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Light-related Loci Controlling Seed Germination in L*er* × Cvi and Bay-0 × Sha Recombinant Inbred-line Populations of *Arabidopsis thaliana*

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• *Background and Aims* Dormancy is a complex trait finely regulated by hormones and environmental factors. The phytochromes that sense red:far-red (R:FR) are the sole photoreceptors involved in the termination of dormancy and the induction of germination by light. The aims of this study were to identify and characterize loci controlling this process in seeds of *Arabidopsis thaliana*.
• *Methods* Recombinant inbred lines (RILs) derived from Landsberg *erecta* and Cape Verde Islands (L*er* × Cvi), and Bayreuth and Shahdara (Bay-0 × Sha) were used to map loci related to light effects in seeds previously exposed to chilling and after-ripening periods.
• *Key Results* Substantial genetic variation was found between accessions of *A. thaliana* in the induction of germination by light. Twelve loci were identified under R, FR or darkness, some of which were novel loci: *DOG8*, *DOG9*, *DOG13*, *DOG14* and *DOG15* detected in the L*er* × Cvi RIL population; and *DOG10*, *DOG11* and *DOG12* mapped in the Bay-0 × Sha RIL population. Furthermore, independent loci were mapped for the induction of germination by low fluence (*DOG-LF1* and *DOG-LF2*) and very low fluence of light (*DOG-VLF1*) in the L*er* × Cvi RIL population. Several loci were confirmed and characterized after different after-ripening and chilling treatments through near-isogenic lines (NILs) and heterogeneous inbred families (HIFs).
• *Conclusions* The results show that one group of loci act in a wide range of environmental scenarios, whereas a smaller group of loci are relevant only under a narrower set of conditions when the influence of the most-prevalent loci is reduced as a consequence of changes in the physiological status of the seeds. In addition, the identification of specific loci controlling the action modes of the phytochromes improves our understanding of the two independent signalling pathways that promote germination in response to light.

Key words: *Arabidopsis thaliana*, dormancy, germination, phytochromes, very-low-fluence response (VLFR), low-fluence response (LFR), natural genetic variation, quantitative trait loci (QTL), recombinant inbred line (RIL), near-isogenic line (NIL) heterogeneous inbred family (HIF).

INTRODUCTION

Dormancy is a most important seed characteristic since it defines the conditions required for germination (for thorough discussions of this concept see Baskin and Baskin, 2004, and Finch-Savage and Leubner-Metzger, 2006). It is modulated by a considerable number of factors acting from the early stages of seed development, and is eventually terminated by particular environmental cues. Since these cues are related to ecological scenarios likely to be favourable for the future development of the new individual, dormancy plays a crucial role in the adjustment of plant populations to their habitat (Benech-Arnold *et al.*, 2000; Finch-Savage and Leubner-Metzger, 2006). There are usually large differences in the behaviour of different species, and even between populations of the same species established in diverse environments, and it is likely that adaptation has resulted in divergent responses to the environment (Donohue *et al.*, 2005). Consequently, the induction and loss of dormancy following seed dispersal can be modulated by a variety of environmental factors acting through many apparently different physiological mechanisms or, at least, adaptations of the same mechanism controlled through natural allelic variation in key regulatory genes (Finch-Savage and Leubner-Metzger, 2006).

In natural populations, dormancy is commonly deep at the time of seed dispersal, thus severely restricting the possibilities of germination. After the seeds reach the soil, the dormancy can be progressively reduced under the influence of a variety of factors, among which temperature is of paramount importance, modulating seed responses to signals such as light or alternating temperatures that can terminate dormancy and initiate germination (Benech-Arnold et al., 2000). Depending on the conditions, the seeds can subsequently enter into secondary dormancy and so the possibilities of germination are again reduced (Finch-Savage and Leubner-Metzger, 2006). Available information on both physiological effects and gene expression patterns is consistent with the view that different environmental factors remove successive blocks to germination and need to be experienced by the seeds in the appropriate sequence (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007). Genetic variation in dormancy may thus result from different combinations of alleles affecting diverse stages in the processes leading to germination. Genetic variation for seed dormancy and germination has been studied for a long time in several crops (Foley and Fennimore, 1998) and wild populations of different species, such as *Arabidopsis thaliana* as a model system (Evans and Ratcliffe, 1972; Napp-Zinn, 1975; Ratcliffe, 1976). Understanding the

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variation is complicated by the interaction between environmental and genetic factors. Quantitative trait mapping is a useful approach to analyse such complex processes. Studies of dormancy quantitative trait loci (QTLs) using recombinant inbred lines (RILs) that originated from three independent populations of *A. thaliana* have identified several loci that produce small effects in populations of L*er* × Col RIL (van der Schaar *et al.*, 1997) and large effects in populations of L*er* × Cvi and L*er* × Sha RIL (Alonso-Blanco *et al.*, 2003; Clerkx *et al.*, 2004). The fine mapping of one of the *delay of germination* loci (*DOG1*) mapped in the RIL population of L*er* × Cvi and the isolation of a loss-of-function *dog1* mutant in the Col background, displaying reduced dormancy, has led to the identification and cloning for the first time of a seed-dormancy gene accounting for variation that occurs in natural populations (Bentsink *et al.*, 2006).

The identification of several loci (*DOG1* to *DOG7*) and the molecular characterization of *DOG1* represent highly significant contributions that open the way for a substantial advance in our understanding of the genetic and molecular bases of seed dormancy. Nevertheless, it should be noted that in previous mapping studies with the L*er* × Cvi RIL population, germination was tested by incubating the seeds under a long photoperiod of white light (Alonso-Blanco *et al.*, 2003). Under natural conditions, low temperatures and light commonly act sequentially on dormancy. The low temperature effects are integrated over a relatively long time and increase the sensitivity of the seeds to light, a factor that terminates dormancy and initiates germination (Benech-Arnold *et al.*, 2000; Finch-Savage and Leubner-Metzger, 2006). Therefore, to further advance our mechanistic understanding of the genetic architecture of dormancy, it is necessary to separately analyse the effects of low temperature and light in seed germination.

