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Cold and Light Control Seed Germination through the bHLH Transcription Factor SPATULA

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Summary

Background: Plants integrate signals from the environment and use these to modify the timing of development according to seasonal cues. Seed germination is a key example of this phenomenon and in *Arabidopsis* is promoted by the synergistic interaction of light and low temperatures in dormant seeds. This signaling pathway is known to converge on the regulation of the gibberellin (GA) biosynthetic genes GA3 oxidase (*GA3ox*), whose expression is transcriptionally induced by light and cold in imbibed seeds. However, the molecular basis of this response has until now been unknown.
Results: Here we show that the bHLH transcription factor SPATULA is a light-stable repressor of seed germination and mediates the germination response to temperature. Furthermore, SPT is required in dormant seeds for maintaining the repression of *GA3ox* transcription. We also show that the related protein PIL5 represses seed germination and *GA3ox* expression in the dark.
Conclusions: We conclude that SPT and PIL5 form part of a regulatory network coupling seed germination and *GA3ox* expression to light and temperature signaling in the seed.

Introduction

A key feature of plant adaptive fitness is the ability to synchronize the onset of vegetative and reproductive development with seasonal changes in the environment. The commencement of vegetative development is controlled by a period of quiescence in the mature seed; this period is known as seed dormancy. During dormancy, seed germination does not occur even though local conditions are capable of supporting radicle emergence from the seed coat. The period of dormancy of many plant seeds is terminated by environmental signals including light, temperature, and nutrient availability, a system adapted to the promotion of germination only when conditions are optimal for seedling establishment and reproductive success. In particular, the role of light and temperature in the promotion of germination in dormant seeds is highly conserved among seed plants from angiosperms to gymnosperms, demonstrating the importance of germination control as a vital adaptive trait in plants [1].

Like those of many plant species, dormant seeds of the model plant *Arabidopsis* require both light and cold for germination, whereas neither treatment applied alone effectively stimulates germination. The most active dormancy-breaking wavelengths are in the red region of the spectrum, and the signal is transduced via the phytochromes [2, 3]. No regulators of the seed response to temperature have yet been described. It is well established that synthesis and perception of the phytohormone gibberellin (GA) is essential for light and cold responses leading to seed germination. GA is required for seed germination [4, 5], and applied GA can break seed dormancy in *Arabidopsis* and partially substitute for cold or light treatments [6]. Furthermore, GA biosynthesis is regulated by both light and temperature at the level of transcription in the imbibed seed. The final step in active GA biosynthesis is catalyzed by the enzyme gibberellic acid 3-oxidase (*GA3ox*), and importantly, the expression of both seed-expressed *GA3ox* isoforms is promoted by both light and low temperatures (known as cold stratification) [7–9]. Light and cold have also been shown to increase the levels of bioactive gibberellins in the seed [9–11].

Here we describe the role of the basic helix-loop-helix transcription factor SPATULA (SPT) in the control of the germination of dormant seeds by light and temperature. We show that SPT is a multifunctional transcription factor, acting as a light-stable repressor of *GA3ox* expression controlling seed responses to cold stratification and, to a lesser extent, red light. SPT also acts as a key regulator of carpel development [12] and, as shown here, of the expansion of cotyledons and petals. In addition, we characterize the role of the related transcription factor PIL5 in germination control and find that although not required for seed dormancy in the light, PIL5 is vital for the repression of germination in the dark after cold stratification.

Results

SPATULA Controls the Germination Response to Cold and Light

To identify factors controlling seed germination, we isolated mutants in uncharacterized regulatory genes represented in seed EST collections and microarray data. One of these was the bHLH transcription factor *SPATULA (SPT)*, previously characterized for its role in fruit development [12, 13]. *spt* mutants are known to exhibit short siliques with a reduced pollen-transmitting tract, yet the expression of SPT in the seed during germination (Figure 1A), as well as vegetative and non-fruit reproductive tissues, indicated a wider role for SPT than previously described. In order to further investigate the role of SPT in the seed, we obtained an allele designated *spt-10* from the Cold Spring Harbor collection [14].

