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Research Article

Phytochromes mediate germination inhibition under red, far-red, and white light in *Aethionema arabicum*

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Abstract

The view on the role of light during seed germination stems mainly from studies with Arabidopsis (*Arabidopsis thaliana*), where light is required to initiate this process. In contrast, white light is a strong inhibitor of germination in other plants, exemplified by accessions of *Aethionema arabicum*, another member of *Brassicaceae*. Their seeds respond to light with gene expression changes of key regulators converse to that of *Arabidopsis*, resulting in opposite hormone regulation and prevention of germination. However, the photoreceptors involved in this process in *A. arabicum* remain unknown. Here, we screened a mutant collection of *A. arabicum* and identified *koy-1*, a mutant that lost light inhibition of germination due to a deletion in the promoter of *HEME OXYGENASE 1*, the gene for a key enzyme in the biosynthesis of the phytochrome chromophore. *koy-1* seeds were unresponsive to red- and far-red light and hyposensitive under white light. Comparison of hormone and gene expression between wild type and *koy-1* revealed that very low light fluence stimulates germination, while high irradiance of red and far-red light is inhibitory, indicating a dual role of phytochromes in light-regulated seed germination. The mutation also affects the ratio between the 2 fruit morphs of *A. arabicum*, suggesting that light reception via phytochromes can fine-tune several parameters of propagation in adaptation to conditions in the habitat.

Introduction

Seed germination, a bottleneck in the plant life cycle, is controlled by environmental factors like temperature, humidity, nitrate, and light (reviewed by Vleeshouwers et al. 1995; Finch-Savage and Leubner-Metzger 2006). Light quality and quantity provide information to the seed about the coverage by soil, the canopy shade, or day-length (Pons 2000; Chen et al. 2014). Due to experiments with the classical model

plants in germination research, like Arabidopsis (*Arabidopsis thaliana*) or lettuce (*Lactuca sativa*), light has long been considered to alleviate dormancy and stimulate germination, and the molecular basis of this regulation has been extensively studied (Vleeshouwers et al. 1995). The requirement for light induction to seed germination has been considered as a depth-sensing mechanism of small-seeded plants to avoid germination deep underground if the resources would not allow them to reach the surface (Seo

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et al. 2009). However, seed germination response to white light can also be neutral, or even negative, when white light inhibits the process (Takaki 2001; Yang et al. 2020). Photoinhibition of germination, termed negative photoblasty, has been observed in several, phylogenetically distant taxa across angiosperms (Koller 1957; Botha and Small 1988; Thanos et al. 1991, 1994; Carta et al. 2017; Vandelook et al. 2018; Mérai et al. 2019). White light was found to cause delay or partial inhibition of germination in tomato (*Solanum lycopersicum*) variants, while it strongly inhibits the germination of watermelon (*Citrullus lanatus*) seeds (Yaniv and Mancinelli 1968; Botha and Small 1988). The light-inhibited germination is interpreted as an adaptive trait to avoid germination on the surface in open and arid habitats like coastal dunes, deserts, shrublands, or grasslands (Pons 2000; Carta et al. 2017). *Aethionema arabicum*, belonging to the *Brassicaceae* and originating from semi-arid open habitats, provides an opportunity to study the largely unknown molecular basis of negative photoblasty. Some accessions of this species have light-neutral seed germination, but seed germination of accession from Cyprus (CYP) is strongly inhibited by continuous white light and long-day conditions, while seeds germinate under short days despite strong light illumination (Mérai et al. 2019). We speculated that the light-inhibited germination might be a day-length measuring mechanism to ensure the proper timing of germination in early spring, matching the germination temperature optimum at 14 °C, and proposed *A. arabicum* as a suitable model plant to study the rewiring of the light response in seeds (Mérai et al. 2019). The germination of CYP seeds is strongly inhibited by white, red, far-red, and blue light, indicating the involvement of phytochromes and possibly other photoreceptors in the process (Mérai et al. 2019). However, the specific photoreceptors mediating germination inhibition in *A. arabicum* are unknown.

