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Functional diversity of phytochrome family in the control of light and gibberellin- mediated germination in Arabidopsis

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Abstract:	In several species, seed germination is regulated by light in a way that restricts seedling emergence to the environmental conditions that are likely to be favourable for the success of the new individual, and therefore this behaviour is recognised to have adaptive value. The phytochromes are one of the most relevant photoreceptors involved in light perception by plants. We explored the redundancy and diversity functions of the phytochrome family in the control of seed responsiveness to light and gibberellins (GA) by using a set of phytochrome mutants of Arabidopsis. Our data show that, in addition to the well known role of phyB in the promotion of germination in response to high Red to Far-Red ratios (R/FR), phyE and phyD stimulate germination at very low R/FR ratios, probably by promoting the action of phyA. Further, we show that phyC regulates negatively the seed responsiveness to light, unravelling unexpected functions for phyC in seed germination. Finally, we find that seed responsiveness to GA is mainly controlled by phyB, with phyC, phyD and phyE having relevant roles when acting in a phyB deficient background. Our results indicate that phytochromes have multiple and complex roles during germination depending on the active photoreceptor background.

Title: Functional diversity of phytochrome family in the control of light and gibberellin-mediated germination in Arabidopsis

Running Title: Diversity of phytochrome function in germination

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ABSTRACT

In several species, seed germination is regulated by light in a way that restricts seedling emergence to the environmental conditions that are likely to be favourable for the success of the new individual, and therefore this behaviour is recognised to have adaptive value. The phytochromes are one of the most relevant photoreceptors involved in light perception by plants. We explored the redundancy and diversity functions of the phytochrome family in the control of seed responsiveness to light and gibberellins (GA) by using a set of phytochrome mutants of Arabidopsis. Our data show that, in addition to the well known role of phyB in the promotion of germination in response to high Red to Far-Red ratios (R/FR), phyE and phyD stimulate germination at very low R/FR ratios, probably by promoting the action of phyA. Further, we show that phyC regulates negatively the seed responsiveness to light, unravelling unexpected functions for phyC in seed germination. Finally, we find that seed responsiveness to GA is mainly controlled by phyB, with phyC, phyD and phyE having relevant roles when acting in a *phyB* deficient background. Our results indicate that phytochromes have multiple and complex roles during germination depending on the active photoreceptor background.

Keywords: Arabidopsis, duplicated genes, germination, hormones, light quality

INTRODUCTION

The time and place of germination occurrence has major effects on plant fitness by conditioning the future environment for plant establishment and reproductive growth (Donohue et al. 2005, Finch-Savage & Leubner-Metzger 2006). Seeds sense and integrate a number of cues that provide information about the environment. In response to these cues, the rate of germination is higher when the conditions are likely to be favourable for the success of the new individual. For numerous species, the ratio of red (R, 600-700 nm) to far-red (FR, 700-800 nm) light (R/FR), perceived by the phytochrome system, is a signal of outmost relevance for the control of germination in the field, since it provides the seed with information related to potential competition typical of vegetational canopies (Casal & Sánchez 1998, Deregibus et al. 1994, Giordano, Sánchez & Austin 2009, Vázquez-Yañez & Smith 1982). Furthermore, weed seeds that acquired high light sensitivity during the burial in the soil are capable to germinate with the absorption of very few photons perceived by the phytocrome system during tillage in agricultural fields (Botto, Sánchez & Casal 1998; Botto, Scopel & Sánchez, 2000).

Phytochromes are synthesised in the inactive form, Pr, (absorption maximum in R) and are transformed by light into the active form, Pfr (absorption maximum in FR). The reaction is photoreversible and the final proportion of active phytochrome (Pfr/P) depends on the R/FR ratio of the incident light (Casal et al. 2003, Kendrick & Spruit 1977). The Arabidopsis genome encodes five phytochromes (phyA-phyE) that have arisen through a series of gene duplications (Mathews & Sharrock 1997, Sharrock & Quail 1989). phyB has a prominent role as the photoreceptor regulating the R/FR reversible responses for germination (Botto, Sánchez & Casal 1995, Shinomura et al. 1994). phyE contributes to this regulation in *phyA phyB* double mutant seeds (Henning et al. 2002), indicating redundancy in phytochrome functions during the R-mediated control of germination.

Interactions among phytochromes in FR-mediated germination are more complex, and they are dependent on the frequency and duration of the FR treatment. In this context, it is well known that phyA is the main photoreceptor promoting germination by FR (Botto et al. 1995, Botto et al. 1996, Shinomura et al. 1996). Whereas phyE is necessary for phyA-mediated induction of seed germination by continuous FR, phyB inhibits the action of phyA in the promotion of germination when seeds are irradiated with a pulse of FR (Henning et al. 2001, Henning et al. 2002). In contrast with the aforementioned phytochromes, the role of phyC in the control of seed germination is unknown.

The environmental conditions during after-ripening and also those experienced by the mother plant during seed development modulate the contribution of phytochromes to germination (Donohue et al. 2012, Donohue et al. 2007). Therefore, phytochrome control of germination in natural environments depends on the previous life history of the organism. In addition, it has been demonstrated that, in Arabidopsis, mutations in phyA, phyB and phyD can affect plant fitness through germination timing (Donohue et al. 2012), suggesting that the action of phytochromes on germination strongly influences post-germination traits and natural selection. The phytochrome system shows a remarkable functional diversity during plant development. For example, phyB, phyE and phyD participate in the R/FR reversible response for internode elongation and flowering (Devlin, Patel & Whitelam 1998, Devlin et al. 1999), however, in the seeds, phyB and phyE but not phyD contribute to R-mediated germination (Henning et al. 2002).

Phytochromes require GA to promote germination, since mutants impaired in GA synthesis are not able to germinate, even after R irradiation (Derkx & Karssen 1993, Hilhorst & Karssen 1988). R-mediated germination involves an increase of active GA in the seed (Oh et al. 2006, Seo et al. 2009), through the activation of the expression of genes involved in GA synthesis, which are controlled by phyB and another unknown type II phytochrome (Yamaguchi

et al. 1998). In addition, it has been demonstrated that the promotion of germination by FR involves the regulation of the expression of genes of that participate in the GA metabolic pathway (Arana et al. 2007, Ibarra et al. 2013), suggesting that it is also associated with an increment of the synthesis of active GA. Moreover, R and FR increase germination sensitivity to GA, which involves the degradation of PIL5 though the action of phyA and phyB (Oh et al. 2004, Oh et al. 2006). Interestingly, whereas PIL5 is the main factor acting during the promotion of germination by R (Oh et al. 2009), the phyA pathway induced by a FR pulse is partially independent of PIL5 (Ibarra et al. 2013), indicating differences in the signaling pathways of phyA and the type II photoreceptors which control the GA response during germination.

Using a set of double, triple and quadruple phytochrome mutants of *Arabidopsis thaliana*, we investigated the roles and interactions of the five phytochromes in the control of seed germination, focusing particularly on the action of phyC. We have also evaluated the role of the different phytochromes in the modulation of the sensitivity to GA in the seeds.