In the field, light cues elicit responses that reflect different environmental situations (Casal and Sánchez, 1998). Promotion of germination by light through phytochromes includes two types of responses or modes of action (Casal *et al.*, 1998). First, germination can respond to the ratio between red (R, 600–700 nm) and far-red (FR, 700–800 nm) spectral bands, which is perceived mainly through phytochromes B and E: this is called the low-fluence response (LFR; Botto *et al.*, 1995; Shinomura *et al.*, 1996; Henning *et al.*, 2002). The LFR modulates germination according to the density of the vegetation covering the soil. Opening a gap in the canopy increases the R/FR ratio that reaches the seeds and this light signal will promote germination in a site where the future seedling will not experience competition from larger, established plants (Deregibus *et al.*, 1994; Insausti *et al.*, 1995). Second, germination can also be promoted through phytochrome A (Botto *et al.*, 1996; Shinomura *et al.*, 1996) by a response requiring very small amounts of photons (millisecond exposures to sunlight): this is the very-low-fluence response (VLFR). The VLFR has been shown to participate in the stimulation of germination caused by soil disturbances such as those produced by mouldboard ploughing (Scopel *et al.*, 1994; Botto *et al.*, 1998*a*). The VLFR can be saturated by small amounts of Pfr (the active form of phytochrome) established by a saturating pulse of FR (Scopel *et al.*, 1991; Botto *et al.*, 1996). Whether a particular seed can be stimulated by a LFR or a VLFR depends on its dormancy status, which is affected by the conditions during burial in the soil (Botto *et al.*, 1998*b*). Extreme sensitivity to light (the capacity to be induced to germinate by a VLFR) is found in seeds with shallower dormancy than those requiring larger Pfr levels and that are promoted by a LFR. The fact that each photoresponse is mediated by different photoreceptors suggests the possibility of additional sources of genetic variation, which therefore require investigation.

In this study, we report on the natural genetic variation for the light responses of seeds of L*er* × Cvi and Bay-0 × Sha RIL populations and their interactions with after-ripening and incubation temperatures before light treatments. Twelve loci were identified under R, FR or darkness in both RIL populations. These include the previously identified *DOG1*, *DOG2*, *DOG3* and *DOG7* (Alonso-Blanco *et al.*, 2003) and five additional novel loci (*DOG8*, *DOG9*, *DOG13*, *DOG14* and *DOG15*) mapped in the L*er* × Cvi RIL population, and three loci (*DOG10*, *DOG11* and *DOG12*) identified in the Bay-0 × Sha RIL population. Moreover, *DOG-LF1* and *DOG-LF2* were mapped in the LFR promotion of seed germination, and *DOG-VLF1* was mapped in the VLFR promotion of seed germination.

MATERIALS AND METHODS

Plant material and growth conditions

L*er* and Cvi parental lines and a set of 162 RILs (Alonso-Blanco *et al.*, 1998), and the Bay-0 and Sha parental lines and a set of 165 RILs (Loudet *et al.*, 2002) were obtained from ABRC (University of Ohio). Plants of RILs and parental genotypes were cultivated in a growth chamber at 22°C under long-day conditions (16 h white light, PAR = 100 μmol m^{-2} s^{-1} and 8 h dark). Plants of different genotypes were grown together and their mature seeds were harvested on the same day at the moment that all siliques had senesced. Seeds of each genotype were harvested as single bulk samples of 3–6 plants and stored in open tubes inside a closed box containing silica gel at room temperature until used for germination experiments. *DOG1* and *DOG2* were confirmed and characterized in different light conditions using NIL *DOG17-1* (generously provided by M. Koornneef, Wageningen University, The Netherlands) and NIL *42*, NIL *45* and NIL-*EDI* (generously provided by C. Alonso-Blanco, Centro Nacional de Biotecnología, Madrid, Spain), respectively. In addition, we selected the *dog1* mutant in the Col background from the Salk line 000867 (http://signal.salk.edu) previously isolated and characterized as a non-dormant line by Bentsink *et al.* (2006). To confirm the presence and the allelic effects of *DOG10*, *DOG11* and *DOG12*, we developed HIFs from individual RILs that still segregate in a single marker and limited genomic region following protocols published previously (Tuinstra *et al.*, 1997). *DOG10* was confirmed using RIL61 (for marker MSAT2·36) and RIL84 (for marker MSAT2·41); *DOG11*

was confirmed using RIL350 (for marker MSAT5·9); and *DOG12* was evaluated using RIL111 (for marker MSAT4·18) and RIL57 (for marker MSAT4·9). For each of these lines, 20 F_7 seeds were germinated and the plants were genotyped individually, with two or three plants fixed with the Bay-0 or the Sha alleles at the respective segregating marker being selected. Harvested seeds from these plants were then used for the germination experiments.

Light conditions and germination experiments

Samples of 25 seeds were sown in clear plastic boxes (40 × 33 × 15 mm, width × breadth × height), each containing 3 mL of 0·8 % (w/v) agar in demineralized water. To avoid undesired effects of relatively high proportions of Pfr present in dry seeds, seeds imbibed for 2 h were irradiated with a saturated pulse of FR (15 min) to establish a minimum photoequilibrium into the seeds in order to reduce germination in darkness by as much as possible. The boxes were then wrapped with black plastic and incubated at 7°C in darkness for 0, 3 or 10 d. After that, the seeds were irradiated for 15 min with a saturated pulse of FR with a RG9 filter establishing a Pfr/P ratio of 0·03 (42 μmol m^{-2} s^{-1}) or a saturated pulse of R establishing a Pfr/P ratio of 0·87 (35 μmol m^{-2} s^{-1}; Casal *et al.*, 1991). Intermediate ratios of Pfr/P were provided by different R + FR mixtures of a single saturated pulse of light, as described previously (Casal *et al.*, 1991; Botto *et al.*, 1996). Following the light treatments, the boxes containing the seeds were again wrapped in black plastic and incubated at 25°C for 3 d before germination was determined. The criterion for germination was the emergence of the radicle.

Seeds with high sensitivity to Pfr/P were obtained before the light treatments by incubation for 3 d at 7 °C followed by 8 h at 35 °C (Cone *et al.*, 1985; Botto *et al.*, 1996). The short heat treatment enhances the light sensitivity to the FR pulse without affecting the response to the R pulse (Cone *et al.*, 1985; Botto *et al.*, 1996), in contrast to the effect of imbibition of seeds for long periods at high temperatures that allows them to enter secondary dormancy (Finch-Savage and Leubner-Metzger, 2006). In this experiment, the classical low-fluence R/FR reversible response (i.e. LFR) was calculated as the difference between the percentage of germination in R minus the FR pulse, and the VLFR was calculated as the difference between the percentages of germination after the FR pulse minus germination in darkness (Casal *et al.*, 1998).

Mapping and QTL analysis

Each QTL mapping experiment consisted of two (first experiment) or three (second and third experiments) randomized complete blocks (each block being a different day of sowing). The QTL analysis was based on the average percentage of germination for each RIL. The percentage of germination was probit-transformed to normalize the data (Cone and Kendrick, 1986). The *S* statistic Qstats package of QTL Cartographer (Wang *et al.*, 2004) was used to test the normality of the distributions. ANOVA was used to partition variance into sources originating from after-ripening (A), chilling (C), light (L) and error. In this model, we excluded the RIL genotype factor in order to conduct a robust analysis for the three environmental factors. The general linear model module of the InfoStat statistical package was used (Grupo InfoStat, 2002).