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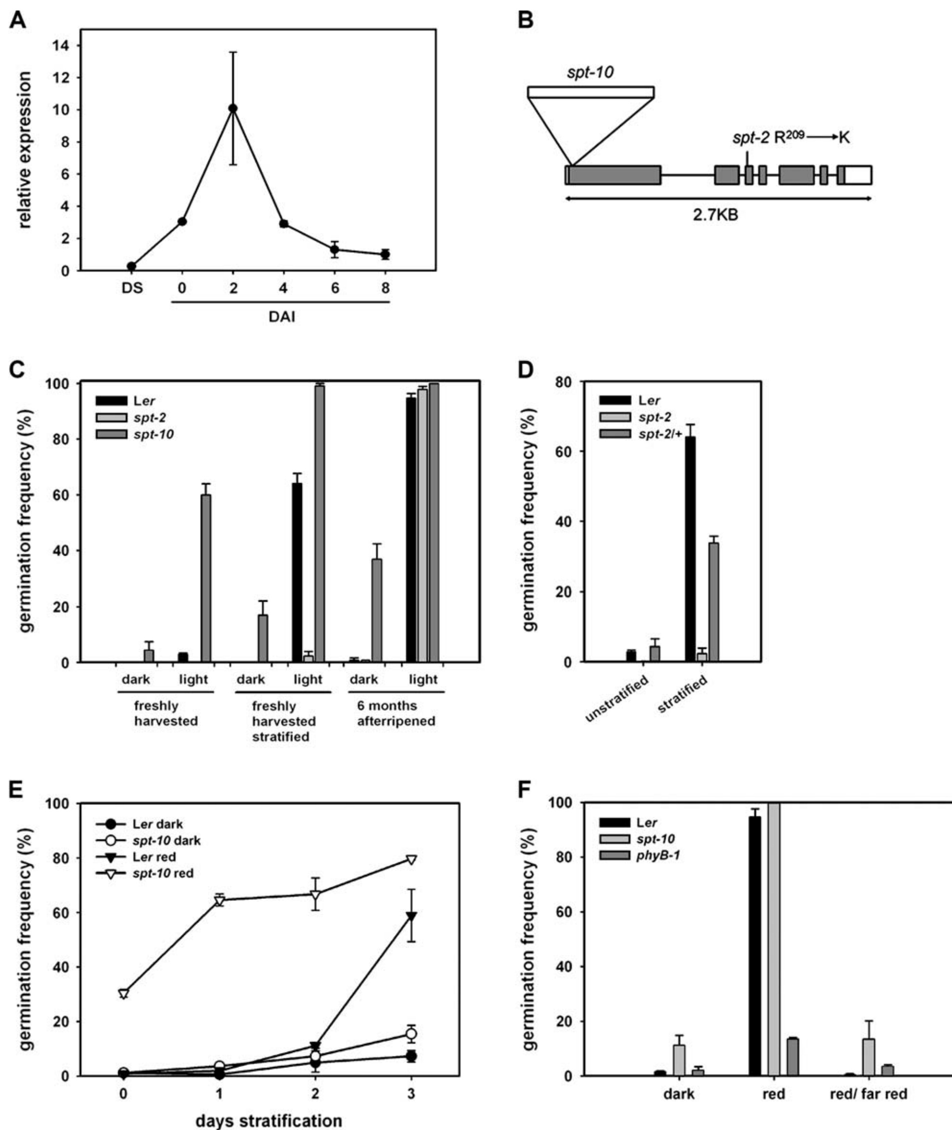

Figure 1. SPATULA Is Expressed in Imbibed Seed and Controls the Response to Stratification

(A) Real-time RT-PCR showing SPT expression during seed imbibition and germination. DS, dry seed; DAI, days after imbibition. Data points represent the mean and standard deviation of three replicates.

(B) Scheme illustrating the position of the *spt-2* and *spt-10* mutants.

(C) The germination phenotype of freshly harvested and after-ripened wild-type, *spt-2*, and *spt-10* seeds in response to cold stratification and white light.

(D) The response of freshly harvested seeds heterozygous for the *spt-2* mutation to stratification in white light.

(E) The response of freshly harvested L*er* and *spt-10* seed to stratification in the dark and after red-light treatment.

(F) Far-red reversal of red-light-induced germination in L*er*, *spt-10*, and *phyB-1*. Error bars for (B)–(F) represent the standard deviation of 3–5 replicate seed batches.

This contains a stable transposon insertion in the first exon, and the full-length transcript could not be detected. The siliques of *spt-10* closely resemble those of the previously described *spt* loss-of-function mutants *spt-1* and *spt-3*. The second, *spt-2*, has been previously characterized and is predicted to result in an amino acid substitution in the putative DNA binding domain of SPT (Figure 1B). Interestingly, *spt-2* mutants exhibit a stronger fruit phenotype than putative *spt* null alleles, suggesting that *spt-2* has a dominant-negative effect on fruit development [12].

Using *spt-10* and *spt-2*, we analyzed the role of SPT in the control of seed dormancy and germination. Freshly harvested wild-type (L*er*) seed exhibited dormancy and did not germinate without both light and cold stratification (Figure 1C). Although freshly harvested *spt-10* mutant seeds were mostly dormant in the dark, they displayed a consistent, strong reduced-dormancy phenotype in the light: i.e., in the presence of light, *spt-10* seed did not require cold stratification for germination (Figure 1C). In contrast, the germination of freshly harvested *spt-2* seeds resembled wild-type in both the light and the dark. After cold stratification, both the wild-type and the *spt-10* mutant germinated at high frequency in the light, whereas *spt-10* also germinated at low frequency in the dark. Surprisingly, freshly harvested

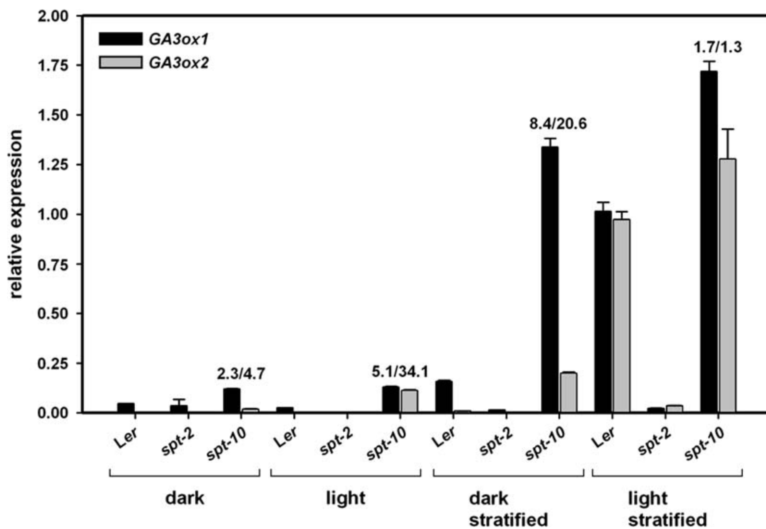

Figure 2. Real-Time RT-PCR Showing the Transcript Abundance of *GA3ox1* and *GA3ox2* in Imbibed Seeds prior to Germination

Figures above the *spt-10* data show the relative increase in the *spt-10* mutant in *GA3ox1* and *GA3ox2* expression, respectively, as compared to the wild-type under the same conditions. Expression is shown relative to the expression in light-treated stratified wild-type seeds, which was set to 1. Error bars represent the standard deviation of three determinations.

spt-2 seeds were found to be completely unresponsive to stratification, both in the presence of light and in the dark. Seeds heterozygous for *spt-2* showed a stratification response that was intermediate between that of *spt-2* and the wild-type (Figure 1D); hence, we concluded that in the context of seed germination *spt-2* behaves as a semi-dominant gain-of-function mutant.

