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Research

Gene expression analysis of light-modulated germination in tomato seeds

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Summary

• Tomato (*Solanum lycopersicum*) seed germination can be inhibited by continuous irradiation with far-red light (FRc) and re-induced by a subsequent red light pulse.

• In this study, we carried out a global transcript analysis of seeds subjected to FRc inhibitory treatment, with and without a subsequent red light pulse, using potato cDNA microarrays. We also identified and characterized genes involved in light-modulated germination as elements of the phytochrome signalling pathway.

• Microarray data showed that the inhibition of germination by FRc involves the induction of a large number of genes and the repression of a significantly smaller quantity. Multivariate analysis established an underlying pattern of expression dependent on physiological treatment and incubation time, and identified different groups of genes associated with dormancy maintenance, inhibition and promotion of germination. We showed that *ELIP*, *CSN6*, *SOS2* and *RBP* are related to the photocontrol of germination. These genes are known to participate in other physiological processes, but their participation in germination has not been suggested previously.

• Light quality regulates the tomato seed transcriptome during phytochrome-modulated germination through changes in the expression of certain sets of genes. In addition, *ELIP* and *GIGANTEA* were confirmed as components of the phytochrome A signalling pathway during FRc inhibition of germination.

Abbreviations: ABA, abscisic acid; *CSN6*, *COP9-signalosome subunit 6*; EKO, ent-kaurenoic acid oxidase; *ELIP*, *early light-induced protein*; FR, far-red light; FRc, far-red light continuous irradiation; *fri1-1*, phytochrome A mutant, *far-red insensitive 1-1*; FRp, far-red light pulse; GAI, gibberellin insensitive; HIR, high-irradiance response; LFR, low-fluence response; MM, Money maker; Pfr, far-red light-absorbing form of phytochrome; Pfr : Pt, Pfr form to total phytochrome (Pfr + Pr forms) protein ratio; Pr, red light-absorbing form of phytochrome; PHY, phytochrome apoprotein; phy, phytochrome holoprotein; PLACE, Plant *Cis*-acting Regulatory DNA Elements; R, red light; *RBP*, *RNA-binding protein ABA-regulated*; RGA, repressor of *ga1-3*; Rp, red light pulse; SGED, Solanaceae Gene Expression Database; SGN, SOL Genomics Network; sqRT-PCR, semiquantitative reverse transcriptase-polymerase chain reaction; TAIR, The *Arabidopsis* Information Resource; UTR, untranslated region; VLFR, very low-fluence response.

Introduction

Seed germination is a critical step in the life cycle of plants. Seeds have mechanisms able to detect environmental cues that promote germination when the circumstances are likely to be favourable for the development of the future plant. Light is one of the main factors carrying rich information providing precise cues about the prevalent environmental conditions, and controls seed germination in many species (Bewley & Black, 1994). Germination can be promoted or inhibited by

light. Promotion is a consequence of the formation of the far-red light (FR)-absorbing form of phytochrome (Pfr), the active form of phytochromes (phy), mainly phyA, phyB and phyE (Botto *et al.*, 1995, 1996; Shinomura *et al.*, 1996; Hennig *et al.*, 2002). In contrast, the inhibition of germination by light depends on prolonged exposure to continuous FR (FRc) mediated by phyA (Casal & Sánchez, 1998; Casal *et al.*, 1998), or to the effect of blue light mediated by an as yet unidentified photoreceptor (Bewley & Black, 1994; Gubler *et al.*, 2008). These responses of phytochromes participate in the perception by the seeds of extremely important aspects of the environmental scenario. Although sensing of the red light (R) to FR ratio (mainly through phyB and phyE) allows the seeds of many species to detect the opening of gaps in the canopy (Vázquez-Yañez & Smith, 1982; Deregibus *et al.*, 1994; Insausti *et al.*, 1995), the intervention of phyA enables the seeds to perceive the exceedingly small amounts of photons reaching them during soil disturbances by tillage operations (Scopel *et al.*, 1991; Botto *et al.*, 1998). In addition, phyA, when exposed for long periods to far-red rich light, is thought to participate, interacting with stable phytochromes, in the perception of the presence of a closed canopy (Casal *et al.*, 1998).

Most of our knowledge about the molecular aspects of phytochrome action is related to the promotion of germination by the R–FR reversible low-fluence response (LFR) mediated by stable phytochromes (Casal & Sánchez, 1998). In the promotion of germination by the Pfr form of stable phytochromes, gibberellins (GA) and abscisic acid (ABA) are central players. Pfr influences the synthesis, catabolism and sensitivity to GA (Toyomasu *et al.*, 1993, 1998; Yamaguchi *et al.*, 1998; Arana *et al.*, 2006), and also the metabolism of ABA (Seo *et al.*, 2006). In *Arabidopsis thaliana*, PIL5 (a phytochrome-interacting basic helix–loop–helix protein) is an important element relating phytochrome promotion of germination to GAs (Oh *et al.*, 2004, 2006). Promotion of germination by phyB involves the degradation of PIL5, which results in transcriptional repression of GAI (GA insensitive) and RGA (repressor of *ga1-3*), and also the stimulus of *de novo* GA biosynthesis, leading to the degradation of RGL2 and other DELLA proteins (Oh *et al.*, 2007). In addition, GA decrease ABA levels by repressing the expression of ABA biosynthetic genes and activating the expression of an ABA catabolic gene (Oh *et al.*, 2007). Conversely, ABA decreases GA levels by repressing GA biosynthetic genes (Seo *et al.*, 2006). Recently, a new negative regulator involved in the phytochrome-mediated promotion of germination has been characterized (Kim *et al.*, 2008). SOMNUS (SOM) encodes a nucleus-localized CCCH-type zinc finger protein that interacts physically with PIL5 and regulates ABA and GA metabolism, partially through a DELLA-independent pathway. However, very little is known about the molecular mechanisms related to phyA action. The photocontrol of germination by phyA is an unexplored area that requires investigation. Under certain circumstances, germination is promoted by phyA through the very low-fluence response (VLFR) that is saturated by very small amounts of Pfr (Botto *et al.*, 1996). The Pfr form of phyA also induces the degradation of PIL5, but the signalling elements downstream in the pathway from the promotion of GA synthesis and ABA catabolism are not identical to those in the pathway initiated by phyB. Other factors, in addition to GAI and RGA, participate differentially in these pathways (Oh *et al.*, 2007). Even less information is available on the inhibition of germination by FRc mediated by phyA.

The germination of tomato seeds is controlled by phytochromes (Mancinelli *et al.*, 1966; Shichijo *et al.*, 2001). They can germinate in darkness as a result of Pfr formed during ripening and present in the dry seed (Bewley & Black, 1994). The removal of Pfr by a FR pulse (FRp) can inhibit germination if given within a certain period of time after the start of imbibition. However, 24 h or more after the start of imbibition, FRp alone is not sufficient for inhibition. By contrast, at that imbibition time, FRc can inhibit germination through a high-irradiance response (HIR) (Shichijo *et al.*, 2001). Tomato seeds lacking functional phyA (*fri1-1*, *far-red insensitive 1-1*) are less inhibited by FRc. As this inhibitory action of FRc through phyA has not been found, so far, in *Arabidopsis*, tomato seeds are a good system to study a process that influences germination in many species and is thought to be of considerable biological importance (Casal & Sánchez, 1998).

In this work, we explored the changes in gene expression related to the control of germination by light in tomato seeds. Transcriptional arrays were used to describe a general view of gene expression associated with light treatments that promote or inhibit germination. In addition, we documented, for the first time, the association of a specific group of genes with the inhibition of germination by FRc displaying HIR in a phyA-dependent fashion.

