

Analysis of Natural Allelic Variation Controlling *Arabidopsis thaliana* Seed Germinability in Response to Cold and Dark: Identification of Three Major Quantitative Trait Loci

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ABSTRACT Light and temperature are key external factors in the control of *Arabidopsis thaliana* seed germination and dormancy mechanisms. Perception and response to these stimuli have to ensure that seedling emergence and growth occur at the most advantageous time for correct establishment. Analysis of over 300 *Arabidopsis* accessions identified 14, from 12 different geographical locations, that were able to germinate to greater than 20% at 6°C in the dark. This natural variation was exploited to identify genetic loci responsible for cold-tolerant, dark germination. A quantitative trait loci approach was used on recombinant inbred line progeny of a cross between Bay-0 and Shahdara. Six distinct quantitative trait loci were identified, three of which were major loci, each responsible for 17–25% of the phenotypic variability in this trait. Parental phenotypes indicated that the majority of the cold-tolerant, dark-germination characteristics are related to light responses. Validation of the three major loci using heterogeneous inbred families confirmed the feasibility of fine mapping and cloning the genes at the quantitative trait loci responsible for cold-tolerant, dark germination.

INTRODUCTION

To ensure that seedlings emerge in conditions favourable for mature plant establishment, environmental cues are integrated into seed dormancy and germination mechanisms (Penfield et al., 2005). Such factors have been shown to condition the seed both directly and via the mother plant (Baskin and Baskin, 1998). In particular, light and temperature are major signals that influence germination control (Donohue et al., 2007).

Germination in the dark will occur for some seeds within a population, excepting a few plant species (Shinomura, 1997). For *Arabidopsis thaliana*, the ability to germinate in the dark can be either dependent on the light perceived by the embryo during its development on the mother plant or light-independent (McCullough and Shropshire, 1970; Hayes and Klein, 1974). The effect of light on seed germination is mainly conveyed by photoreceptors called phytochromes which are synthesized in a biologically inactive red-light (R)-absorbing form—Pr—and converted by R to the active far-red (FR)-absorbing form—Pfr (Borthwick et al., 1952). Activated phytochromes are translocated from the cytoplasm

to the nucleus, where they modulate the activity of transcription factors, thus initiating transcriptional cascades (Ma et al., 2001; Tepperman et al., 2001). In *Arabidopsis*, five genes encode phytochrome apoproteins and PhyA, B and E have been shown to stimulate germination (Shinomura et al., 1994, 1996; Poppe and Schäfer, 1997; Hennig et al., 2002). PhyA mediates the high irradiance response in FR light (Casal and Sánchez, 1998) and the very low fluence response that is activated by a broad spectrum of light (Shinomura et al., 1996), whereas PhyB and E intervene in the R-to-FR reversible low-fluence response (Shinomura et al., 1994; Hennig et al., 2002). Dark germination resulting from preconditioning of developing seeds by light appears to act principally through PhyB (Shinomura et al., 1994; Shinomura, 1997).

A number of phytohormones act as internal regulators of the ability of a seed to germinate or remain dormant: gibberellins

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(GA), ethylene, and brassinosteroids having a positive effect and abscisic acid (ABA) repressing germination (Bentsink and Koornneef, 2002; Kucera et al., 2005). In particular, the balance between the antagonistic effects of GA and ABA determines whether germination will take place, and light has been shown to regulate some of the genes involved in GA or ABA metabolism via phytochrome (Yamaguchi et al., 1998; Oh et al., 2006, 2007; Seo et al., 2006; Yamauchi et al., 2007). In addition, a short cold treatment during imbibition, known as stratification, stimulates *Arabidopsis* germination and has been shown to increase GA biosynthesis through the transcriptional regulation of *GA3ox* genes (Yamaguchi et al., 2001, 2004). The requirement for stratification involves the repression of *GA3ox* expression by the basic helix-loop-helix (bHLH) transcription factor SPATULA (Penfield et al., 2005). Furthermore, germination in the dark after stratification has been shown to be dependent on the expression of another bHLH transcription factor—PIF3-like 5 (PIL5)—which also negatively regulates seed germination by inhibiting *GA3ox* expression (Penfield et al., 2005), in addition to stimulating the expression of two repressors of GA responses and increasing ABA levels in the dark (Oh et al., 2007).

In contrast to stratification, which involves a short cold period during seed imbibition, germination at low temperature can result in chilling injury that prevents seedling development (Nomura et al., 2001). Increased tolerance of germination and emergence at low temperature can enable seedlings to be established in advance of other plants and can be advantageous in wild species competing for light and nutrients (Boyd et al., 2007). In direct-seeded crop species sown in the spring, cold-sensitive germination can significantly affect the number of plants successfully established, as well as the synchronization and subsequent uniformity of a crop (Foolad et al., 1998). Improving crop tolerance to low temperature and the identification of target genes is a major goal for crop breeders worldwide (Nomura et al., 2001). Germination responses to low temperature are, however, not well understood, but the ability to germinate at low temperature probably involves the induction of cold-acclimation mechanisms. Cold acclimation is generally associated with changes in gene-expression levels (Fowler and Thomashow, 2002; Maruyama et al., 2004) and the cold-inducible C-repeat binding factors (CBF) 1, 2, and 3 have been shown to be key transcriptional regulators in this response (Gilmour et al., 1998; Jaglo-Ottosen et al., 1998; Liu et al., 1998; Stockinger et al., 1997). The expression of *CBFs 1-3* is modulated by light through PhyB, and the rate of accumulation of CBF transcripts in response to cold is reduced in complete darkness (Fowler et al., 2005; Kim et al., 2002). In addition, a recent study indicates that phytochrome activity is directly affected by temperature and may, therefore, integrate both temperature and light signals into germination-control mechanisms (Heschel et al., 2007).