MATERIAL AND METHODS

Plant material

All the mutants used were previously obtained in the Columbia background, as follows: *phyA211* (Reed et al. 1994), *phyB9* (Reed et al. 1993), *phyD-201* and *phyE-201* (Strasser et al. 2010, Wollenberg et al. 2008), *phyC-2* (Monte et al. 2003) and their combinations and genotyping details as described previously (Iñigo et al. 2012, Strasser et al. 2010, Wollenberg et al. 2008). Plants of the wild type and the different phytochrome mutants were grown at 23°C, under long day (LD) conditions. Seeds were simultaneously collected, after-ripened for 2 months at room temperature and then used for the experiments. At least 3 independent seed batches, coming from independently grown and harvested events, were used for the experiments. For specific

experiments, wild type and *phyB* mutants were collected, after-ripened for 2 months at room temperature and then stored in plastic polypropylene tubes at 4-8°C for four years, until their use. Wild type and *phyC* seeds shown in Fig. S1 were collected, after-ripened for 5 months at room temperature and then used for the experiments.

Germination assays

In order to measure germination responsiveness to light, seeds (25 seeds per genotype in each experiment) of the wild type (Columbia) and phytochrome mutants were imbibed into clear plastic boxes (42 x 30 mm^2 x20mm) on two layers of filter paper containing 1ml of distilled water. Seeds were then treated with a FR pulse (25 min, calculated Pfr/P=0.03) in order minimise the quantities of Pfr formed during their development in the mother plant. The clear boxes where wrapped in black plastic sheets and all the seeds were cold stratified for 48h at 4°C in darkness. At the end of the stratification, they were exposed for 24h either to hourly pulses of 3 min of R, FR or mixtures of R plus FR that provided a series of calculated Pfr/P, whereas control seeds remained in darkness. After light treatments, seeds were incubated in darkness at 22°C for 4 days, until the measurement of germination. The handling of the seeds during all the experiment was performed in absolute darkness. Experiments were repeated at least four times.

In order to evaluate the promotion of germination by GA, seeds of the wild type or the different phytochrome mutants (25 seeds per genotype for each experiment) were imbibed into clear plastic boxes, on two filter paper sheets containing 1ml of a solution with Paclobutrazol 4ppm (FLUKA) in combination with different concentrations of GA_{4+7} (SIGMA). Control seeds were incubated on 1ml of a solution with Paclobutrazol 4ppm, without GA. Seeds were then treated with a saturating FR pulse (25 min, calculated Pfr/P=0.03) in order minimise the quantities of Pfr formed during the development of the seed in the mother plant before the starting of the experiment. The boxes were then wrapped in black plastic sheets and cold stratified at 4°C for

48h. Then a group of seeds for each GA concentration were irradiated during 24h with hourly pulses of 3 min of R (calculated Pfr/P=0.87), whereas another group received hourly pulses of 3 min of FR (calculated Pfr/P=0.03). A third group was kept in darkness. Seeds then were incubated in darkness at 22°C for 4 days, until germination counting. The handling of the seeds during all the experiment was performed in absolute darkness. Experiments were repeated at least four times for each seed batch analysed.

To test for significant differences in responses to light between the phytochrome mutants and the wild type, we conducted a series of separate two-way analysis of variance (two-way ANOVAs) for each wild-type and mutant pair, using the angular transformation of the percentage of germination and the GraphPad Prism Software. We considered that the loss of the functional phytochrome/s caused a significant alteration of germination response to light when the treatment-by-genotype interaction from the two-way ANOVA was significant at $p<0.05$.

We performed similar analysis to test the effect of light on the sensitivity of germination to GA: we conducted a series of separate two-way ANOVAs comparing, within each genotype, the responsiveness to GA under the different light treatments. A significant light treatment-by-GA concentration interaction from the ANOVA ($p<0.05$) indicates that the active phytochrome/s of each mutant background are influencing the light-mediated GA response. Bonferroni post-tests were run in order to asses the differences between the mutants and wild type for each Pfr/P proportion, and the differences between R and FR for each GA concentration. The Shapiro-Wilks W statistic was used to test the normality for residuals using the STATGRAPHICS.PLUS software. In most cases, residuals were normally distributed with exception of *phyA phyB phyD phyE* and *phyA phyC* (light experiments), and *phyB phyE* and *phyB phyD* (GA experiments). For those cases, we conducted a series of nonparametric Kruskal-Wallis tests to confirm the

significance of the result. In all the cases, no differences with the Bonferroni post-test were found (significant differences are indicated with asterisks inside each figure pannel~~data not shown~~).

Biomolecular Fluorescence Complementation (BiFC)

In order to generate the vectors for the infiltration of *A. benthamiana* leaves, the corresponding fragments of nEYFP and cEYFP (pSAT4 vector series, Steven Rothstein, University of Guelph Stanton Gelvin, Purdue University) were adapted for their fusion with the fragments of the different phytochromes, then were joint by PCR to the rbsc-terminator, and placed after the 35S promoter of the pCHF5 vector. Full-length versions of PHYA and PHYC cDNAs were amplified from Col-0 seedlings and then cloned into the described vectors. The general procedure for the infiltration of *N. benthamiana* leaves was as described in Iñigo et al. (2012). After the infiltration, the plants were grown for 2 days in continuous light, and then were transferred to darkness for 1 day, in order to avoid PHYA and PHYC degradation. Leaf discs were analyzed under a Zeiss LSM710 confocal microscope at 150X (wave length: excitation 488nm, emission: 540 nm).

Light sources

R light (35 µmol. $m^{-2}.s^{-1}$, calculated Pfr/P=0.87) was provided by a diode panel (660nm). FR light (40 µmol. $m^{-2}.s^{-1}$, calculated Pfr/P=0.03) was provided by 150-W incandescent internal reflector lamp filtered through an RG9 Schott glass filter (Mainz, Germany) and a 10-cm water filter. Intermediate calculated Pfr/P were established by mixtures of R plus FR (15-40 mmol. $m^{-2}.s^{-1}$) as described in (Casal et al. 1991, Yanovsky, Whitelam & Casal 2000).

RESULTS

192 ***Germination responses to Pfr/P in phytochrome mutants***

193 Although it has long been known that phyA and phyB regulate seed responsiveness to
194 light (Botto et al. 1995, Botto et al. 1996, Shinomura et al. 1994) with a contribution of phyE and
195 phyD (Henning et al. 2001, Henning et al. 2002), little is known about their individual roles and
196 interaction in the control of germination. Germination of wild type seeds was promoted by light
197 treatments establishing Pfr/P ratios between 0.03 and 0.87, and this effect was completely
198 abolished in the quadruple *phyA phyB phyD phyE* mutant (Fig. 1 A). This indicates that some of
199 these phytochromes, or their combined action, are required for the control of germination at
200 different Pfr/P ratios. To better understand the function of different phytochromes in the
201 promotion of germination, we examined their relative contributions using a set of simple, double,
202 triple and quadruple mutants.