Materials and Methods

Plant material and seed production

Plants of *Solanum lycopersicum* L. cv. Money maker (MM) wild-type (WT) and PHYA mutant, *fri1-1*, were grown in organic matter-enriched soil from seeds kindly donated by Richard Kendrick (van Tuinen *et al.*, 1995). Seeds were extracted from the mature fruits, sequentially treated with 250 mM HCl and 10% v/v commercial sodium hypochlorite solution, and finally washed with distilled water. Seeds were dried at room temperature and stored in hermetic dry storage.

Light sources and germination conditions

Red light (35 μmol m^{-2} s^{-1}) was provided by 40-W fluorescent lamps (Philips 40/15, Philips Electronic Inc., Sao Paulo, Brazil) in combination with a red acrylic filter, giving Pfr : total phytochrome (Pt) = 0.83. FRc light (100 μmol m^{-2} s^{-1}) was

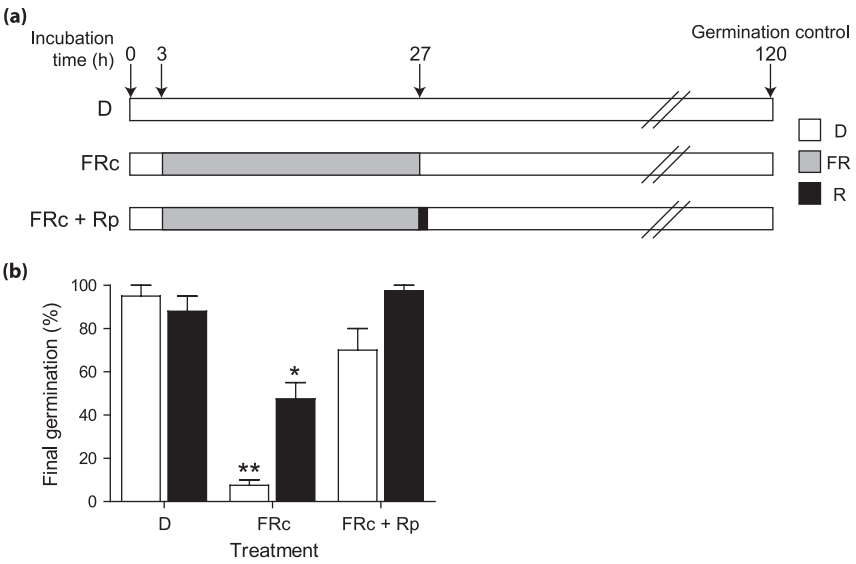


Fig. 1 Light treatments of microarray samples and seed physiological response. (a) Experimental protocol for darkness (D), continuous far-red light (FRc) and continuous far-red light followed by a red pulse (FRc + Rp). (b) Germination of tomato (*Solanum lycopersicum*) cv. Money maker (MM, open bars) and phytochrome A mutant *far-red insensitive 1-1* (*fri1-1*, filled bars) seeds. Bars represent means of 10 biological replicates ± standard error. Significant differences between means: *, $P < 0.05$; **, $P < 0.001$.

provided by a set of 150-W incandescent lamps in combination with a red acetate filter, six 2-mm-thick blue acrylic filters (Paolini 2031, Acrílicos Anbyn, Buenos Aires, Argentina) and a 10-cm-thick water filter.

MM and *fri1-1* seeds were incubated on distilled water-saturated cotton wool in clear plastic boxes wrapped in black plastic sheets at 25°C. For microarray samples, after dark incubation for 3 h, seeds were treated as follows: (1) 24 h of FRc; (2) 24 h of FRc, followed by a 10-min red light pulse (Rp), FRc + Rp; and (3) complete darkness (Fig. 1a). After light treatments, seeds were returned to darkness until germination counting. Samples were obtained at the following times after the start of incubation: (1) 6, 9, 12, 24 and 27 h for FRc samples; (2) 30 and 51 h for FRc + Rp samples; and (3) 3, 9, 12 and 24 h for darkness samples (Table S1, see Supporting Information).

For the validation of microarray data, seeds were kept in complete darkness (for germination control) or incubated for 3 h in darkness, and then exposed to a 30-min Rp followed by incubation in darkness for 21 h. Seeds subjected to Rp were treated with a 1-h FRp or 24 h of FRc to distinguish between the effect of Pfr reversion and HIR (Fig. 6a, see later). Samples were taken at 24, 33 and 45 h after the start of incubation for both genotypes and treatments.

Arabidopsis thaliana ecotype Columbia WT and *sos2-2* mutant seeds were sown on agar–agar 1% w/v in clear plastic boxes, irradiated with FRp (to reduce Pfr levels), immediately treated with a 30-min Rp and incubated in darkness at 25°C until germination counting.

RNA isolation

For microarray samples, whole seeds were ground in liquid nitrogen, and extracted with 2 vol of extraction buffer [1% sodium dodecylsulphate (SDS), 6% *p*-aminosalicylic acid, 1% NaCl, 6% water-saturated phenol and 2% 2-mercaptoethanol] and 2 vol of 125 : 24 : 1 acid : phenol : chloroform (with isoamyl alcohol) solution, pH 4.5 (Ambion, Austin, TX, USA). The aqueous phase was sequentially extracted with 1 vol acid : phenol : chloroform solution and 1 vol chloroform. Nucleic acids in the aqueous phase were precipitated with 0.15 vol 3 M NaCl and 2.5 vol 100% ethanol at –80°C for 20 min. After centrifugation, the pellet was washed with 70% ethanol, resuspended in RNase-free water and precipitated with 1 vol 4 M LiCl overnight at 4°C. LiCl was removed after centrifugation; the pellet was rinsed with 2 M LiCl and resuspended in RNase-free water. Total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and cleaned up with RNeasy Plant Mini Kit columns (Qiagen, Valencia, CA, USA). Message RNA was amplified with a Message Amp II aRNA Amplification Kit (Ambion) and concentrated by vacuum centrifugation.

For microarray data validation, three biological replicates of treated MM and *fri1-1* seeds were ground in liquid nitrogen and extracted with a NucleoSpin RNA Plant Kit (Macherey Nagel, Düren, Germany). All kits were used according to the manufacturers' instructions.

Microarray hybridizations

Five micrograms of amplified RNA per biological replicate were submitted to the Expression Profiling Service of the NSF Potato Genome Project (TIGR Solanaceae Genomics Resource, J. Craig Venter Institute, Rockville, MD, USA; formerly TIGR, USA) for hybridization to the TIGR Potato 10K cDNA microarrays, chip scanning and data accumulation (GenePix Pro 4.0 software, MDS Analytical Technologies, Sunnyvale, CA, USA). Two independent biological replicates

(consisting of 10 subsamples each) were submitted for each light treatment (Table S1), and the resulting expression datasets are publicly available at the Solanaceae Gene Expression Database (SGED) (http://www.tigr.org/tigr-scripts/tdb/sol/study/sol_study.pl).

Microarray data handling and analysis

To study differences in the number of modified genes in some treatments, we counted the clones that had twofold or greater difference in relative expression values between treatments using autofilters from the Data menu of the Excel program. Multivariate analysis (correspondence analysis and canonical discriminant analysis) were performed with PC-ORD software (McCune & Mefford, 1999) using the normalized dataset provided by the Expression Profiling Service. The C17 treatment sample (Table S1) was identified as an outlier in preliminary tests, and was removed from subsequent analyses. Canonical discriminant analysis was used to obtain the genes that best discriminated between physiological treatments, and the selection was performed according to the square of the value of the canonical coordinates associated with the axis. The 868 selected genes had a value for some of the coordinates of greater than 0.79. A new correspondence analysis was performed with these genes, followed by grouping using Ward's clustering algorithm.

Raw data resulting from the scanning of the microarrays was used to search for genes differentially expressed in every hybridization. Microarray data analysis was performed with the free software genArise developed at the Computing Unit of the Cellular Physiology Institute of the National University of Mexico (UNAM; www.ifc.unam.mx/genarise/). We analysed genes whose *z*-score was greater than +2 for over-expressed genes and less than −2 for down-regulated genes. Gene annotation was determined using the GenePix Array List version 3 for the TIGR Potato cDNA Microarray (http://www.tigr.org/tdb/sol/sol_ma_layout.shtml).

Semiquantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR) validation of microarray data

For sqRT-PCR of the selected genes, 1 μg of total RNA was used as template to synthesize cDNA using SuperScript III (Invitrogen). Primers for PCR were generated using FastPCR software version 4.0.27 (R. Kalendar, Institute of Biotechnology, University of Helsinki, Finland) with sequence data from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/) and the SOL Genomics Network (SGN) (http://www.sgn.cornell.edu/) (Mueller *et al.*, 2005). Primers for *GIGANTEA* were kindly gifted by Mariana Rutitzky and Marcelo Yanovsky (IFEVA-CONICET, FAUBA, Buenos Aires, Argentina), and for *SlSOS2* by Andres Belver (Estación Experimental del Zaidín, CSIC, Granada, Spain). Gene-specific primers are given in Table S10 (see Supporting Information). PCRs were performed in a mixture containing 1 μl of first-strand cDNA in a final volume of 25 μl. The number of PCR cycles for each gene was determined such that the amount of product was in the linear range of amplification. PCR amplifications were separated in a 2% agarose gel and transferred to Hybond N^+ Nylon Membrane (GE Healthcare Life Sciences Corporation, Buckinghamshire, UK) using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). A specific probe for each gene was generated and hybridized with an AlkPhos Direct Labelling and Detection System Kit (GE Healthcare Life Sciences Corporation). A chemifluorescent signal was generated using an ECF™ substrate reaction for *c.* 4–24 h, detected using a Storm 840 scanner (Molecular Dynamics, GE Healthcare Life Sciences Corporation) and analysed by the ImageQuant 5.2 program (Molecular Dynamics). The results are plotted as the expression ratio of the selected genes to actin. All kits and reagents were used according to the manufacturers' instructions.

Search for *cis*-regulatory sequences

The search for *cis*-regulatory sequences located in the region upstream of the translational start site in the selected genes was performed using the Database of Plant *Cis*-acting Regulatory DNA Elements (PLACE) (http://www.dna.affrc.go.jp/PLACE/index.html) (Higo *et al.*, 1999) with sequences obtained from the Unigene Database of SGN (http://www.sgn.cornell.edu/) (Mueller *et al.*, 2005) when possible, the GenBank Database or its homologue in *A. thaliana* [The *Arabidopsis* Information Resource (TAIR); http://www.arabidopsis.org/]. The sequences used for the subsequent analysis were as follows: for *GIGANTEA*, a 318-bp fragment of SGN-U329770 from SGN, the only available tomato Unigene in the database that matches the region upstream of the translational start site of *AtGIGANTEA*; for *ELIP* (*early light-induced protein*), a 155-bp assembly prepared from the putative promoter region of *LeELIP* (AY547273) found in GenBank and its best matched Unigene, SGN-U338643, from SGN; for *CSN6* (*COP9-signalosome subunit 6*), a 300-bp fragment of the translational upstream region of the gene *AtCSN6A* (AT5G56280) from TAIR, which is the best match for the putative tomato *CSN6*; for *RBP* (*RNA-binding protein ABA-regulated*), a 267-bp assembly prepared from the potato Unigene SGN-U274180 from SGN (best match to the microarray clone sequence annotated as *RBP*) and the 5′ untransformed region (UTR) of the putative *A. thaliana RBP* (AT2G37510) from TAIR (matched to the potato Unigene); for *SOS2*, a 319-bp fragment of SGN-U322214 from SGN, a tomato Unigene showing high identity with the microarray clones annotated as *SOS2*-like and the 5′ UTR sequence of the serine/threonine kinase family members *AtSOS2* (AT5G35410) and *AtCIPK6* (AT4G30960). References for the relevant *cis*-sequences are given in Table S11 (see Supporting Information).

Results

Germination of tomato seeds is inhibited by FRc and promoted by a subsequent Rp

WT tomato cv. MM and *fri1-1* mutant seeds were exposed, after 3 h of incubation in darkness, to FRc, FRc followed by Rp (FRc + Rp) or kept in darkness (Fig. 1a). Seeds of both genotypes germinated close to 100% in darkness (Fig. 1b). After FRc, almost 90% of WT seeds showed inhibited germination, but the germination of *fri1-1* seeds was reduced by only 40% under the same light conditions, indicating the participation of phyA in this response. When Rp was applied after FRc, the germination of the seeds was promoted in a similar proportion in both genotypes (Fig. 1b).

Global gene expression changes are related to the inhibitory and promoting effects of light on tomato seed germination

A microarray study was conducted to compare global gene expression patterns related to the light-modulated germination in WT and *fri1-1* tomato seeds. Because tomato microarrays were not available at the start of the study, gene expression data were obtained by hybridizing tomato samples to potato arrays. Recent bibliography has indicated that potato and tomato have a high degree of transcript sequence conservation (*c.* 78%), and that reliable gene expression values can be derived from microarrays of closely related species (Rensink *et al.*, 2005; Oshlack *et al.*, 2007). The analysis was conducted using microarrays containing ~10 000 potato cDNA clones and samples harvested at different times after the start of the light treatments from seeds displaying the behaviour described in Fig. 1b. A significant signal was obtained from 79.8% of the genes represented by the clones in the potato cDNA array over all the hybridizations made, in agreement with the previously observed extent of conservation among transcript sequences of the two species (Rensink *et al.*, 2005).

To select the genes up- and down-regulated under inhibitory and inductive light conditions (FRc and FRc + Rp, respectively), we identified the genes with a twofold or larger change in expression ratio. A large number of genes were associated with the light modulation of seed germination in tomato seeds (Fig. 2). In WT seeds, 12 h after the beginning of incubation, FRc induced the expression of close to 200 genes, compared with darkness; whereas only eight genes were repressed in the same light conditions (Fig. 2a; Table S2, see Supporting Information). About 18% of the genes induced by FRc were associated with transcriptional and translational regulatory machineries, such as nucleic acid binding factors, translation initiation and elongation factors, ribosomal proteins, DNA/RNA helicases and splicing factors (Fig. 2c). In *fri1-1* mutant seeds, compared with WT, FRc induced differentially the expression of 55 genes and repressed 18 genes (Fig. 2b; Table S2).

The re-induction of germination by Rp after FRc (FRc + Rp treatment), compared with FRc, induced a rapid increase in the expression of more than 100 genes, 3 h after Rp, and close to 300 genes 21 h later (Fig. 2d; Table S3, see Supporting Information). A more reduced group of genes with a similar temporal pattern of gene expression was repressed by Rp following FRc (Fig. 2f; Table S4, see Supporting Information). In *fri1-1* mutant seeds compared with WT, the number of genes differentially induced or repressed by Rp was reduced (Fig. 2e,g). Only 17 genes were induced early, whereas 15 different genes were up-regulated 21 h later, in *fri1-1* seeds (Fig. 2e; Table S3). A reduced group of genes was repressed 3 h after Rp; however, this number increased up to fourfold after 21 h (Fig. 2g; Table S4). In general, the differential expression of the early-responsive genes was maintained later in time for the different groups of genes (Fig. 2d,f,g).

Different patterns of gene expression are associated with the inhibitory and promoting effects of light on tomato seed germination

Multivariate analysis was applied for all genes expressed on the microarrays using WT and *fri1-1* seeds harvested at different times under FRc, FRc + Rp or kept in darkness (Table S1). The matrix of genes and treatments with expression values as entries, obtained from cDNA microarray hybridizations, was subjected to a rank transformation, followed by a correspondence analysis, to uncover the patterns of expression related to our experimental conditions (Perelman *et al.*, 2003). The results revealed clear differences between light treatments (Fig. 3a). The first dimension of the analysis (Axis 1) grouped RNA samples according to the conditions of the seeds (darkness, FRc and FRc + Rp groups), and explained 31.3% of the degree of correspondence between genes and treatments. Genotype was not identified as a determining factor in the observed pattern. The results suggest that a significant group of genes was responsible for the light responses of the seeds described in Fig. 1b.