Next, we examined the germination of after-ripened wild-type, *spt-2*, and *spt-10* seeds. After 6 months storage, L*er* still required light for germination but no longer required cold stratification (Figure 1C). Loss of *SPT* appeared to affect the light dependency of after-ripened seed; unlike wild-type seed, after-ripened *spt-10* mutant seed germinated at a significant rate in the dark. Strikingly, *spt-2* seeds stored for a similar period now behaved like the wild-type and germinated at high frequency in the light, but not in the dark. Hence, we concluded that the primary consequence of the *spt-2* mutation is the attenuation of the stratification response because *spt-2* only negatively affects germination when stratification is essential. Indeed, experiments on partially after-ripened *spt-2* seed confirmed that no promoting effect of stratification is ever observed on the germination of the *spt-2* mutant seed (data not shown). The *spt* mutant phenotypes were not maternally inherited, ruling out a role for SPT in the seed-coat control of dormancy ([15]; data not shown). These data show that SPT has an important function in the control of seed germination in response to cold stratification; as such, it is the first regulatory gene to be described with such a role. Of equal importance, the *spt-2* mutant shows that germination promotion by stratification and after-ripening are genetically separable in *Arabidopsis*.

To further enhance our understanding of the role of SPT in the control of dormancy breakage, we analyzed the germination of freshly harvested wild-type and *spt-10* mutants over a range of stratification times in the dark or after 10 s of red light (Figure 1E). Little wild-type germination was seen in the dark, and 3 days of stratification only promoted a modest increase in *spt-10* germination in the absence of light. A combination of 3 days of stratification and red light was required for significant germination of L*er*. In the *spt-10* mutant, red light promoted significant germination without any cold stratification at all, and short periods of chilling promoted high levels of germination. Hence, we concluded that the primary consequences of loss of *spt-10* function were a lack of a chilling requirement for germination and hypersensitivity to applied chilling. This was manifested predominantly in the elevation of the *spt-10* germination response to light (Figure 1E). Red-light-induced germination in *spt-10* was fully far-red reversible (Figure 1F). However, *spt-10* seeds also exhibited a low rate of germination in the dark, and this low rate was unaffected by a far-red pulse, demonstrating that one function of SPT is to repress germination to a small but significant extent in the absence of light. Thus, SPT has a role in coupling seed germination to the light response.

SPATULA Is a Repressor of *GA3ox* Expression in Dormant Seeds

One of the key targets of light and cold signaling in the seed is the promotion of GA biosynthesis through the transcriptional regulation of *GA3ox* [7, 9]. We used real-time RT-PCR to determine the expression of both *GA3ox1* and *GA3ox2* in imbibed wild-type and *spt* mutant seeds 24 hr after imbibition at 20ºC or 24 hr after transfer to 20ºC after 3 nights of stratification (Figure 2). In contrast to previous analyses, we found that both *GA3ox1* and *GA3ox2* required the synergistic effect of light and low temperatures for high expression in dormant L*er* seeds. This correlates well with observed germination under these conditions (Figure 1C). In unstratified seeds exposed to light, no increase in *GA3ox* expression was observed in the wild-type, yet in the *spt-10* mutant 5- and 20-fold increases were observed in *GA3ox1* and *GA3ox2,* respectively, when compared to L*er*. This correlates well with the germination phenotype of the *spt-10* mutant and demonstrates that SPT functions as a repressor of both *GA3ox1* and *GA3ox2* expression in dormant seeds in the light. *spt-10* also had increased *GA3ox* transcript levels in dark-stratified seeds, which in the *spt-10* mutant also exhibit slightly increased germination compared to the wild-type. When stratified seeds were exposed to light, a large increase in *GA3ox* expression was seen in L*er* and *spt-10*, but not the