Mapmaker/EXP 3·0 (Lander *et al.*, 1987) was used to construct the linkage map. Linkage groups were verified with a minimum LOD = 3 and a maximum distance = 50 cM (Kosambi function). Marker segregation data for the L*er* × Cvi (Alonso-Blanco *et al.*, 1998) and the Bay-0 × Sha (Loudet *et al.*, 2002) RIL populations were obtained at http://www.arabidopsis.org/. The composite interval mapping procedure of QTL Cartographer (Zeng, 1994) was used for QTL analysis. QTL co-factors were initially selected by using forward–backward stepwise multiple regression. Mapping was conducted with a walking speed = 0·5 cM and a window size = 3 cM. LOD thresholds for each trait were calculated with 5000 permutations (Doerge and Churchill, 1996) and ranged between 2·5 and 2·7 ($P = 0·05$).

Two-way interactions among the identified QTLs were tested by ANOVA using the corresponding two markers as random factors. The analysis was performed using the computer program EPISTAT (Chase *et al.*, 1997) with LOD thresholds corresponding to a significant value of $P < 0·001$. Ten-thousand trials were used in Monte Carlo simulations performed with EPISTAT in order to establish the statistical significance of the LOD scores for the detected interactions (Chase *et al.*, 1997).

For each putative QTL, the interaction QTL × E (environment) was tested by repeated-measures of two-way ANOVA using the segregation of the corresponding marker and the environmental conditions in which seeds were exposed as classifying factors ($P < 0·005$). The general linear model module of the InfoStat statistical package was used (Grupo InfoStat, 2002).

RESULTS

Phenotypic variation in responses of germination to light after different chilling and after-ripening periods

The induction of germination after a single R or FR pulse was analysed in 15- or 90-d after-ripened seeds of L*er* and Cvi followed by 0, 3 or 10 d of incubation at 7 °C. L*er* and Cvi seeds after-ripened for 15 d without chilling did not germinate (Table 1). L*er* seeds incubated for 3 d at 7 °C germinated close to 75 % and 0 % after R and FR pulses, respectively. In contrast, Cvi seeds displayed a deep dormancy and a smaller response to the light stimulus than the L*er* seeds (Table 1). When chilling was extended to 10 d, the difference in germination between R- and FR-treated seeds was reduced in both Cvi and L*er* accessions (Table 1). L*er* R-treated seeds with after-ripening for 90 d showed large germination values independent of the chilling period, and FR-treated seeds required 10 d of chilling to reach similar values compared with R (Table 1). For Cvi seeds, the promotion of germination required a period of chilling and the response was almost independent of the R and FR pulse (Table 1). Significant

TABLE 1. *Summary statistics for seeds of the Ler × Cvi RIL population that had been after-ripened for 15 or 90 d, then induced to germinate by chilling (0, 3 or 10 d) and then subjected to a saturated pulse of light (R or FR)*

	After-ripening period										
	15 d					90 d					
	Red pulse			Far-red pulse			Red pulse			Far-red pulse	
	Chilling (d)										
	0	3	10	0	3	10	0	3	10	0	3	10
L*er*	0	74 ± 9	97 ± 4	0	1 ± 1	79 ± 3	74 ± 9	77 ± 7	94 ± 4	1 ± 1	47 ± 9	79 ± 3
Cvi	0	13 ± 4	68 ± 2	0	0	60 ± 0	0	30 ± 12	68 ± 2	0	13 ± 4	60 ± 0
RIL mean	23 ± 2	64 ± 3	83 ± 2	0·5	11 ± 1	78 ± 3	33 ± 3	64 ± 3	83 ± 2	8 ± 1	11 ± 1	72 ± 2
RIL max / min	98 / 0	100 / 0	100 / 7	16 / 0	97 / 1	100 / 7	98 / 0	100 / 0	100 / 9	78 / 0	97 / 1	100 / 6
V_g	833·5	1024·8	443·8	3·5	282·5	588·4	961·7	1021·5	469·8	173·9	281·2	776·2
V_e	14·2	40·3	41·2	0·9	28·7	50·2	29·2	29·7	27·5	10·9	18·7	32·1
H^2	0·98	0·96	0·92	0·79	0·91	0·92	0·97	0·97	0·94	0·94	0·94	0·96

Vg, component of variance due to among-RIL variation.
V_e, component of variance due to within-RIL variation.
H^2, broad-sense heritability calculated as the proportion of total variance ($V_g + V_e$) attributable to genotype (V_g).

differences were not found between germination in darkness and after a FR pulse in seeds of L*er* and Cvi accessions (data not shown).

Seed germination of L*er* × Cvi RILs showed transgressive variation in both directions compared with the parental lines (Table 1). The heritability index was over 0·79, indicating that a high proportion of the phenotype was explained by the genotype in our experimental conditions (Table 1). Three-way ANOVA analysis showed significant effects for the environmental factors (i.e. after-ripening, chilling and light) in modulating the germination behaviour of the RILs (see Supplementary Information Table S1, available online). Significant interactions between chilling and light, and chilling and after-ripening were detected (Supplementary Information Fig. S1 and Table S1). Overall, the results indicate that enough genetic variation exists between RILs to map the loci controlling termination of dormancy by light after the seeds had experienced different after-ripening and chilling conditions.

Mapping loci for light induction of germination for seeds of Ler × *Cvi RIL population after exposure to different chilling and after-ripening treatments*

QTL analysis detected six loci in seeds after-ripened for 15 or 90 d and incubated for 0, 3 or 10 d at 7 °C and then exposed to a single pulse of R or FR. The loci have been named following the criteria of Alonso-Blanco *et al.* (2003). Four of them were located at the same positions and showed similar additive effects to those previously identified: *DOG1*, *DOG2*, *DOG3* and *DOG7* (Fig. 1, and Alonso-Blanco *et al.*, 2003). *DOG1*, *DOG2* and *DOG7* were the most prevalent QTLs, affecting the light response of seeds in a large number of after-ripening and chilling conditions, whereas *DOG3* was detected only in R-treated and non-chilled seeds after-ripened for 15 d (Fig. 1). The L*er* alleles at *DOG1* and *DOG7* increased germination up to 36 % and 23 %, respectively, and the Cvi alleles at *DOG2* increased germination up to 25 % (Table 2). Two novel QTLs were mapped at chromosome 3: *DOG8* and *DOG9* (Fig.1). *DOG8* was detected in R-treated seeds incubated at 7 °C for 10 d, and *DOG9* was mapped in non-chilled and FR-treated seeds after-ripened for 90 d. Cvi alleles at *DOG8* and *DOG9* loci increased germination (Fig. 1). *DOG9* co-localized with *DOG6*, but they are probably distinct loci because they showed opposite allelic effects (Fig. 1, and Alonso-Blanco *et al.*, 2003). The QTL mapping analysis explained up to 65 % or 34 % of the total phenotypic variation displayed in R and FR-treated seeds, respectively (Table 2).

Analysis of QTL × E interactions showed that four of the six loci (*DOG1*, *DOG2*, *DOG3* and *DOG7*) had significant effects depending on the after-ripening, chilling and light conditions experienced by the seeds ($P < 0{\cdot}005$, Table 2). Interactions of these QTL with after-ripening confirm previous results (Alonso-Blanco *et al.*, 2003).