In order to identify and classify the most significant genes related to the light responses of tomato seeds, the groups of samples sorted in Fig. 3a were used to perform a canonical discriminant analysis. Using Ward's clustering algorithm, the 868 genes selected by canonical discriminant analysis were arranged in five clusters according to the expression patterns and physiological responses displayed by the seeds (Fig. 4). Cluster 1 grouped 72 genes showing an increase in expression with incubation time independent of light treatment (Table S5, see Supporting Information). Cluster 2 included 54 genes down-regulated within the first 12 h of incubation, without any influence of light or darkness conditions (Table S6, see Supporting Information). Late embryogenesis proteins were found exclusively in this group, representing more than 7% of the total number of genes. The 127 genes in Cluster 3 were up-regulated by FRc after 12 h; in this group were found genes related to ABA, ethylene and brassinosteroid signalling pathways and

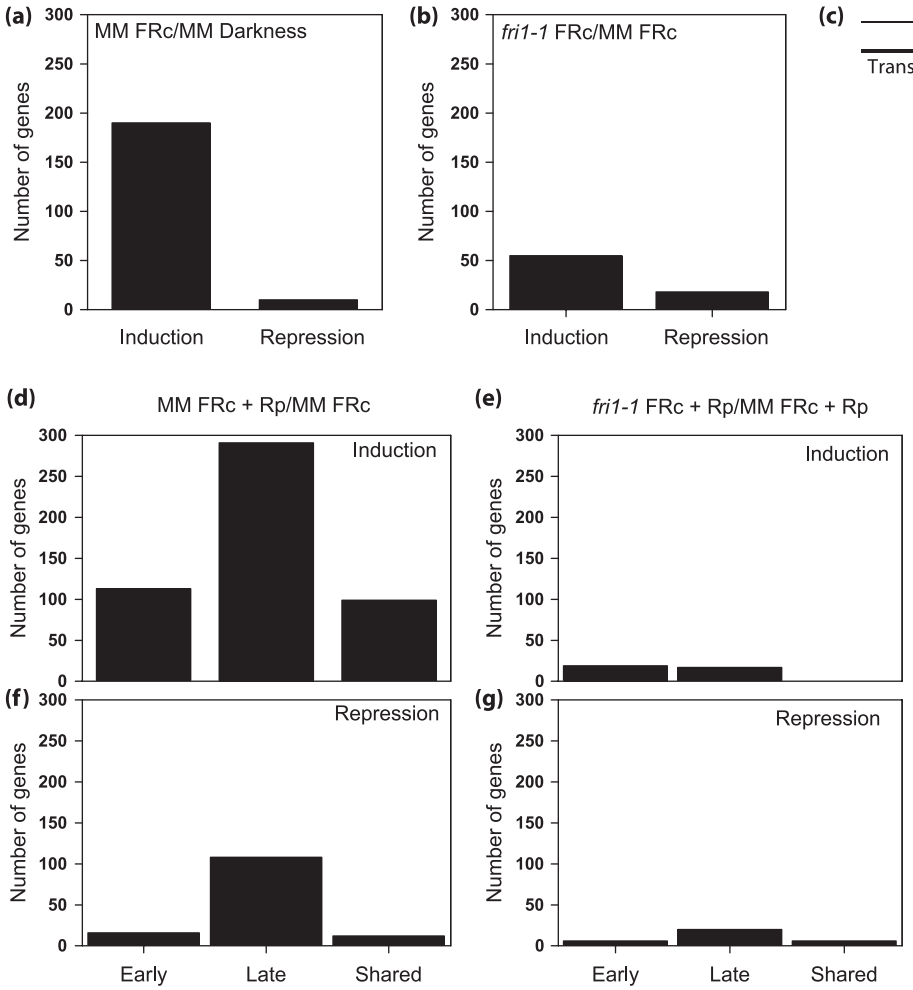


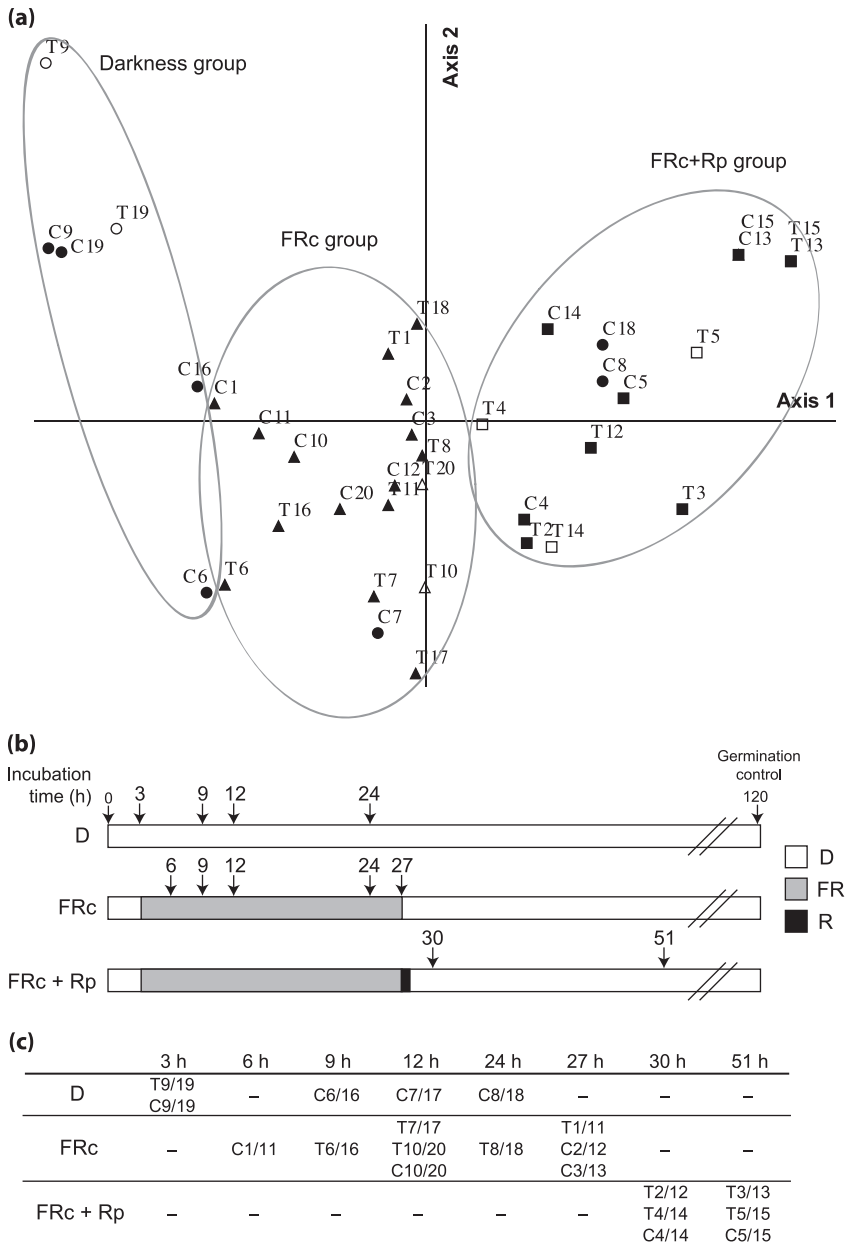
Fig. 2 Modulation of gene expression. (a, b) Number of genes induced or repressed by continuous far-red light irradiation (FRc) of tomato (*Solanum lycopersicum*) cv. Money maker (MM) (a) and *far-red insensitive 1-1* (*fri1-1*) (b) seeds with respect to the behaviour of MM seeds in darkness (a) or FRc (b), respectively. (c) Most represented functional categories of genes induced by FRc in MM seeds (shown in Table S2, see Supporting Information). (d–g) Number of genes induced or repressed by continuous far-red light followed by a red pulse (FRc + Rp) for MM (d, f) and *fri1-1* (e, g) seeds with respect to the behaviour of MM seeds in FRc (d, f) or FRc + Rp (e, g), respectively. Genes included in each group are shown in Tables S3 and S4 (see Supporting Information).

enzymes related to polyamine synthesis (Table S7, see Supporting Information). By contrast, 615 genes down-regulated by FRc and promoted by Rp were included in Clusters 4 and 5 (Tables S8 and S9, respectively; see Supporting Information). The differences in expression between FRc and darkness were evident after 9 h for the genes in Cluster 4 and after 21 h for those in Cluster 5. The genes in Cluster 4 were related to cell wall extensibility, RNA metabolism, synthesis of GA, cytokinins and ethylene, and catabolism of ABA; genes associated with endosperm weakening were represented in Cluster 5.