Interplay between the various factors that affect germinability at low temperatures in the dark make it a complex genetic trait, appropriate for quantitative trait locus (QTL)

analysis, and cold-tolerant germination has previously been the subject of QTL analyses in rice and tomato (Foolad et al., 1998; Nomura et al., 2001). A number of studies have identified QTL loci in *Arabidopsis* that affect seed dormancy and germination traits (Van Der Schaar et al., 1997; Alonso-Blanco et al., 2003; Clerckx et al., 2004). *Arabidopsis* accessions exhibit extensive diversity in their ability to germinate at different temperatures (Schmuths et al., 2006). Moreover, natural variation has previously been demonstrated to modify PhyA (Malooof et al., 2001) and PhyC (Balasubramanian et al., 2006) activity in this species. In the present study, we have analyzed over 300 *Arabidopsis* accessions for their germination capacities in the cold and dark. QTL mapping was carried out using a recombinant inbred line (RIL) population generated from a cross between Bay-0 and Shahdara (Loudet et al., 2002) and three major QTL were identified. These have been validated genetically and physiologically using Near-Isogenic Lines (NILs) derived from Heterogeneous Inbred Families (HIFs).

RESULTS

Natural Variation in Germinability at Low Temperature in the Dark among *Arabidopsis* Accessions

Screening of *Arabidopsis* accessions for cold-tolerant germination and light requirement was carried out on 309 seed lots from the Versailles Biological Resource Centre (<http://dbsgap.versailles.inra.fr/vnat/>). Seeds from the majority of accessions did not germinate significantly at 6°C in the dark; however, for 15 accessions, seeds germinated to greater than 20%. These were retested with seeds from plants cultivated together and the phenotype was confirmed for 14 accessions (Table 1). Interestingly, 236AV and 271AV, both denoting to the Shahdara population, but corresponding to different single-seed descent (SSD) lines available from different stock centres, varied in their germinability at 6°C in the dark, with the 236AV genotype consistently showing higher germination levels. Comparison of germination percentages with the climatic conditions of the habitats from which accessions were harvested did not indicate a correlation ($R^2 < 0.1$) with either the amount of sunshine or minimum temperatures (Table 1).

Comparison of Germination Characteristics in Bay-0 and Shahdara Accessions

For one accession—Shahdara (236AV)—a RIL population was available which was derived from a cross with the accession Bay-0 (Bayreuth, 41AV) (Loudet et al., 2002) which did not germinate at 6°C in the dark, even after 16 d (Figure 1A). Tests on Bay-0 seeds left for different periods in the light (5 min to 1 h) prior to transfer to 6°C did not stimulate germination significantly (maximum $4 \pm 2\%$). Nonetheless, comparison of the germination rates of the two accessions at 6°C in the light showed that both were able to germinate completely, although the rate of Bay-0 germination was consistently slightly

Table 1. *Arabidopsis* Accessions Capable of Germinating in the Cold and Dark, and Details of the Habitats from which They Were Harvested.

Versailles number	Accession	Harvest site	Climate (monthly average)		Germination at 6°C in the dark (% , ±SE)
			Minimum temperature (°C)	Minimum sunshine (h)	
3	Arg-4	Argentolle, France	1.9	20	66 ± 4
25	Jea	St Jean Cap Ferrat, France	5.8	47	64 ± 3
46	Ko-2	Copenhagen, Denmark	-0.2	10	42 ± 3
107	KI-2	Cologne, Germany	0.9	14	28 ± 3
110	Bs-1	Basel, Switzerland	-0.7	18	26 ± 4
116	Do-0	Donsbach, Germany	-0.7	14	98 ± 1
119	Li-2	Limburg, Germany	-0.3	14	36 ± 1
168	Su-0	Southport, UK	2	16	64 ± 3
170	Mc-0	Mickles Fell, UK	1.1	14	45 ± 3
180	Blh-1	Bulhary, Czech Republic	-1.9	17	89 ± 5
181	Blh-2	Bulhary, Czech Republic	-1.9	17	70 ± 6
236	Shahdara	Shakdara river, Tajikistan	-18.1	30	54 ± 1
255	Niigata	Niigata, Japan	-0.2	32	95 ± 2
271	Shahdara	Shakdara river, Tajikistan	-18.1	30	28 ± 2

Germination percentages are means of three individual measurements. Similar results were obtained in three individual experiments. Climatological data are cited on the Versailles Biological Resource Centre website (<http://dbsgap.versailles.inra.fr/vnat/>) and were obtained by Cramer and Leemans (2001).