203 Germination of *phyB* mutants strongly decreased under light regimes establishing Pfr/P
204 ratios between 0.2 and 0.87, whereas germination of *phyA* mutants was significantly reduced in
205 comparison with the wild type at Pfr/P ratios between 0.03 and 0.33 (Fig. 1 B). On the other
206 hand, germination of *phyE* and *phyD* single mutants was similar to wild type in the 0.66-0.87
207 Pfr/P range, but decreased strongly at lower Pfr/P values between 0.03 and 0.33 for *phyE* and
208 0.03-0.22 for *phyD* (Fig. 1 C). This indicates that phyB is relevant in a wide range of Pfr/P ratios,
209 whereas phyD and phyE are relevant to promote germination at very low Pfr levels.

210 Because the action of phytochromes in germination responses are often hierarchical
211 (Heschel et al. 2007, Heschel et al. 2008), we hypothesized that phyE and phyD functions could
212 be hidden in our experimental conditions by the action of phyB. Therefore, we studied the action
213 of phyE and phyD in a *phyB* mutant background. Whilst single *phyD* and *phyE* mutants showed
214 maximal germination rates in response to Pfr/P ratios ranging from 0.66 to 0.87, this effect was
215 lost in the *phyB phyD* and *phyB phyE* double mutants (Fig. 2 A-E). Moreover, *phyB phyD*, *phyB*

phyE and *phyB phyD phyE* mutants showed a similar responsiveness pattern than *phyB* to Pfr/P ranges between 0.22 and 0.87 (Fig. 2 and ~~no significant genotype x light treatment interactions and Bonferroni post-tests in two-ways ANOVAs when germination of *phyB* is compared to *phyB phyD*, germination of *phyB* is compared to *phyB phyE* or germination of *phyB* is compared to *phyB phyD phyE*, data not shown~~Table S1). On the other hand, at lower than 0.22 Pfr/P ratios, the reduced germination of *phyE* and *phyD* mutants was not observed in the absence of phyB. Taken together these results indicate that phyB is the main photoreceptor controling germination at high Pfr/P and that phyE and phyD are required for germination at ranges that include very low Pfr/P, probably promoting the action of phyA

Role of phyC in the stimulus of germination.

In order to study the role of phyC in light-mediated germination, chilled *phyC* seeds were induced to germinate under light treatments that established different Pfr/P ratios, and compared with the wild type. Surprisingly, *phyC* germination values were higher at the whole range of Pfr/P ratios with the exception of 0.87 (Fig. 3 A). Moreover, the action of phyC on germination was independent of the degree of seed dormancy, since experiments with seed batches after-ripened for 5 months at room temperature show that *phyC* seeds still keep higher values of germination than the wild type (Fig. S1). Taken together, these results indicate that phyC antagonises the promotion of germination by light.

In order to investigate the interaction of phyC with phyA and phyB, which are the main photoreceptors that control light-mediated seed germination (Botto et al. 1995, Botto et al. 1996, Shinomura et al. 1994), we evaluated the germination of phyC in the *phyA* and *phyB* background, through the analysis of light responsiveness of double *phyA phyC* and *phyB phyC* mutant seeds. Germination of *phyA phyC* seeds was very low for treatments that established Pfr/P ratios below

0.33, but was similar to *phyC* single mutants at higher Pfr/P ratios (0.66-0.87). On the other hand, *phyB phyC* double mutants showed just a small decrease in germination at Pfr/P ratios of 0.87 probably due to the *phyB* mutation (Fig. 3 B). Since the *phyC* mutation affected mostly germination at Pfr/P ranges overlapping phyA, our results suggest that the negative effect of phyC on germination is achieved, at least in part, by blocking the action of phyA.

Role of different phytochromes in the control of seed responses to GA

Light perceived through the phytochrome system increases the content of GA in the seed (Oh et al. 2006, Seo et al. 2009) and stimulates its sensitivity during germination (Oh et al. 2009, Yang et al. 1995) indicating that phytochromes regulate germination, at least in part, through changes in GA metabolism and signaling. To dissect the phytochrome effect on GA-sensitivity from GA-synthesis, we tested the response to exogenous GA in the presence of the GA-synthesis inhibitor Paclobutrazol (PAC). Hourly pulses of R increased the responsiveness of wild type seeds to exogenous GA in a FR reversible manner (Fig. 4 A). This effect was clearly observed at 0.1 and 1 µM of GA whereas concentrations equal or above 10 µM saturated the response to GA, yielding around 100% germination independently of the light treatments. On the other hand, R light did not stimulate GA response in the quadruple *phyA phyB phyD phyE* (Fig. 4 B), suggesting that the action of phyA, phyB, phyD and / or phyE is required for the R-mediated modulation of GA responsiveness in the seeds.

Surprisingly, *phyA, phyC, phyD* and *phyE* single mutants showed a similar R-FR reversible response to the wild type (Fig. S2 A-D). In contrast, the *phyB* mutation abolished the effect of R-FR reversible response at 0.1µM GA and severely reduced the promotion of germination at 1 µM (Fig. 5 A). These results suggest that phyB is the main photoreceptor that controls germination responsiveness to GA in the lower range of concentrations assayed (0.1-1

μM). Moreover, both wild type and *phyB* seeds that were after-ripened for one up to four years showed a similar pattern of light-mediated regulation of GA response (Fig. S3 A-B), indicating that phyB action on GA sensitivity does not change substantially with after-ripening or ageing.

The fact that the *phyB* mutant showed a R-FR reversible response to GA which was lost in the quadruple *phyA phyB phyD phyE* seeds, suggests that other phytochromes different to phyB could be involved in responsiveness to GA. Hence, we assayed the effect of phyD and phyE in the R/FR control of seed responsiveness to GA in a *phyB* mutant background (Fig. 5 B-D). Loss of phyE function further decreased GA sensitivity, since the *phyB phyE* seeds lost the R-mediated stimulus of GA response at 1 μM, which was still present in *phyB* seeds (Fig. 5 A-B). Furthermore, at 10 μM GA, R-mediated germination response of *phyB phyE* seeds was significantly lower than in *phyB* ($p < 0.1$) (Fig. 5 A-B). We conclude that phyE is involved in the regulation of seed GA responsiveness, but unlike phyB, phyE controls the sensitivity in the range of medium to high GA concentrations. On the other hand, *phyB* and *phyB phyD* mutants did not yield differences in the responsiveness to GA when seeds were treated with R. However, seed sensitivity was increased in the *phyB phyD* mutant when treated with FR, suggesting than phyD negatively regulates GA responsiveness to FR in the absence of phyB (Fig. 5 C) and this may indicate a negative action of phyD on phyA.

The data shown above indicates that whereas phyE promotes the R-mediated response to GA for germination, phyD has a negative effect in the FR-mediated germination. Furthermore, the *phyB phyD phyE* triple mutant displayed a significant larger GA sensitivity germination in R than in FR (10 μM GA, Fig. 5 D) suggesting that phyA and/or phyC could be controling the responsiveness to GA. Since the action of phyA is usually not FR reversible (Casal & Sánchez 1998), we predicted that phyC is playing a role in the modulation of GA responsiveness for germination under R. In fact, the R/FR response to GA for the *phyB phyC phyD phyE* quadruple

mutant was significantly smaller than in the triple mutant *phyB phyD phyE* (Fig. 5 E, $p < 0.05$) demonstrating that phyC is involved promoting the GA sensitivity in response to R. Further, phyC on its own was not able to induce GA-sensitivity in response to R pulses (Fig 4B) also suggesting an interaction between phyA and phyC signaling.