Identification of genes regulated by the inhibitory and promoting effects of light on tomato seed germination

Using the genArise program, we conducted a search of genes with differential expression between FRc and FRc + Rp. After careful stringent analysis, we selected some of the most significant genes whose expression was differentially modulated by light. In particular, we were interested in genes affected by FRc in one direction and by the subsequent Rp in the opposite direction, as these genes are good candidates for participation in the antagonism between the inhibitory action of prolonged FR (mediated by phyA) and the promotional effects of the Pfr form on germination. Of those genes whose expression was decreased by FRc and promoted after Rp, we found a group that had been associated previously with germination. This group included genes related to cell wall proteins (β-1,4-glucanases, β-1,3-glucanases, β-mannosidase, several polygalacturonases, expansins, some xyloglucan endotransglycosylases) and enzymes involved in the synthesis of active GA-like ent-kaurenoic acid oxidase (EKO) (Figs 5a, S2, S3a; see Supporting Information).

A second group of genes, not previously associated with the inhibition of germination, included *GIGANTEA* and *ELIP*,



	3 h	6 h	9 h	12 h	24 h	27 h	30 h	51 h
D	T9/19 C9/19	–	C6/16	C7/17	C8/18	–	–	–
FRc	–	C1/11	T6/16	T7/17 T10/20 C10/20	T8/18	T1/11 C2/12 C3/13	–	–
FRc + Rp	–	–	–	–	–	–	T2/12 T4/14 C4/14	T3/13 T5/15 C5/15

Fig. 3 Multivariate analysis of microarray data. (a) Correspondence analysis, applied to the rank-transformed expression values of genes represented on the microarray, grouped Money maker (MM, filled symbols) and *far-red insensitive 1-1* (*fri1-1*, open symbols) RNA samples according to their physiological treatments (darkness, D, circles; continuous irradiation with far-red light, FRc, triangles; and FRc followed by a red pulse, FRc + Rp, squares). (b) Sampling times (indicated by arrows) for the different treatments. (c) Table indicating the treatment and sampling times of every RNA sample. Complete references are given in Table S1 (see Supporting Information).

which were both up-regulated by FRc and down-regulated by Rp, and *CSN6*, *RBP* and *SOS2*, which were down-regulated by FRc and up-regulated by Rp (Figs 5b,c, S3b,c). The behaviour of these five genes is interesting, because it is consistent with that expected for participants in the antagonism between two of the modes of action of phytochrome (HIR and LFR), which inhibit and promote germination, respectively (Casal & Sánchez, 1998). To test for this possibility, we determined, using sqRT-PCR, the temporal changes in expression of these five genes in seeds of WT and *fri1-1* treated as shown in Fig. 6a. In order to maximize the detection of gene expression associated with antagonism, we treated the seeds after 3 h of dark incubation with Rp, which established a high Pfr : Pt ratio, promoting LFR that was completed in the following 21 h in darkness, as shown by the fact that germination escaped inhibition with FRp (Fig. 6c). If, instead of FRp, the seeds received FRc, germination was significantly inhibited, displaying HIR. Using this experimental protocol, the expression of both *GIGANTEA* and *ELIP* was significantly increased by FRc compared with FRp (Fig. 6b). *GIGANTEA* showed a peak of transcript accumulation at 9 h after the beginning of FRc treatment, whereas the expression of *ELIP* was high and constant all the time. Most significantly, FRc treatment did not change the expression of these genes in *fri1-1* seeds. These results show that the effect of FRc on the expression of *GIGANTEA* and *ELIP* requires the presence of functional

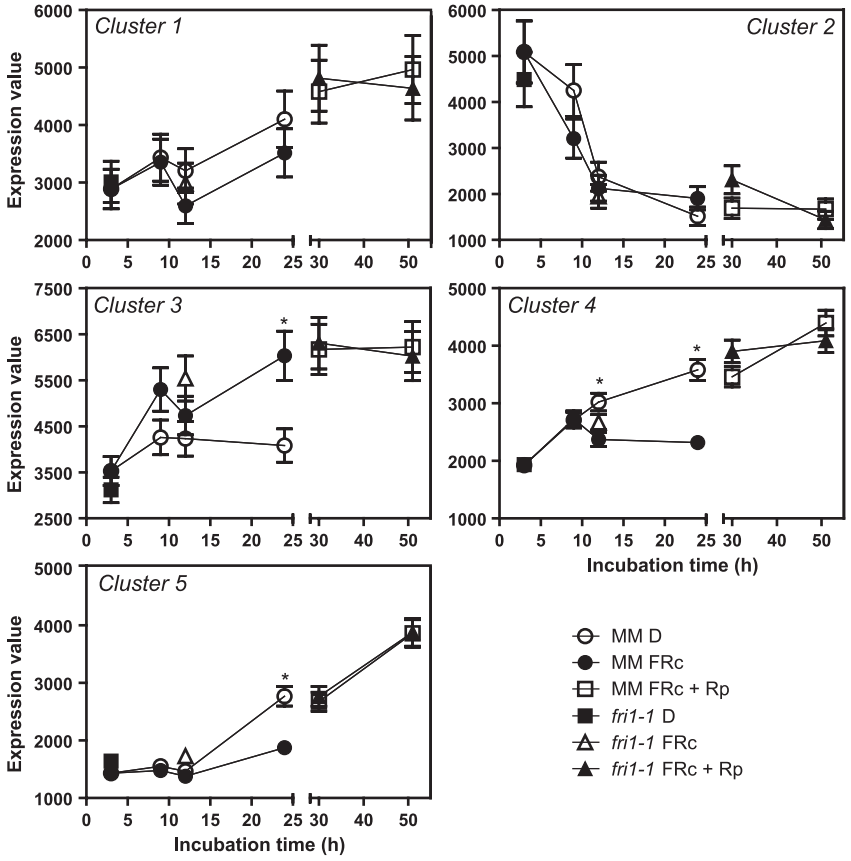


Fig. 4 Average expression of the gene clusters obtained from canonical discriminant analysis. Asterisks show significant differences between means ($P < 0.05$). The genes included in each cluster are shown in Tables S5–S9 (see Supporting Information).

phyA, and therefore the changes in transcript abundance of these genes have the characteristics of HIR. To our knowledge, these are the first genes associated with HIR in seeds.

In contrast, the transcript levels of *CSN6*, *RBP* and *SOS2* did not show any difference in expression between FRc and FRp treatments, suggesting that they are not implicated in the inhibition of germination by FRc. Therefore, the differences in expression observed in the microarray experiment suggest that these genes mediate the promotion of germination in tomato seeds by the action of Pfr of the phytochromes. In the case of *SOS2*, this interpretation of the data was reinforced by a complementary analysis using *A. thaliana* seeds, showing that the *sos2-2* mutant germinated significantly less well than WT under Rp (Fig. 6d).