slower than that of Shahdara (Figure 1B). Fresh Shahdara seed lots were unable to germinate at 25°C in the dark (data not shown). The cold treatment might, therefore, stratify seeds and remove dormancy. Dormancy is lost during dry seed storage—a process known as after-ripening—and seed lots that had been after-ripened for two different time periods were examined. Shahdara seeds were able to grow at 20°C in the dark, unlike those of Bay-0 (Figure 1C), and the longer the period of after-ripening, the higher the level of germination. To determine whether the light requirement for germination in the Bay-0 accession is related to differences in GA biosynthesis or perception in the dark compared to Shahdara, the effect of exogenous GA was examined. Incorporation of GA in growth media stimulated Bay-0 germination in a concentration-dependent manner (Figure 2); GA also increased Shahdara germinability.

QTL Mapping Using a RIL Population

The ability to germinate at 6°C in the dark was analysed using F₇ seed lots from 417 Bay-0×Shahdara RILs and the parental lines cultivated together. Interestingly, these Shahdara seed lots showed increased germination at 6°C in the dark compared to those analyzed previously (Table 1), with germination at 88–100% (Figure 1A and data not shown); Bay-0 germination was unaltered. The L-shaped distribution of phenotypes indicated a bias in the population towards the Bay-0 phenotype, with 38% of the population having a seed germinability of 10% or less at 6°C in the dark (Figure 3) and the average germination of the RIL population was 35%. The heritability of cold, dark germination capacity was 0.96, based on the three replicates obtained from seed batches harvested from

plants cultivated together. This probably represents an overestimation of the heritability value, since it does not take into account variation arising from maternal environmental effects which can be observed when seed batches cultivated independently are used.

F₆ plants from the RIL population had previously been genotyped for a set of 38 physically anchored microsatellite markers (Loudet et al., 2002; www.inra.fr/vast/). Using these genotyping data available for 411 of the RILs (Loudet et al., 2002), six QTLs termed CDG (Cold-tolerant Dark Germination) were mapped which had LOD scores greater than 2.2 (Table 2); in total, these explained 67% of the total phenotypic variance. Three QTLs made high contributions to cold-tolerant germination in the dark (>17% of the total variance each), and were highly significant (score above 20 LOD). The Bay-0 allele was responsible for reducing germination at all three of these major QTLs and in four out of the six QTLs detected, accounting in total for 63% of the variance (Table 2). The segregation of the two remaining QTLs (CDG-5 and CDG-6), though associated with very small effects, could theoretically result in some transgression. However, the restriction of the trait measured by the limits of the germination percent range in the conditions chosen probably prevented the observation of such a phenomenon. As no significant epistatic interactions were detected at the 1% error rate (data not shown), there is no clear explanation for the bias observed towards the Bay-0 non-germinating phenotype in the RIL population (Figure 3). Detailed analysis of the three CDG loci relative effects did, however, indicate that a threshold effect might occur; the effect of adding one Bay-0 allele at one of the three CDG QTLs in a genetic background which was already fixed with two Bay-0 alleles at the

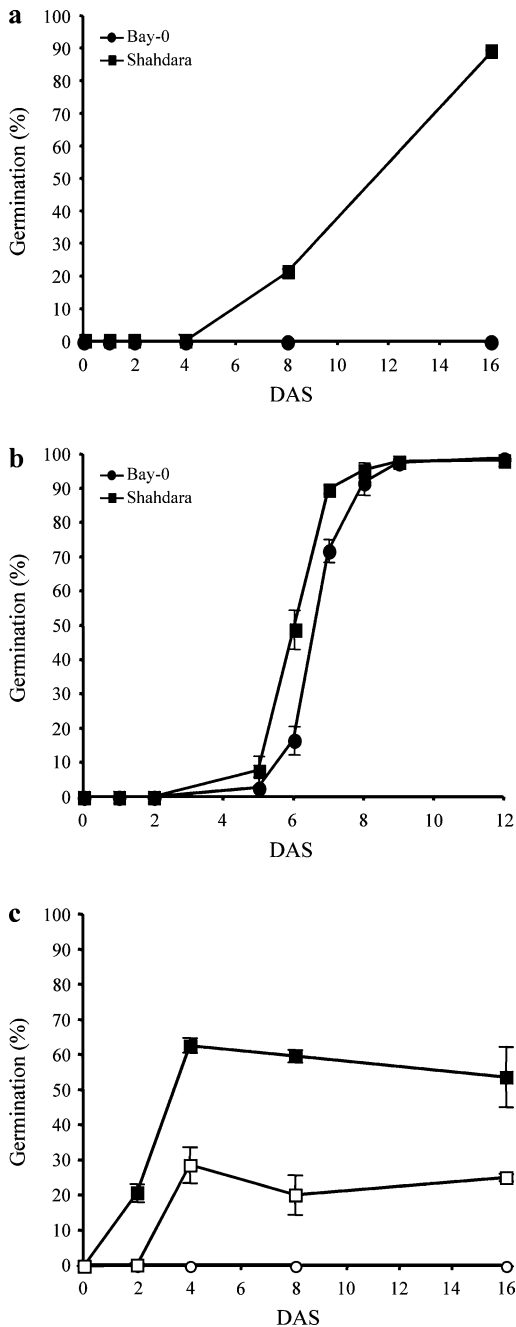


Figure 1. Light-Dependence and Cold Sensitivity of Bay-0 and Shahdara Seed Germination.