Plants perceive and transduce light signals through photoreceptors. Red and far-red spectra (600 to 750 nm) are received by phytochromes (reviewed by Gyula et al. 2003; Li et al. 2011). UV-A and blue light (320 to 500 nm) perception is mediated by 3 flavin-based photoreceptor families, the cryptochromes (crys), phototropins (phots), and the Zeitlupe family (ztl, fkf1, and lkp2) (reviewed by Christie et al. 2015). Additionally, UV-B light (282 to 320 nm) is perceived by UV RESISTANCE LOCUS 8 (UVR8) (Rizzini et al. 2011). Blue light can be involved in the control of dormancy induction in cereals via cryptochrome CRY1 and in the control of dormancy alleviation in *Arabidopsis* via phyB (Shropshire et al. 1961; Barrero et al. 2014; Stawska and Oracz 2019). However, the connection between light and seed germination is most prominent for longer wavelengths perceived by phytochromes, extensively studied for decades (Casal and Sanchez 1998; Yang et al. 2020).

The light-sensing chromophore of all phytochromes in land plants is an open-chain tetrapyrrole called phytochromobilin (Lamparter 2004). It is synthetized in the plastids from 5-aminolevulinic acid in a series of enzymatic reactions

including an oxidative cleavage of a heme intermediate into biliverdin IX by a ferredoxin-dependent heme oxygenase (HO). Biliverdin is reduced to PΦB by the PΦB synthase. The chromophore is then exported to the cytosol and assembles autocatalytically with the phytochrome proteins, forming the photoreversible holophytochromes (Brown et al. 1990; Mahawar and Shekhawat 2018). Arabidopsis has 5 phytochromes that play a role in diverse signaling pathways, e.g. regulating seed germination, seedling de-etiolation, shade avoidance, flowering time, and hormonal metabolism (Franklin and Quail 2010). In the darkness, phytochromes are synthetized in the inactive P_r form that converts to the active P_{fr} form upon absorbing red light (660 nm). The active P_{fr} form initiates the signaling cascade leading to the phytochrome response (Furuya and Schäfer 1996). P_{fr} is inactivated quickly upon far-red light (730 nm) absorption or by spontaneous relaxation via slower dark reversion (Mancinelli 1994; Quail 1997). The light-labile phyA is the only receptor that perceives and mediates responses in far-red light, while phyB-phyE are considered light-stable red-light receptors, with a predominant role of phyB (Dehesh et al. 1993; Nagatani et al. 1993). Much insight into their function stems from phytochrome-deficient mutants, in which either the apoproteins or the chromophore are missing. Single *phya*, *phyb*, etc. mutants allow the functional characterization of the individual phytochromes (Koornneef et al. 1980; Quail et al. 1995), whereas mutations affecting the chromophore biosynthesis pathway result in a loss or severe reduction of all photoreversible phytochromes and are impaired in photomorphogenesis (reviewed by Terry 1997). This was shown for mutants in several plant species lacking HO and PΦB synthase (Koornneef et al. 1980, 1985; Chory et al. 1989; Kraepiel et al. 1994; Lamparter et al. 1996; van Tuinen et al. 1996; Weller et al. 1996, 1997; Izawa et al. 2000; Sawers et al. 2004). Assuming that loss of genes encoding signaling or other regulatory components would result in an easily scoring phenotype, we generated a mutant collection in *A. arabicum* and screened for germination of light-exposed seeds. Here, we present *koy-1*, a chromophore-deficient HO mutant in *A. arabicum* that has lost the inhibition of seed germination under red, far-red, and white light and shows a shifted fruit-morph ratio, revealing novel phenotypes never observed in chromophore mutants of other species.

Results

Screen for irradiation-induced mutants with hyposensitive seed germination in response to white light

The strong inhibition of seed germination in an *A. arabicum* accession originating from CYP by light, independent of wavelength (Mérai et al. 2019), allowed to characterize this feature with a straightforward genetic approach. Defects in light perception or signal pathways to the hormonal control of the inhibition should result in seeds able to germinate

under light, and the respective mutations are expected to help identify important components of the regulation. Therefore, we prepared a mutant collection of *A. arabicum* in the background of the CYP accession as wild type (WT). As the thick seed coat and mucilage would limit the uptake of chemical mutagens, we irradiated seeds with fast neutrons, planted them individually, and collected their seeds, representing the M2 generation. At least 30 seeds per lines were assayed at optimal temperature (14 °C) for germination under 160 μmol m^{-2} s^{-1} light intensity, conditions under which less than 1% of the WT seeds germinate. Among approximately 1,300 lines screened, we found 4 well-established lines as primary mutant candidates. These were further propagated for 2 generations to confirm the heritability of the mutant phenotype. In the following, we present a mutant for which we identified the genetic defect and the possible role of the gene product. We named the mutant *koyash-1* (*koy-1*), after the god of the sun in Turkic mythology.