Two-way interactions were analysed among the six QTL identified. *DOG1* interacted significantly with *DOG2*, *DOG7* and *DOG8*, while *DOG2* interacted with *DOG7* ($P < 0{\cdot}0005$, Table 2). The interactions between *DOG1*,

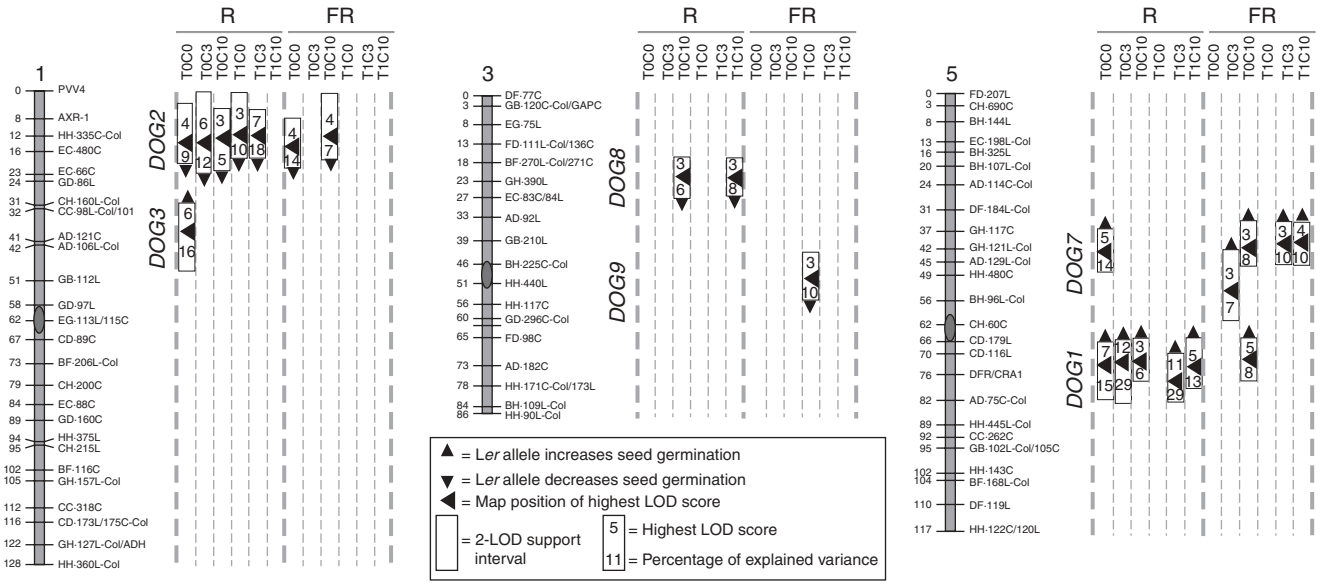

FIG. 1. QTL mapping for the induction of seed germination by a R or FR pulse in the L*er* × Cvi RIL population. Seeds were stored at 25 °C for 15 d (T0) or 90 d (T1), and then incubated for 0, 3 or 10 d (C0, C3 or C10, respectively) at 7 °C followed by a pulse of R or FR. Only linkage groups with significant QTLs are shown. Rectangles show the two-LOD support interval; the arrowheads inside the rectangles point to the position of the highest LOD score; the numbers above the arrowheads indicate the LOD score; the numbers below the arrowheads indicate the percentage of explained variance for each QTL. The arrowheads outside the rectangles show the allelic effects of each QTL: the arrowheads point upwards when the L*er* alleles increased the average value of the trait and downwards when the Cvi alleles increased the average value of the trait.

DOG2 and *DOG7* alleles were also detected previously (Alonso-Blanco *et al.*, 2003). A novel QTL × QTL interaction was detected between *DOG1* and *DOG8*. The reaction norms of *DOG1* × *DOG8* interaction showed that the Cvi alleles at *DOG1* and the L*er* alleles at *DOG8* decrease the light sensitivity to R (Supplementary Information Fig. S2).

Mapping loci for light induction of seed germination in the Bay-0 × Sha RIL population

Ninety-day after-ripened seeds of Bay-0, Sha and their RILs were incubated for 3 d at 7 °C and then exposed to a R or FR pulse (i.e. identical environmental conditions to those for T1C3R and T1C3FR in Fig. 1). Bay-0 seeds showed a deep dormancy whilst Sha seeds germinated by 98 % and 15 % after a R or FR pulse, respectively (Supplementary Information Table S2). Thus, Bay-0 and Sha differed considerably in their seed dormancy status, displaying a wide genetic variation in their responses to light. The QTL analysis mapped *DOG10* and *DOG11* in R- and in FR-treated seeds with a higher contribution under FR and *DOG12* in R-treated seeds (Fig. 2). The Sha alleles at *DOG10* and *DOG12* and the Bay-0 alleles at *DOG11* promoted seed germination (Fig. 2 and Supplementary Information Table S3). The genetic component of the total phenotypic variance was similar between R and FR treatments (46 % and 49 %, respectively; Supplementary Table S3). Neither a significant two-way QTL × QTL interaction, nor a QTL × E interaction were detected.

An objective of this study was to evaluate the presence of common loci controlling the termination of dormancy by light in accessions of *A. thaliana* originating from diverse locations and displaying different levels of dormancy. Linkage map positions between L*er* × Cvi and Bay-0 × Sha RIL populations were aligned with known physical positions for markers and with estimated physical positions for the QTLs. *DOG1* and *DOG11* at chromosome 5 overlapped between RIL populations for R-treated seeds incubated 3 d at 7 °C (Figs 1 and 2).

Mapping loci for LFR and VLFR on seed germination in the Ler × *Cvi RIL population*

It is known that germination may be promoted by two different modes of action of the phytochromes depending on the seed sensitivity to R and FR. When germination is promoted by an R pulse and cancelled by a subsequent pulse of FR, the light response is R/FR reversible and classified as a low-fluence response (LFR). Under certain conditions, a pulse of FR can promote germination above the level of dark-imbibed seeds and the response induced is known as a very-low-fluence response (VLFR; Casal *et al.*, 1998). To maximize the VLFR induction of seed germination, L*er*, Cvi and RIL seeds after-ripened for 1 year were incubated for 3 d at 7 °C followed by 8 h at 35 °C, and then treated with R, FR or kept in darkness (Supplementary Information Table S4). L*er* seeds displayed a larger LFR compared with Cvi seeds (59 vs. 39 %, respectively), and Cvi seeds showed an enhanced VLFR with respect to L*er* seeds (14 vs. 3 %, respectively, Supplementary Information Table S4). The loci detected

TABLE 2. *Summary of QTL detected for seeds in the L*er × *Cvi RIL population that had been after-ripened 15 or 90 d, then induced to germinate by chilling (0, 3 or 10 d) and then subjected to a saturated pulse of light (R or FR)*

