role of the L*er*/Cvi allelic variation in affecting dormancy is discussed in the context of current knowledge of Arabidopsis germination.

TO survive in a particular location, plants have developed mechanisms that regulate seed germination at the most convenient season of the year. One such mechanism for proper timing of seed germination is seed dormancy, which can be defined as the temporary failure of an intact viable seed to complete germination under favorable conditions (Bewley 1997). Large variations, which are considered adaptations to particular environments, exist for this seed characteristic among and within plant species (Baskin and Baskin 1998). Therefore, seed dormancy is an important adaptive trait that is a primary component of the different life history strategies (winter and spring habits) of annual plants. In addition, seed dormancy is also an important agronomical trait since preharvest sprouting, problems with uniform germination, and some seed processing properties (like malting in barley) are traits largely determined by seed dormancy characteristics (Bewley 1997).

Seed dormancy is a very complex trait due first to the complex genetic structure of the seed. Seeds consist of three parts with different genetic compositions: the embryo and endosperm of zygotic origin and the seed coat or testa derived from maternal tissues. The three structures together determine the germination and dormancy behavior of seeds. Germination begins with the uptake of water by the quiescent seed and ends with the elongation of the embryonic axis, leading to the protrusion of the radicle through the seed coat (Bewley and Black 1994). In the case of dormancy, this is established during seed development and may involve any of the seed structures. Thus, seed dormancy is first classified into two overall categories: so-called embryo and seed-coat-imposed dormancy (Bewley and Black 1994). Second, seed dormancy is influenced by environmental factors, such as light and temperature, during seed development on the mother plants, during seed storage, and during germination. In addition, seed dormancy disappears or is released during dry storage of the seeds, the time needed for that being referred to as the "after-ripening requirement." These characteristics make seed dormancy a trait that is difficult to quantify because even different seeds from the same genotype may lose their dormancy at different times. The measurement of seed dormancy is best achieved by estimating the after-ripening requirement of a large number of seeds and requires germination assays at different times during seed storage to determine the "average" after-ripening requirement. In this way the "degree" or "strength" of the seed dormancy can be precisely estimated.

Despite the fundamental and applied importance of

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seed dormancy, little is known about the molecular mechanisms underlying this trait, due to its genetical complexity and the large environmental effects (Bewley 1997). However, in the past decade the model annual plant *Arabidopsis thaliana* has been shown to be an ideal species in which to perform genetic analyses because of the resources developed by the international community, including the availability of its complete genome sequence (Meinke *et al.* 1998). Furthermore, it has been shown that this species is also suitable for an effective analysis of seed dormancy (for a recent review see Bentsink and Koornneef 2002). A large number of mutations affecting seed dormancy and germination have been generated artificially and the genetic, physiological, and molecular characterizations of these mutations are starting to shed light on the complexity of its regulation. For instance, mutants in genes such as *ABA-INSENSITIVE3* (*ABI3*; Ooms *et al.* 1993; Nambara *et al.* 1995), *FUSCA3* (*FUS3*; Bäumlein *et al.* 1994), and *LEAFY COTYLEDONS* (*LEC1* and *LEC2*; Meinke *et al.* 1994) with defective seed maturation are nondormant, indicating that dormancy is part of the developmental program established during the later phases of seed development. Nongerminating mutants affected in the biosynthesis of the plant hormone gibberellin (GA; Koornneef and Van Der Veen 1980) and the nondormant mutants deficient in abscisic acid (ABA; Koornneef *et al.* 1982) have shown the important and opposite roles of these two phytohormones. Embryonic ABA has been correlated with the induction of dormancy, and it has been determined that the GA requirement for dormancy release and germination is abolished in the absence of ABA, indicating that GAs are needed to counteract the ABA dormancy effects. Moreover, the characterization of reduced seed dormancy mutants affected in the maternally inherited testa pigmentation has revealed that the GA requirement for seed germination is determined not only by the embryonic ABA but also by the testa characteristics (Debeaujon and Koornneef 2000). Light-induced stimulation of seed germination is affected in phytochrome photoreceptor-deficient mutants (Casal and Sánchez 1998) and a phytochrome effect has also been suggested in the onset of dormancy on the mother plant (McCullough and Shropshire 1970; Hayes and Klein 1974). Moreover, several genes encoding transcription regulators such as *D*OF *a*ffecting *g*ermination (*DAG*; Papi *et al.* 2000; Gualberti *et al.* 2002), *FUS3* (Luerssen *et al.* 1998), *LEC1* and *LEC2* (Lotan *et al.* 1998; Stone *et al.* 2001), and several genes with unknown functions such as those disrupted in the *r*educed *do*rmancy 1–4 mutants (*rdo*; Léon-Kloosterziel *et al.* 1996; Peeters *et al.* 2002) have been implicated.

In addition to artificially induced mutations, genetic variation for seed dormancy and germination characteristics has been described for a long time among Arabidopsis wild populations (Kugler 1951; Lawrence 1976; Ratcliffe 1976). Arabidopsis accessions collected at different geographical locations show a quantitative pattern of variation for light requirement (Kugler 1951; Napp-Zinn 1975) and for the after-ripening requirement (Lawrence 1976; Ratcliffe 1976). The genetic analysis of this natural variation has been attempted in some early studies. Kugler (1951) showed that the light dependency for germination of the accession Hannovrisch Münden (Hm) was recessive in crosses with the dark-germinating accessions Stockholm (St) and Haarlem (Haa). Further analysis of F_3 families derived from the cross Hm × St by Napp-Zinn (1975) suggested that three loci determined the light requirement difference between both parents. However, the dissection of the multifactorial genetic variation into the individual loci has become feasible only recently by using quantitative trait loci (QTL) mapping procedures. This approach has been applied in the study of seed dormancy variation in crop species by analyzing crosses between cultivated varieties such as wheat and barley (Anderson *et al.* 1993; Ullrich *et al.* 1993; Romagosa *et al.* 1999; Kato *et al.* 2001), crosses between domesticated species and their wild relatives such as wild rice (Cai and Morishima 2000), or crosses between wild relatives such as wild oat (Fennimore *et al.* 1999). In addition, it has been used in Arabidopsis to analyze a cross between the two most widely used laboratory accessions, Landsberg *erecta* (L*er*) and Columbia (Col), which show a low level of dormancy (van der Schaar *et al.* 1997). In this study, despite the small parental differences, the combination of recombinant inbred lines (RILs) and multiple QTL model (MQM) mapping methods allowed the identification of 14 loci of small effect, accounting for the dormancy and germination differences between both accessions. Once the main QTL have been identified, the individual loci can be further characterized and fine mapped by developing near-isogenic lines (NILs) with monogenic differences. This approach can be efficiently used in model species such as Arabidopsis since the analysis can be easily followed up to the molecular level, enabling the identification of the genes underlying the genetic variation at individual QTL (Alonso-Blanco and Koornneef 2000; Remington *et al.* 2001). Thus, the analysis of this source of genetic variation constitutes an important resource for the functional analyses of seed dormancy. The study of more dormant accessions might contribute to the identification of novel loci and/or alleles, since most mutant analyses have been performed in the low-dormancy accessions. In addition, the identification of the genes accounting for the variation among Arabidopsis populations will contribute to the understanding of the ecological and evolutionary mechanisms involved in the development of different life history strategies of annual plants and in adaptation to different environments. Thus, the analysis of another important life history trait, "flowering time," identified major genetic determinants of the existing natural varia-

tion in Arabidopsis and the respective genes have been cloned (Michaels and Amasino 1999; Sheldon *et al.* 1999; Johanson *et al.* 2000; El-Assal *et al.* 2001).

In the present work we have analyzed two Arabidopsis accessions differing largely in their seed dormancy behavior: the low-dormancy laboratory strain L*er* and the very dormant strain Cape Verde Islands (Cvi). We have identified the loci accounting for the after-ripening requirement using a set of RILs derived from a cross between both accessions. Four major-effect QTL were confirmed and further characterized genetically and physiologically by analyzing NILs carrying specific Cvi introgression fragments in a L*er* genetic background. The possible role of this allelic variation in seed dormancy is discussed in the context of the current knowledge of Arabidopsis germination.

MATERIALS AND METHODS

Plant materials: The Arabidopsis accessions L*er* from Northern Europe (Rédei 1992) and Cvi from the tropical Cape Verde Islands (Lobin 1983), and a set of 161 RILs derived from crosses between them, were analyzed for their seed dormancy behavior. These lines were previously described and characterized using amplified fragment length polymorphism (AFLP) and cleaved amplified polymorphic sequence (CAPS) markers (Alonso-Blanco *et al.* 1998a).

Construction of dormancy NILs: Eleven NILs were constructed by the introgression of seed-dormancy-increasing Cvi alleles into a L*er* genetic background through phenotypic and genotypic selection in three backcross generations. RILs CVL-49, CVL-122, CVL-128, and CVL-160 were used as starting material, selected on the basis of their phenotype and genotype as lines with strong seed dormancy and with different combinations of alleles at the six QTL genomic regions where Cvi alleles that increase dormancy were mapped. Introgression lines were derived from each RIL after two backcross generations and two further selfing generations as follows: RILs were backcrossed to L*er* and small populations of 100–120 $BC1F_2$ plants were obtained and their F_3 seeds tested for germination. The 2 plants with the highest seed dormancy from each population were backcrossed once more to L*er*, and F_3 seeds from 100 to 120 $BC2F_2$ plants were again assayed for dormancy. A total of 44 plants with different degrees of seed dormancy (3–7 from each of the eight populations) were selected, selfed, and genotyped genome-wide with 182 AFLP and CAPS markers chosen from the L*er*/Cvi genetic map (Alonso-Blanco *et al.* 1998a). From those plants, 10 introgression lines were selected as pre-NIL and were used to develop, by marker-assisted selection in a further backcross generation, the 11 final NILs carrying different combinations of high-dormancy Cvi alleles at one to three delay of germination (*DOG*) QTL regions. These lines contain Cvi introgression fragments of 10–50 cM and were named NIL DOG followed by the number(s) of the QTL for which the Cvi allele was expected to be introgressed. When several NILs with overlapping fragments that were expected to carry the same Cvi dormancy alleles were obtained, these lines were named with an additional code. Thus, 8 NILs carrying a single introgression fragment were constructed as well as 3 other lines carrying two introgression fragments. In addition, a NIL carrying Cvi alleles in a single genomic region of ~15–20 cM, where a decreasing dormancy Cvi allele has been mapped in the present work, was previously developed (NIL 45 described in Swarup *et al.* 1999).