While WT seed germination is already strongly reduced at a white light intensity of 100 μmol m^{-2} s^{-1}, *koy-1* seeds germinate close to 100% at up to 166 μmol m^{-2} s^{-1} (Fig. 1A). Using a set-up extending the intensity range, *koy-1* seeds were fully inhibited only at 450 μmol m^{-2} s^{-1} (Fig. 1A). WT and *koy-1* germinate over 90% in darkness (Fig. 1, A and B), excluding differences in germination potential between the seed batches. The optimal temperature for seed germination with an optimum range between 11 and 14 °C and a low germination percentage at 8 or 20 °C is also similar between WT and *koy-1* (Supplemental Fig. S1), with slightly lower germination at 17 °C. Therefore, the mutation in *koy-1* changes only the response to light and not to temperature.

Light spectra specificity of the *koy-1* mutant phenotype

The germination of WT seeds is strongly inhibited by continuous red, far-red, and blue light in a dosage-dependent manner (Fig. 1, B–E). The effective intensity of blue and red light is comparable: germination is substantially inhibited at 50 μmol m^{-2} s^{-1} and completely abolished at 80 μmol m^{-2} s^{-1} and above (Fig. 1, C and E). Interestingly, the inhibition by far-red light is 4 orders of magnitude stronger, resulting in complete inhibition at and above 0.02 μmol m^{-2} s^{-1} intensity (Fig. 1D). *koy-1* seeds could completely germinate under red and far-red illumination even up to very high intensities (220 or 1 μmol m^{-2} s^{-1}, respectively). On the contrary, the inhibition by blue light is stronger for *koy-1*: complete inhibition of mutant seeds is achieved at 56 μmol m^{-2} s^{-1} blue light, whereas 37% of WT seeds germinate under these conditions (Fig. 1E). These data indicate that germination control in *koy-1* mutant seeds is unresponsive to inhibition by red and far-red light, while the seeds respond to blue light.

Previously, we showed that the light-sensitive germination of WT seeds might have evolved as a day-length sensing mechanism, allowing germination on short days in spring or autumn and preventing it under long-day conditions (Mérai et al. 2019). Applying the high light intensity of 450 μmol m^{-2} s^{-1} that inhibits even *koy-1* germination (Fig. 1A), we could show that the diurnal regulation was abolished in the mutant: *koy-1* seeds germinated irrespective of the day-length regimes and were fully inhibited only in continuous light (Fig. 1F).