Characterization of *cis*-regulatory sequences in promoter regions of selected genes

The genes mentioned above were searched for *cis*-regulatory sequences located in the region upstream of the translational start site. We based our search on sequences from tomato or Solanaceae (*GIGANTEA*, *ELIP* and *SOS2*), *A. thaliana* (*CSN6*) or a combination of both databases (*RBP*). We identified sequences related to tissue-specific (endosperm and embryo factors), light-regulated and hormone-regulated (GA, ABA, ethylene and auxin) expression (Table 1; Fig. S4, Table S11, see Supporting Information). The promoter sequences of the five selected genes have *cis*-regulatory elements involved in endosperm-specific expression and, in addition, *CSN6* and *SOS2* have embryo-specific gene expression elements. As expected, we identified sequences involved in light-mediated activation of expression. In four of the five selected genes (except *ELIP*), we identified *cis*-regulatory sequences related to hormone activity. The promoter region of *GIGANTEA* has elements involved in GA signalling repression and ABA response activation (in agreement with the gene expression analysed previously). We found GA and ABA signalling activation elements in *CSN6*, *RBP* and *SOS2* regions upstream of the translational start site, and, in addition, we identified GA repression and auxin and ethylene activation of response elements. The presence in these genes of elements involved in the action of several hormones is a reflection of their participation in the integration of signals of diverse origin.

Discussion

Throughout their life cycle, plants continually respond to signals that provide useful information for their adjustment to the environment. In particular, seeds exhibit a variety of responses to external cues that promote germination when the

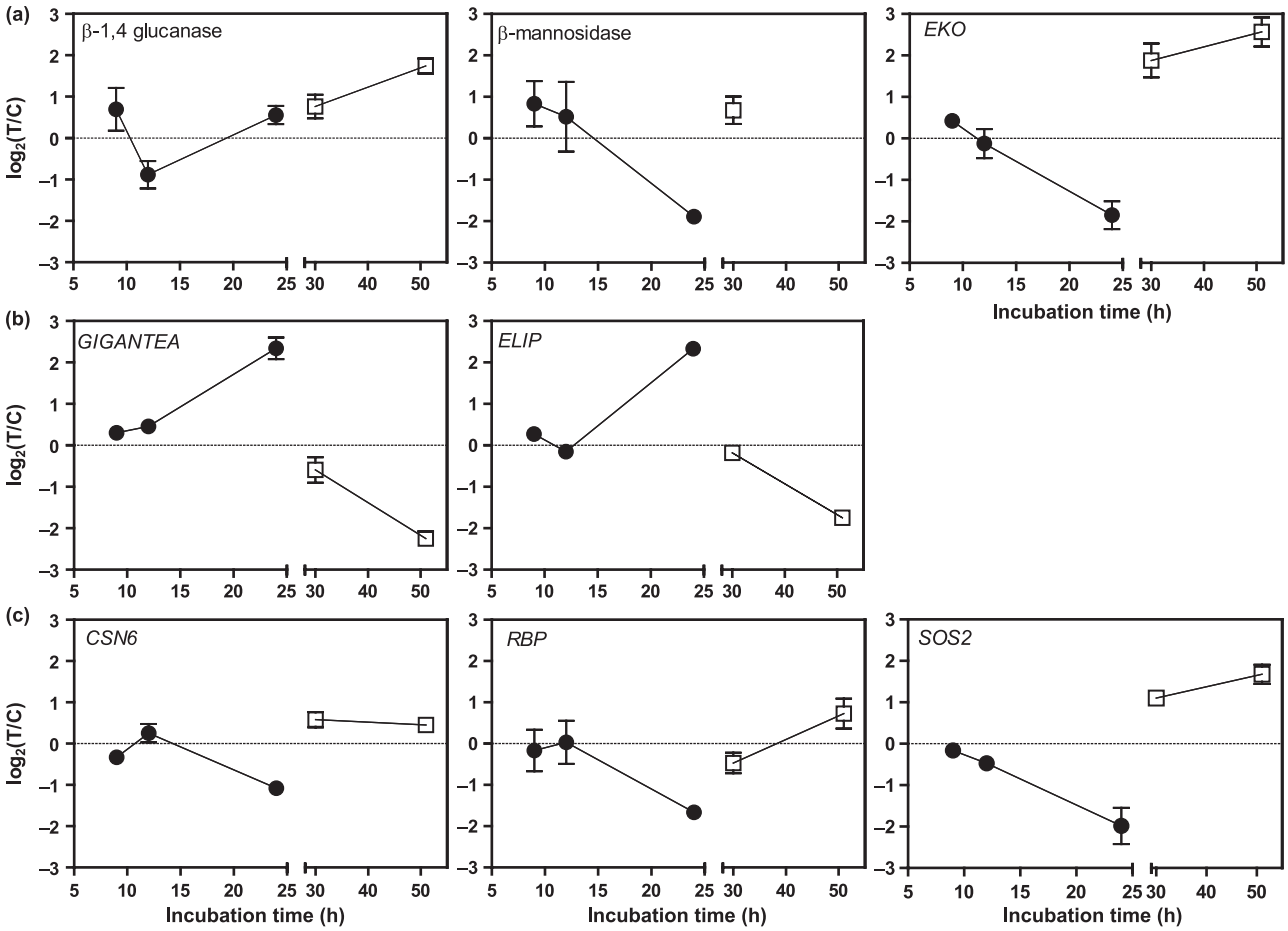
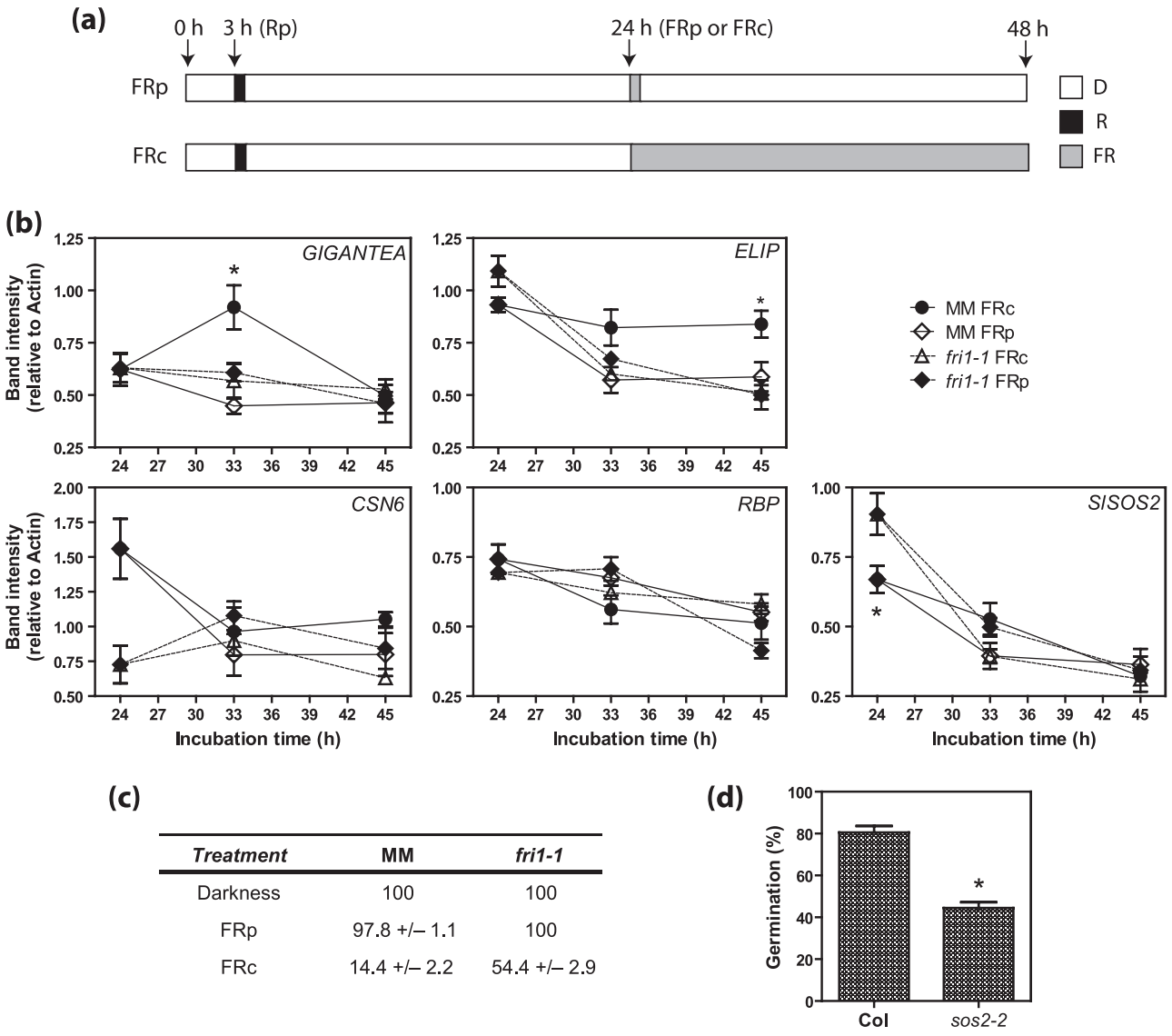