Growth conditions: All plants were grown in an air-conditioned greenhouse (temperature 22°–25°) supplemented with additional light to provide a day length of 14 hr. Genotypes to be compared were grown together in single experiments and their mature dry seeds were harvested on the same day at the moment that all siliques had senesced. To largely reduce the environmental effects on seed dormancy due to local greenhouse environmental differences affecting the mother plants, seeds from each genotype were harvested as single or multiple bulks of 3–12 plants (as specified in the text). Seeds were harvested in cellophane bags and stored together in a cardboard box at room temperature. F_1 hybrid seeds from reciprocal crosses between the parental lines were obtained by emasculation of flowers and hand pollination.

RIL evaluation: The complete set of RILs, the parental lines, and reciprocal F_1 hybrids were grown in a single experiment. A total of 12 plants per RIL and 24 plants of the parental lines and their hybrids were grown in two blocks. Blocks were divided in rows of 12 plants, and 6 plants of each RIL were grown per block in half a row, lines being completely randomized. To reduce developmental and environmental effects on seed dormancy, the onset of flowering was synchronized, since the RIL population shows large variation for flowering initiation (Alonso-Blanco *et al.* 1998b). For that, RILs were planted at three consecutive weeks according to their flowering times. The seeds of all genotypes were harvested on the same day in a single seed bulk per RIL and four seed bulks from 6 plants for the parental lines and F_1 hybrids.

NIL evaluation: All the NILs carrying Cvi alleles at the dormancy QTL regions and the parental lines were grown together in a design similar to that described for the RIL evaluation, but consisting of four blocks with six plants. The seeds of each genotype were harvested in four seed bulks of three plants corresponding to the different blocks.

Seed dormancy measurements and germination assays: The percentage of germinating seeds of a genotype at a particular time of seed storage was taken as a measurement of the degree of dormancy at that particular time. In each experiment, germination was tested for the various genotypes in at least six different time points of dry storage from the harvest date until 100% of the seeds germinated in most genotypes. Curves of germination percentage on the time of storage provided the kinetics of seed dormancy of a genotype.

In addition, the seed dormancy of a genotype was estimated in a single parameter as the number of *d*ays of *s*eed *d*ry *s*torage ("after ripening") required to reach 50% germination ($DSDS_{50}$). To estimate the $DSDS_{50}$ value of each genotype, all the measurements of germination proportions at the various times during seed storage were used for probit regression on a logarithm time scale applying the regression module of the statistical package SPSS, version 10.0.6.

Germination tests in water under white light were performed at each time point by incubating seeds during 1 week as follows: Between 50 and 100 seeds of a genotype were evenly sown on a filter paper soaked with 0.7 ml demineralized water in a 6-cm petri dish. Petri dishes were placed in moisture chambers consisting of plastic trays containing a filter paper saturated with tap water and closed with transparent lids. Moisture chambers were stored for 1 week in a climate chamber at 22°–25° illuminated with 38-W Philips TL84 fluorescent tubes at 8 W m^{-2} with a light period of 16 hr followed by 8 hr of darkness. After that, the total number and the number of germinating seeds was scored and the percentage of germinating seeds was calculated.

Germination of the parental lines and NILs was also assayed under five different test conditions known to enhance germi-

nation or to break seed dormancy (see Introduction). Germination was analyzed after a cold treatment by placing moisture chambers in a cold room at 6° for 7 days before being transferred into the illuminated 22°–25° climate chamber. Seed germination was tested in the presence of three chemical compounds by soaking the filter paper in the corresponding solution: 10 μM of gibberellins 4 and 7 (GA_{4+7}; Duchefa, The Netherlands; Koornneef and Van der Veen 1980), 10 μM of norflurazon (NOR; Chem Service, West Chester, PA), which is an inhibitor of abscisic acid biosynthesis (Chamovitz *et al.* 1991), or 10 mM KNO_3 (Derkx and Karssen 1993). Concentrations for NOR, GA_{4+7}, and nitrate were selected from preliminary concentration response analyses as the lowest concentration with maximum effect on the seed germination of L*er* and Cvi parental lines. GA_{4+7} was dissolved in a few drops of 1 M KOH and then diluted to 10 μM with phosphate citrate buffer pH 5 containing 3.3 mM K_2HPO_4 $3H_2O$ and 1.7 mM citric acid. NOR was dissolved in a few drops of acetone and then diluted with water to 1 μM final concentration. Germination was also assayed after removal of the seed coat under a stereomicroscope by scratching the seeds carefully with two needles.

For every genotype and condition, three to four germination tests at each storage time point were performed using a single or different seed bulks. The average germination percentage at each time point of seed storage was calculated, as well as the standard error, to obtain an estimate of the measurement error. Since in the present study we used seed bulks from various plants, variation among plants within a genotype due to greenhouse environmental effects on the mother plants is negligible, and variation among genotype means is interpreted as the genetic variation component of the total phenotypic variation.

QTL analyses: For each RIL, the proportion of germination at 1, 3, 6, 10, 15, and 21 weeks of seed storage was estimated from three replicates of the germination tests performed with a seed bulk of 12 plants. The proportions of germination were used to estimate the $DSDS_{50}$ value of the RILs. $DSDS_{50}$ values were transformed ($\log_{10}$) to improve the normality of the distribution, and transformed data were used to perform QTL analysis. The mean germination percentages of the RILs at each time point of seed storage were calculated and transformed by the angular transformation (equals arcsin $\sqrt{\ }$) and these data sets were used separately for QTL analyses at the six different time points of seed storage. A set of 99 markers covering most of the Arabidopsis genetic map at average intervals of 5 cM was selected from the L*er*/Cvi RIL map (Alonso-Blanco *et al.* 1998a). The computer software MapQTL version 4.0 (van Ooijen 2000) was used to identify and locate QTL on the linkage map by using interval mapping and MQM mapping methods as described in its reference manual (http://www.plant.wageningen-ur.nl/products/mapping/mapqtl/). In a first step, putative QTL were identified using interval mapping. Thereafter, one marker at each putative QTL (between 4 and 7, depending on the trait) was selected as a cofactor and the selected markers were used as genetic background controls in the approximate multiple QTL model of MapQTL. To refine the mapping and to identify linked QTL, cofactor markers at each QTL were moved one by one around the putative QTL position, finally selecting the closest markers to the QTL, *i.e.*, those maximizing the logarithm-of-odds (LOD) score. LOD threshold values applied to declare the presence of a QTL were estimated by performing the permutation tests implemented in MapQTL version 4. The quantitative trait data of the RILs were permuted 1000 times over the genotypes, and empirical LOD thresholds corresponding to the genome-wide significance $\alpha = 0.05$ were estimated to be between 2.5 and 2.7 for the various data sets. Two-LOD support intervals were established as an ≈95% QTL confidence interval (van Ooijen 1992). The estimated additive genetic effect and the percentage of variance explained by each QTL, and the total variance explained by all the QTL affecting a trait, were obtained with MapQTL in the final multiple-QTL model in which one cofactor marker was fixed per QTL. Additive genetic effects presented correspond to the differences between the estimated means of the two homozygous RIL genotypic groups at each particular QTL. A positive additive effect implies that Cvi genotypes have higher germination (lower dormancy) than L*er*, while negative effects indicate that Cvi genotypes have lower germination (higher dormancy). All the statistical comparisons were based on the transformed data, but none of the conclusions was changed when using the original data. Therefore, additive effects presented are estimated with the original scale data as merely orientative.

Since 116 of the RILs carry L*er* cytoplasm and 45 carry Cvi cytoplasm, cytoplasmic genetic effects were analyzed in the RIL population using the cytoplasmic genotype as a factor in one-way ANOVA and in multiple-factor linear models in combination with the nuclear QTL markers affecting each trait.

Two-way interactions among the QTL identified were tested by ANOVA using the corresponding two markers as random factors. In addition, two-way interactions were searched for among all pairwise combinations of the 99 nuclear markers as well as the cytoplasmic genotype, using the computer program EPISTAT (Chase *et al.* 1997) with log-likelihood ratio (LLR) thresholds corresponding to a significance of $P < 0.001$. Ten thousand trials were used in Monte Carlo simulations performed with EPISTAT to establish the statistical significance of the LLR values for the interactions detected (Chase *et al.* 1997).

The overall genotype by storage time (G × ST) interaction was tested for the percentage of germination by two-factor ANOVA using genotypes (RILs) and time points of seed storage as classifying factors. For each putative QTL, QTL × ST interaction was tested by repeated-measures ANOVA using the corresponding marker and the time of seed storage (repeated measurements of the RILs) as between and within classifying factors ($P < 0.005$). The general linear model module of the statistical package SPSS version 10.0.6 was used for ANOVA analyses.

Molecular markers: The introgression lines containing Cvi dormancy alleles were genotyped using AFLP marker analysis, which was performed according to Vos *et al.* (1995). Nine primer combinations chosen from the L*er*/Cvi molecular map (Alonso-Blanco *et al.* 1998a) were used to amplify 182 polymorphic bands with known genetic location and that covered most of the genetic map at intervals of 1–15 cM.

CAPS and microsatellite markers previously mapped in the L*er*/Cvi RILs and/or the L*er*/Col RILs (Alonso-Blanco *et al.* 1998a; The Arabidopsis Information Resource, http://www.arabidopis.org) were used for marker-assisted selection of the final NILs carrying Cvi dormancy alleles. CAPS markers were analyzed according to Konieczny and Ausubel (1993) and microsatellite markers according to Bell and Ecker (1994). The following markers linked to the *DOG* QTL regions were used: DFR, MBK, nga129, and g2368 for *DOG1* and *DOG7* QTL region; PVV4, AXR1, and PhyA for *DOG2*; g2395 for locus *DOG3*; nga151 for *DOG4*; B9-1.8 for *DOG5*; and TOPP5 for *DOG6*. Other CAPS and microsatellite markers were used to genotype in genomic regions where undesired Cvi alleles had to be removed.