DISCUSSION

Light is the one of the major relevant environmental cues for the seeds and the phytochromes are the most important photo-sensory mechanism during germination. In fact, the quintuple phytochrome mutants show a null or reduced germination capacity, depending on accession background (Hu et al. 2013, Strasser et al. 2010), and show a complete absence of light induced germination (Strasser et al. 2010). It has been shown that phyA is the main photoreceptor that modulates germination at very low Pfr/P ratios as those established by a saturating FR pulse, and also that phyB and phyE are involved at higher Pfr/P, like those established in the R/FR reversible photoresponse (Botto et al. 1995, Botto et al. 1996, Henning et al. 2001, Henning et al. 2002, Poppe & Shäfer 1997, Shinomura et al. 1994, Shinomura et al. 1996). Here we show novel roles for phyC, phyE and phyD in the modulation of seed responsiveness to light and GA, and explore the interactions among these phytochromes when phyA and/or phyB are absent.

In particular, we find that phyE and phyD are important contributors to germination at low to middle Pfr/P (0.03-0.33), and are partially redundant with phyB at higher Pfr/P (Fig. 2 A-C). phyE and phyD belong to the type II functional group of photoreceptors that regulate the R/FR photoreversible low-fluence response (Mathews & Sharrock 1997, Rockwell, Su & Lagarias 2006), and the fact that they influence germination at ranges that include extremely low Pfr/P as those established by FR pulses suggests that they can influence the action of phyA (Fig. 6). The possibility that phyE contributes to the phyA-mediated promotion of seed germination

under FR pulses is in accordance with Henning et al (2002), who showed that phyE is necessary for phyA-mediated germination under continuous FR. On the other hand, our results constitute the first evidence for the phyD involvement on the induction of germination at very low Pfr/P. Henning et al. (2001) showed that phyD negatively regulates the phyA-mediated promotion of germination by a FR pulse. The different roles of phyD shown by these authors and this study may be due to the characteristics of the FR treatments, or the conditions of maturation of the seeds in the mother plant.

In addition, we found that the contribution of phyC on light-mediated germination is dependent on the active phytochrome background. Unexpectedly, in wild type seeds, phyC is a negative regulator of germination in a wide range of Pfr/P ratios operating, at least in part, through blocking phyA action (Fig. 3 A-B). On the other hand, in *phyB phyD phyE* triple mutant seeds, phyC appears to act together with phyA in the promotion of the R/FR reversible response, at very low Pfr ranges (Fig. 2 C: percent of germination in *phyB phyD phyE*= 9,6 % and 38,5% at Pfr/P= 0.03 and 0.22, respectively, $p < 0.0001$). These results are consistent with the role for phyC and phyA in the stimulus of the responsiveness to GA during germination in *phyB phyD phyE* seeds (Fig. 5 D-E).

How phyC controls the light and GA sensitivity during germination is not yet understood. In previous reports, it has been suggested that the formation of heterodimers between phyC and phyB or phyD are essential for the action of phyC and that phyC is non-functional in the absence of other phytochromes (Clack et al. 2009, Hu et al. 2013). The dual role of phyC depending on the presence of other phytochromes suggests that the promotion or inhibition of seed germination may be associated to the capacity to form homo or heterodimers. In fact, the promotion of germination by R in the *phyB phyD phyE* mutant seeds and the requirement of both phyA and phyC for the R/FR GA-sensitivity response, may suggest a positive activity of phyA Pfr and

phyC Pfr heterodimers. phyA and phyC protein to protein interactions were not detected by immunoprecipitation and two-hybrid experiments (Clack 2009). However, preliminary data from bimolecular fluorescence complementation assays (BiFC) indicate a possible interaction between phyC and phyA apoproteins (Fig. S4). Although this interaction seems weak compared to phyC and phyB interaction, we cannot rule out the involvement of low levels or phyA-phyC heterodimers in the interactions observed between this two phytochromes. However, to determine its relevance in vivo needs further experimentation. These data raises an attractive hypothesis that suggests that seed germination is regulated at different and complex levels by the phytochrome family based on gene redundancy and hierarchical relationships between different members, and also in their capacity for Pfr dimerisation.

It is known that phytochromes influence the sensitivity of seeds to GA (Oh et al. 2004, Yang et al. 1995). We demonstrate that R light fails to increase the sensitivity to GA in *phyA phyB phyD phyE* mutant seeds suggesting that different combinations of phytochromes are necessary for this response (Fig. 4). In fact, phyB is necessary for the R- mediated germination at very low and middle concentrations of GA (0.1-10µM, Fig. 5 A), and we observed a sustained role of phyB in the control of GA sensitivity, independently of the after-ripening period (Fig. S3). These results suggest that even after a significant decrease in dormancy phyB is still the major phytochrome regulating the sensitivity to GA. On the other hand, we observed that other simple phytochrome mutations did not affect seed responsiveness to GA (Fig. S2). However, we found that, in a *phyB* mutant background, phyE has a redundant positive contribution for the R-stimulus of GA response during germination (Fig. 5 B) and phyC is a contributor to R-mediated GA responsiveness when phyB, phyE and phyD are absent (Fig. 5 D-E) evidencing a remarkable redundancy of phytochrome functions in the control of GA signaling.

Previous studies that point to the role of separate phytochromes in the regulation of GA responsiveness for germination are scarce (Yang et al. 1995, Strasser et al. 2010). Strasser et al. (2010) reported that the quintuple phytochrome mutant had a null germination in absence of exogenous GA, and this indicates that the phytochromes are required for the promotion of GA synthesis/sensitivity during germination. On the other hand, Yang et al (1995) observed no differences in the responsiveness to GA of *phyB* mutants when compared with the wild type. The differences between Yang et al (1995) and the data reported here may be due to the characteristics of the experimental conditions. For example, whereas we reduced the Pfr formed during the development of the seed by a FR treatment shortly after the beginning of imbibition, Yang et al. (1995) did not. It is possible that the presence of Pfr from some of the stable phytochromes might have influenced the results.

We found that the role of some of the phytochromes in light-mediated germination coincided with their strong relevance on the control of GA responsiveness in the seed, as in the case for phyB (Fig. 1 B and Fig. 5 A). But interestingly, in some other cases, we do not find a direct association between the control of light responsiveness and the sensitivity to GA for germination. The latter observation is valid for phyC, phyE and phyD which, although they show a relevant role in the control of seed responsiveness to light, simple *phyC*, *phyE* and *phyD* mutants show a similar GA responsiveness for germination than the wild type (Figs. 1, 3 and 2S). This indicates a diversification in phytochrome pathways for the control of germination, where some of them exert a control at least in part through the modulation of GA responsiveness in the seed, but others influence pathways different to those of GA signaling (Fig. 6).