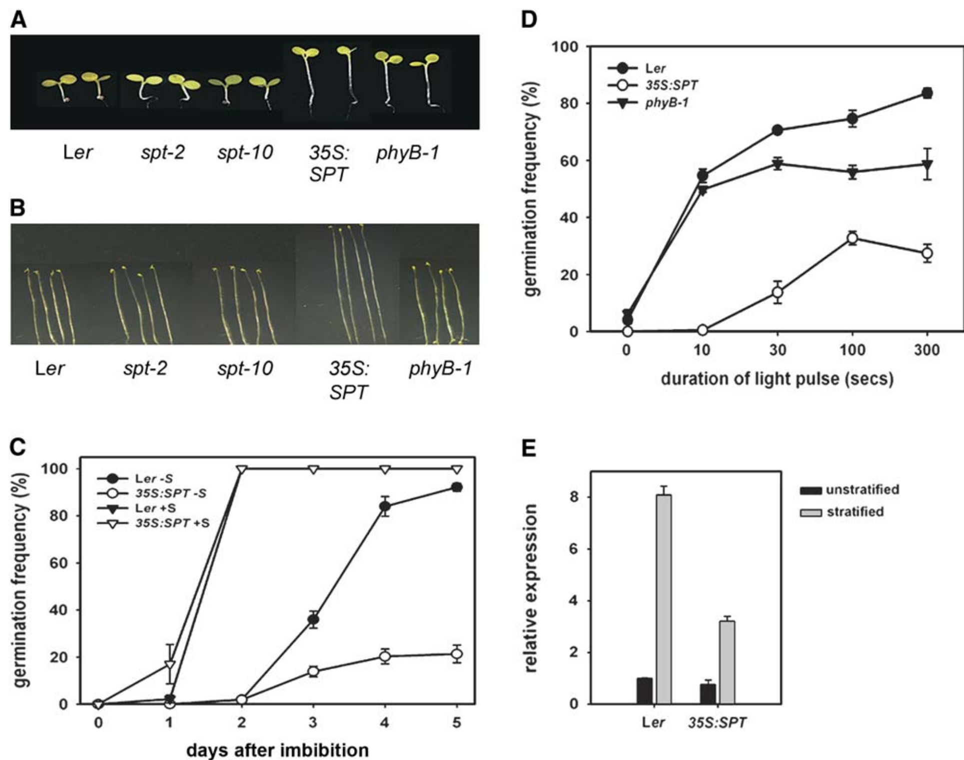

Figure 3. The Overexpression of *SPT*

(A) The seedling morphology of *spt* mutants and overexpressors after 5 days of growth in white light.

(B) Dark-grown seedling morphology of the wild-type, *35S:SPT,* and the *spt* mutants after 5 days.

(C) The germination of 1-week-after-ripened L*er* and *35S:SPT* seed in white light. −S, without 3 nights of stratification; +S, with 3 nights of stratification.

(D) The germination of freshly harvested stratified wild-type, *phyB-1*, and *35S:SPT* seeds in the dark and after red-light pulses of increasing duration. Data points represent the mean and standard deviation of three repilicates.

(E). The expression of *GA3ox* in imbibed seeds of the wild-type and *35S:SPT* after 24 hr in white light at 20ºC, with and without 3 nights of prior stratification. Error bars represent the standard deviation of three replicate determinations.

germination-unresponsive *spt-2* mutant. These findings confirm that a SPT-dependent pathway negatively regulates *GA3ox* expression in imbibed seeds.

SPATULA Overexpression Disrupts the Light Response in Seeds and Seedlings

We investigated the effect of SPT overexpression on seed germination and plant growth. Our data demonstrate that *35S:SPT* seedlings display a phenotype that is consistent with the proposed role of SPT in the response of seed germination to cold and light. Fifteen independent transgenic lines containing the full-length *SPT* cDNA fused to a double Cauliflower Mosaic Virus (CaMV) 35S promoter displayed a clear long-hypocotyl phenotype when grown in white light and the dark (Figures 3A and 3B). The light-grown phenotype closely resembles that of *phyB* loss-of-function mutants [16]. In addition, when partially after-ripened wild-type and *35S:SPT* seeds germinated in white light, a clear increase in seed dormancy was observed in *SPT* overexpressors compared to wild-type lines treated identically (Figure 3C). However, unlike that of *spt-2*, the germination of *SPT*-overexpressing seeds was clearly restored by a combination of stratification and constant white light. However *35S:SPT* seeds were less sensitive to germination promotion by pulses of red light after cold treatment (Figure 3D). When *GA3ox* expression was measured in dormant wild-type and *35S:SPT* lines 24 hr after imbibition in the light, low expression was observed in unstratified seeds of both genotypes (Figure 3E). In *35S:SPT*, *GA3ox* levels increased after stratification, but this increase was reduced at least 2-fold in comparison to the wild-type. Hence, we concluded that although *SPT* overexpression is insufficient to repress germination after stratification and constant white light, it does still attenuate *GA3ox* expression under these conditions.

SPATULA Regulates the Seedling Response to Red Light

Because SPT plays a significant role in moderating seed light responses, we tested whether SPT is also important in light signaling in seedlings (Figure 4). Little difference between the wild-type and the *spt* mutant hypocotyl response to increasing white, blue, or far-red light was seen, and although *35S:SPT* hypocotyls were longer than wild-type ones under all of these conditions, they were fully responsive to increasing white, blue, and far-red light (Figure S1 in the Supplemental Data available with this article online). Under red light *spt-2* seedlings appeared to have very slightly shorter hypocotyls than the wild-type under red light, whereas *spt-10*

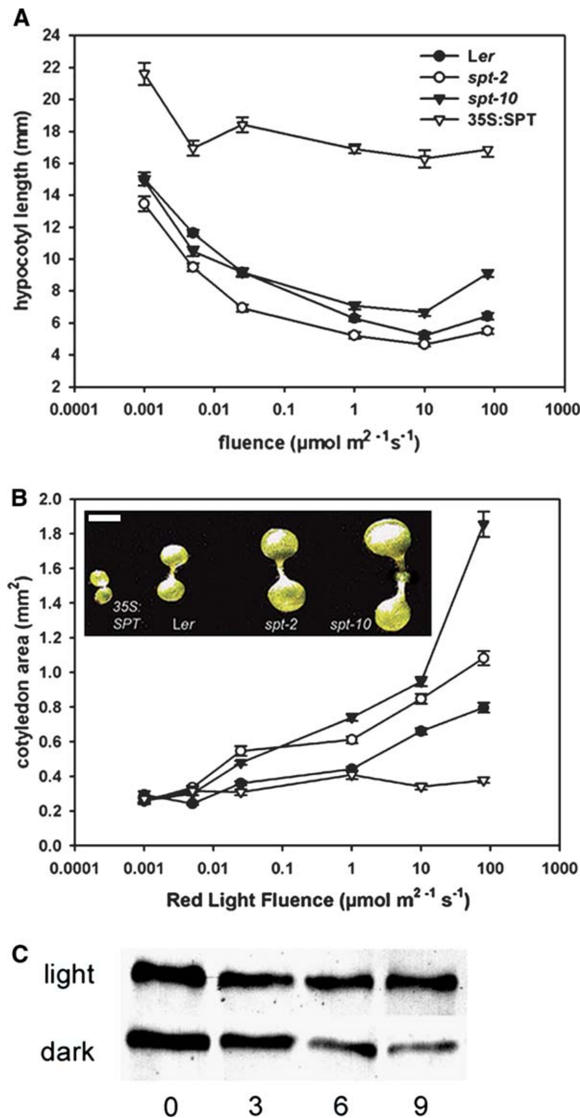

Figure 4. The Phenotype of Wild-Type, *spt* Mutant, and *SPT*-Overexpressing Seedlings after 5 Days of Growth under Red Light

(A) Hypocotyl length under red light.