RESULTS

Seed dormancy behavior of L*er*, Cvi, and their recombinant inbred lines: The seed dormancy behavior of L*er*, Cvi, and reciprocal F_1 and F_2 hybrid seeds was analyzed

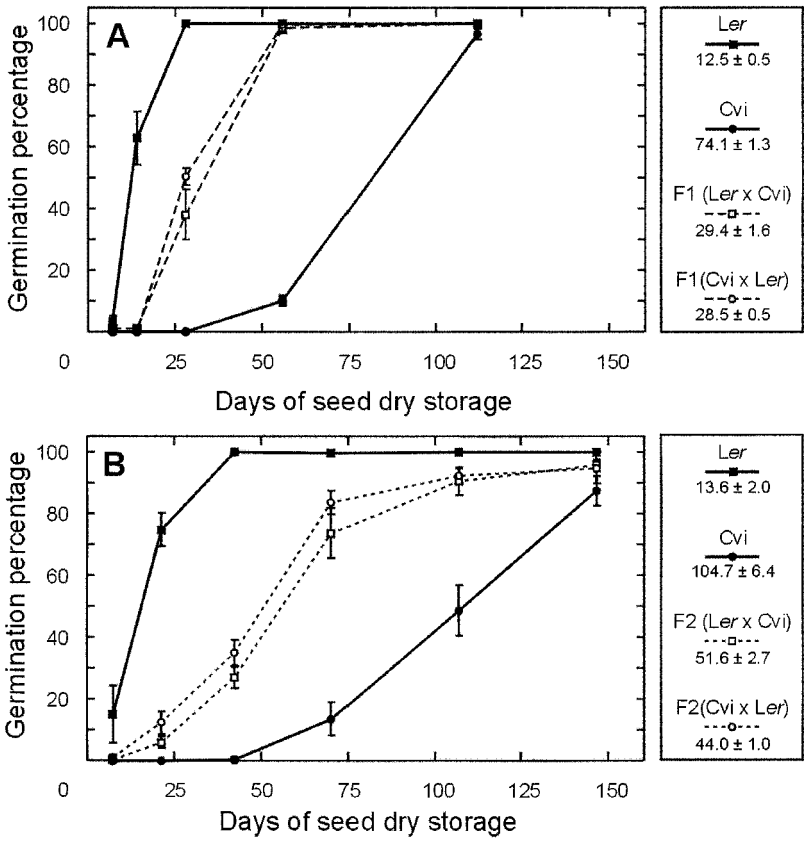


FIGURE 1.—Dormancy/germination behavior of L*er*, Cvi, and their reciprocal F_1 and F_2 hybrid seeds during dry storage. Percentages of germination in water under white light were estimated at different times of seed dry storage and germination curves for each genotype are presented. Plants grown in two different experiments (A and B) are shown to indicate the environmental effects on germination and to illustrate that genotypes from different experiments cannot be directly compared. The after-ripening requirement was estimated for each genotype in each experiment as the $DSDS_{50}$ value (±SE) given in the legends. In A, the mean germination percentage (±SE) of four different seed bulks of two plants for the parental lines and of four seed bulks of three different crosses for the F_1 hybrid seeds is shown. In B, each data point corresponds to the mean germination percentage (±SE) of four seed bulks of six plants.

by characterizing their germination phenotypes during seed dry storage time. Germination was tested at six different times of storage from the harvest date until all the seeds geminated, and curves of germination percentage during storage were obtained (Figure 1). From these, the number of *d*ays of *s*eed *d*ry *s*torage (after ripening) required to reach 50% germination ($DSDS_{50}$) was estimated for each genotype as a single measurement of seed dormancy. Although $DSDS_{50}$ values vary among experiments where plants were grown at different times of the year (in the same greenhouse), due mainly to environmental effects on the mother plants (Figure 1, A and B), L*er* seeds in general germinated 100% after 6 weeks of storage, while Cvi seeds required at least 15 weeks to do so. When comparing plants grown together, the $DSDS_{50}$ values of Cvi were at least five times higher than L*er* values, L*er* varying from 12 to 17 days in different experiments and Cvi $DSDS_{50}$ values ranging from 74 to 185 days. Therefore, L*er* and Cvi differ largely in their seed dormancy, Cvi seeds being much more dormant than L*er* seeds. Maternal genetic effects on the dormancy variation were not detected as deduced from phenotypic comparisons of reciprocal F_1 and F_2 seeds (Figure 1). F_1 hybrid seeds obtained using L*er* as mother plants (or F_2 derived seeds) did not differ significantly from F_1 hybrid seeds obtained using Cvi as mother plants (or, respectively, from F_2 seeds) either in the $DSDS_{50}$ or in the germination percentage at any tested time. In addition, the reciprocal F_1 and F_2 seeds showed $DSDS_{50}$ values intermediate between the parental values, indicating an overall additive effect of L*er* and Cvi alleles.

The genetic variation for seed dormancy of L*er* and Cvi was further analyzed by studying germination during seed storage of 161 RILs derived from crosses between both parental lines (ALONSO-BLANCO *et al.* 1998a). As shown in Figure 2, some transgression in both directions was detected for the $DSDS_{50}$ value in the RIL population, indicating that both parental lines carry alleles increasing and decreasing seed dormancy. Analysis of the frequency distributions of germination percentages after 1, 3, 6, 10, 15, and 21 weeks of seed storage (Figure 3) shows the kinetics of dormancy of the RILs. The mean germination of the RIL population gradually increased from 1.9% in 1-week-old seeds up to 94.7% in 21-week-old seeds. However, considerable phenotypic variation is present in the RIL population at each time point of seed storage, and transgression in both parental directions is observed at different times of storage; transgression toward reduced dormancy could be detected during the first three weeks of storage, whereas transgression over the Cvi parent appeared detectable after 15 weeks of seed storage (Figure 3). Genotype × storage time interactions were significant ($P < 0.001$) for any comparison of RIL germination percentages among times of seed storage, showing that RILs respond differently to seed storage. Therefore, the phenotypic effects of the dormancy allelic variation are expressed differently during seed storage.

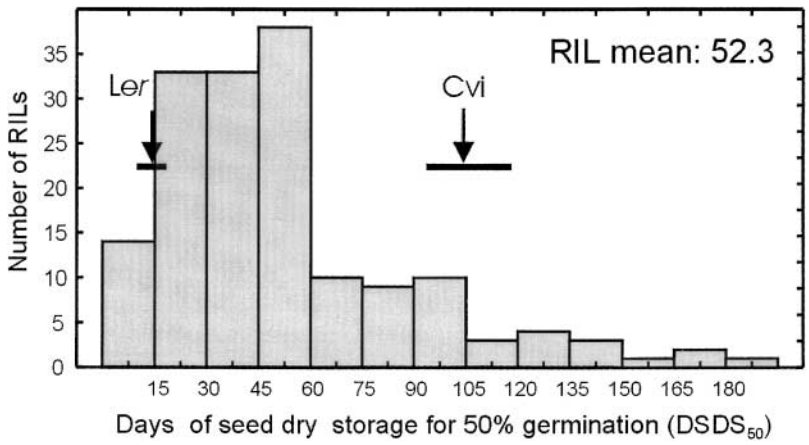


FIGURE 2.—Frequency distribution of after-ripening requirements ($DSDS_{50}$ values) of the L*er*/Cvi RILs. Arrows depict the mean $DSDS_{50}$ of the parental lines estimated in four different seed bulks of 6 plants, and horizontal bars represent their range of variation (same parental lines as shown in Figure 1B). The $DSDS_{50}$ value of the RILs was estimated as a single value from a single seed bulk of 12 plants. RIL mean: average $DSDS_{50}$ of the RIL population.

Mapping seed dormancy loci in the L*er*/Cvi RIL population: To identify the loci that control the L*er*/Cvi seed dormancy variation, QTL analysis was performed using the RIL phenotypic values of the time of seed storage required for 50% germination ($DSDS_{50}$). Conservatively, seven QTL located on all chromosomes except chromosome 2 were identified, their total additive effects accounting for 61.4% of the after-ripening requirement phenotypic variation (Figure 4). These loci have been named *DOG* 1–7, the locus number denoting their relative effect from larger to smaller. Cvi alleles at six loci increased the time of seed storage required for seed germination (increased dormancy) and only Cvi alleles at *DOG2*, located on the top of chromosome 1, reduced dormancy as compared with the L*er* allele. Three of the loci, *DOG1–DOG3*, showed large additive effects (each explaining >10% of the phenotypic variation) and together accounted for ~60% of the additive genetic variance (38.2% of the total variance). The region of chromosome 5 containing *DOG1* and *DOG7* appeared especially complex; the single QTL additive effects of these loci, which, using the MQM module of MapQTL, could be located 20 cM apart, might be underestimated.

To further characterize the *DOG* loci genetically, we used the RIL germination percentages at the six different times of seed storage (1, 3, 6, 10, 15, and 21 weeks) for QTL analyses. Thus, the QTL responsible for the germination variation at each time of seed storage could be identified and their additive genetic effects followed during storage (Table 1 and Figure 5). A conservative total number of seven QTL, corresponding to the same seven genomic regions previously identified using the $DSDS_{50}$ values, were identified from the six germination assays. No other significant QTL could be detected consistently in more than one germination assay. Therefore, the detected loci affecting germination are the same *DOG* loci affecting the after-ripening requirement variation. As shown in Figure 5 and Table 1, the germination percentages at each time of seed storage detected between two and seven significant QTL, their combined additive effects accounting for between 27.8 and 64.4% of the total variance. Consistently with the effects of the *DOG* loci on the after-ripening requirement, Cvi alleles at six of the seven QTL decreased the percentage of germination (increased dormancy) while only Cvi alleles at *DOG2* increased the percentage of germination, as compared with L*er* alleles. Similar to the $DSDS_{50}$ analysis, the region on chromosome 5 between map positions 56 (BH.96L-Col) and 95 (GB.102L-Col) appeared as a complex region containing at least two genetically linked loci, *DOG1* and *DOG7*, with phenotypic effects in the same direction.

Since the RILs were obtained from reciprocal crosses (ALONSO-BLANCO *et al.* 1998a), maternal cytoplasmic effects on the seed dormancy parameters could be analyzed but no significant effect was detected either as additive or as interacting with any of the nuclear markers.