Germination was determined after incubation at either 6°C (A and B) or 20°C (C) in either the dark (A and C) or under constant light (B). Rates were scored based on the number of seeds with protruding radicles compared to the total number of seeds sown. Seed lots used were obtained from plants cultivated with F₇ RILs (A and B) or had been after-ripened (4°C, 30% relative humidity) (C) for 3 years (open symbols) or 4 years (closed symbols). Bay-0, circles; Shahdara, squares; DAS, days after sowing. Error bars represent SE values (*n* = 3) and where not visible, are smaller than the data symbol.

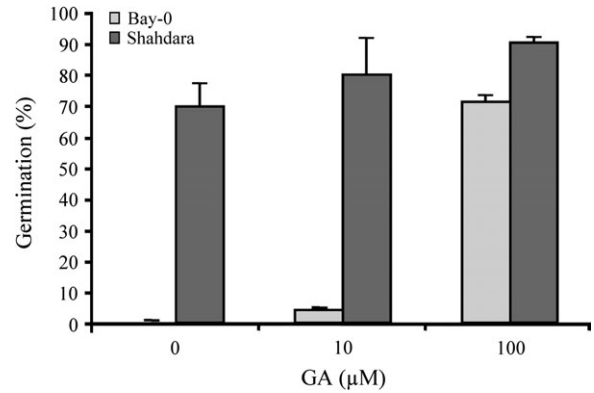


Figure 2. Stimulation of Bay-0 and Shahdara Cold, Dark Germination by Gibberellins.

Germination percentages were scored after 16 d in the dark based on the number of seeds with protruding radicles compared to the total number of seeds sown. GA, gibberellins 4 and 7. Error bars represent SE values (*n* = 3).

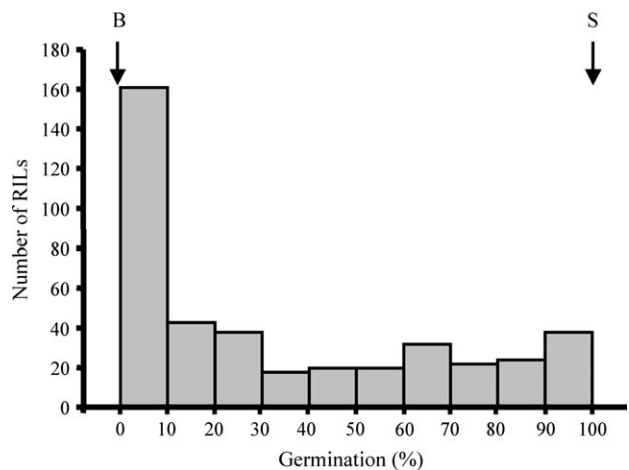


Figure 3. Frequency Distribution of the Mean Germination Percentage for Bay-0×Shahdara Recombinant Inbred Lines at 6°C in the Dark.

Arrows depict the means of the parental lines (S, Shahdara; B, Bay-0). RILs, recombinant inbred lines.

other CDG loci was relatively small. This was probably why one of the first-order interactions (CDG1×CDG3) and the second-order CDG1×CDG2×CDG3 interaction were close to the 0.05 significance level, but clearly above 0.01.

Confirmation of Three Major QTLs Using Near-Isogenic Lines

F₆ RILs were chosen which were heterozygous around CDG-1, CDG-2, or CDG-3, but fixed as homozygous in the rest of the genome. This corresponded to RILs 046, 128, and 194 for CDG-1, RILs 010, 102 and 422 for CDG-2 and RILs 090, and 156 for CDG-3 (Figure 4A–4C). F₇ offspring from each of these RILs

Table 2. Characteristics of Mapped QTLs that Account for Germination at Low Temperature in the Dark in the Bay-0×Shahdara Population.

QTL	Chromosome	Marker	1-LOD support interval (cM)	LOD score	% of variance	Additive effect
CDG-1	1	MSAT1-10	9-14	32	25	-33.6
CDG-2	2	MSAT2-36	21-37.5	24.3	19	-29.2
CDG-3	4	MSAT4-15	35-41	22.3	17	-28.4
CDG-4	3	MSAT3-21	43-63	3.3	2	-10.2
CDG-5	5	MSAT5-9	44-58	3.3	2	+9.4
CDG-6	4	MSAT4-8	0-12.5	2.3	2	+9.2
% of total variance					67%	

CDG, cold-tolerant, dark germination. The position in centiMorgans (cM) is that from the first marker on the chromosome. The additive effect represents the mean effect in % germination of the replacement of both Shahdara alleles by Bay-0 alleles at the QTL.