Genetic characterization of *koy-1*

Backcrosses of *koy-1* with the CYP WT in both directions showed recessive inheritance of the light-unaffected germination, suggesting mapping the causative mutation by searching for a homozygous polymorphism distinguishing mutant from WT siblings (Supplemental Table S3). We processed pools of 18 *koy-1* seedlings and 64 WT progeny derived from the same M1 parent for DNA isolation and Illumina sequencing and mapped the reads to PacBio contigs of the CYP accession genome. We called single nucleotide polymorphisms (SNPs) and InDel variations in the *koy-1* pool, filtered against the control pool, and asked for overlap with genic features. Thereby, we identified a 324 bp deletion in the promoter region of the HO gene *AearHO1* (Fig. 2, A and B), a gene closest to the Arabidopsis ortholog *AtHO1* according to phylogenetic and protein sequence analysis (Fig. 2, C and D). The gene encodes a HO, an enzyme involved in the biosynthesis of the phytochrome-associated chromophore, for which all enzymes and corresponding genes are known (Kohchi et al. 2001; Terry et al. 2002). In *Arabidopsis*, there are 4 HOs: group I with *AtHO1* (also called *HY1*), *AtHO3*, and *AtHO4*, and group II with *AtHO2* (Fig. 2C and Supplemental Fig. S2). The *Arabidopsis hy1/ho3/ho4* triple mutant shows severe symptoms with growth abnormalities, whereas milder effects in the single mutants indicate partial redundancy among the members of the group I (Emborg et al. 2006). Some *Brassicaceae* species have 3 HOs, while other angiosperms often have only 2 HO genes, corresponding to *HO1* and *HO2*, respectively (Fig. 2C and Supplemental Fig. S2). In *A. arabicum*, representing the earliest diverged sister group within the *Brassicaceae* (Mohammadin et al. 2017), only 2 HOs could be identified with a protein Blast search, one of each HO group (Fig. 2C and Supplemental Fig. S2). The mutation in *koy-1* has removed the *AearHO1* promoter region between −90 and −414 bp upstream of the start codon, including the transcriptional start site identified by transcriptome analysis in the WT (Fernandez-Pozo et al. 2021) and several binding sites for transcription factors (Fig. 2, B and E). The expression level of *AearHO1* in seedlings of *koy-1* is reduced to 15% (Fig. 2F). The remaining transcripts are possibly initiated by alternative transcriptional start sites, such