Analysis of QTL × ST interactions showed that five of the seven detected loci have significantly different additive effects at the various times of seed storage (Table 1). However, the relative effects of these QTL showed different trends during storage (Figure 5). Thus, the seven QTL could be classified in three different classes according to the behavior of their additive genetic effects during seed storage:

i. *DOG1*, *DOG3*, and *DOG6* show a larger effect in the germination assays carried out during the first 6 weeks of storage, their maximum appearing between weeks 3 and 6; thereafter their additive effects decreased.
ii. *DOG2* and *DOG7* show a behavior complementary to the previous class since they have a larger effect in the assays performed after week 6. These loci show small effects in the germination assays performed during the first 3 weeks and their relative additive effect increased gradually until reaching its maximum between weeks 10 and 21 (Figure 5).
iii. *DOG4* and *DOG5* showed no interaction with the environments, appearing as small-effect loci in all assays. Therefore, the seven QTL behave differently

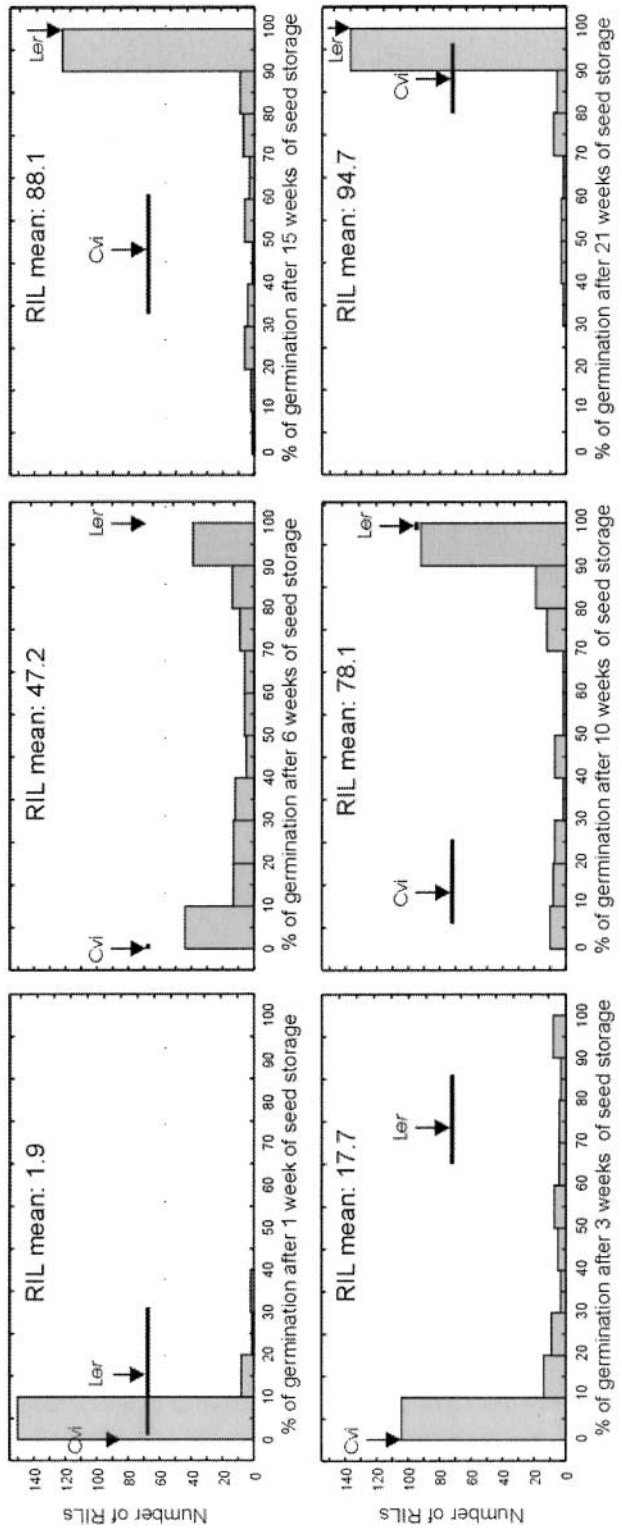


FIGURE 3.—Frequency distributions of mean germination percentage of the L*er*/Cvi RILs at 1, 3, 6, 10, 15, and 21 weeks of seed dry storage. Arrows depict the means of the parental lines estimated from four different seed bulks of 6 plants, and horizontal bars represent their range of variation (same parental lines as shown in Figures 1B and 2). Mean germination percentages of RILs are estimated from a single seed bulk of 12 plants. RIL mean: average germination percentage of the RIL population.

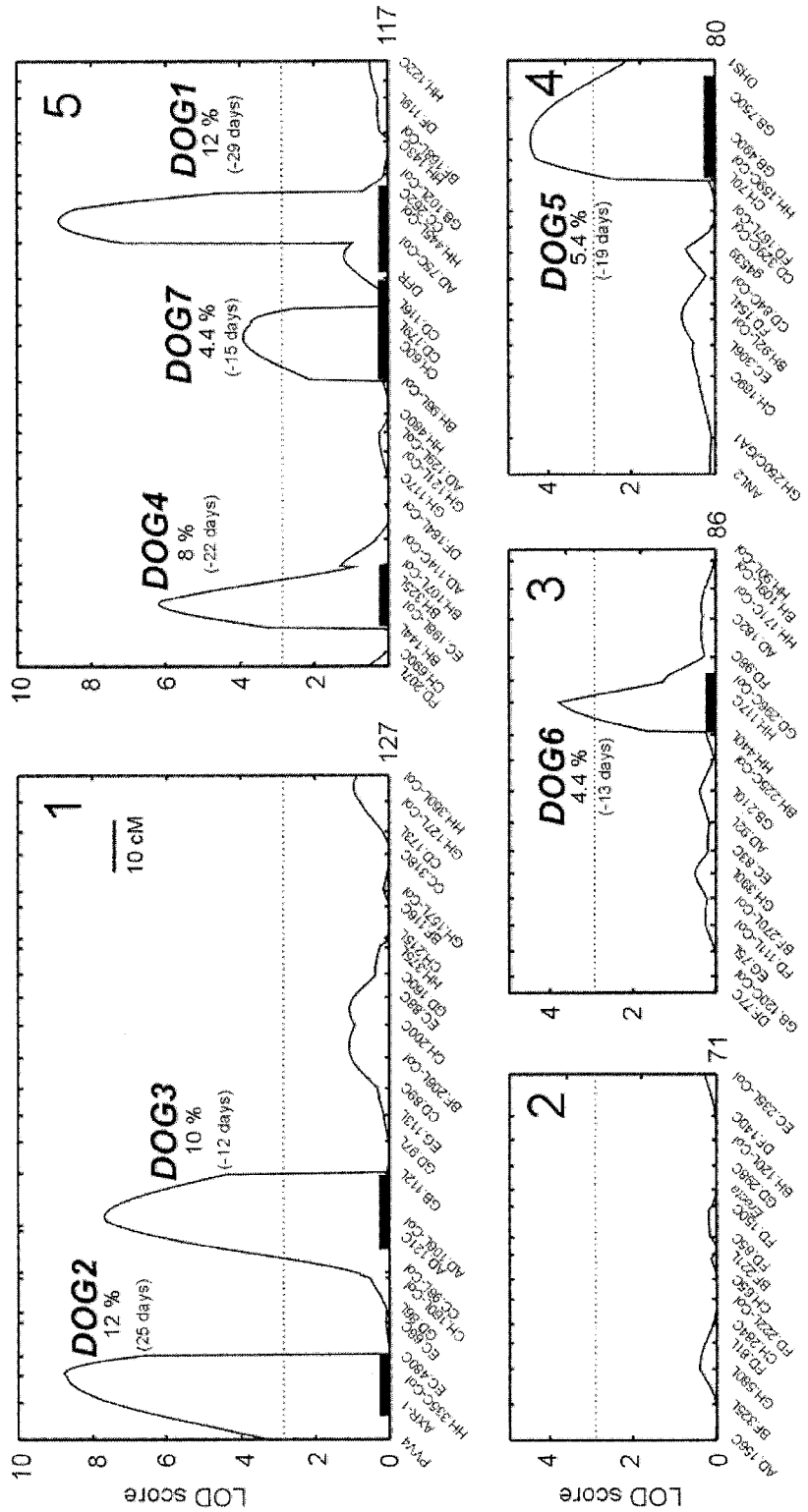


FIGURE 4.—QTL likelihood maps for the after-ripening requirement ($DSDS_{50}$ values) of the L*er*/Cvi RILs. The abscissas correspond to the genetic maps in centimorgans, the linkage group number being indicated in the top right corner of each map. The horizontal dashed line indicates the LOD score threshold of 2.7. Two-LOD support intervals are shown as solid bars along abscissas. For each QTL, its name, additive genetic effect, and the percentage of the total variance explained by its additive effect are given. A negative additive effect indicates that Cvi genotypes have a higher $DSDS_{50}$ mean (a higher dormancy) than that of L*er*, while positive effects imply that Cvi genotypes have the lower $DSDS_{50}$ mean.

TABLE 1

QTL for seed germination/dormancy detected at different times of seed dry storage

QTL			Time of seed storage												
			1 wk		3 wk		6 wk		10 wk		15 wk		21 wk		
Closest marker	QTL name	Map position	% of explained variance	Additive effect	% of explained variance	Additive effect	% of explained variance	Additive effect	% of explained variance	Additive effect	% of explained variance	Additive effect	% of explained variance	Additive effect	QTL × ST interaction
			27.8		52.4		64.4		58		46.2		37.6		
HH.335C-Col	*DOG2*	1–12.1	NS	NS	6.8	14.7	13.0	28.9	11.6	21.5	13.7	15.3	10.2	6.4	*
AD.106L-Col	*DOG3*	1–41.7	14.4	−3.3	21.8	−26.8	10.3	−28.1	NS	NS	NS	NS	NS	NS	*
HH.117C	*DOG6*	3–56.3	5.3[a]	−2.1	7.1	−15.0	4.7	−17.3	2.6[a]	−10.1	NS	NS	NS	NS	*
HH.159C-Col	*DOG5*	4–61.0	NS	NS	3.8[a]	−11.7	3.2	−12.5	5.4	−13.9	6.9	−10.6	10.8	−7.5	NS
EC.198L-Col	*DOG4*	5–12.8	4.7[a]	−2.4	6.7	−16.7	5.4	−18.9	6.6	−16.9	8.9	−14.5	9.1	−8.8	NS
BH.96L-Col to CD.179L	*DOG7*	5–55.5 66.3	NS	NS	NS	NS	4.1	−17.4	2.6[a]	−11.1	5.2	−10.3	NS	NS	*
DFR		5–75.5	—	—	—	—	—	—	—	—	—	—	8.0	−7.1	
AD.75C-Col to GB.102L-Col	*DOG1*	5–82.1 94.9	10.1	−3	19.1	−22.6	16.8	−38.8	18.7	−32.3	7.3	−12.9	NS	NS	*

The closest marker to each QTL at each storage time is shown and its location is indicated by the linkage group followed with the map position. Additive effects are given as the difference between the means of the two RIL genotypic groups (a negative value implies that Cvi alleles reduce germination, *i.e.*, increase dormancy, as compared to L*er* alleles; a positive value indicates that Cvi alleles increase germination). For the DOG1/DOG7 region, an interval spanning the position of the cofactors used in the different analyses is given. QTL interacting with seed storage time are indicated by * ($P < 0.005$). QTL × ST interactions were tested using only the four data sets collected between 3 and 15 wk of seed storage, at which considerable variation was detected. NS, not significant.