(B) Cotyledon expansion under red light. The scale bar represents 1 mm. Data points represent the mean and standard deviation of 20–25 seedlings at each fluence.

(C) Western blots showing the stability of SPT-HA in light-grown seedlings maintained under white light or transferred to the dark after the addition of 100 μM cycloheximide.

appeared slightly longer than the wild-type at higher red-light fluences. *35S:SPT* exhibited a small degree of red-light-controlled hypocotyl inhibition, a response that saturated at 0.01 $\mu mol \cdot m^{-2} \cdot s^{-1}$. *35S:SPT* hypocotyls were otherwise completely insensitive to increasing fluence rates of red light (Figure 4A). Again, this phenotype resembles that of *phyB* loss-of-function mutants and suggests that SPT overexpression disrupts light-stable phytochrome signaling. During the course of these experiments, it was noticed that under red light significant differences in cotyledon expansion exist among the wild-type, *35S:SPT*, and *spt* mutants (Figure 4B). Wild-type cotyledon size increased with increasing red-light fluence. This effect was abolished in *35S:SPT*, whereas the cotyledons of both *spt-2* and *spt-10* showed an exaggerated response compared to that of the wild-type. Hence, SPT is also required for normal seedling responses to red light. In this case, in contrast to carpel development and seed germination, the *spt-2* mutant phenotype resembles, and is weaker than, that of *spt-10*, suggesting that *spt-2* is functioning as a conventional loss-of-function allele in red-light-exposed cotyledons. This situation was also found to be the case in petals, where both *spt-2* and *spt-10* were found to have increased petal expansion compared to the wild-type (Figure S2). Therefore, the genetic behavior of the *spt-2* allele appears to be tissue specific and may point to a dual role for SPT as both activator and repressor of gene expression, as has been observed for PIF3 [17].

SPATULA Is a Light-Stable Protein

Of the group of bHLH transcription factors involved in phytochrome signaling in *Arabidopsis*, PIF3 and PIL5 are known to be highly unstable in the light, whereas HFR1 is reported to be stabilized in the dark ([18, 19]; Shen et al., abstract, Arabidopsis 2005, Madison, WI). We speculated that SPT protein stability may be light regulated. To address this, we overexpressed SPT with a C-terminal 12× hemagglutinin (HA) epitope tag. These transgenic plants exhibited a similar phenotype to those overexpressing SPT alone (data not shown). Using the HA tag, we monitored the abundance of the SPT protein in the presence of cycloheximide in 5-day-old light-grown seedlings either maintained in the light or transferred to darkness (Figures 4C and 4D). In the light, very little degradation of the SPT-HA protein was seen, confirming that SPT is light stable, whereas in the dark the protein abundance fell steadily. Similar results were obtained with three independent repeats of this experiment. SPT-HA could not be detected in dark-grown seedlings (data not shown). These data fit well with our previous observations that SPT function in the repression of germination is most important in the light, but the weak phenotype present in dark-imbibed seeds after stratification (Figure 1C, Figure 2B) suggests that at least some SPT protein must also persist in wild-type seeds in the dark.

PIL5 Represses Seed Germination and *GA3ox* Expression in the Dark after Stratification

Previously, the PIL5 gene has been shown to be required for normal red and far-red responses in the seed and seedling, but the precise role of PIL5 in dormancy control remains unclear [20, 21]. We obtained the previously characterized *pil5-1* mutant and analyzed the effect of the loss of PIL5 on seed dormancy. Unlike *spt-10*, freshly harvested *pil5-1* seeds exhibited normal dormancy in both the dark and the light (Figure 5A). However, after stratification treatment, *pil5-1* seeds germinated at high frequency whether they were maintained in the dark or exposed to light. In agreement with previous experiments, freshly harvested wild-type (Col-0) seed required both light and stratification for germination. The response of freshly harvested *pil5-1* seed to increasing stratification time both in the dark and after a 10 s pulse of red light was investigated. *pil5-1* seed showed no germination without stratification, and wild-type germination increased with stratification as long