as the CAAT and TATA boxes between −414 and −500 bp upstream of the deletion (Fig. 2, B and E). Genetic analysis in back-crossed progeny confirmed the co-segregation of the *AearHO1* promoter deletion with the ability to germinate in light, the phenotype of *koy-1* and the co-segregation of the deletion with the long hypocotyl phenotype under far-red light (Supplemental Tables S3 and S4).
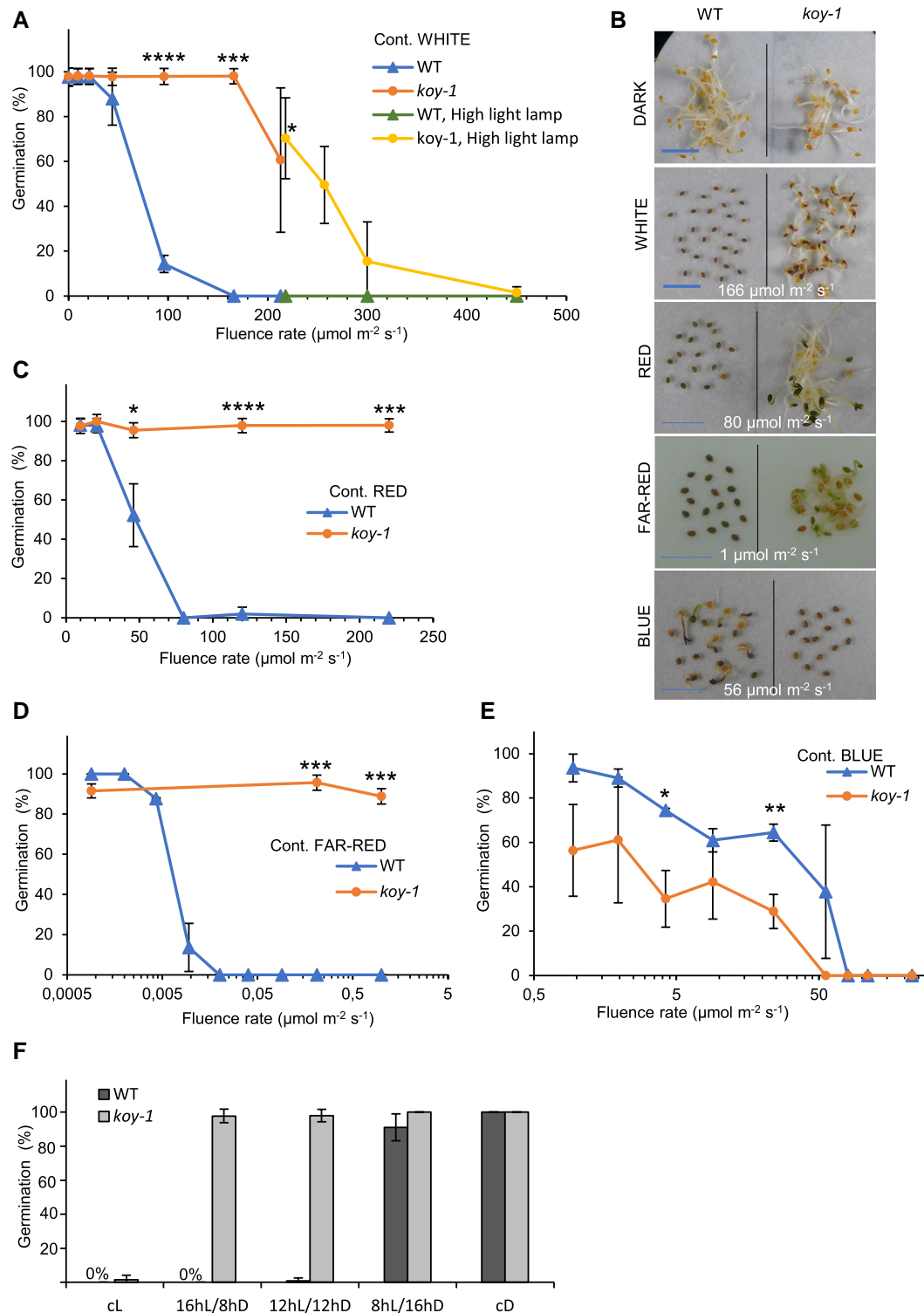
Figure 1. Germination response of WT and *koy-1* mutant seeds to light intensity and light color. Total germination of WT and *koy-1* seeds after 6 d under different intensities of continuous white **A, B)**, red **B, C)**, far-red **B, D)**, and blue **B, E)** light. Asterisks indicate significant differences at **P < 0.01, ***P < 0.001, and ****P < 0.0001 values tested by Welch's *t*-test. **F)** Diurnal regulation of seed germination under 450 μmol m^{-2} s^{-1} white light. Daily dark and light regime is indicated as hour D and L. cL and cD indicate constant light and dark, respectively. **A, C–F)** Error bars indicate the standard deviation of 3 biological replicates.
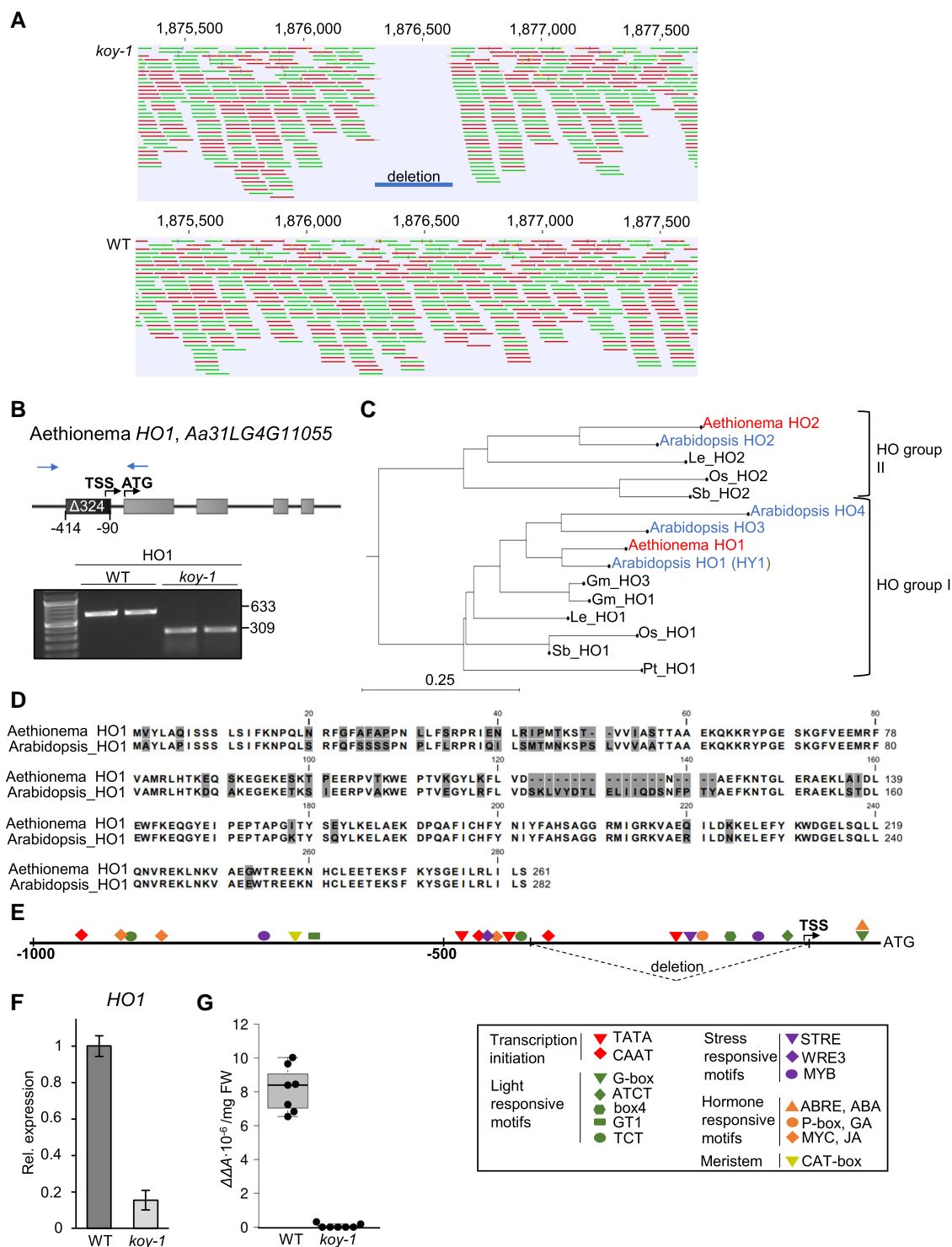
Figure 2. Identification of *koy-1* as mutation of the HEME OXYGENASE 1 gene. **A)** Visualization of the mapped reads around the *HO1* gene in the *koy-1* and WT DNA pools. Green and red bars represent forward and reverse reads, respectively. **B)** Simplified phylogenetic tree of genes of the HO group generated with the Neighbor Joining method. Scale bar indicates *P* distance. A complete version and a description are provided in Supplemental Figs. S2 and Supplemental Data Set S1. **C)** Schematic representation of the *AearHO1* gene with the promoter deletion and its confirmation by PCR. **D)** Protein sequence alignment of Arabidopsis and Aethionema HO1. **E)** Consensus motifs in the region 1 kb upstream of the start codon, identified by PlantCARE. **F)** Relative expression of *AearHO1* in dark-grown seedlings, normalized to the average WT level. Error bars indicate the standard deviation of 3 biological replicates. **G)** In vivo spectroscopy of photoreversible phytochromes in etiolated seedlings. Center lines show the medians; box limits indicate the 25th and 75th percentiles, whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. $N = 7$. Note, that in *koy-1* samples 5 out of 7 were under detection level.