[a] QTL detected with LOD scores between 2 and 2.7.

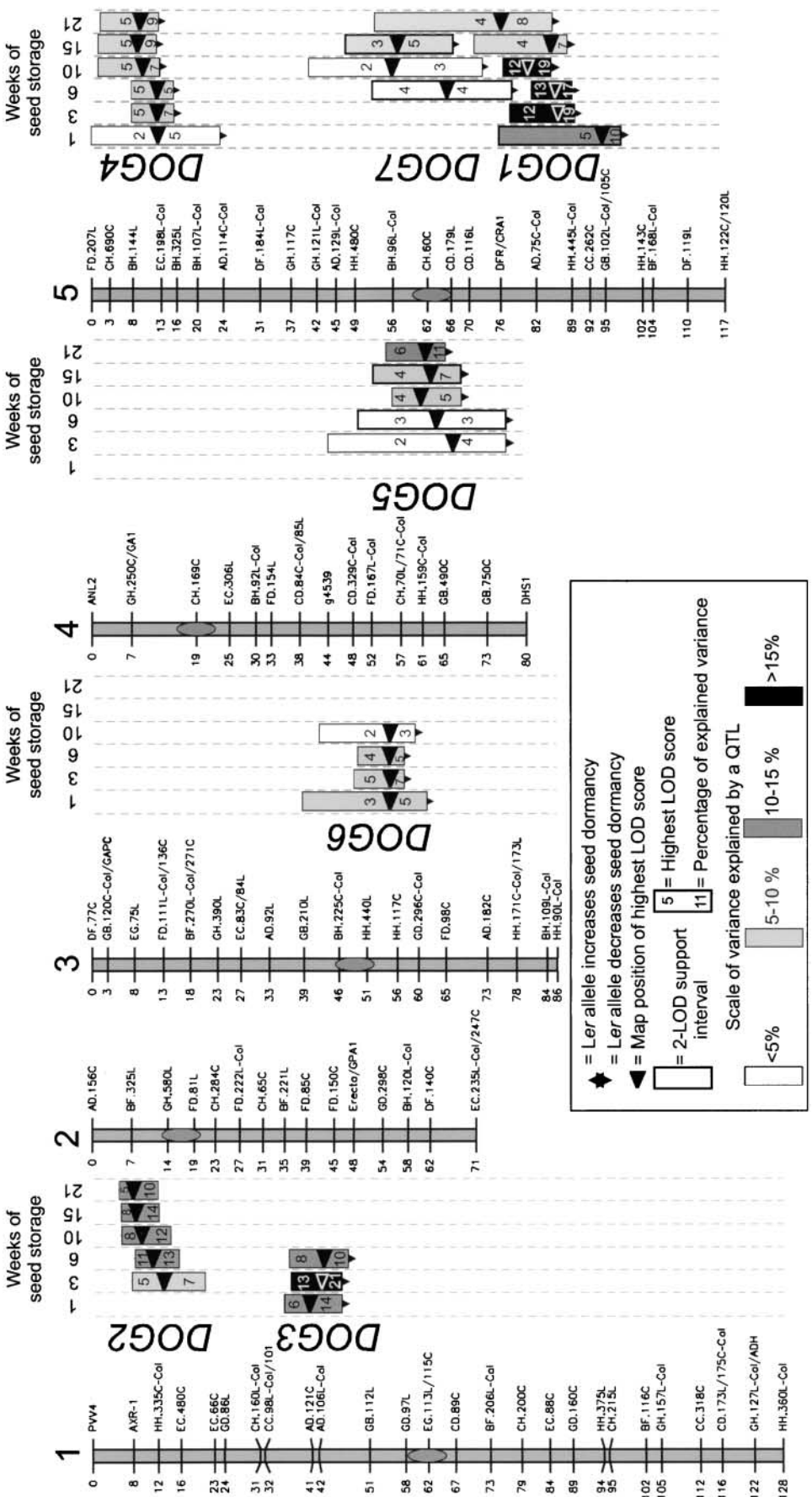


FIGURE 5.—QTL mapping for germination of the L*er*/Cvi RILs at different times of seed storage. The significant QTL detected in the six germination assays performed at different times of seed storage are depicted close to the L*er*/Cvi genetic maps of the five linkage groups.

TABLE 2

Two-way QTL interactions

Two-way interaction	Time of seed storage															
	1 wk			3 wk			6 wk			10 wk			15 wk			21 wk		
	P	Class	R^2	*P*	Class	R^2	*P*	Class	R^2	*P*	Class	R^2	*P*	Class	R^2	*P*	Class	R^2
DOG1 × *DOG2*	NS	—	—	NS	—	—	NS	—	—	0.001	B	6.9	0.001	B	6.7	0.009	B	4
DOG1 × *DOG3*	0.012	A	4.1	0.002	A	6.3	0.003	A	5.5	0.001	C	6.9	0.001	C	7.3	0.002	C	5
DOG1 × *DOG4*	NS	—	—	NS	—	—	NS	—	—	NS	—	—	0.006	B	4.8	0.003	B	5
DOG1 × *DOG6*	0.01	A	4.3	0.001	A	7.0	NS	—	—	NS	—	—	NS	—	—	NS	—	—
DOG1 × *DOG7*	NS	—	—	NS	—	—	NS	—	—	0.008	B	4.4	0.004	B	5.2	NS	—	—
DOG2 × *DOG7*	NS	—	—	NS	—	—	NS	—	—	0.002	B	6.3	0.009	B	4.4	NS	—	—
DOG3 × *DOG4*	NS	—	—	NS	—	—	NS	—	—	NS	—	—	NS			0.007	C	4
DOG3 × *DOG7*	0.002	A	6.2	0.001	A	6.7	0.003	A	5.8	0.005	C	5	0.009	C	4.4	NS	—	—
Percentage of total explained variance			35			57			67			64			51			3

Significant genetic interactions were classified as: A, the two alleles reducing dormancy interact synergistically; B, the two alleles increasing dormancy interact synergistically; C, L*er* alleles at *DOG3* increase dormancy (opposite to its nondormant additive effect detected in the QTL mapping) in the presence of Cvi alleles at *DOG1*, *DOG4*, or *DOG6*. NS, not significant ($P > 0.01$).

genetically, suggesting that they might participate in different aspects of seed dormancy.

Two-way interactions were analyzed among the seven dormancy QTL identified. When testing the interactions using the transformed germination percentages at each of the six different times of seed storage, several interactions involving all QTL except *DOG5* appeared as significant ($P < 0.005$; Table 2). Two-way interactions were also scanned throughout the genome by analyzing all pairwise combinations of markers, but no significant genetic interaction was consistently found in several germination assays. The interactions detected at <6 weeks of storage time (when seeds are mostly dormant) corresponded to synergies between nondormant alleles; in contrast, most interactions detected in the germination assays after long storage times (when most seeds germinate) showed synergies between dormant alleles. These interactions might be interpreted as a consequence of the limited measurement scale of percentages. In addition, epistatic effects of most loci were detected only in the same germination assays at which additive effects were previously found. However, *DOG3* also showed particular interallelic interactions in the assays performed after 10 weeks of seed storage (Table 2; Figure 6). The interactions between *DOG3* and *DOG1*, *DOG4*, and *DOG7* detected allele effects at *DOG3* in short seed storage times that differed from those in long ones. This is illustrated in Figure 6 for the *DOG1* × *DOG3* interactions detected with the germination percentages in the six storage times. On average, *DOG3*-L*er* alleles reduced dormancy in the first two assays, similar to the *DOG3* additive effects estimated in the QTL mapping analyses. In contrast, in the later assays, *DOG3*-L*er* alleles increase dormancy in the presence of dormant alleles at the interacting QTL. Depending on the time of storage and the genotype at several interacting loci, this conditional *DOG3* allele effect appears not simply due to the genetic linkage of *DOG2*; L*er* alleles of *DOG2* increase dormancy and have a maximum additive effect after long periods of seed storage (Figure 5) because similar significant *DOG1* × *DOG3* interactions appear when considering only RILs with the same allele at *DOG2*. Furthermore, this *DOG1* × *DOG3* interaction was the only significant interaction detected among the seven QTL when using the $DSDS_{50}$ values ($P = 0.0002$), *DOG3*-L*er* alleles also showing in this case opposite effects depending on the genotype at *DOG1*. These interactions suggest that *DOG3* alleles affect the *DOG1* effects, although we cannot discard more complex explanations.