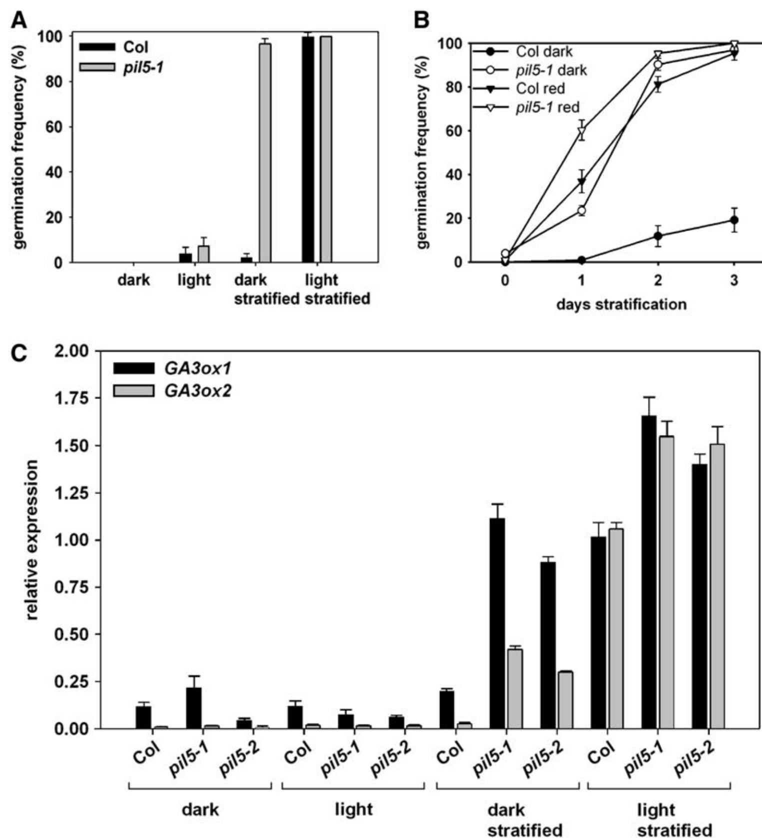

Figure 5. The Role of PIL5 in the Control of Seed Germination by Llight

(A) The germination of freshly harvested wild-type and *pil5-1* mutant seeds.

(B) The germination of freshly harvested wild-type and *pil5-1* mutant seeds to increasing durations of cold stratification in the dark or after a 10 s pulse of red light. Error bars for (A) and (B) represent the standard deviation of three replicate seed batches.

(C) Real-time RT-PCR showing the expression of *GA3ox1* and *GA3ox2* in freshly harvested seeds of wild-type, *pil5-1*, and *pil5-2* after 24 hr of imbibition at 20°C, with or without 3 days of cold stratification. Expression is shown relative to stratified wild-type seeds in the light, which was set to the value of one. Error bars represent the standard deviation of three replicate determinations.

as a red-light pulse was provided (Figure 5B). Strikingly, *pil5-1* germination in the dark increased with increasing stratification time in a manner closely resembling that of the wild-type in the light. Therefore, PIL5 is essential to the prevention of germination in the dark after chilling but, in contrast to SPT, has no role in the chilling response itself.

Given the close homology of SPT and PIL5, we tested to see if PIL5 was also necessary for the repression of *GA3ox* expression in imbibed seeds by using *pil5-1* and a second allele, *pil5-2* (see Experimental Procedures; Figure 5C). In unstratified seeds, levels of *GA3ox* expression in the *pil5* mutants resembled those of the wild-type. However, in the dark after stratification, a clear increase in *GA3ox* expression was seen in the *pil5* mutants but not in the wild-type. Even in stratified seeds in the light, the transcript levels of *GA3ox1* and *GA3ox2* in the *pil5* mutants exceeded that in wild-type. So, like SPT, PIL5 also functions as a repressor of *GA3ox* expression, and PIL5 function is the key to maintaining low *GA3ox* expression in the dark, particularly after chilling.

Discussion

SPT Is a Regulator of Cold Stratification

We have shown that SPT is a key repressor of germination in dormant seeds and that it regulates seed responses to environmental signals. SPT primarily regulates the cold response; loss of SPT allows germination without stratification (in the presence of light), whereas the gain-of-function *spt-2* mutation eliminates the stratification response altogether (Figure 1C). Importantly, the *spt-2* mutation does not affect germination when stratification is not required, as in after-ripened seeds. This is the first demonstration that cold stratification and after-ripening represent distinct, genetically separable pathways in plants. The genetic behavior of the *spt-2* mutation is unusual in that in different tissues it was found to have a dominant-negative effect (carpels), gain-of-function effect (seeds), and loss-of-function effect (cotyledons, petals). This may point to an interaction of SPT with a suite of tissue- or response-specific cofactors that confer either transcription activation or repression qualities to the complex.

Stratification-dependent germination in the dark is a phenotype diagnostic for mutants impaired in the light-stable phytochrome control of seed germination, as exemplified by the *pil5* mutants (100% germination in the dark after stratification; Figure 5A). The role of SPT in light signaling appears to be 2-fold: first, there is an indirect effect due to the perturbation of the stratification response; second, there is a direct role in the phytochrome response, as indicated by the germination of after-ripened *spt-10* seeds in the dark and the low level of dark germination seen in freshly harvested *spt-10* seeds after stratification (Figures 1C and 1E). The overexpression of SPT also supports a role for SPT in the repression of seed germination. *35S:SPT* seeds showed increased seed dormancy in the light and perturbed light-stable phytochrome signaling in seeds and seedlings. Although SPT overexpression is not sufficient to prevent germination in response to cold stratification, it does still repress *GA3ox* expression under these conditions. This effect does not seem sufficient to block

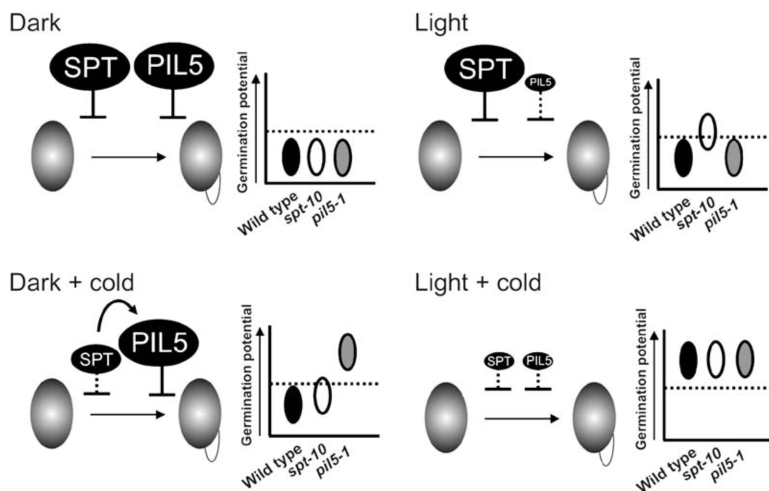

Figure 6. A Model Representing the Control of Seed Germination by SPT and PIL5 after Light and Cold Treatment

The relative repressive activity of SPT and PIL5 under each condition is represented by changes in the size of either protein symbol. A given population of seeds exhibits a range of germination potentials, depicted by the ovals (right), and if this exceeds a critical threshold (shown by the dashed line) in an individual seed, then germination will occur [26]. The regulation of PIL5 activity is regulated at the level of protein stability by light; the factors affecting SPT activity are the focus of further study. In dark stratified seeds SPT activity appears to be dependent on PIL5 (denoted by arrow).

germination, however. SPT is the first regulatory gene described to control cold responses in the seed.