The role of HO1 in the biosynthesis of the phytochrome chromophore suggested that the strongly reduced expression (Fig. 2F) would reduce the amount of light-responsive phytochrome. Indeed, photoreversible phytochrome was hardly detectable in koy-1 by in vivo spectroscopy, in contrast to a phytochrome signal of >8 $\Delta\Delta A \times 10^{-6}$/mg fresh weight in the WT (Fig. 2G). In summary, the deletion at the HO1 gene is limiting the amount of chromophore and photoreversible phytochrome and thereby the mutation in koy-1 that is responsible for the altered light responsiveness of the seeds.

Physiological characterization of koy-1

Lack of chromophore and active phytochromes is expected to affect light responses other than that on seed germination control. Therefore, we compared the hypocotyl elongation in darkness and under monochromatic light of different intensities. WT and koy-1 mutant seedlings show the expected etiolated phenotype with elongated hypocotyls in darkness (Fig. 3A). The blue light suppressed the hypocotyl elongation to a similar extent in WT and koy-1 seedlings (Fig. 3, A and B). In contrast, in koy-1, the red and far-red light failed to inhibit hypocotyl elongation, indicating that the reception of red and far-red light and its downstream effects on seedling development are strongly affected (Fig. 3, B–D).

To characterize further photomorphogenic traits, we determined chlorophyll and anthocyanin accumulation in seedlings grown under white, red, blue, and far-red light or darkness. The chlorophyll content was significantly lower in koy-1 seedlings under red light, although the residual level might indicate a limited response (Fig. 3E). Anthocyanin accumulated in Aethionema seedlings under all light conditions, unlike in Arabidopsis, where red light does not induce this pigment (Neff and Chory 1998). However, anthocyanin induction in koy-1 mutants was significantly reduced under red and far-red light (Fig. 3F), further supporting the lack of perception of this light color by the mutant.

Light-induced gene expression and hormonal changes in WT and koy-1 seeds

To investigate the molecular basis of the light-inhibited seed germination in Aethionema, we examined the expression of key regulator genes under different light exposure and the amounts of hormones regulating germination. At imbibition, WT and koy-1 seeds were illuminated for 24 h under 80 μmol m^{-2} s^{-1} red light or 1 μmol m^{-2} s^{-1} far-red light. Under these conditions, the germination of WT seeds is inhibited, while all koy-1 seeds are germinated after 3 d. As the germination of koy-1 seeds is hypersensitive to blue light, we chose 56 μmol m^{-2} s^{-1} blue light intensity, when WT seeds still retain 37% germination while the koy-1 seeds are fully inhibited (Fig. 1E). Seeds imbibed in darkness were used as control and harvested for sample preparation 24 h after imbibition like all others.