Genetical and physiological characterization of the *DOG* loci: To characterize the various loci, 12 introgression lines carrying one or two Cvi genomic fragments around the *DOG* QTL regions into an otherwise L*er* genetic background were developed by phenotypic and genotypic selection (see MATERIALS AND METHODS). These near isogenic lines were thoroughly genotyped

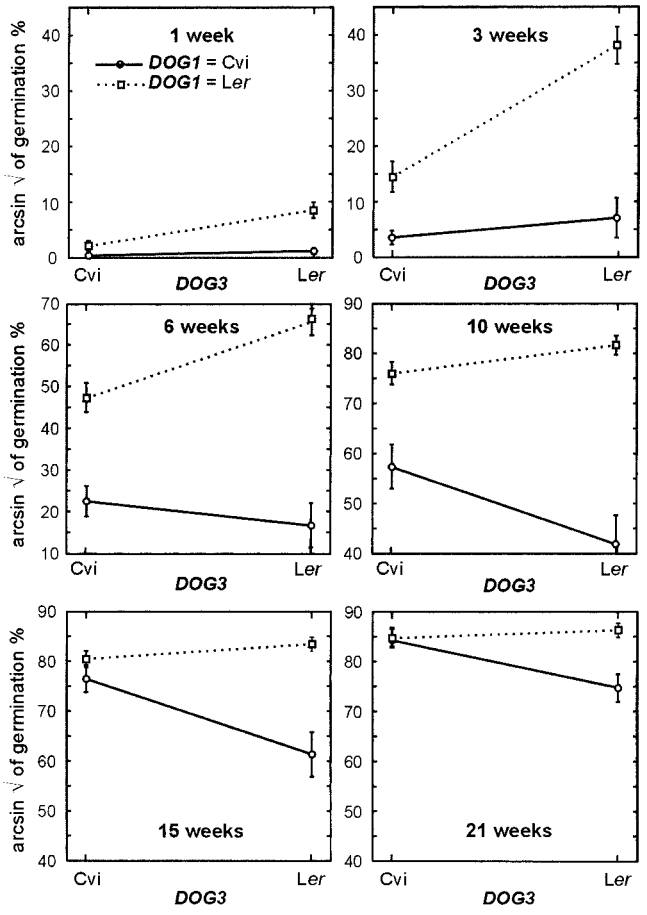


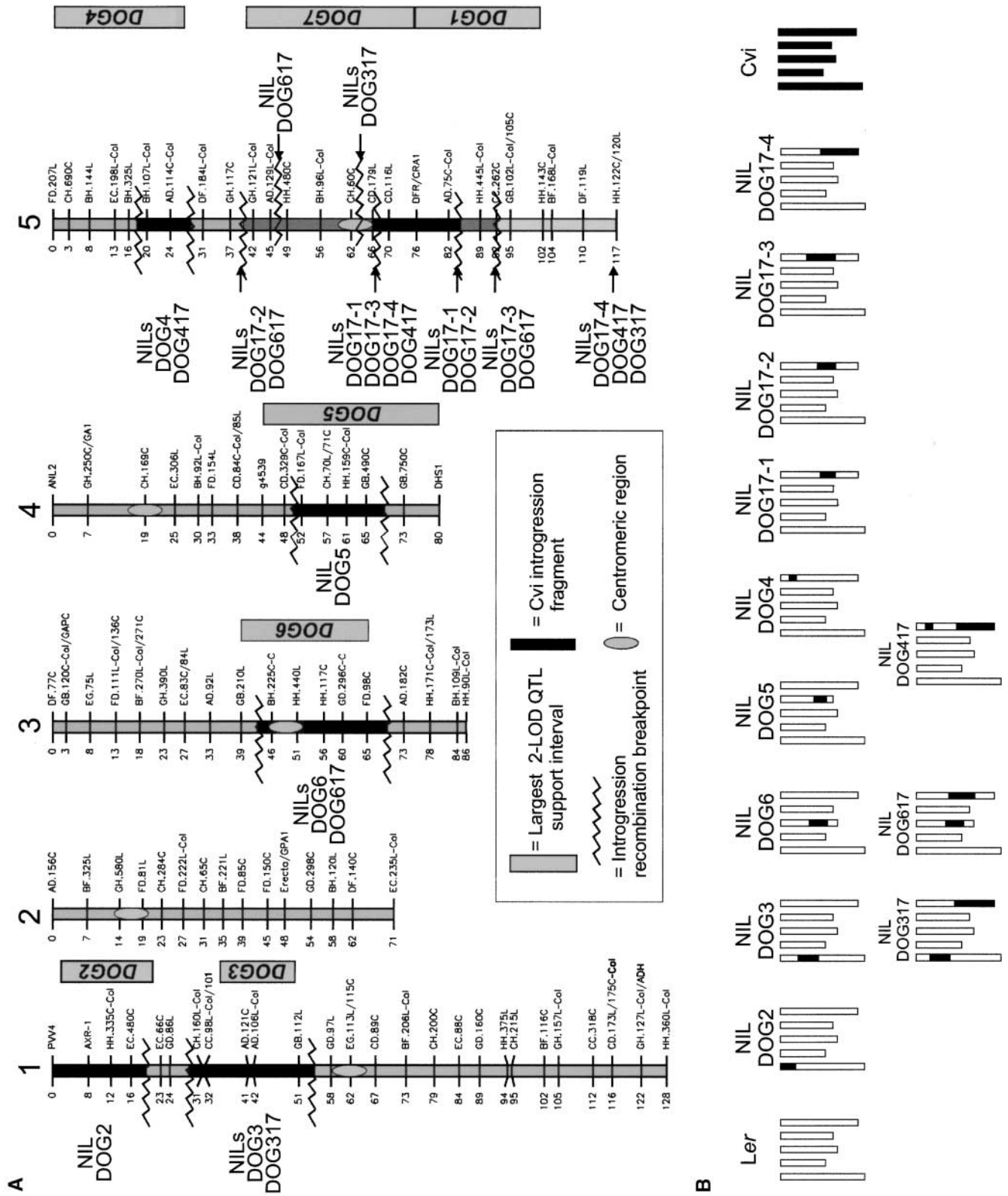
FIGURE 6.—Two-way interaction between *DOG1* and *DOG3*. Each graph corresponds to the representation of the mean (±SE) germination of the four genotypic RIL classes after 1, 3, 6, 10, 15, and 21 weeks of seed storage.

and the genetic position and size of the introgressions were determined (Figure 7). Nine of the lines carried single Cvi introgressions in the six genomic regions containing the identified *DOG* loci (NILs named according to a single QTL mapped around the introgression region as DOG2, DOG3, DOG4, DOG5, and DOG6; or, according to the combination of *DOG1* and *DOG7* QTL present in some NILS named as DOG17-1, DOG17-2, DOG17-3, and DOG17-4). In addition, three other NILs carried a Cvi introgression fragment of the complex region *DOG1/DOG7* and a second introgression in the region of *DOG3*, *DOG4*, or *DOG6* (NILs named after all QTL involved as DOG317, DOG417, and DOG617).

The germination behavior of these lines was analyzed in water under light (Figure 8) aiming to (i) confirm the existence of the QTL according to their effects in a L*er* genetic background and (ii) in some cases determine the genetic interactions between the largest-effect QTL *DOG1* and the remaining loci.

The dormancy behavior of the single introgression lines measured by curves of germination percentage over time of seed dry storage and $DSDS_{50}$ values enabled confirmation of several loci (Figure 8). Four very dormant NILs, DOG17-1–DOG17-4, carrying introgressions of slightly different sizes around the *DOG1* and *DOG7* QTL (Figures 7 and 8) were analyzed and compared. NIL DOG17-1 carried the smallest Cvi introgression of ~20 cM and was only slightly less dormant than Cvi (Figure 8B). Therefore in this small region between positions 65 and 85 cM of chromosome 5 we could assign the strongest Cvi dormant alleles and confirm the locus *DOG1*. However, it is not known if the strong dormancy of this line is determined by a single locus, *DOG1*, or by the two linked QTL *DOG1* and *DOG7* previously mapped in that region. Since the complexity shown in the RIL mapping experiments suggests that this region contains more than one closely linked locus, at this stage we do not claim that the dormancy difference between this line and L*er* is monogenic, since both QTL might participate. For this reason, we named this line DOG17-1; further mapping is needed to establish whether Cvi alleles at more than one locus are introgressed. This line shared its lower recombination breakpoint with NIL DOG17-2, whose 50-cM introgression included an additional 30-cM region not present in NIL DOG17-1, which spans the 2-LOD support interval of *DOG7* (Figure 7). These two NILs did not differ significantly in their germination behavior (Figure 8B), leading to the conclusion that no dormancy QTL is detectable in the dormant background shared with NIL DOG17-1 introgression, located in the region between the upper breakpoints of both lines (between positions 40 and 65 cM of chromosome 5). Another line, DOG17-3, shared the upper recombination breakpoint with NIL DOG17-1 but carried an additional distal region of ~10 cM. These lines did not differ significantly in their dormancy behavior ($P < 0.05$) as well. A fourth line, NIL

FIGURE 7.—Genotype of the dormancy QTL NILs. (A) Graphical representation of the Cvi introgression fragments of the 12 NILs in relation to the map position of the dormancy QTL. Support intervals for each QTL are represented as shaded blocks at the right side of each chromosome and correspond to the largest 2-LOD support interval estimated in the QTL mapping experiments. Cvi introgression fragments are depicted as solid or shaded (when overlapping fragments were available) blocks on the chromosomes. The name(s) of the NIL(s) carrying an introgression fragment is shown at the left of the corresponding fragment. For the DOG1/DOG7 region on chromosome 5, overlapping introgression fragments were developed and, in this case, the name of the NILs refers to recombination breakpoints of the introgression fragments instead of referring to the introgression itself, which is shown by an arrow pointing to the corresponding breakpoint. (B) Graphical genotype of the parental lines L*er* and Cvi, the 9 NILs carrying a single Cvi introgression fragment (top row) and the 3 NILs with two introgression fragments (bottom row).