Fig. 5 Microarray data from selected genes. Expression values for genes with known (a) and unknown (b, c) response in germination. Symbols represent means ± standard error of $\log_2$ of the expression ratio between treatments. Circles, MM FRc/D; squares, MM FRc + Rp/FRc. D, darkness; FRc, continuous irradiation with far-red light; FRc + Rp, FRc followed by a red pulse; MM, cv. Money maker.

situation is likely to be favourable for the development of the future plant. Light is one of the most relevant signals for the fine-tuning of germination, promoting or inhibiting this process (Casal & Sánchez, 1998; Benech-Arnold *et al.*, 2000). Promotion is caused by the Pfr form of phytochromes, mainly phyA, phyB and phyE (Casal & Sánchez, 1998; Henning *et al.*, 2002). In seeds of several species, in which germination is promoted in darkness as a result of Pfr formed during ripening (MacCullough & Shropshire, 1970; Bewley & Black, 1994), germination can be inhibited by short exposures to FR that lead to the reversion of Pfr of the stable phytochromes, or by prolonged exposures to FR through the action of phyA (Casal & Sánchez, 1998; Shichijo *et al.*, 2001). The WT tomato seeds used in this study showed a very high germination rate in darkness, which was reduced sharply by prolonged FRc. Although, in darkness, *fri1-1* mutant seeds germinated to the same extent as WT, they were much less inhibited by FRc, confirming previous observations made by Shichijo *et al.* (2001) (Fig. 1b). Rp following FRc restored the high germination values in both genotypes, showing that the final outcome depends on the antagonistic influence of the persistently stimulated phyA and Pfr of other phytochromes.

Although promotion by the active form of the stable phytochromes, particularly phyB, has received attention from several research groups, the molecular aspects of the inhibition of germination by light have hardly been investigated to date. The inhibition of germination by FRc enhances the expression of a significantly larger number of genes in comparison with those that are repressed (Fig. 2a), and a similar observation was made when germination was re-induced by Rp subsequent to FRc (Fig. 2d,f). It is worth noting, however, that one way in which FRc can influence the abundance of transcripts may be the prevention of the degradation of the mRNAs present in dry seeds. Our experimental conditions did not allow an evaluation of the importance of that pathway. The number of genes under the control of phyA in seeds treated with FRc or FRc + Rp was considerably smaller than those dependent on the action of other phytochromes (Fig. 2b,e,g). Thus, under our conditions, the modulation of gene expression was mostly a result of changes in the level of Pfr of stable phytochromes,



Treatment	MM	*fri1-1*
Darkness	100	100
FRp	97.8 +/– 1.1	100
FRc	14.4 +/– 2.2	54.4 +/– 2.9

Fig. 6 Validation of gene expression patterns by semiquantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR). (a) Experimental protocol used for the validation of microarray data. Tomato (*Solanum lycopersicum*) seeds were incubated for 3 h in darkness before irradiation with a 30-min red light pulse (Rp), returned to darkness for 21 h and then subjected to a 1-h far-red light pulse (FRp) or 24 h of continuous far-red light irradiation (FRc). Samples were taken at the beginning of light treatment (24 h) and after 9 h (33 h) and 21 h (45 h). (b) sqRT-PCR analysis of expression of *GIGANTEA*, *ELIP*, *CSN6*, *RBP* and *SlSOS2* under high-irradiance response (HIR) treatment. Plots show mean expression relative to actin (in relative units) of three independent biological replicates ± standard error, and asterisks show significant differences between means ($P < 0.05$). (c) Final germination under HIR (FRc) and FRp treatments of cv. Money maker (MM) and *far-red insensitive 1-1* (*fri1-1*) seeds used in (b). (d) Germination of *Arabidopsis thaliana* wild-type (WT) Columbia (Col) and *sos2-2* mutant irradiated with Rp. Asterisks show significant differences between means ($P < 0.001$).

which also explains why the correspondence analysis did not show a clear effect of genotype when comparing WT with *fri1-1* seeds (Fig. 3). Finch-Savage *et al.* (2007) showed in *Arabidopsis thaliana* seeds that the different factors that can decrease dormancy must operate in an appropriate sequence in order to be able to remove successive blocks to germination. Each one generates both common and unique patterns of change in gene expression. As an increase in Pfr is usually at the end of this sequence, many of the molecular events required for germination have already taken place, and this is reflected in the small number of genes affected by light compared with those affected during previous imbibition at low temperatures (Finch-Savage *et al.*, 2007). In agreement with these observations, our results demonstrate that the signalling pathway initiated by phyA in response to FRc does not regulate the expression of all activities required for germination controlled by Pfr of stable phytochromes, affecting only a relatively small number of genes. This may favour a rapid reactivation of

Table 1 *Cis*-regulatory sequences identified in the promoter regions of the selected genes

Classification		Selected genes				
		GIGANTEA	*ELIP*	*CSN6*	*RBP*	SOS2
Tissue-specific expression	Endosperm-specific	X	X	X	X	X
	Embryo-specific			X		X
Light-regulated expression	Light response activation	X	X	X	X	X
	Repression in darkness	X			X	X
Hormone-regulated expression	GA signal activation			X	X	X
	GA signal repression	X		X		X
	Abscisic acid signal activation	X		X	X	X
	Auxin signal activation				X	X
	Ethylene signal activation	X		X		X

germination when environmental light changes establish a sufficiently large R : FR ratio reaching the seeds, which can suppress the inhibitory effect of FRc.