		T0 = 15 d after-ripening						T1 = 90 d after-ripening						
		Red pulse			Far-red pulse			Red pulse			Far-red pulse			
		Chilling (d)												
		0	3	10	0	3	10	0	3	10	0	3	10	QTL × E
DOG1 (DFR) C5·72 cM	LOD	6·75	11·5	3·4			5·26		11·26	4·91				<0·0001
	Add %	24	36	15			18		35	16				
	Var %	15	29	6			8		29	13				
	Interval cM	66–82	65–83	66–77			66–76		71–82	65–77				
DOG2 (HH·335) C1·15 cM	LOD	3·68	5·60	3·17	4·28		3·80	2·77	7·12					<0·0001
	Add %	−19	−22	−13	−14		−17	−18	−25					
	Var %	9	12	5	14		7	10	10					
	Interval cM	6–16	0–22	6–22	12–24		0–20	0–15	6–15					
DOG1 × *DOG2*	Var %		8	9			10		8	9			10	
DOG3 (AD·121) C1·38 cM	LOD	5·76												0·0050
	Add %	25												
	Var %	16												
	Interval cM	31–49												
DOG7 (nga139) C5·42 cM	LOD	5·38				3·27	3·64					3·50	4·26	0·0013
	Add %	22				21	23					10	20	
	Var %	14				7	8					10	10	
	Interval cM	38–46				42–58	34–46					34–46	34–46	
DOG1 × *DOG7*	Var %					12								
DOG2 × *DOG7*	Var %		11	14						9		12	14	
DOG8 (GH·390) C3·20 cM	LOD			2·97						3·05				NS
	Add %			−14						−11				
	Var %			6						8				
	Interval cM			16–27						20–27				
DOG1 × *DOG8*	Var %									6				
DOG9 (HH·440) C3·49 cM	LOD										2·93			NS
	Add %										−11			
	Var %										10			
	Interval cM										43–54			
Complete model	Var %	65	60	40	14	19	33	10	47	39	10	22	34	

The closest marker to each QTL is shown in the first column, and its location is indicated by the linkage group followed by the map position. Additive effects are given as the difference between means of the two RIL genotypic groups (a positive value implies that L*er* alleles induce germination more in comparison with Cvi alleles, and a negative value indicates the opposite). NS, not significant.

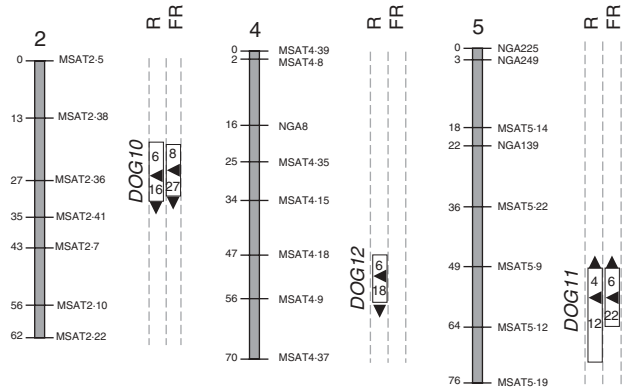

FIG. 2. QTL mapping for the induction of seed germination by a R or FR pulse in the Bay-0 × Sha RIL population. Seeds were stored at 25 °C for 90 d and then incubated for 3 d at 7 °C, followed by a pulse of R or FR. Only linkage groups with significant QTLs are shown. The arrowheads outside the rectangles show the allelic effects of each QTL: the arrowheads point upwards when the Bay alleles increased the average value of the trait and downwards when the Sha alleles increased the average value of the trait. For other details see Fig. 1.

in the QTL mapping analysis were named *delay of germination related with low-fluence* (*DOG-LF*) or *delay of germination related with* the *very-low-fluence* (*DOG-VLF*). *DOG-LF1*, located to the 22–30 cM region of chromosome 2, and *DOG-LF 2*, located to the 115–125 cM region of chromosome 1, mapped for the LFR; and *DOG-VLF1*, located at the 0–18 cM of chromosome 5, mapped for the VLFR (Fig. 3 and Supplementary Information Table S5). *DOG-VLF1* co-localized with *DOG4* but it is likely to correspond to different loci because they showed opposite allelic effects (Fig. 3, and Alonso-Blanco *et al.*, 2003). Neither a significant two-way QTL × QTL interaction among the three QTLs, nor a QTL × E interaction among modes of action were detected.

Furthermore, the loci identified in R- and/or FR-treated seeds after-ripened for 15 or 90 d were not detected in seeds after-ripened for 360 d and incubated for 3 d at 7 °C followed by 8 h at 35 °C (compare R and FR conditions in Table 2 and Supplementary Information Table S5). *DOG13* and *DOG15* were mapped in R-treated seeds, suggesting that prolonged after-ripening and/or high incubation temperature are responsible for detection of different loci. In addition *DOG14*, mapped at the bottom of chromosome 2, contributed to the control of germination in darkness (Supplementary Information Table S5).

Genetic and physiological characterization of DOG1*,* DOG2*,* DOG10*,* DOG11 *and* DOG12

Seed germination was tested for NILs carrying a short DNA fragment of Cvi, inserted in the uniform background of L*er*, in the region of *DOG1* or *DOG2* treated with R, FR or kept in darkness. NIL *DOG17-1* seeds showed significantly reduced germination after R or FR, but not in darkness, compared with L*er* seeds ($P < 0{\cdot}001$, Fig. 4), confirming the participation of *DOG1* in the termination of dormancy by light. The Cvi alleles enhanced germination at *DOG2* in R ($P = 0{\cdot}0103$ for NIL *EDI*) or FR-treated seeds ($P = 0{\cdot}0046$ for NIL *45*, and $P = 0{\cdot}0002$ for NIL *EDI*, Fig. 4). These results show that *DOG2* is contained in the shorter Cvi region included in the NIL *EDI*. To analyse the effects of chilling on the expression of *DOG1* and *DOG2*, 120-d after-ripened seeds were incubated for 0, 3 or 10 d at 7 °C followed by a saturating pulse of R or FR, or kept in darkness (Fig. 5). The results showed that longer periods of chilling increased the L*er* additive effects at