In recent years, important progress has been achieved in the identification of molecular components acting downstream the phytochrome system for the control of GA metabolism/responsiveness in the seed. For example, in the light, levels of active GAs are

regulated epigenetically by phyB though the activation of histone arginine demethylases JMJ20 and JMJ22 (Cho et al. 2012). On the other hand, the phytochromes interact with PIL5 protein activating its degradation and this is mediated, at least in part, by phyA and phyB (Oh et al. 2006). PIL5 inhibits germination through binding to DELLA promoters and activating DELLA expression (Oh et al. 2004). Consistently, phyA and phyB-mediated germination involves the down-regulation of DELLA proteins (Ibarra et al. 2013, Oh et al. 2006, Piskurewicz et al. 2009). Noteworthy, although phyB-mediated control of seed transcriptome during R-mediated germination is mainly dependent on down-regulation of PIL5 (Oh et al. 2009), global expression patterns in phyA-dependent germination include just a percentage (c.a. 45%) of PIL5- regulated genes (Ibarra et al. 2013). Furthermore, whereas phyB mainly singals in endosperm, phyA, and others phytochromes, signals in the embryo, indicating a spatial diversification of phytochrome functions during germination (Lee et al. 2012). It still remains to be addressed whether R-mediated control of germination by other type II photoreceptors such as phyE, phyD and phyC is mostly dependent on PIL5, or whether they show an extensive diversification in their signaling pathways compared to phyB, as it was shown for phyA (Ibarra et al. 2013). In addition, their role in embryo or endosperm signaling remains still unknown.

Phytochrome activity in the natural environment is regulated by factors such as soil water availability and the life history of the organism (Botto, Scopel & Sánchez 2000, Donohue et al. 2012), and the role of the different phytochromes are dependent on temperature during the imbibition and germination (Heschel et al. 2008, Heschel et al. 2007). The diversification in phytochrome functions and the activation of different light signaling pathways dependent on each phytochrome member may provide to the seeds the ability to respond and adjust the timing and place of germination to different light environments of ecological relevance.

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557

558 **LEGENDS**

559 **Fig. 1**. Induction of germination by light in the wild type compared with *phyA phyB phyD phyE*
560 quadruple mutant (A), *phyA* and *phyB* simple mutants (B) and *phyE* and *phyD* simple mutants
561 (C). Seeds were imbibed for 45 min at room temperature, treated with a FR pulse and chilled at
562 4°C for 3d. Then, seeds were exposed to hourly pulses at different R/FR ratios for 24h, which
563 established different calculated Pfr/P proportions. After light treatments, seeds were then kept at
564 23°C in darkness for 4 d when germination was evaluated. Graphs indicate media ± S.E.M from
565 at least 4 independent experiments. Two-way ANOVA results of the comparison between each
566 mutant and the wild type are indicated at the upper side of each panel. Asterisks show significant
567 differences between each mutant and the wild type at each Pfr/P proportion, after Bonferroni post
568 test analysis ($p < 0.05$). For comparison, the wild type germination data were included in each
569 panel. The control in darkness (D) is indicated in the left section of the X axes.
570 **Fig. 2.** Induction of germination by light in the wild type and *phyB* simple mutant compared with
571 *phyB phyD* double mutant (A), *phyB phyE* double mutant (C) and *phyB phyE phyD* triple mutant
572 (D). For comparison, the wild type and *phyB* germination data were included in each panel. The
573 experimental protocol, statistic analysis and references are the same than those described in Fig. 1
574 **Fig. 3.** Induction of germination by light in the wild type compared with the simple *phyC* mutant,
575 the double *phyA phyC* mutant (A), and double *phyB phyC* mutants (B). Two-way ANOVA results
576 of the comparison between the wild type and *phyC*, *phyA phyC* vs *phyC* (A) or *phyB phyC* vs.
577 *phyC* are indicated at the upper side of each panel. For comparison, the wild type and *phyC*
578 germination data were included in each panel. The experimental protocol and references are
579 similar to the described in Fig. 1.
580 **Fig. 4**. Germination dose-response curves to gibberellins (GA) in the wild type (A) and *phyA*
581 *phyB phyD phyE* quadruple mutant (B). Seeds were imbibed in solutions with paclobutrazol 4

582 ppm plus different GA concentrations for 45 min at room temperature, treated with a FR pulse
583 and chilled for 3 d. Then two groups of seeds were irradiated with hourly pulses of R, or with
584 hourly pulses of R followed by FR for 24 h. A third group of seeds was kept in darkness as
585 control. After light treatments, seeds were then kept at 23°C in darkness for 4 d when
586 germination was evaluated. Graphs indicate media ± S.E.M from at least 4 independent
587 experiments. Two-way ANOVA results of the comparison between R and FR treatment for each
588 genotype are indicated at the upper side of each panel. Asterisks show significant differences
589 between R and FR for each genotype at different GA concentrations after Bonferroni post test
590 analysis ($p < 0.05$). Control without GA (PAC) is indicated in the left section of the X axes.
591 **Fig. 5**. Germination dose-response curves to GA in *phyB* (A), and different double (B and C),
592 triple (D) and quadruple (E) mutants in the *phyB* background. The experimental protocol,
593 statistical analysis and references are the same than those described in Fig. 4.
594 **Fig. 6.** Model of phytochrome action in seed germination induced by light and GA. phyA and
595 phyB are the central photoreceptors promoting germination under very low and high Pfr/Pr
596 photequilibrium, respectively. PhyE and phyD contributes mainly to phyA-mediated germination.
597 Furthermore, phyB operates in a wide range of GA concentrations to increase GA sensitivity of
598 the seeds, meanwhile phyC and phyE only have effects at a medium range of GA concentrations.
599 Gray and black connectors indicate the photoreceptors effects on light and GA seed sensitivities,
600 respectively. Numeric references in the graph indicate previous references for the phytochrome
601 interactions: [(1)] Botto, Sánchez & Casal 1995, Shinomura et al. 1994, [(2)] Henning et al. 2001, [(3)]
602 Botto et al. 1995, Botto et al. 1996, Shinomura et al. 1996, [(4)] Henning et al. 2002.
603 .