The expression level of AearHO1 in WT and mutant seeds was independent of the light type but reduced to 10% to 13% in all koy-1 mutant samples compared to the WT (Fig. 4A), confirming the effect of the promoter deletion. AearCHS, encoding chalcone synthase and a commonly used light-regulated marker gene, was strongly induced by all light conditions in WT seeds, as expected. In koy-1 seeds, only blue light induced CHS to a level comparable to the WT, confirming that the severe loss of phytochrome does not allow to respond to red and far-red light (Fig. 4B).

The expression of AearPhyA and AearPhyB was significantly downregulated in the WT under all light conditions (Supplemental Fig. S3). However, the expressional changes also occur under red light in the koy-1 mutant, indicating that not all response to red light is abolished (Supplemental Fig. S3). Among genes encoding the blue light receptors, AearCRY1 is the only one that responds significantly different between WT and koy-1 mutants under blue illumination (Supplemental Fig. S4).

Seed germination is induced by shifting the hormonal balance toward higher gibberellin (GA) and lower abscisic acid (ABA) levels. The effect of light is exerted via transcriptional regulation of many genes involved GA and ABA metabolism, but in A. arabicum often in the opposite direction compared to light-dependent germination in A. thaliana, resulting in a shifted hormonal balance toward ABA under white light (Mérai et al. 2019). Therefore, we tested the transcript levels of key regulatory genes and the hormonal levels in WT and koy-1 mutant plants under red, far-red, or blue light. The expression of the GA biosynthetic enzyme AearGA3ox1 is strongly reduced in far-red light in WT seeds but increased in koy-1 seeds upon red and far-red treatment (Fig. 4C). Transcripts for the GA degradation enzyme AearGA2ox3 increased significantly under red and far-red light in WT seeds only (Fig. 4D). Blue light significantly increased the AearGA3ox1 and AearGA2ox3 transcripts to a similar level in WT and koy-1 (Fig. 4, C and D). In line with the transcriptional data, we detected a significant decrease in GA$_4$ hormone levels in WT seeds under far-red light. In contrast, GA$_4$ is significantly increased in koy-1 seeds under red and far-red light (Fig. 4E).