PIL5 Is a Repressor of Seed Germination in the Dark

PIL5 is absolutely required in dormant seeds for repressing germination in the dark after cold stratification (Figure 5A). This is consistent with previous observations that the *pil5-1* mutant will germinate after an inhibitory pulse of far-red light and that the PIL5 protein is unstable in the light ([21]; Shen et al., abstract, Arabidopsis 2005, Madison, WI). Hence, the presence of the PIL5 repressor is expected to be restricted to the dark or to low red/far-red ratios. This could explain why the *spt-10* phenotype is manifested predominantly in the presence of light because the overlapping activity of PIL5 in darkness could repress germination in the absence of SPT (Figure 6). Interestingly, the *pil5-1* single mutant will germinate close to 100% of seedlings in the dark after stratification, even though SPT also plays a small role in this response. This suggests that this particular activity of SPT is PIL5 dependent (denoted by the arrow between SPT and PIL5 in dark-stratified conditions; Figure 6).

SPATULA and PIL5 Negatively Regulate *GA3ox* Expression in Imbibed Seed

Studies have previously shown that both *GA3ox1* and *GA3ox2* are regulated by light [7, 8] and that *GA3ox1* but not *GA3ox2* is induced by cold stratification in the dark [9]. We have convincingly demonstrated that although a small induction of *GA3ox1* is observed in stratified seeds in the dark, in both Col-0 and L*er* maximum expression of the two *GA3ox* isoforms depends on the synergistic interaction of both light and cold signaling (Figures 2 and 5). This regulation required the repressive action of both SPT and PIL5 under environmental conditions consistent with their proposed role in germination repression. However, further work is required to establish whether the repression of seed germination by SPT and PIL5 is directly mediated by their effect on *GA3ox* expression. In fact, *spt-2* mutant germination is only partially restored by exogenous GA (data not shown), suggesting that further targets for SPT exist in the seed and remain to be discovered. Furthermore, the germination of the strongly dormant Cape Verde Islands ecotype of *Arabidopsis* responds poorly to GA [22], suggesting that germination promotion of dormant seed requires more than this phytohormone.

Conclusions

Temperature and light are conserved regulators of seed germination across the plant kingdom. We have shown that light and temperature signaling are tightly coupled at the molecular level in seeds. It is likely that SPT and PIL5 are members of a regulatory network integrating environmental signals and endogenous cues and that further members of this network remain to be discovered. SPT is also required for phytochrome responses in the seedling and regulates floral organ development. If SPT is typical of the PIF/PIL transcription-factor group, then it is likely that further roles outside of the seedling remain to be discovered for these genes as well.

Experimental Procedures

Plant Material

spt-2 and *pil5-1* and *phyB-1* seeds were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC) and have been previously described [13, 16, 21]. The *spt-10* insertion line corresponds to line ET7451 from the Cold Spring Harbor enhancer-trap collection [14]. The presence of the insertion was followed by the *spatula* carpel phenotype, and the absence of the wild-type *SPT* transcript. *pil5-2* corresponds to line SALK_131872. This line segregates kanamycin resistance 3:1 and exhibits similar seed and seedling phenotypes to *pil5-1* (data not shown).

Germination Assays

Seed for germination assays was harvested from plants grown simultaneously in glasshouse conditions with supplementary lighting to ensure a 16 hr photoperiod. The term "freshly harvested" refers to seeds collected from siliques that had just changed from green to brown. These were sown within 48 hr of harvest for germination assays. Both L*er* and Col0 were found to be dormant at this time. Seeds were sown in 0.9% (wt/vol) water-agar medium and stratified where indicated in the dark at 4°C–6°C. Wrapping plates immediately in three layers of foil after sowing but before imbibition was found to be essential to retain the light requirement for germination in the wild-type (data not shown). Germination was scored by radicle emergence after 5 days on five batches of 40–100 seeds from each genotype, each batch being obtained from one individual plant. Growth conditions under white light were 20°C for a 16 hr photoperiod at a photon fluence rate of 75 $\mu mol \cdot m^{-2} \cdot s^{-1}$. For experiments with red light, seeds imbibed in the dark were warmed to ambient

temperature and exposed to a pulse of continuous monochromatic red LEDs (PEAK 660 nm, 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$) as indicated before being rewrapped and incubated in the dark at 20ºC for 5 days. Seed after-ripening took place in dark storage in the laboratory, typically at 18ºC–20ºC. All data points represent the mean and standard error of five seed batches (three seed batches for the red/far-red reversibility experiment). Experiments were repeated several times, and similar results were obtained.