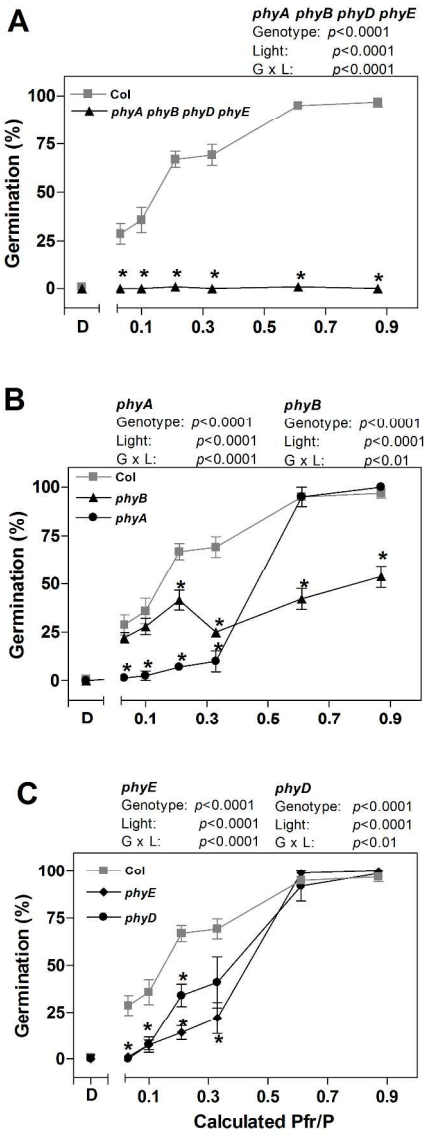

Fig. 1

Induction of germination by light in the wild type compared with phyA phyB phyD phyE quadruple mutant (A), phyA and phyB simple mutants (B) and phyE and phyD simple mutants (C).
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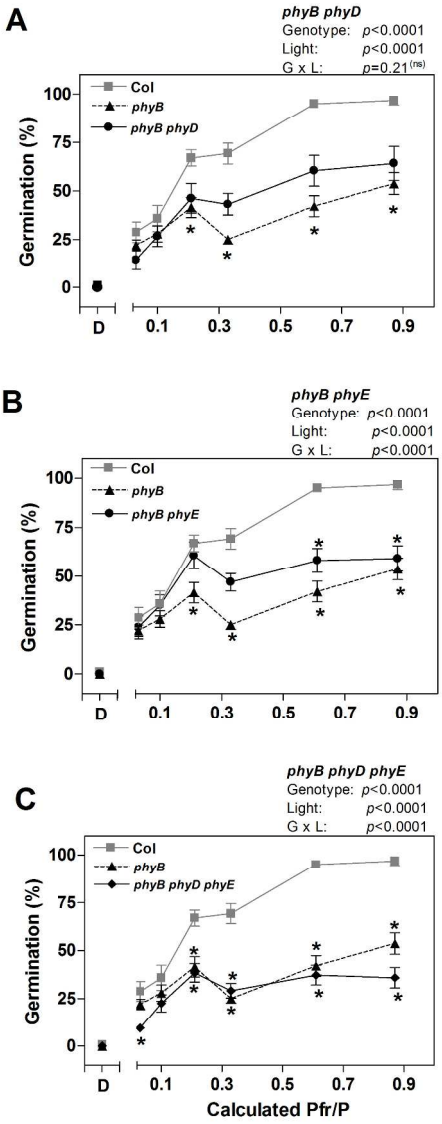

Fig. 2

Induction of germination by light in the wild type and phyB simple mutant compared with phyB phyD double mutant (A), phyB phyE double mutant (C) and phyB phyE phyD triple mutant (D).
207x364mm (300 x 300 DPI)

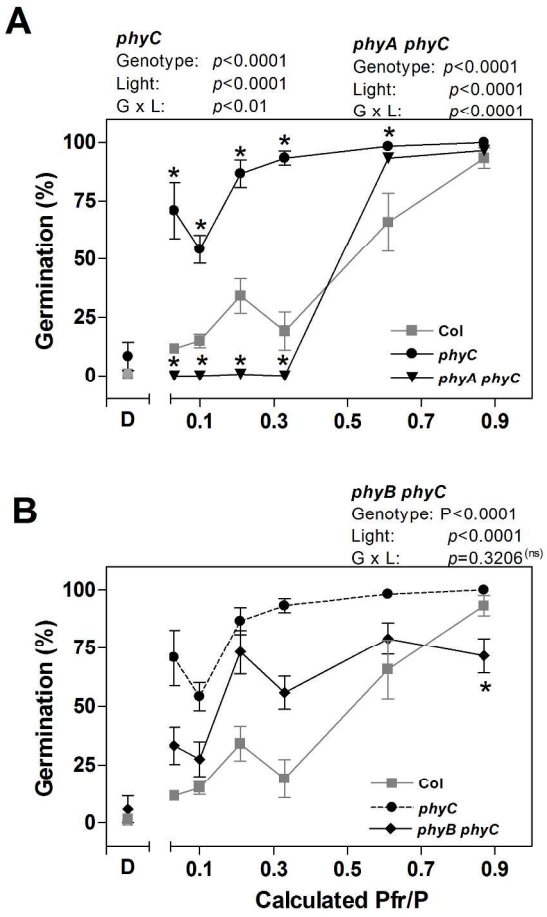

Fig. 3

Induction of germination by light in the wild type compared with the simple phyC mutant, the double phyA phyC mutant (A), and double phyB phyC mutants (B).
187x301mm (300 x 300 DPI)

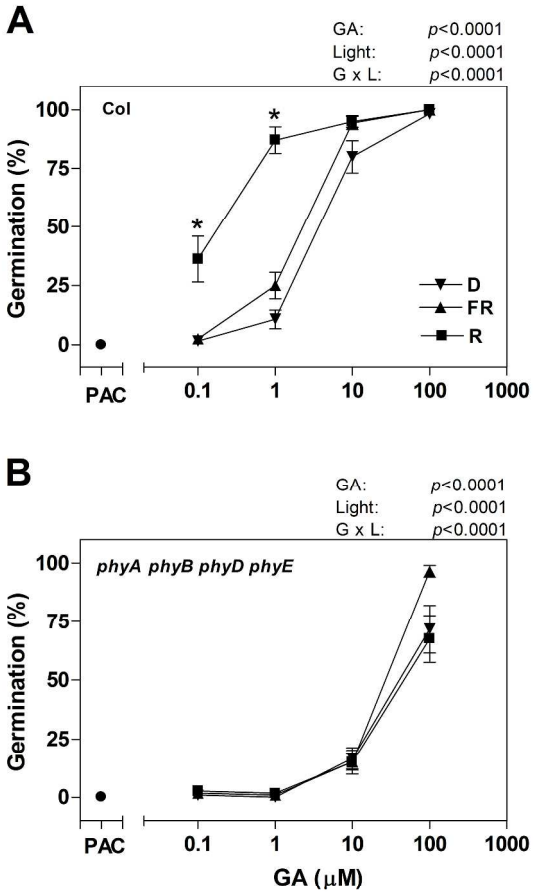

Fig. 4

Fig. 4. Germination dose-response curves to gibberellins (GA) in the wild type (A) and phyA phyB phyD phyE quadruple mutant (B).
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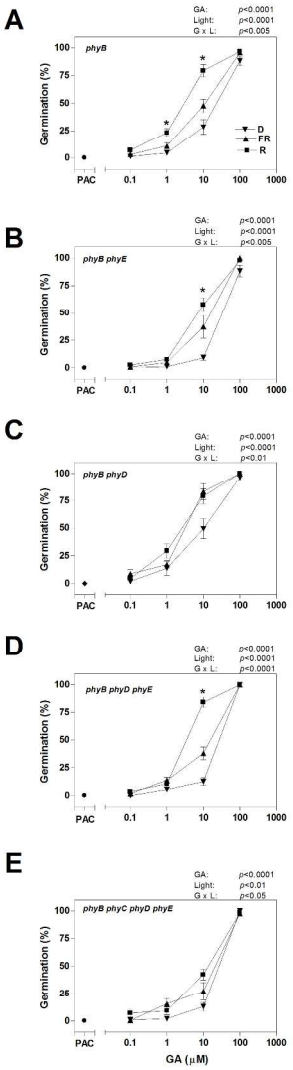

Fig. 5

Germination dose-response curves to GA in phyB (A), and different double (B and C), triple (D) and quadruple (E) mutants in the phyB background.
179x367mm (300 x 300 DPI)