were genotyped with appropriate markers in the region of interest, as described by Loudet et al. (2005), and HIF members that were fixed for the markers tested as either the Bay-0 or Shahdara allele were used as NILs. These NILs were subsequently phenotyped for germination at 6°C in the dark using seed harvested from each individual (F_3 seed) (Figure 4D-4F). The three HIFs developed for CDG-1 all showed a difference in the level of germination at 6°C in the dark between the Bay-0 and Shahdara alleles. Only HIF046, however, exhibited the expected higher level of germination associated with the Shahdara allele, in agreement with the QTL analysis indicating that the Bay-0 allele reduced germination at this locus. The HIF046 would be appropriate, therefore, for fine mapping of the QTL. Similarly, the CDG-2 QTL was confirmed by HIF010 and HIF422, and the CDG-3 QTL by HIF156. Furthermore, the difference between homozygous Bay-0 and Shahdara genotypes for these four HIFs were similar to the allelic effects predicted for each loci by QTL analysis.

DISCUSSION

Light and temperature are two major constraints on seed germination, yet our understanding of how these environmental factors are integrated into germination mechanisms is limited. To determine the extent of *Arabidopsis* variability in germination capacity at chilling temperatures in the dark, seed lots from over 300 accessions were examined. Analysis of the germination of 73 accessions in the light (long-day conditions) at 10, 18, and 26°C has previously shown that large natural variations in seed germinability occur in *Arabidopsis* with respect to temperature (Schmuths et al., 2006). In contrast, here, only a few accessions showed significant germination at 6°C, with only 14 accessions having greater than 20% germination (Table 1). As the minimal temperature used in germination tests by Schmuths et al. (2006) was 10°C, the comparatively limited degree of variation observed in our study could reflect the strong repression of germination in *Arabidopsis* at chilling temperatures. Nonetheless, Bay-0 seed lots that did not germinate at 6°C in the dark showed high levels of germination at

6°C in the light (Figure 1A and 1B) and, conversely, no germination at 25°C in the dark (data not shown). In addition, comparison of the germination percentages in the 36 lines common to our analyses and those by Schmuths et al. (2006) found no correlation between our results and those obtained at 10°C for seeds preconditioned during their development at either 14 or 22°C ($R^2 = 0.09$ and 0.02 , respectively). The absence of germination for the majority of accessions, therefore, probably reflects a strong light requirement for germination in *Arabidopsis*.

A period of chilling during seed imbibition is known to abolish seed dormancy (Cone and Spruit, 1983) and one effect of the cold treatment could be to stimulate Shahdara germination by removing dormancy. In effect, germination was not observed for fresh Shahdara seed lots in the dark at 25°C, but was consistently high at 6°C (data not shown and Figure 1A). Accordingly, after-ripened Shahdara seeds showed higher levels of germinability at 20°C, which increased with the period of after-ripening (Figure 1C). The cold treatment could modulate the balance between ABA and GA in Shahdara so that germination becomes possible without a light stimulus, suggesting a modification of light requirement. Furthermore, modification of this balance by exogenous GA application stimulated both Bay-0 and Shahdara germination in the dark (Figure 2), indicating that GA biosynthesis or perception is a limiting factor for dark germination.

Variation was observed between two different lines corresponding to Shahdara (236AV and 271AV). These genotypes originated from the NASC and ABRC stock centres, respectively, and had been produced by SSD from the Shahdara seeds collected initially. The variability between the two different Shahdara accessions, therefore, is probably due to variability between the original two individuals chosen for SSD and indicates that variation for cold-tolerant germination in the dark exists within the Shahdara population in the wild, although it is possible that the genetic variation appeared and was fixed during the few cycles of SSD at one or other stock centre. Furthermore, differences were found in the germination capacity of different seed lots for the Shahdara accession (236AV).