Seedling Growth Assays

For all experiments with seedlings, 20–30 seeds were sown on Gilroy-phytagel or water-agar plates. In the fluence response assays germination was stimulated by a pulse of white light after a 4 day period of stratification at 4ºC. Plates were then kept in darkness or transferred to the appropriate light treatment after a 24 hr period. For hypocotyl and cotyledon measurements of seedlings 7 days after imbibition, we used ImageJ to measure to the nearest 0.5 mm. For de-etiolation experiments, seedlings kept at 20ºC were exposed to continuous monochromatic red LEDs (PEAK 660 nm, 0–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$), far-red LEDs (PEAK 756 nm, 0–120 $\mu mol \cdot m^{-2} \cdot s^{-1}$), blue LEDs (PEAK 439 and 455 nm, 0–120 $\mu mol \cdot m^{-2} \cdot s^{-1}$), or white light provided by fluorescent tubes (Sylviana; PEAK 434, 455, 631, and 707 nm, 0–100 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Construction of *SPT*-Overexpressing Plants

The *SPT* cDNA was obtained as a pBLUESCRIPT clone from a cDNA library constructed from 2-day-old germinating seeds (I.A.G., unpublished data) and was confirmed to be full length via sequencing with standard primers. The sequence is 100% identical to that described in Genbank entry AF319540. Using standard molecular-biology techniques, we excised the *SPT* cDNA as a BamHI EcoRI fragment and cloned it into the pGREENII-0029 35S vector, containing a double cauliflower mosaic virus (CaMV) 35S promoter [23]. This was transformed into Agrobacterium strain GV3101 and into *Arabidopsis* Landsberg *erecta* by the floral-dip method. Twenty independent transgenic lines were obtained, and all lines that were confirmed to be bona-fide *SPT* overexpressors exhibited the described seedling and dormancy phenotypes.

RNA Extractions and Real-Time RT-PCR

Unless otherwise stated, chemicals were purchased from Sigma (Poole, UK). RNA was isolated from dry, imbibed, and germinating seeds via a protocol based on a borate extraction [24]. In brief, 150 mg of seed (based on dry seed weight) was ground and extracted with 1 ml of frozen XT buffer (0.2M sodium borate, 30 mM EGTA, 1% SDS, 1% sodium deoxycholate, 2% polyvinylpyrollidone, 10 mM DTT, and 1% IGEPAL [pH 9.0]) in a pestle and mortar. This was allowed to thaw and was treated with 40 μl proteinase K (PCR grade, Roche, UK) for 90 min at 42ºC; precipitation on ice followed for 1 hr with 80 μl 2M potassium chloride. The supernatant was collected after centrifugation at 4ºC. The RNA was precipitated from the supernatant at −20ºC for 2 hr with 360 μl 8M lithium chloride. The RNA was collected by centrifugation at 4ºC and redissolved in 100 μl water. The RNA was further purified via the clean-up protocol of the RNeasy Plant RNA isolation kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was synthesized with 5 μg of total RNA in 20 μl reactions, Superscript II Reverse Transcriptase (Invitrogen) and random primers according to the manufacturer's instructions, and 180 μl water was added before the PCR step.

Real-Time RT-PCR was performed with SYBR-green as described [25]; 2 μl of the diluted cDNA template was used, along with the following primers for the *SPT*, *GA3ox1*, and *GA3ox2* cDNAs: SPTF, 5′-CCTTACTTCACCCGTGGAGATG-3′; SPTR, 5′-GCGTTGGAATGACCAATGTTC-3′; GA3OX1F, 5′-AAGTGGACCCCTAAAGACGATCT-3′; GA3OX1R, 5′-GTCGATGAGAGGGATGTTTTCAC-3′; GA3OX2F, 5′-TGAGTTCCTCACCGGAAGTCTT-3′; and GA3OX2R, 5′-CGAGCCGCCTTGAGCTT-3′. All data points represent the mean and standard deviation of three independent determinations.

Plasmid Construction and Generation of *SPT-12xHA*-Overexpressing Lines

To construct the HA-tagged *SPT*, we amplified the cDNA of *SPT* (accession number AF319540) by PCR with pfu-Turbo DNA polymerase (Stratagene, La Jolla, CA).

Primers were 5′-GCG**ACGCGT**AATTACTACTACCATGATATCACAGAGAGAAGAA-3′ and 5′-GCG**GGGCCC**AGTAATTCGATCTTTTAGGT-3′. Bold indicates the introduction of MluI and ApaI restriction sites, respectively. The PCR product was sequenced, cut at the introduced restriction sites, and ligated into the binary plasmid pGT35SHA (R.K. and I.A.G., unpublished data), containing a double 35S enhancer and in frame with a 12xHA epitope tag. This was introduced into *Agrobacterium* strain GV3101::pMP90, which was used to transform plants of *A. thaliana* ecotype Landsberg erecta via the floral-dip method. Transgenic plants were selected for basta resistance on soil by spraying with KASPAR (Certis, Sutton on Derwent, UK). Three independent lines were produced, all of which displayed the long hypocotyl phenotype described for *35S:SPT*.

Protein Extractions and Western Blotting

T2 seedlings were grown in continuous white light (50 $\mu M \cdot m^{-2} \cdot s^{-1}$) at 20ºC for 5 days on filter paper placed on 1/2 MS plates. 1 ml of 100 μM cycloheximide (Sigma, Poole, UK) was added to the surface of the filter papers, and plates were placed either in continuous white light or in darkness at 20ºC. Seedlings were harvested at 0, 3, 6, and 9 hr after treatment. For extraction of total protein, approximately 100 seedlings were ground in a mortar and pestle under liquid nitrogen, 200 μl extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA, 50 mM NaCl, 0.7% [w/v] SDS, 1 mM DTT, and 1 mM PMSF) and protease inhibitor cocktail (Sigma, St. Louis, MO) were added, and then the mixture was heated for 10 min at 65ºC and clarified by centrifugation at full speed for 10 min in a microfuge. Protein extracts were separated by SDS-PAGE (10%) and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). A rat anti-HA monoclonal antibody 3F10 (Roche, Penzberg, Germany) was applied in a dilution of 1:5000. The immunoreactive polypeptides were visualized with an alkaline-phosphatase-conjugated goat anti-rat antibody (abcam, Cambridge, UK).

Supplemental Data

Supplemental Data including two additional figures are available with this article online at http://www.current-biology.com/cgi/content/full/15/22/1998/DC1/.

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