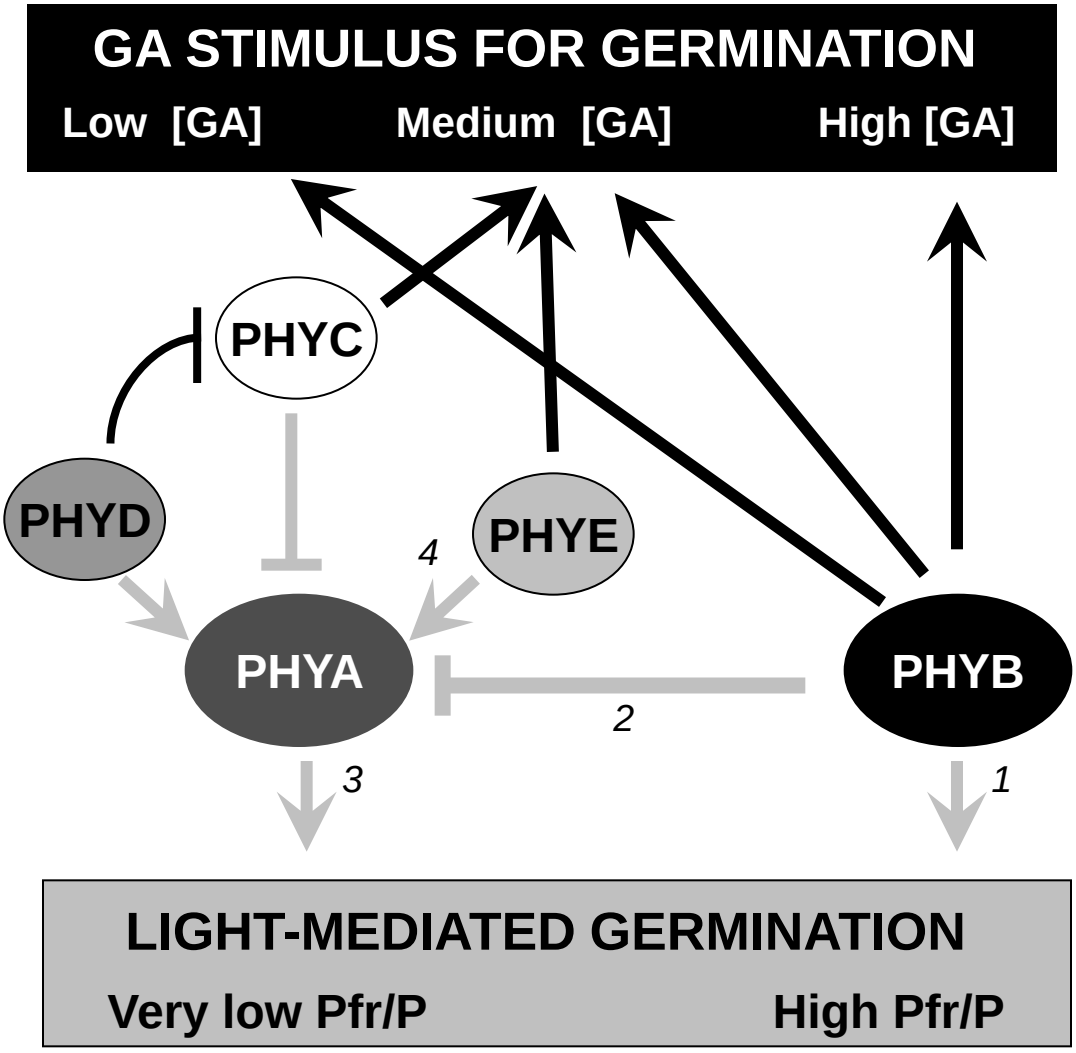
GA STIMULUS FOR GERMINATION
Low [GA]
Medium [GA]
High [GA]
PHYC
PHYD
PHYE
PHYA
PHYB
4
2
3
1
LIGHT-MEDIATED GERMINATION
Very low Pfr/P
High Pfr/P

Fig. 6

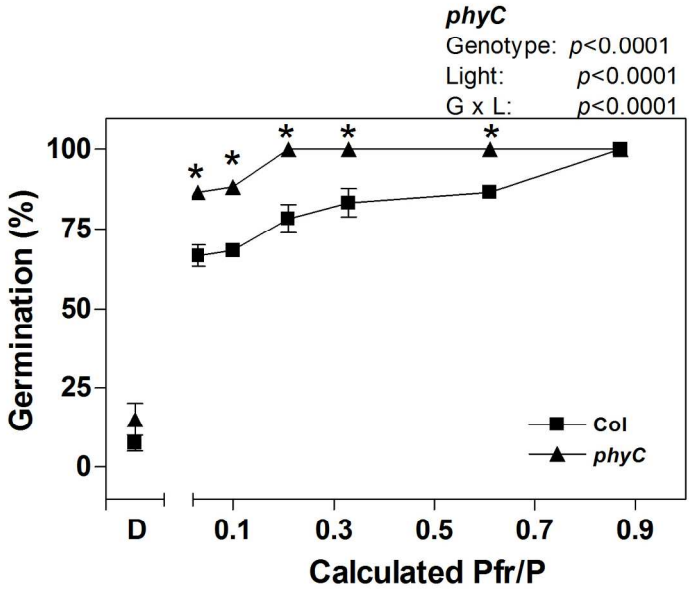

Fig. S1

Induction of germination by light in the wild type compared with the simple phyC mutant.
173x150mm (300 x 300 DPI)

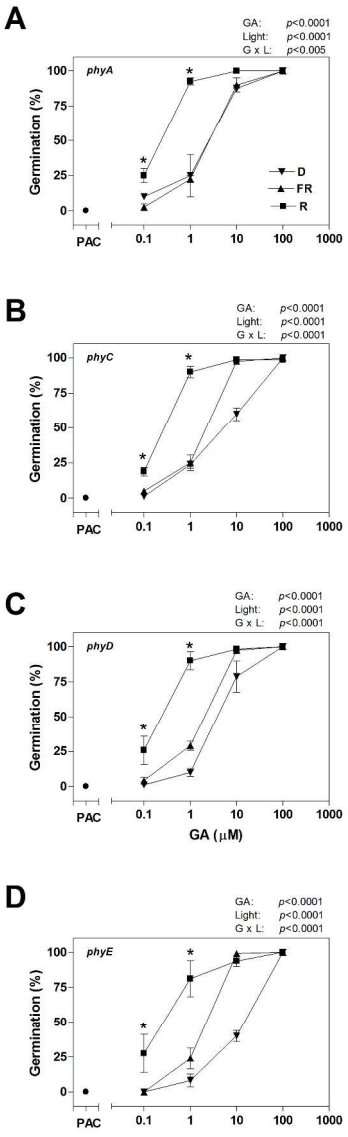

Fig. S2

Germination dose-response curves to gibberellins (GA) in the simple phyA (A), phyC (B), phyD (C) phyE (D) mutants.
193x366mm (300 x 300 DPI)