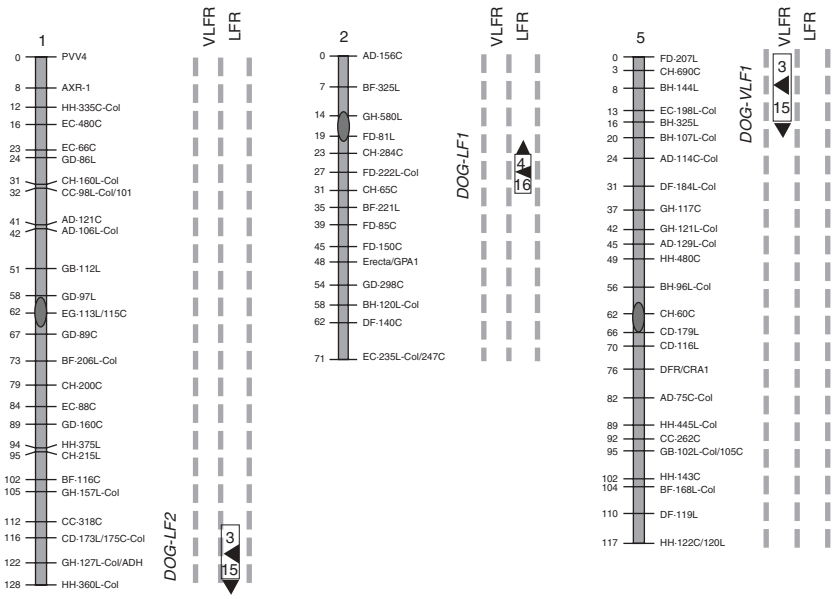

FIG. 3. QTL mapping for the very-low-fluence response and the low-fluence response (VLFR and LFR, respectively) in the L*er* × Cvi RIL population. Seeds were stored at 25 °C for 360 d, incubated for 3 d at 7 °C followed by 8 h at 35 °C, and then exposed to a pulse of R or FR, or kept in darkness. Only linkage groups with significant QTLs are shown. For details see Fig. 1.

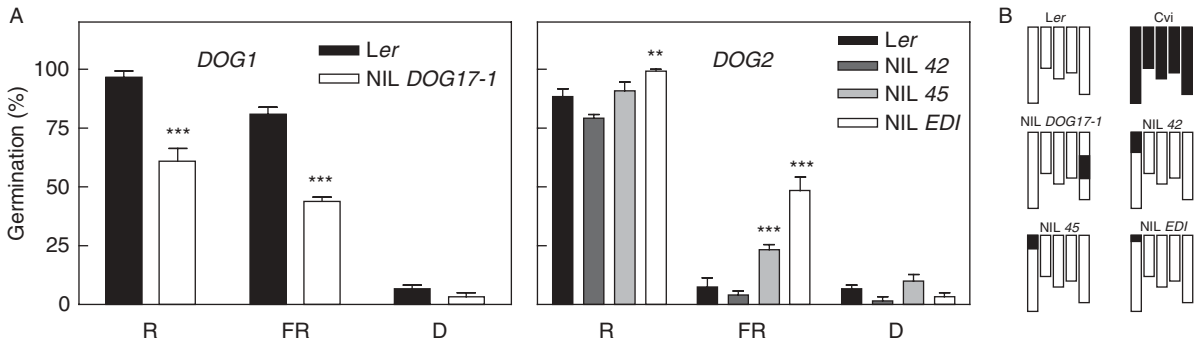

FIG. 4. (A) Confirmation and characterization of *DOG1* and *DOG2* in the promotion of germination by light. Seeds were stored at 25 °C for 90 d (*DOG1*) or 45 d (*DOG2*) and then incubated 3 d at 7 °C, followed by a pulse of R or FR, or kept in darkness (D). Seeds were harvested simultaneously from plants grown in identical environmental conditions, and therefore the NILs are directly comparable with the L*er* control. Values are means of 3–6 replicates ± s.e. Only significant differences between means are shown: ***, $P < 0{\cdot}01$; **, $P < 0{\cdot}05$ (Student's *t*-test). (B) Graphical representation of the genotypes of the parental lines L*er*, Cvi and the four NILs carrying a single Cvi introgression fragment.

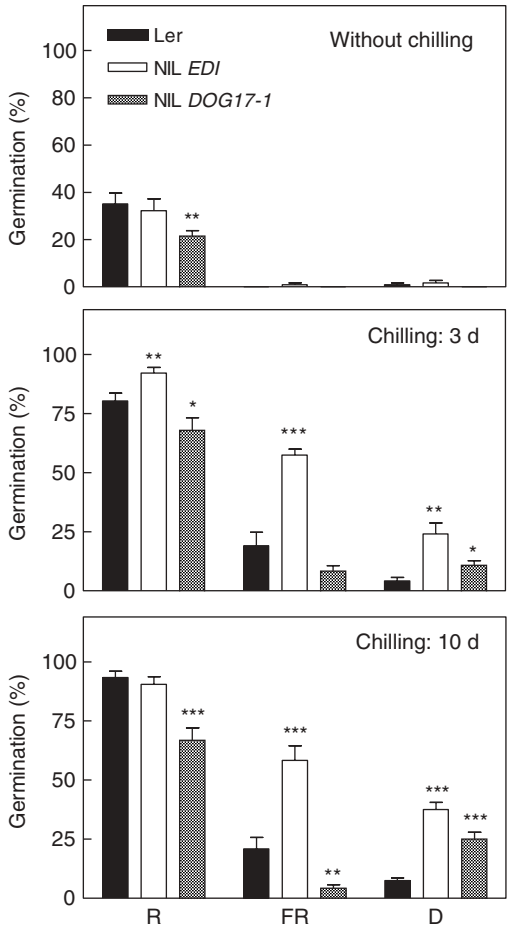

FIG. 5. Chilling effects on the promotion of germination by light of *DOG1* and *DOG2*. Seeds were stored at 25 °C for 120 d and then incubated at 7 °C for 0, 3 or 10 d, followed by a pulse of R or FR, or kept in darkness (D). Seeds were harvested simultaneously from plants grown in identical environmental conditions, and therefore the NILs are directly comparable with the L*er* control. Values are means of 6–9 replicates ± s.e. Only significant differences between means are shown: ***, $P < 0{\cdot}01$; **, $P < 0{\cdot}05$; *, $P < 0{\cdot}10$ (Student's *t*-test).

DOG1 in seeds exposed to R and FR (Fig. 5). Significant effects of *DOG1* alleles in seeds treated with a saturating pulse of FR were detected only when longer periods of chilling were used. This suggests that when the Pfr level of the seeds is very low the influence of *DOG1* can be perceived if dormancy is substantially reduced. In contrast, seeds incubated in darkness showed a null or opposite contribution of the allelic effects, suggesting that this locus may be particularly linked to the promotion of germination by light (Fig. 5). The promotion of germination by Cvi alleles at *DOG2* was manifested in seeds incubated at low temperatures and their effects were independent of the light treatment (Fig. 5).

In addition, the role of *DOG1* was studied in the promotion of germination by single light pulses that established a series of Pfr/P ratios. The germination of wild-type (WT, Columbia accession) and *dog1* seeds plotted against Pfr/P showed two phases separated by a plateau (Fig. 6). The first phase, at lower Pfr/P, corresponds to the VLFR and the second one to the LFR. In seeds not exposed at 7 °C before the light pulse, the VLFR occurred below Pfr/P = 0·20 and the LFR was observed between Pfr/P = 0·33 and Pfr/P = 0·85. Increasing the light sensitivity of seeds by a previous incubation at 7 °C for 7 d reduced the VLFR range to Pfr/P = 0·03 and Pfr/P = 0·10 in *dog1* and WT seeds, respectively (Fig. 6). The higher germination response of *dog1* seeds compared with WT seeds was a consequence of the enhancement of the VLFR in the mutant genotype (Fig. 6). In *dog1* seeds, the slope of the VLFR was 320–410 % higher than in the WT (Fig. 6).