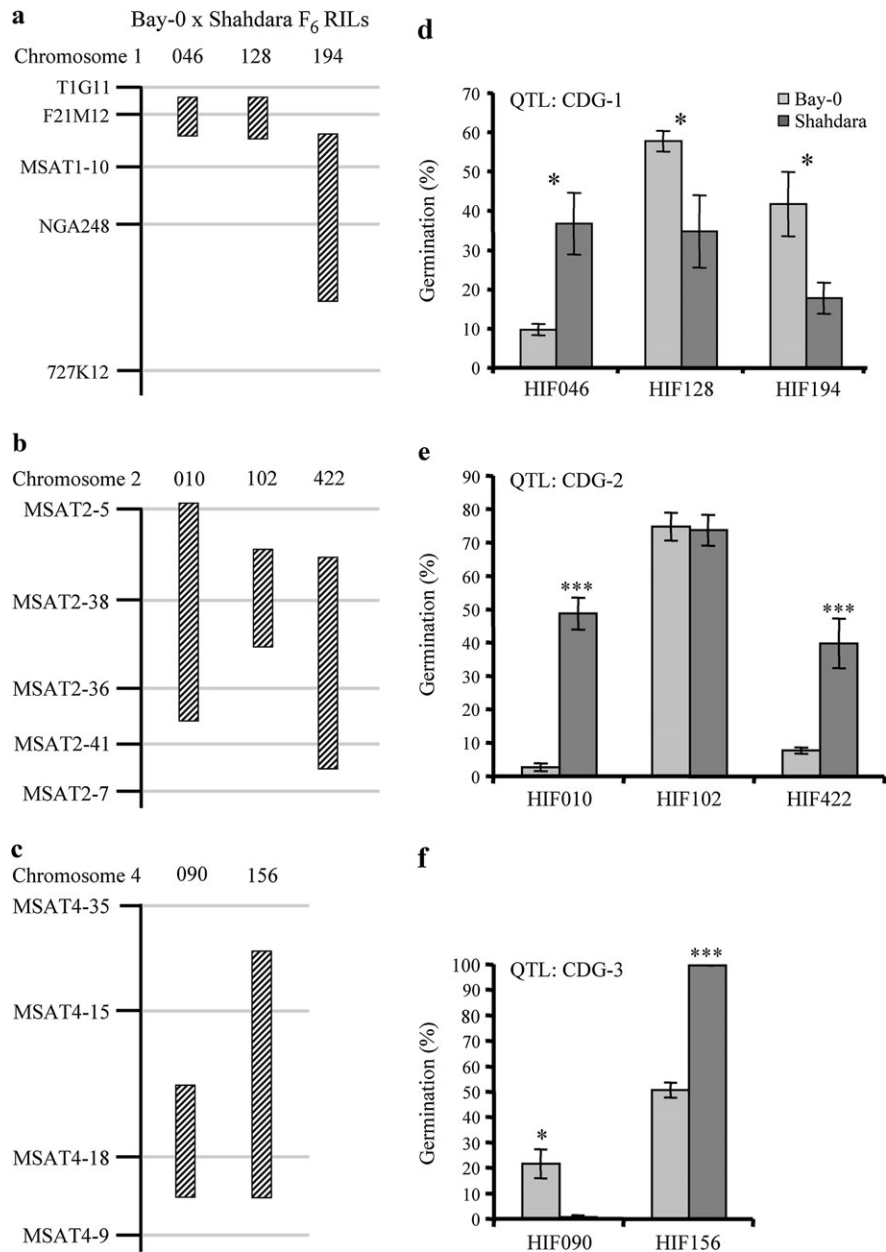


Figure 4. Confirmation of the Major QTLs CDG-1, CDG-2, and CDG-3 by Comparison of the Phenotypes of Near-Isogenic Lines Derived from Heterogeneous Inbred Families (HIF).

(A–C) F₆ recombinant inbred lines (RILs) from the Bay-0×Shahdara cross which show residual heterozygosity in the region of the QTL for CDG-1, CDG2, and CDG3, respectively. Black lines represent chromosomes with markers and intervals indicated to the right. Marker positions and identity can be found at www.inra.fr/vast/ in the MSAT database. Numbers designate the RIL, with the hatched bar beneath indicating the region which is still heterozygous. Recombination breakpoints delimiting heterozygous regions are arbitrarily depicted in the middle of the marker interval.

(D–F) Comparison of germination at 6°C in the dark for HIFs which segregated in the region shown in (A) to (C) and which had been fixed for the Bay-0 or the Shahdara allele. Error bars represent SE values ($n = 3$). Significance in t -tests at *5% level or ***0.1% level.

Notably, the lots grown with other accessions germinated less well than those cultivated with F₇ RILs (Table 1, Figure 1A and data not shown). A preconditioning effect on seed germination by the temperature at which the mother plant was cultivated has been shown to influence germination capacity and plants culti-

vated at lower temperatures produce seed that germinate less well (Schmuths et al., 2006; Donohue et al., 2007). The differences in temperature and range of temperatures between the glasshouses used to generate the different seed lots could, thus, have caused the differences noted in germination capacity.

To determine the genetic basis of the ability of some *Arabidopsis* accessions to germinate at chilling temperatures in the dark, this trait was analyzed in a RIL population derived from a cross between Bay-0 and Shahdara. These accessions showed patent differences in their seed germination capacity at 6°C in the dark, Bay-0 being unable to germinate (Figure 1A) and Shahdara showing 28–100% germination (Table 1, Figure 1A and data not shown). This was clearly explained genetically as the majority of the six QTLs mapped (CDG-1 to -6; Table 2) showed negative allelic effects; the Shahdara allele increased germination capacity relative to the Bay-0 allele, resulting in a complex genetic basis for what might appear to be a simple trait variation between the parents. Only two minor QTLs showed allelic effects that contrasted with the strong parental phenotypes and would probably have caused some transgression if trait range limits and precision were sufficient. Additional QTLs might have been detected if multiple RIL seed lots had been used that had been generated from plants cultured independently.