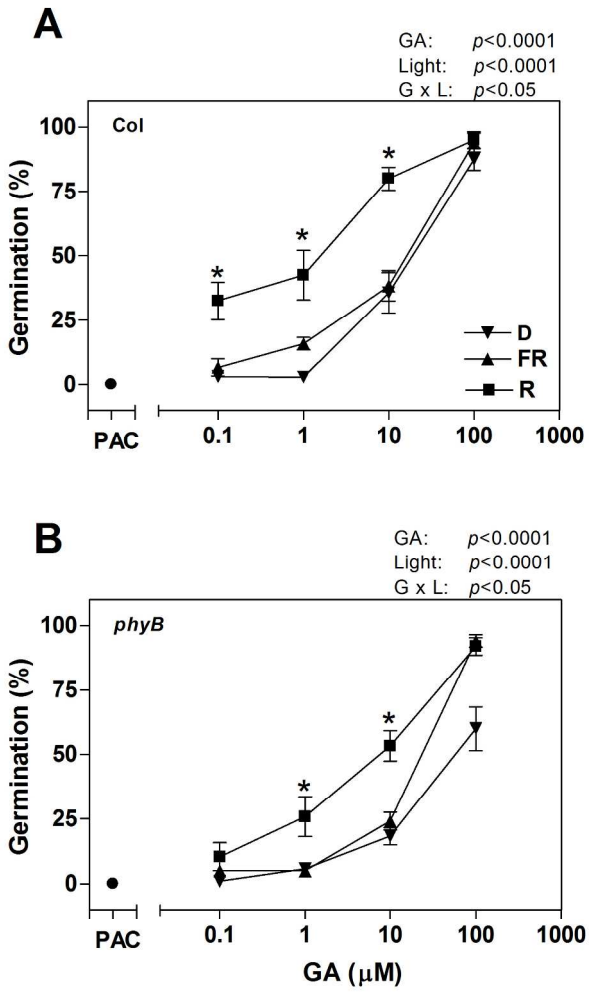

Fig. S3

Germination dose-response curves to GA in the wild type (A) and the simple phyB (B) mutant, after 4 years of storage.
188x269mm (300 x 300 DPI)

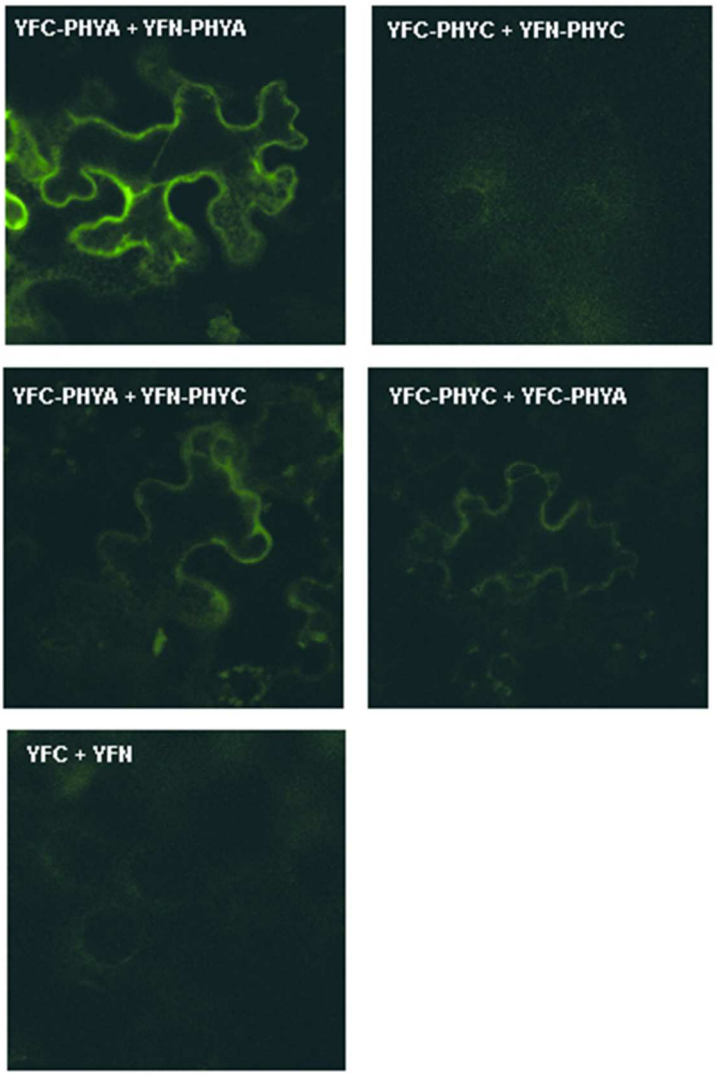

Fig. S4. Physical interaction between PHYA and PHYC.
149x200mm (72 x 72 DPI)

Table S1

Genotype vs. *phyB*	G x L interaction	Pfr/P 0.03	0.1	0.21	0.33	0.61	0.87
phyB phyD	1,76[a]	1,269[a]	0,195[a]	0,374[a]	1,85[a]	2,176[a]	1,559[a]
phyB phyE	1,10[a]	0,136[a]	0,611[a]	2,445[a]	2,45[a]	1,809[a]	0,640[a]
phyB phyE phyD	1,2[a]	2,271[a]	0,887[a]	0,421[a]	0,527[a]	0,736[a]	2,202[a]

Arana et al. Diversity of phytochrome functions in germination

LEGENDS OF SUPPLEMENTARY FIGURES

Fig. S1. Induction of germination by light in the wild type compared with the simple *phyC* mutant. The seed batches were stored for 5 months at room temperature before the experiments. Two-way ANOVA of the comparison between the wild type and *phyC* is indicated at the upper side of the panel. The experimental protocol and references are similar to the described in Fig. 1.

Fig. S2. Germination dose-response curves to gibberellins (GA) in the simple *phyA* (A), *phyC* (B), *phyD* (C) *phyE* (D) mutants. The experimental protocol and references are similar to the described in Fig. 4.

Fig. S3. Germination dose-response curves to GA in the wild type (A) and the simple *phyB* (B) mutant, after 4 years of storage. Seeds were treated as described in Fig. 3. The experimental protocol and references are similar to the described in Fig. 4.

Fig. S4. Physical interaction between PHYA and PHYC. BiFC assays testing the interactions between PHYA and PHYA homodimers (YFC-PHYA + YFN-PHYA), PHYC and PHYC homodimers (YFC-PHYC + YFN PHYC), PHYA and PHYC heterodimers (YFC-PHYA + YFN PHYC and YFC-PHYC + YFC-PHYA) in *N. Benthamiana* leaf cells. The negative control (YFC + YFN) is included in the figure. The experiments were performed with dark-adapted *N. Benthamiana* plants, in order to avoid phyA or phyC degradation.

Table S1: Test for significant differences between *phyB* and mutants sharing a *phyB* background. The table shows the results from the two-way ANOVAs for the comparison between *phyB* and mutants sharing a *phyB* background. F ratios for the interaction genotype x light (G x L) are indicated in the first column. Bonferroni post-

tests comparing the germination response to light of each mutant vs. *phyB*. t ratio[illegible] indicated below each Pfr/P value.

[illegible] 0.05