To confirm the position of the QTLs mapped in the Bay-0 × Sha RIL population, HIFs were developed from individual RILs that still segregate in a single marker of interest. *DOG10* was confirmed at the MSAT2·36 marker. The Sha alleles increased germination by 27 % in R-treated seeds ($P < 0{\cdot}0001$, Fig. 7). R- and FR-treated seeds of the same HIF exposed after different after-ripening periods confirmed the additive effects of Sha at the MSAT2·36 marker (data not shown). The promotion of germination by the Bay-0 alleles at *DOG11* was associated with the MSAT5·9 marker and their effects were detected in FR-treated seeds

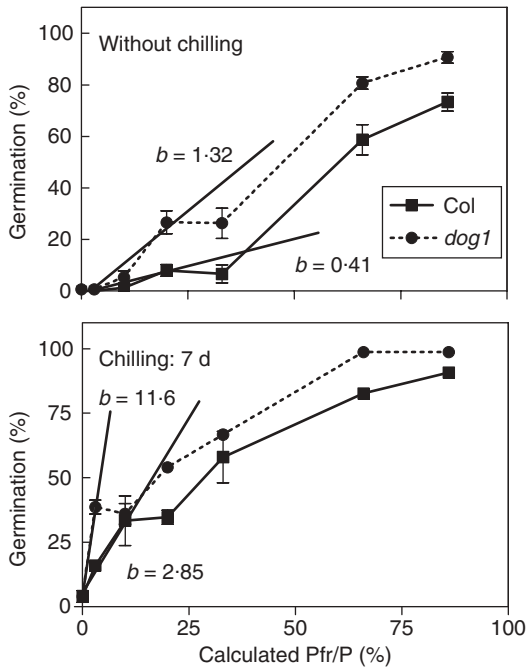

FIG. 6. Germination of wild-type (Columbia, Col) and *dog1* mutant seeds plotted against different Pfr/P ratios provided by a light pulse. Seeds were stored at 25 °C for 50 d and then incubated at 7 °C for 0 or 7 d, followed by a pulse of light that established different calculated Pfr/P ratios. The slopes of the regression line for the VLFR are indicated with the letter *b*. Values are means of 3–6 replicates ± s.e.

($P < 0{\cdot}0046$) but not in seeds kept in darkness (Fig. 7). We could not confirm that *DOG12* is associated with either the MSAT 4·18 or the MSAT4·9 markers (Fig. 7).

DISCUSSION

Light is a crucial factor that controls seed behavior, enhancing the chances of germination in environmental situations favourable for the future development of a new plant. We found substantial genetic variation between accessions of *A. thaliana* in the responses of germination to light. Seeds of L*er* and Sha showed low levels of dormancy and a clear R/FR response, whereas Cvi and Bay-0 genotypes showed deeper dormancy. QTL mapping using recombinant inbred lines of L*er* × Cvi and Bay-0 × Sha allowed us to identify 12 loci under R or FR light pulses, or under darkness (Figs 1, 2 and Supplementary Information Table S5). Interestingly, the loci identified in younger seeds after-ripened for no more than 90 d were not detected in older seeds incubated at 35 °C for 8 h after chilling, suggesting that prolonged after-ripening and/or high temperatures during incubation make the seeds sensitive to the influence of different light-related loci such as *DOG13* and *DOG15* (Supplementary Information Table S5). In addition, *DOG-LF1*, *DOG-LF2* and *DOG-VLF1* influenced the variation in LFR and VLFR, indicating specific and independent molecular points of control for the two modes of action of the phytochromes that promote seed germination (Fig. 3).

It was found that several loci mapped in a wide range of situations characterized by different combinations of after-ripening, low temperature and light, whereas a different set of loci influenced germination only within a narrow set of conditions and apparently when the influence of the most-prevalent QTLs was reduced. These results suggest that dormancy relief is a process finely regulated by a defined hierarchy of genes expressed in a temporal sequence associated with the physiological status of the seeds. *DOG1* and *DOG2* were prevalent loci mapped almost exclusively in seeds treated with R after a wide range of after-ripening and chilling conditions; whereas also *DOG3* appeared in R-treated seeds but only in a narrow window of conditions such as short after-ripening and no chilling (Fig. 1, and Alonso-Blanco *et al.*, 2003). Furthermore, a novel group of loci including *DOG8* and *DOG9* was also mapped in particular environments, suggesting that they act into specific signalling events

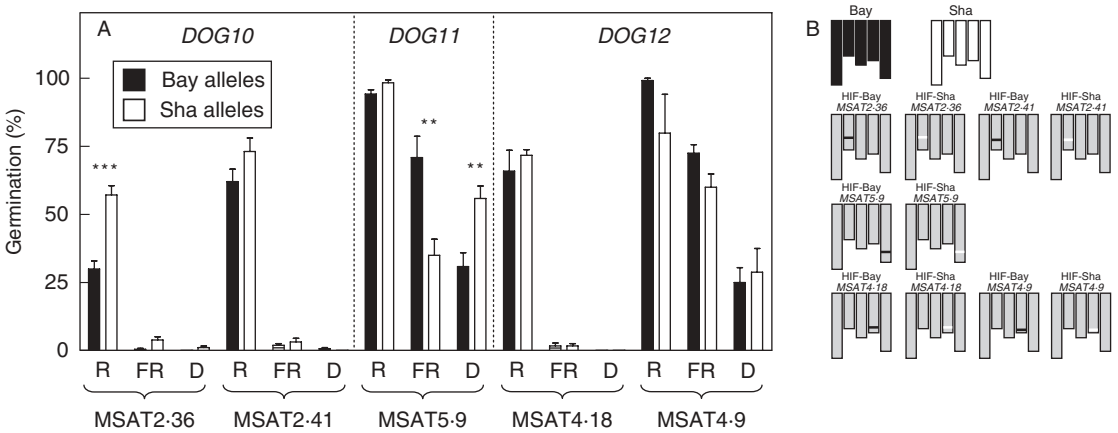

FIG. 7. (A) Confirmation and characterization of *DOG10*, *DOG11*and *DOG12* in the promotion of germination by light using heterogeneous inbred families (HIFs). Seeds were stored at 25 °C for 180 d (*DOG10*), 210 d (*DOG11*) and 300 d (*DOG12*), and then incubated for 3 d at 7 °C followed by a pulse of R or FR, or kept in darkness (D). Seeds were harvested simultaneously from plants grown in identical environmental conditions, and therefore the HIFs are directly comparable at the same marker. Values are means of 6–18 replicates ± s.e. Only significant differences between means are shown: ***, $P < 0{\cdot}01$; **, $P < 0{\cdot}05$ (Student's *t*-test). HIFs carried on Bay and Sha alleles at the indicated marker are shown. (B) Graphical representation of the genotypes of the parental lines Bay, Sha and the 5 HIFs segregating at the marker of interest as Bay (black) or Sha (white), and homozygous elsewhere (grey).

induced by R or FR, respectively (Fig. 1). For example, *DOG8* required a long incubation at low temperature to be influential (Fig. 1). Although environment-specific loci were found, we cannot discount the fact that those loci could display their actions in other environmental scenarios that determine a similar physiological status in the seeds (Finch-Savage *et al.*, 2007). In fact, comparing global transcript expression patterns of Cvi seeds related with after-ripening, low temperature, nitrate and light stimuli, Finch-Savage *et al.* (2007) found that the gene expression profiles of seeds were grouped according to the depth of dormancy rather than to the dormancy-relieving treatments.