The variability in RIL germination showed a strong bias towards non-germination, as demonstrated by the L-shaped frequency distribution (Figure 3). The high number of individuals found at the lower limit could be indicative of epistatic interactions between major QTLs; however, analysis of the RILs using the 'Pair-Scan' function from the WebQTL program did not detect any significant epistatic interactions at the 1% error rate (data not shown). Another possible explanation for this distribution of phenotypes is that the variation measured for germination percent was not linearly related to the contribution of individual QTLs and showed a threshold effect, so that the 0–10% phenotypic class actually grouped individuals with different intrinsic genotypic and additive values. This might also be accompanied by interactions with the maternal genome, which can be involved in the establishment of traits related to seed germination (Boyd et al., 2007). The RILs were generated from a cross with Bay-0 as the mother plant and, thus, all have Bay-0 cytoplasm. This could also explain why only a fraction of the RILs with the Shahdara allele at the three major QTLs were close to the Shahdara-phenotypic value.

The observations that cold treatment or after-ripening is required for Shahdara germination in the dark (Figure 1C and data not shown) and that Bay-0 can be stimulated to germinate in the cold and dark by the exogenous GA (Figure 2) indicate that these suppress dormancy and the light requirement for germination. The CDG loci identified could, therefore, be involved in dormancy mechanisms and correspond to loci previously identified in QTL analyses of dormancy and germination traits. A QTL for dark germination has previously been identified in *Arabidopsis* on chromosome 2 and this co-localized with a smaller QTL for germination in the light (Van Der Schaar et al., 1997). This QTL was situated between 39 and 59 cM and is unlikely, therefore, to correspond to CDG-2. Nonetheless, CDG-1 was localized to the same genomic region as the *Arabidopsis* delay of germination loci, DOG2 and DOG3 (Alonso-Blanco et al., 2003), and QTL that affect controlled de-

terioration or germination speed (Clerx et al., 2004). No previously identified locus that affects germination characteristics has been assigned to the regions corresponding to CDG-2 or CDG-3. Moreover, analysis of low-temperature germination has previously been used to identify QTLs in rice and tomato (Foolad et al., 1998; Nomura et al., 2001) and three or five QTLs, respectively, have been identified. It will be interesting to see whether common mechanisms are employed in these species and *Arabidopsis* for germination at chilling temperatures.

Three of the CDG loci were major QTLs and are ideal candidates for QTL cloning, the first step of which is usually to confirm and mendelize the QTL. Several independent HIFs were used to validate each of the three loci (Figure 4). All the HIFs did not show the predicted higher levels of germination at 6°C in the dark for the Shahdara fixed NIL compared to the Bay-0 fixed NIL. As no significant strong epistatic relationships involving the QTLs were detected, and specifically no reversed effect due to epistasis, the simplest explanation is that the heterozygous region segregating in each HIFs does not necessarily include the QTL itself. Different HIFs could include different linked loci with contrasted effects within the QTL interval; the position of the heterozygous regions varies and the exact location of their borders between adjacent markers is not known precisely. As in similar multiple-HIF tests published previously (Calenge et al., 2006; Loudet et al., 2005; Perchepepied et al., 2006), at least one HIF confirmed each locus and could be used to fine-map the three different loci. Nonetheless, based on our knowledge of the factors that can affect cold-tolerant germination in the dark, a candidate-gene approach can also be envisaged. Indeed, the strong light requirement highlighted by the germination characteristics of the Bay-0 parent (Figures 1 and 2) together with the negative effect of the Bay-0 allele at these loci suggests that CDG-1 to -3 may result from variation in genes that bypass the light requirement for germination. Interestingly, the gene for the phytochrome PhyB implicated in the induction of germination by light is found in the interval of the major QTL CDG-2. Furthermore, the *PIL5* gene is also found in the interval encompassing CDG-2 and stratified seeds of *pil5-1* mutants germinate in the dark (Penfield et al., 2005).

The identification of novel loci that modify development and growth of seedlings in response to light and temperature signals will be important for improving our understanding of the incorporation of these stimuli into germination control. Furthermore, such loci should contribute to our ability to select varieties of direct-seeded crops with cold-tolerant germination. The three major QTLs identified in this study are potential candidates for such a strategy and fine-mapping and cloning of the loci are underway.

METHODS

Plant Material

Seeds for accessions were obtained from the Versailles Biological Resource Centre for *Arabidopsis* (<http://dbsgap.versailles.>

inra.fr/vnat/) and corresponded to stocks 1AV to 319AV (Versailles codes), excepting numbers 73, 93, 148, 270, 277, 292, 293, 300, 310 and 311. The Ko-2 (#46AV), Kl-2 (#107AV), Bs-1 (#110AV), Do-0 (#116AV), Li-2 (#119AV), Su-0 (#168AV), Mc-0 (#170AV), Blh-1 (#180AV), Blh-2 (#181AV), Shahdara (#236AV), and Shahdara (#271AV) accessions are equivalent to the NASC # N1288, N1278, N996, N1112, N1312, N1540, N1362, N1030, N1054, N929, and CS6180 stocks. Seeds from accessions cultivated together were harvested from plants grown in a glasshouse (18–28°C) with a minimum photoperiod of 13 h assured when required by supplementary lighting. Plants were grown in compost (Tref substrates, Rotterdam, Netherlands) and watered with Plan-Prod nutritive solution (Fertil, Boulogne-Billancourt, France). Seeds from the complete Bay-0×Shahdara RIL set (<http://dbsgap.versailles.inra.fr/vnat/>) were phenotyped using the F₇ seeds produced from plants cultivated together in a glasshouse, as described in Loudet et al. (2002), with a temperature range of 19–20°C.