Gene clusters obtained from canonical discriminant analysis also show that the time of incubation is another relevant factor in gene expression regulation during FRc inhibition and Rp re-induction treatments (Fig. 4). The kinetics of average gene expression followed by the clusters offers a good description of when relevant changes occur, and is also consistent with the functioning of several well-known elements involved in the germination process. The pattern of expression of Clusters 1 and 2 (Tables S5, S6) showed no association with light treatment, possibly because some of these genes contribute to the reactivation of metabolism after the start of imbibition (Bewley, 1997). In contrast, genes in Clusters 3, 4 and 5 were related to the control of germination by light. The average expression of Cluster 3 (Table S7) showed a response of its genes to FRc treatment, and this response was not antagonized by the Pfr form of stable phytochromes. By contrast, Clusters 4 and 5 displayed an expression pattern highly related to the final outcome of germination. The steady increase in the expression of the genes included in these groups during incubation in darkness was halted by FRc, and resumed after subsequent Rp. In Cluster 4 (Table S8), the early expression of genes related to the synthesis of GA, cytokinins and ethylene is consistent with the known participation of these hormones in germination. The central role of GA metabolism in the promotion of germination by phyB has been well documented in *Arabidopsis*, in particular the involvement of GA3ox and GA2ox (Yamaguchi & Kamiya, 2002; Cadman *et al.*, 2006). GA3ox also participates in the promotion of germination by light through the LFR and VLFR in *Datura ferox* seeds (de Miguel *et al.*, 2000; Arana *et al.*, 2007). This study showed that another GA-related gene, EKO, seems to be controlled by the Pfr of stable phytochromes, as no effect of phyA was detected (Figs 5a, S3). The genes of Cluster 5 showed a later increase in expression during incubation, and among the genes identified were those related to changes in endosperm and embryo cell walls, involved in the final steps of the germination process (Table S9). Although a few cell wall weakening-related genes were induced earlier (Cluster 4), the strongest response was observed after longer incubation times, with a significant transcript increase in expansins, glucan endo-1,3-β-glucanases, polygalacturonases, xyloglucan endo-transglucosylases and β-1,4-glucanase. The promotion of an endo-β-1,4-mannanase gene by the Pfr of stable phytochromes and the inhibition by FRc has been shown in *Datura ferox* seeds (Sánchez & Mella, 2004). In this study, this pattern of expression was confirmed in a β-1,4-glucanase (Fig. 5). Interestingly, the microarray expression analysis showed that the action of phyA under FRc influenced the expression of β-1,4-glucanase, but not β-mannosidase (Fig. S3). These results demonstrate that different components of the cell wall-degrading system are independently controlled by different phytochromes at the gene expression level, and suggest that germination can be modulated by the interaction of a variety of environmental factors until the very last moment before radicle protrusion.

Our microarray analysis also revealed that a number of genes that have been recognized previously as part of the signalling networks related to light or hormone action in other physiological processes are influenced by the light treatments controlling germination. Some of these include *CSN6* (coding a repressor of photomorphogenesis) (Peng *et al.*, 2001), *RBP* (coding a protein involved in mRNA metabolism probably controlled by ABA) (Hirayama & Shinozaki, 2007) and *SOS2* (coding a protein kinase known to participate in responses to stress and possibly to ABA, as it physically interacts with *ABI2*) (Mahajan *et al.*, 2008), which were down-regulated by FRc and promoted by subsequent Rp in tomato seeds (Fig. 5c). These effects were observed in both WT and *fri1-1* seeds (Figs 6b, S3b); therefore, it can be expected that they might participate in the cross-talk between the signalling networks of some of the stable phytochromes. The involvement of *SOS2* as a positive regulator of germination is also supported by the reduced germination response of *sos2-2* mutant seeds of *Arabidopsis* under Rp (Fig. 6d). *ELIP* (Bruno & Wetzel, 2004), however, is particularly interesting, because its expression is enhanced by FRc and decreased by Rp in WT, but not by FRp

or in the *fri1-1* mutant, indicating that it participates in the antagonism between HIR and LFR. The same responses were also found in *GIGANTEA*, a gene that has been shown previously to be related to germination (Oliverio *et al.*, 2007; Rutitzky *et al.*, 2009), but was not linked to HIR. In spite of the biological importance of HIR, to date no signalling component has been associated with this mode of action of the phytochromes in seeds.

GIGANTEA is a nuclear protein involved in phytochrome signalling that regulates flowering, circadian rhythms and hypocotyl growth under red and blue light in several species, such as *Arabidopsis* (Fowler *et al.*, 1999; Park *et al.*, 1999; Huq *et al.*, 2000; Tseng *et al.*, 2004; Mizoguchi *et al.*, 2005; Paltiel *et al.*, 2006), rice (Hayama *et al.*, 2003), *Medicago truncatula* (Paltiel *et al.*, 2006) and pea (Hetcht *et al.*, 2007). Tseng *et al.* (2004) showed, in *Arabidopsis* seedlings, that GIGANTEA interacts with SPY, a negative regulator of GA signalling. In addition, it has been reported that GIGANTEA participates in the promotion of germination through a phyA-mediated VLFR in *Arabidopsis* seeds (Oliverio *et al.*, 2007). In this study, we have demonstrated that *GIGANTEA* acts in another signalling branch of phyA through HIR that inhibits seed germination under FRc (Fig. 6b). However, our results are in apparent contradiction with those published recently by Rutitzky *et al.* (2009), proposing a positive role of *GIGANTEA* in the promotion of germination under continuous R in tomato seeds (Rutitzky *et al.*, 2009). Unfortunately, we cannot strictly compare our results with those published previously because the experimental protocols for germination were different, and also the magnitude of germination differences between light treatments were not included in Rutitzky *et al.* (2009).

There is evidence pointing to a role of ELIP1 and ELIP2 in the protection of the photosynthetic apparatus and in senescent leaves, although their action mechanisms are not yet clear (Montané & Kloppstech, 2000; Binyamin *et al.*, 2001; Hutin *et al.*, 2003). However, it is known that the expression of both genes is under phytochrome control in etiolated *Arabidopsis* seedlings. Harari-Steinberg *et al.* (2001) showed the regulation of both genes by phyA through HIR, suggesting a role of these proteins in the adjustment of the seedlings to the transition from dark to light conditions. In agreement, we have shown that *ELIP* transcripts are increased by HIR of phyA in the seeds. Regarding our results, we cannot rule out the possibility that ELIP plays some regulatory function affecting germination *per se*, or regulates the signalling network related to the preparation for the next seedling developmental stage. An endosperm-specific *cis*-regulatory sequence in the promoter region (Tables 1, S11; Fig. S4, see Supporting Information) suggests that it might be involved in the control of endosperm softening activities. However, the paucity of information about the functions of this protein prevents us from proposing a firm hypothesis.

The promoter sequences of the five selected genes possess *cis*-regulatory elements involved in tissue-specific, light- and hormone-regulated expression (Tables 1, S11; Fig. S4, see Supporting Information). The presence of endosperm-specific expression element-binding sites in the promoter sequences of the selected genes strengthens their role in the control of germination. In addition, almost all genes have interaction sites for elements related to hormone signalling networks, indicating complex connections between these genes and hormones in the regulation of germination and/or processes occurring at a later stage associated with the development of the seedlings (Mazzella *et al.*, 2005).

The opposing effects of FRc and Rp are a reflection of the antagonism between LFR and HIR modes of action. This antagonism is thought to be part of the mechanisms preventing germination under a dense canopy and allowing germination only on opening of a gap (Casal & Sánchez, 1998). The genes affected by FRc, dependent on phyA and related to HIR, are considerably less numerous than those affected by Pfr of the other phytochromes. This observation suggests that canopy detection depends largely on the functioning of stable phytochromes, and that phyA signalling can modulate this response through a relatively small group of genes. It is known that after-ripening or other processes that decrease dormancy reduce the Pfr requirement for the expression of a number of genes (Cadman *et al.*, 2006). Even under these circumstances, HIR might still permit canopy detection, acting through the control of the expression of a reduced and specific set of genes, such as *GIGANTEA* and *ELIP*, and others yet unidentified.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Multivariate analysis of microarray data.

Fig. S2 Microarray data from selected genes.

Fig. S3 Microarray data from selected genes in *far-red insensitive 1-1* (*fri1-1*) samples.

Fig. S4 *Cis*-regulatory sequence analysis in 5′ promoter regions of the selected genes.

Table S1 Treatments and sampling timing

Table S2 Up- and down-regulated genes under continuous far-red light irradiation (FRc) treatment

Table S3 Up-regulated genes under re-induction treatment

Table S4 Down-regulated genes under re-induction treatment

Table S5 Genes in Cluster 1

Table S6 Genes in Cluster 2

Table S7 Genes in Cluster 3

Table S8 Genes in Cluster 4

Table S9 Genes in Cluster 5

Table S10 Gene-specific primer sequences used in semi-quantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR)

Table S11 Plant *Cis*-acting Regulatory DNA Elements (PLACE) references of *cis*-regulatory sequences identified in the promoter region of the selected genes.

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