The identification of several loci inducing germination through the VLFR (*DOG-VLF1*) and LFR (*DOG-LF1* and *DOG-LF2*) indicates that at least two independent signalling pathways control this process via light (Fig. 3). It is interesting to note that Yanovsky *et al.* (1997) observed significant allelic effects for the VLFR and LFR induction of seed germination between Col and L*er* accessions close to *DOG-LF1*. This is consistent with the fact that each mode of action involves a different photoreceptor (phyB and phyE for LFR, phyA for VLFR) and, consequently, the signalling cascades are not identical. Moreover, each one participates in the perception of particular environmental cues that promote germination. The promotion of germination through the VLFR in response to very brief exposures to light during tillage in agricultural fields (Scopel *et al.*, 1994; Botto *et al.*, 1998*a*) or the perception of high a R/FR ratio associated with the opening of gaps in closed canopies leading to a LFR (Vázquez-Yanes and Orozco-Segovia, 1990; Deregibus *et al.*, 1994; Insausti *et al.*, 1995) are most likely regulated by different genes. It appears that each set of these loci play detectable functions only after proper changes in the light sensitivity of the seeds caused by factors such as after-ripening, chilling or high temperatures. It seems likely, then, that the expression of allelic variations in the VLFR and LFR loci identified in this study may be related to the seasonal patterns of light sensitivity observed in wild populations of seeds in the soil (Derkx and Karssen, 1994; Botto *et al.*, 1998*b*).

DOG1 and *DOG2* were confirmed and characterized by comparing the germination behavior of NIL seeds exposed to several chilling and light treatments. Whereas *DOG2* allelic effects operated under light and darkness conditions, the inhibitory action of Cvi alleles at *DOG1* was confined to seeds irradiated with a R and/or FR pulse (Figs 4 and 5). The results consistently showed that NIL *DOG17-1* seeds germinated more than L*er* seeds in darkness, suggesting an active role of *DOG1* specifically in the induction of germination by light (Fig. 5). The apparent discrepancy of our data for seeds geminating in darkness with those of Bentsink *et al.* (2006) is likely to reflect differences in the quantity of the active form of phytochrome in seeds originated from plants grown in light environments with different R/FR ratios (McCullough and Shropshire, 1970; Wulff, 1995). Our experimental protocol substantially decreased the levels of Pfr derived from the light environment experienced by the seeds during ripening. In addition, we showed that *DOG1* acts as a negative regulator of the VLFR induction of seed germination (Fig. 6). It has been reported that Col and Nossen accessions have reduced VLFR compared with the L*er* accession (Yanovsky *et al.*, 1997; Alconada-Magliano *et al.*, 2005). Our data demonstrate that the loss-of-function of *DOG1* might be one of the changes leading to the increase of the VLFR in nature.

Using heterogeneous inbred families, we were able to confirm the presence and expected additive effects of *DOG10* and *DOG11* located around the MSAT2·36 and MSAT5·9 markers in the QTL analysis of the Bay × Sha RIL population (Fig. 7). Meng *et al.* (2008) found QTLs named *CDG-2* and *CDG-5* that accounted for germination at low temperature in the dark, co-localizing with *DOG10* and *DOG11*, respectively. In addition, *DOG1* and *DOG11* are loci linked at similar positions in the *Ler* × Cvi and Bay-0 × Sha mapping populations, respectively (Figs 1 and 2). In both RIL populations, *DOG1* and *DOG11* explained a significant fraction of the phenotypic variation and displayed opposite additive effects in darkness with respect to the light stimulus (Table 2, Supplementary Information Table S3, and Figs 5 and 7).

Considering the mapping intervals, *PIL5* (At2g20180) appears as a strong candidate gene for *DOG10* and/or *DOG-LF1* (Figs 2 and 3). PIL5 protein, a basic helix–loop–helix transcription factor, is a negative regulator of phytochrome-mediated seed germination (Oh *et al.*, 2004, 2006). When activated by light, the phytochromes accelerate the degradation of PIL5 in seeds, releasing the transcriptional repression of the GA3-oxidase genes and the transcriptional activation of the GA2-oxidase gene, leading to increases in the level of bioactive GA in seeds. Taking into account that there are sufficient polymorphisms at the *PHYB* gene to cause differential plant responses to light (Filiault *et al.*, 2008), we cannot discount *PHYB* (At2g18790) as another candidate gene for *DOG10* and/or *DOG-LF1* into the mapping intervals, and neither can it be discounted that different genes might be responsible for the natural genetic variation at *DOG10* and/or *DOG-LF1* (i.e. *PIL5* and *PHYB*).

CONCLUSIONS

Seed germination is determined by genetic control via complex regulatory networks that continuously integrate environmental signals. The goal of this paper was to explore the natural genetic variation in two populations of recombinant inbred lines in order to identify the loci controlling the induction of germination by light under different chilling and after-ripening treatments. The results presented here establish some novel conclusions. First, one group of loci act in a considerable number of different conditions, whereas a smaller group of light-related loci are effective only under a narrower set of environmental conditions, apparently when the influence of the most-prevalent QTLs is reduced as consequence of changes in the physiological status of the seeds. Second, the identification of specific loci associated with the two action modes of phytochromes that promote germination improves our understanding of the two independent signalling pathways that relate seed germination with specific environmental clues.

SUPPLEMENTARY INFORMATION

Supplementary Information is available online at www.aob.oxfordjournals.org/ as follows. Table S1: three-way ANOVA for germination in the L*er* × Cvi RIL population. Table S2: summary statistics for seed germination (%) in the Bay-0 × Sha RIL population. Table S3: summary of QTLs detected in the Bay × Sha RIL population. Table S4: summary statistics for seed germination (%) in the L*er* × Cvi RIL population. Table S5: Summary of QTLs detected in the L*er* × Cvi RIL population. Fig. S1: environmental interaction factors obtained from ANOVA analysis. Fig. S2: *DOG1* × *DOG8* interaction for 90-d after-ripened seeds incubated for 10 d at 7 °C and then irradiated with a R pulse.

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