A
B
DOG1
DOG2
DOG3
DOG4
DOG5
DOG6
DOG7
NILs DOG4 DOG417
NILs DOG17-2 DOG617
NILs DOG17-1 DOG17-3 DOG17-4 DOG417
NILs DOG17-1 DOG17-2
NILs DOG17-3 DOG617
NILs DOG17-4 DOG417 DOG317
NIL DOG617
NILs DOG317
NIL DOG5
NILs DOG6 DOG617
NILs DOG3 DOG317
NIL DOG2
= Largest 2-LOD QTL support interval
= Cvi introgression fragment
= Introgression recombination breakpoint
= Centromeric region
Ler
Cvi

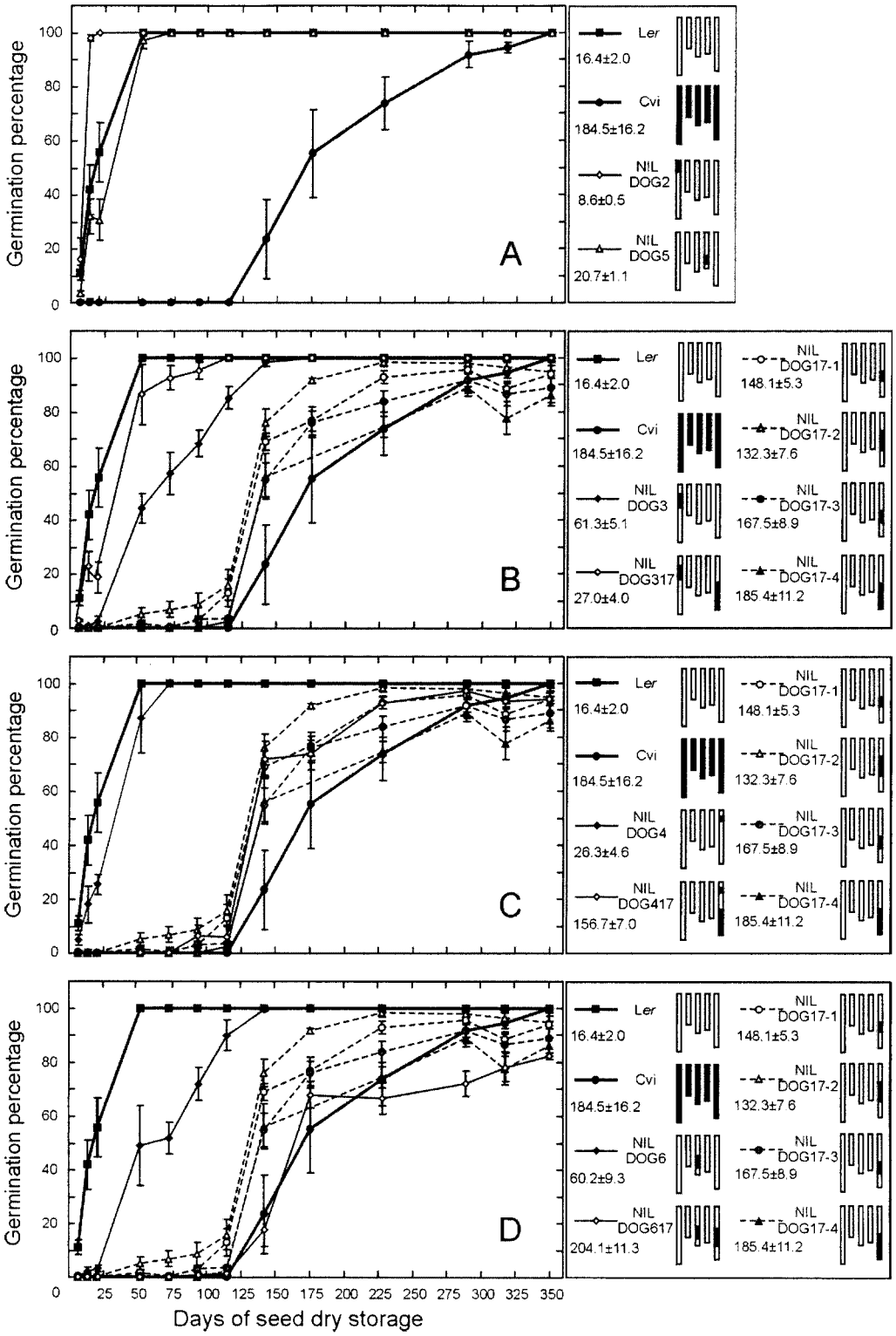


FIGURE 8.—Dormancy/germination behavior of L*er*, Cvi, and NILs during seed dry storage. Percentages of germination in water under white light were estimated at different times of seed dry storage, and curves of germination for each genotype are presented. Mean (±SE) of the germination percentage of four different seed bulks from three plants is shown for each genotype. The graphic genotypes of the lines are shown in the legends together with the estimated $DSDS_{50}$ (±SE). All plants were grown in a single experiment and therefore the various genotypes are directly comparable. (A) Dormancy behavior of the NILs DOG2 and DOG5 compared to that of L*er* and Cvi. (B) Dormancy behavior of NILs DOG3, DOG17-1–DOG17-4, DOG317, and the parental lines. (C) Dormancy behavior of NILs DOG4 and DOG417 compared to that of NILs DOG17-1–DOG17-4 and to that of the parental lines. (D) Dormancy behavior of NILs DOG6 and DOG617 compared to that of NILs DOG17-1–DOG17-4 and to that of the parental lines.

DOG17-4, also had a common upper breakpoint with NIL DOG17-1 but carried an additional 30-cM distal fragment. In contrast, this line was slightly but significantly ($P < 0.05$) more dormant than the other NIL DOG17 (Figure 8B), suggesting that small-effect Cvi alleles increasing dormancy are located on chromosome 5 between positions 85 and 117.

NILs carrying single Cvi fragments around the QTL *DOG2*, *DOG3*, and *DOG6* also differed significantly from L*er* in germination behavior ($P \leq 0.005$; Figure 8, A, B, and D), confirming the lower dormancy Cvi alleles at *DOG2* and the stronger dormancy ones at *DOG3* and *DOG6*. In addition, in a L*er* genetic background, the effects of *DOG6* and *DOG3* appear similar ($DSDS_{50}$ of

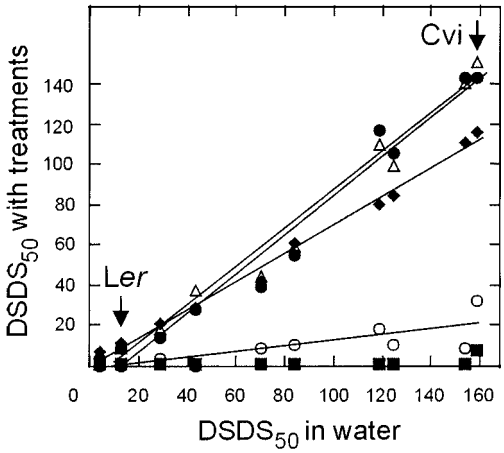


FIGURE 9.—Germination responses of L*er*, Cvi, and NILs to different treatments. Percentages of germination were estimated at 10 different times of seed dry storage in water and in five additional test conditions: in water after a cold treatment (○; R^2, 0.64), with GA_{4+7} (●; R^2, 0.96), with NOR (◆; R^2, 0.99), with KNO_3 (△; R^2, 0.97), and after removal of the seed coat (■). For each genotype, the $DSDS_{50}$ value in the five treatments is plotted against the corresponding value in water and the regression line is fitted for each comparison. Germination percentages were estimated from four different seed bulks of three plants.

60.2 ± 9.3 and 61.3 ± 5.1 for NILs DOG6 and DOG3, respectively) with both showing larger effect than *DOG2*. These results contrast with the relatively low effect of *DOG6* and relatively stronger effect of *DOG2* estimated in the RIL QTL mapping experiments (Figures 4 and 5), indicating the presence of genetic interactions.

NILs carrying single Cvi fragments around the QTL *DOG4* and *DOG5* did not differ significantly from L*er* wild type in their germination behavior (Figure 8, A and C), and therefore we could not confirm these loci. Both loci showed rather small additive effects in the QTL mapping experiments, and in a L*er* background they might become not easily detectable. However, it might also be possible that the introgression fragments of these lines did not include the corresponding loci. In the case of NIL DOG4, the introgression carried only one-third of the region corresponding to the 2-LOD support interval, while in the case of NIL DOG5 this was about half of its interval.

Furthermore, the genetic interactions among *DOG1/DOG7* and *DOG3*, *DOG4*, and *DOG6* in an otherwise L*er* genetic background could be tested by analyzing the three NILs carrying two Cvi introgressions (Figure 8, B–D). NIL DOG417 showed germination behavior similar to that of NIL DOG17-4 (Figure 8C), and therefore *DOG4* could not be confirmed either in a L*er* background or in a *DOG1/DOG7* dormant background. In contrast, NIL DOG617 was the strongest dormancy line ($DSDS_{50}$ = 204 ± 11.3), being significantly more dormant than NIL DOG17-4 and Cvi when comparing germination percentages after storage times >3 months ($P < 0.005$). Therefore, the overall effects of the allelic variation at *DOG1* and *DOG6* in a L*er* background are additive, as deduced from the germination curves of these NILs (Figure 8D). In contrast, the line carrying Cvi alleles at the *DOG1/DOG7* and *DOG3* regions behaved as a nondormant line, not differing significantly from L*er* ($P > 0.05$). Therefore, the alleles at *DOG3* and *DOG1/DOG7* regions strongly interact, confirming the interaction observed in the epistasis analysis of the RIL population. This interaction indicates that the Cvi alleles at *DOG1/DOG7* require L*er* alleles in the *DOG3* region to produce strong dormancy, in agreement with the larger effect of *DOG1* in a *DOG3*-L*er* background than in a *DOG3*-Cvi background as estimated in the RIL population analysis (Figure 6). In other words, Cvi alleles in the *DOG3* region increased dormancy in a *DOG1/DOG7*-L*er* background, but reduced dormancy in a *DOG1/DOG7*-Cvi genetic background.

To characterize physiologically the four dormancy loci confirmed in NILs, the seed germination behavior of the lines L*er* and Cvi was analyzed in four additional physical and chemical treatments known to reduce dormancy (BENTSINK and KOORNNEEF 2002). Germination of the eight NILs showing a dormancy behavior significantly different from L*er* was tested at different times of seed dry storage after a cold treatment or in the presence of nitrate, the hormone GA_{4+7}, or the inhibitor of ABA biosynthesis, NOR. The response of the various genotypes to these treatments was measured by comparing the $DSDS_{50}$ values obtained in water with those obtained with the corresponding treatment (Figure 9). Linear regression models taking the $DSDS_{50}$ in water as an independent variable and the $DSDS_{50}$ with the treatment as dependent variables accounted for considerable variation ($P < 0.005$; R^2 values between 0.64 for cold treatment and 0.99 for NOR), indicating overall linear responses to the treatments. The most effective condition to break the dormancy of these genotypes was a cold treatment, which reduced the $DSDS_{50}$ values of Cvi from 160 to 30 days. In addition, treatment with NOR reduced the seed dormancy of Cvi and of the high-dormancy NILs (Figure 9), showing that the Cvi dormancy can be partly overcome by reducing ABA biosynthesis during seed imbibition. The least effective treatments were GA_{4+7} and nitrate, which showed similar effect, both reducing the dormancy of Cvi seeds ($DSDS_{50}$ values of 142 and 150.3, respectively) rather little. However, GA_{4+7} showed a larger effect than NOR on the seed germination of L*er* and on the low-dormancy NILs. This different response of L*er* and Cvi seeds to GA_{4+7} and NOR suggests that the increased dormancy determined by some Cvi alleles involves a reduction of sensitivity to GA, leading to an increased effect of ABA during imbibition. However, none of the NILs showed an obvious differential response to any of the treatments, measured as deviation from the regression lines.