HIFs were developed from individual F₇ RILs that still segregated in a single and limited genomic region (Loudet et al., 2005; Tuinstra et al., 1997). For each RIL (for example, RIL046), several plants were sown and genotyped individually for the appropriate markers across the segregating region indicated in Figure 4 (F21M12 for RIL046). Two to three independently fixed plants for each allele were chosen and allowed to self-fertilize. Seeds from these plants were then phenotyped as described below to identify the phenotypic effect of Bay-0 versus Shahdara alleles in the segregating region. HIF090 was generated previously (Loudet et al. 2005).

Phenotyping for Germinability in the Cold

To phenotype for seed germinability in the cold and dark, 50–100 surface-sterilized seeds were sown in triplicate on 0.5% (w/v) agarose plates, which were then wrapped in aluminium foil and placed in a lightproof container after a maximum ambient light exposure of 10 min, excepting experiments where the length of exposure to light was investigated. The lightproof container was then placed at 6°C for 16 d, after which germination was scored based on radicle emergence. The 417 RILs were phenotyped in five overlapping sets over a 6-week period, including connecting genotypes as controls. There was no block effect, as all plates were incubated together in a closed container. In the absence of a strong ‘set’ effect, the phenotypic means calculated for each RIL were used for QTL analysis. Viability of seed lots was confirmed either by sowing an additional plate for each seed lot on solid Gamborg B5 media with 29 mM sucrose (Duchefa, The Netherlands) and incubation in a growth chamber, or by subsequent transfer of seeds to a growth chamber, photoperiod 16 h light at 20°C, 8 h dark at 18°C, 70% RH, 250 mmol m⁻² s⁻¹. Accessions whose germination levels at 6°C in the dark were higher than 20% were retested using seed lots from plants that had been grown simultaneously.

Seeds for germination assays at 6°C in the light were sown as described above on 0.5% (w/v) agarose and placed in a growth

chamber at 6°C, under constant light (5 mmol m⁻² s⁻¹). For germination assays in the dark, all plates were sown and placed in at 6°C at the same time and a series of plates were then removed and counted at each individual time point. The GA₄₊₇ (Duchefa, The Netherlands) used in germination assays was prepared as a stock in 50% (v/v) ethanol and added to the concentrations indicated. All plates, including 0 GA, were supplemented with an equivalent volume of 50% (v/v) ethanol. After-ripened seed stocks had been stored at 4°C, 30% RH for either 3 or 4 years prior to use. Germination was scored at regular intervals based on radicle protrusion.

Statistical Analyses and QTL Mapping

The complete set of data obtained using the RILs was included in an analysis of variance (ANOVA) model to determine the specific effects of the ‘genotype’ and ‘environmental’ factors. This ANOVA enabled us to quantify the broad-sense heritability (genetic variance/ total phenotypic variance). Subsequent analyses used mean values from germination assays carried out in triplicate for each line. ANOVA estimations were obtained using the *aov()* function of the S-PLUS version 3.4 statistical package (Statistical Sciences, Seattle, Washington).

The original set of 38 microsatellite markers and the genetic map obtained with MAPMAKER 3.0 (Loudet et al., 2002, <http://dbsgap.versailles.inra.fr/vnat/>) were used in this study. QTL analyses were performed using the Unix version of QTL CARTOGRAPHER 1.14 (Basten et al., 2000). The standard methods for interval mapping (IM) and composite interval mapping (CIM) were used as described previously (Loudet et al., 2002). First, IM (Lander and Botstein, 1989) was carried out to determine putative QTL involved in the variation of the trait. CIM model 6 of QTL CARTOGRAPHER was then performed on the same data: the closest marker to each local LOD score peak (putative QTL) was used as a cofactor to control the genetic background while testing at another genomic position. When a cofactor was also a flanking marker of the tested region, it was excluded from the model. The number of cofactors involved in our model was six. The walking speed chosen for QTL analyses was 0.1 centimorgans. The LOD significance threshold (2.2 LOD) was estimated from several permutation test analyses, as suggested by Churchill and Doerge (1994). Additive effects of detected QTL were estimated from CIM results as representing the mean effect of the replacement of the Shahdara alleles by Bay-0 alleles at the studied locus. The contribution of each identified QTL to the total variance (R²) was estimated by variance component analysis, using phenotypic values for each RIL. The model used the genotype at the closest marker to the corresponding detected QTL as random factors in ANOVA. Only homozygous genotypes were included in the ANOVA analysis. QTL×QTL interactions were searched for in the Anova analysis as well as using the ‘Pair-Scan’ function in WebQTL tool (<http://www.genenetwork.org/>).

As the distribution of the phenotypes across the RIL population was not normal (Figure 3), we improved normality using an arcsine square-root function, often used for the

transformation of traits measured as percentages. Distribution of residuals after transformation was very close to normality, and hence confirmed the validity of the results obtained. All analyses were performed on non-transformed as well as transformed data, with no significant differences obtained. The data presented are from non-transformed data.

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