Therefore, a distinct role in a specific response with respect to the parameters analyzed could not be assigned to any of the four loci represented in these NILs.

Embryo dormancy was also analyzed by testing the germination of the embryos after removal of the seed coats (Figure 9). L*er* parental embryos germinated 100% the first day after seed harvest. However, Cvi embryos germinate ~50% when testas are removed from seeds at day 8 after harvest, indicating that part of the Cvi dormancy is due to the absence of growth potential in the embryo and may be described as pure embryo dormancy. In addition, dormant NILs also showed certain embryo dormancy, although no NIL presented an embryo germination as low as Cvi, indicating that the Cvi embryo dormancy cannot be assigned to a single particular locus but probably requires the effects of Cvi alleles at several loci.

DISCUSSION

Arabidopsis accessions collected from wild populations at different geographical locations differ largely in their seed dormancy (Lawrence 1976; Ratcliffe 1976). For instance, the laboratory strains L*er* and Col behave almost nondormantly when germination is measured in water under white light (van der Schaar *et al.* 1997) while other accessions such as Cvi, originally from Cape Verde Islands, or Enkheim-2, show much stronger seed dormancy under the same conditions (Koornneef *et al.* 2000). To understand the genetic basis of this intraspecific natural variation, we have analyzed the after-ripening requirement variation in a cross between two accessions showing dormancy phenotypic extremes, L*er* and Cvi. L*er* seeds require between 12 and 17 days of seed dry storage for 50% germination, depending on the maternal environment, whereas Cvi seeds need between 74 and 185 days. This 5- to 10-fold L*er*/Cvi difference in seed dormancy measured as the after-ripening requirement in $DSDS_{50}$ values is determined mainly by seven QTL, *DOG1–DOG7*. Four of these loci, *DOG1*, *DOG2*, *DOG3*, and *DOG6*, showed overall large phenotypic additive effects varying between 12 and 25 days of the $DSDS_{50}$ values as estimated in the mapping experiments using a RIL population. The strong additive effect of these four loci was further confirmed in NILs with a L*er* genetic background, but in addition, genetic interactions between these loci are found to participate in this variation. Genetic interactions are detected in the analysis of RILs or by comparing the additive effects of particular loci (such as *DOG2*) in RILs and NILs. These indications of epistasis may represent a scaling artifact due to the limited quantitative scales. However, a different and interesting interaction has been found between the strongest-effect locus *DOG1* and *DOG3*, the strong dormancy of *DOG1*-Cvi alleles appearing conditional upon the *DOG3*-L*er* alleles. Furthermore, we cannot discard the idea that higher-order and more complex interactions are involved, as suggested by the difference between the additive effect of *DOG6* estimated in the RIL population and the NILs. Thus, the overall effect of the Cvi alleles at five of the *DOG* loci increased seed dormancy as compared with the L*er* allele; Cvi alleles at *DOG2* reduce dormancy, and Cvi alleles at *DOG3* either increase or reduce dormancy, depending on the allele at *DOG1*. These additive and epistatic effects of the *DOG* loci explain the extreme parental phenotypes and the transgression in both directions observed in the RIL population. In addition, the estimated effects of the four major effect loci predict a transgressive phenotype more dormant than Cvi when combining Cvi alleles at *DOG1* and *DOG6* and L*er* alleles at *DOG2* and *DOG3*. This is confirmed in the NIL DOG617, which carries two Cvi introgression fragments and thus higher-dormancy alleles at all but the two smaller-effect QTL *DOG4* and *DOG5*.

The various *DOG* loci identified in the present study behave differently genetically in their additive effects during seed storage and in their epistatic effects, suggesting that they might be involved in different aspects of seed dormancy. The genetic and physiological characterizations of Cvi and the NILs carrying Cvi alleles at particular *DOG* regions enable several speculations on the different roles of the L*er*/Cvi dormancy allelic variation. First, the strong dormancy of Cvi is shown to involve not only seed-coat-imposed dormancy but also a certain embryo-imposed dormancy, which is absent in nondormant laboratory strains such as L*er*. This embryo dormancy is found to probably require the effects of Cvi alleles at several *DOG* loci. However, seed-coat-imposed dormancy appears as the major dormancy component since Cvi seeds lose their embryo dormancy 1 month after harvest, while retaining testa dormancy 2 months later. Maternal genetic effects on the dormancy variation were not detected by comparing either reciprocal crosses or the cytoplasms of the RILs, suggesting that the L*er*/Cvi genetic variation affecting the seed-coat-imposed dormancy is determined mainly by the embryo genotype. Thus, it is suggested that this genetic variation is probably involved in the growth potential of the embryo required to overcome the mechanical restraints of the maternal testa. Nevertheless, preliminary analyses of the dormancy of seeds derived from reciprocal crosses between L*er* and NIL DOG2 indicate that *DOG2* has maternal effects (data not shown). Since both genotypes, NIL DOG2 and L*er*, lack embryo dormancy, it is hypothesized that this locus affects the seed-coat-imposed dormancy through the genetic structure of the testa, the maternal tissues surrounding the seeds during their development, or a factor imported from the mother plant. Second, the *DOG* loci may affect the level of either embryo or seed-coat-imposed dormancy in various ways, such as influencing the induction of seed dormancy during the later phases of seed maturation, affecting the mecha-

nisms controlling the release of dormancy during storage, or controlling mechanisms involved in the onset of germination. The behavior of the Cvi accession and the high-dormancy NILs carrying Cvi alleles at *DOG1*, *DOG3*, or *DOG6* resemble the nongerminating mutants deficient in gibberellins (*ga1, ga2*, and *ga3*; Koornneef and van der Veen 1980) or defective in GA signal transduction (*sleepy1*; Steber *et al.* 1998). Gibberellins are required for the onset of germination to counteract the ABA-imposed dormancy (Bentsink and Koornneef 2002). However, in contrast to those GA-related mutants, the germination of Cvi and these NILs can be restored by after-ripening and cold treatment, indicating that a different kind of genetic variation leads to the increased dormancy. The observation that exogenous GA application is less effective in releasing the dormancy of Cvi seeds than is the NOR inhibition of seed ABA biosynthesis during seed imbibition suggests that part of the allelic variation of L*er*/Cvi dormancy at the *DOG* loci might be involved in the mechanisms downstream to GA. Thus, it is speculated that Cvi shows an increased ABA-mediated seed dormancy not determined simply by increased seed ABA synthesis (Jullien *et al.* 2000) or reduction of GA biosynthesis, but by reduction of GA sensitivity. Nevertheless, this function could not be assigned specifically to any of the *DOG* loci. In addition, inhibition of ABA biosynthesis during seed imbibition could only partly overcome the strong dormancy of lines carrying Cvi alleles at *DOG1*, *DOG3*, and *DOG6*, because the seeds of these lines still retain considerable dormancy (Figure 9). Therefore, it is speculated that these loci affect mechanisms that are different or downstream to the ABA-mediated seed dormancy during imbibition. Finally, the strong effect of a cold treatment to reduce the seed dormancy of Cvi and the high-dormancy NILs suggests that cold does not affect GA biosynthesis, as has been proposed for the light-induced germination (Yamaguchi *et al.* 1998). Moreover, the cold temperature mechanism must inactivate not only the mechanisms mediated by the ABA synthesized during seed imbibition but also other ABA-mediated or ABA-independent dormancy mechanisms that are probably involved.

Molecular interpretations of the function of the *DOG* loci require further characterization and, ultimately, gene isolation. Comparison of the map positions of the *DOG* loci with known seed dormancy and germination mutants allows the identification of primary candidate genes for all QTL except *DOG1* (Bentsink and Koornneef 2002). Therefore, *DOG1* is likely to represent a new dormancy locus accounting for an important part of the variation for seed dormancy present in nature. *DOG2* maps close to *aba3*, *phyA*, and *cry2* mutants; *DOG3* maps close to *lec1* and *lec2*; *DOG4* maps between *tt7* and *tt4*; *DOG5* maps around *abi1*; *DOG6* maps adjacent to *rdo1*, *fus3*, and *abi3*; and *DOG7* maps close to *ats, era1*, and *tt3*. The dormancy phenotypic effects of the *DOG* loci are comparable or even stronger than those of the currently available seed dormancy and germination mutants, which facilitates their further genetic and molecular analysis. However, the seed pleiotropic effects of several of the candidate mutants, such as alterations of the pigmentation or shape of the testa or changes in embryo pigmentation, do not appear in the *DOG* NILs. In addition, other seed characteristics found in some germination mutants, like changes in seed sugar composition or effects on hypocotyl elongation, are probably not affected by most of the *DOG* loci, as deduced from the comparison of QTL map positions for the various traits studied in the L*er*/Cvi RIL population. (Bentsink *et al.* 2000; Borevitz *et al.* 2002). Conversely, other traits such as seed size, seed storability, or flowering time might be influenced by some of the *DOG* loci (Alonso-Blanco *et al.* 1998b, 1999; Bentsink *et al.* 2000). We have begun the fine mapping of *DOG1*, *DOG2, DOG3*, and *DOG6* by analyzing crosses between the corresponding NILs and L*er*. Thus, we have discarded the *CRY2* photoreceptor and *ABA3* genes as candidates for *DOG2*, and *ABI3* for *DOG6*, further suggesting that these loci might provide new genes involved in the control of seed dormancy. A previous study of the genetic variation affecting seed dormancy and germination present between L*er* and Col showed that the small phenotypic differences between both accessions were attributable to 14 loci with rather small effects (van der Schaar *et al.* 1997). The seven *DOG* loci identified in the L*er* and Cvi materials all locate in genomic regions containing L*er*/Col QTL, suggesting that allelic series at a limited number of loci might account for the natural seed dormancy variation. However, the molecular isolation of the underlying genes and the identification of the specific allelic variants is still needed to understand the molecular basis of the genetic variation found in these works. Such an endeavor will provide new components and new genetic variants of known components for the subsequent physiological and molecular understanding of seed dormancy. Ultimately, the identification of these loci will initiate the comprehension of the ecological and evolutionary significance of this quantitative natural variation and of the mechanisms involved in the development of different life history strategies for adaptation to the environment.

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