Among the ABA-regulating NCED genes encoding the 9-cis-epoxycarotenoid dioxygenases, which mediate a rate-limiting step of ABA synthesis, AearNCED5 and AearNCED6 were previously found to be induced by white light in the CYP accession of Aethionema (Mérai et al. 2019). The expression of AearNCED5 in WT is increased in all light conditions, but in koy-1 seeds only under blue light (Fig. 4F). The induction of AearNCED6 is specific to far-red light in WT seeds but missing in the mutant (Fig. 4G). In contrast to the opposite light regulation of AearGA3ox1, AearGA2ox3, AearNCED5, and AearNCED6 between Aethionema and Arabidopsis seeds (Seo et al. 2006; Oh et al. 2007; Mérai et al. 2019), the ABA-deactivating enzyme AearCYP707A2 is similarly induced by red or blue light, and reduced by far-red, in WT and koy-1 mutant (Fig. 4H). Expression of DELAY OF GERMINATION-1
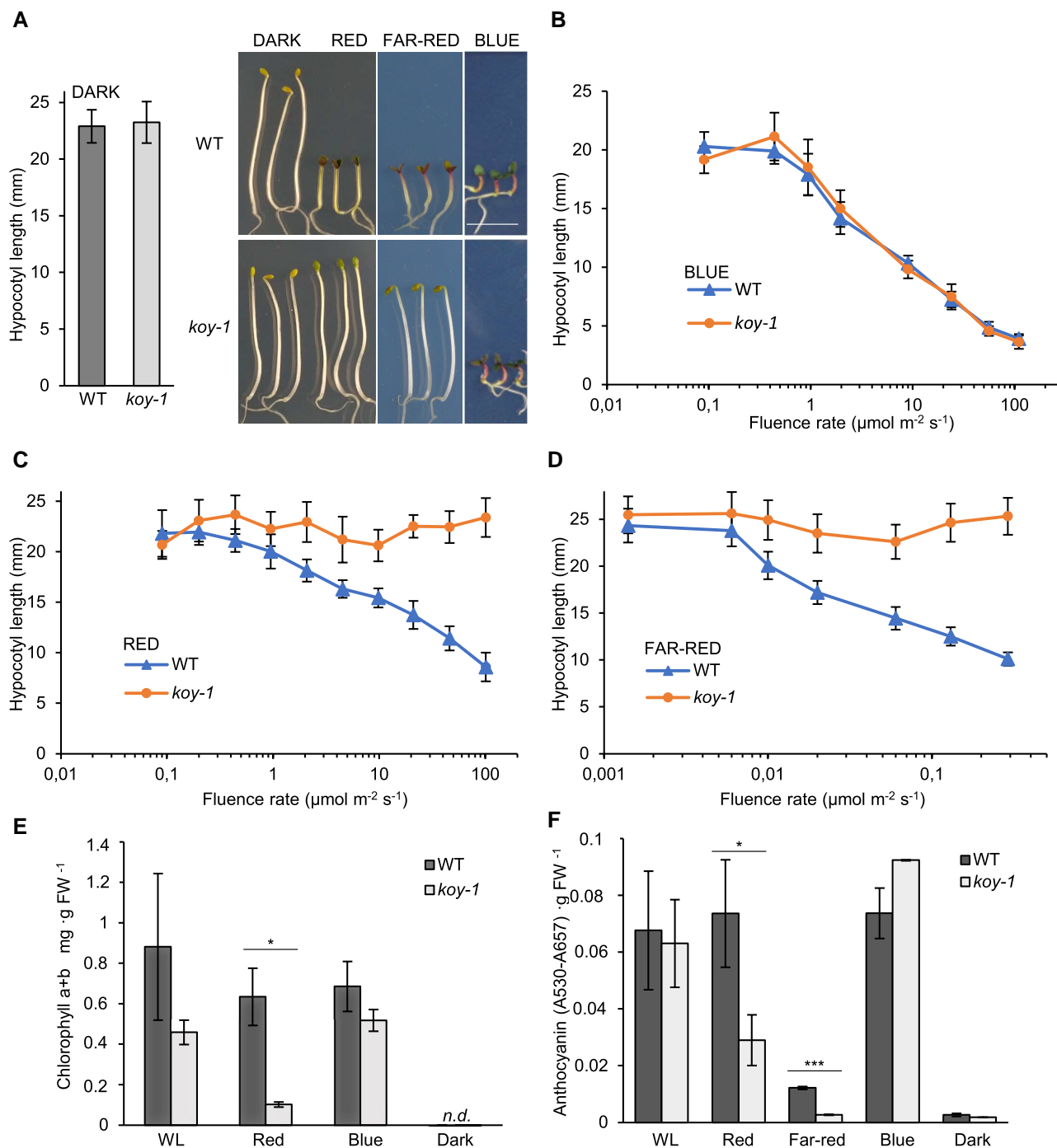
Figure 3. Photomorphogenic phenotypes of WT and *koy-1* mutant seedlings. Hypocotyl length of WT and *koy-1* mutant seedlings in darkness **A)**, in blue **B)**, red **C)**, and far-red light **D)**. Photos in **A)** were taken from seedlings grown at the highest light intensities applied in **B–D)**. Size bar represents 1 cm. Error bars indicate the standard deviation of minimum of 10 hypocotyl lengths. Chlorophyll **E)** and anthocyanin **F)** content was measured in seedlings kept in darkness to induce germination, followed by 4 d of light exposure to 120 μmol m^{-2} s^{-1} white, 9 μmol m^{-2} s^{-1} red, 0.0008 μmol m^{-2} s^{-1} far-red, or 60 μmol m^{-2} s^{-1} blue light intensities. **E and F)** Error bars indicate the standard deviation of 3 biological replicates. Asterisks indicate significant differences at *$P < 0.05$, ***$P < 0.001$ values tested by Welch's *t*-test.

(*DOG1*), an important regulator of seed dormancy via ABA synthesis, parallels that of NCED6 (Fig. 4I). The ABA hormone levels were significantly increased in WT seeds in all light conditions, while only under blue light in mutant seeds (Fig. 4J).

Moreover, the inhibition of de novo ABA synthesis by nor-flurazon completely rescued the WT seed germination both under red and far-red light, indicating a major role of ABA in the phytochrome-mediated germination inhibition
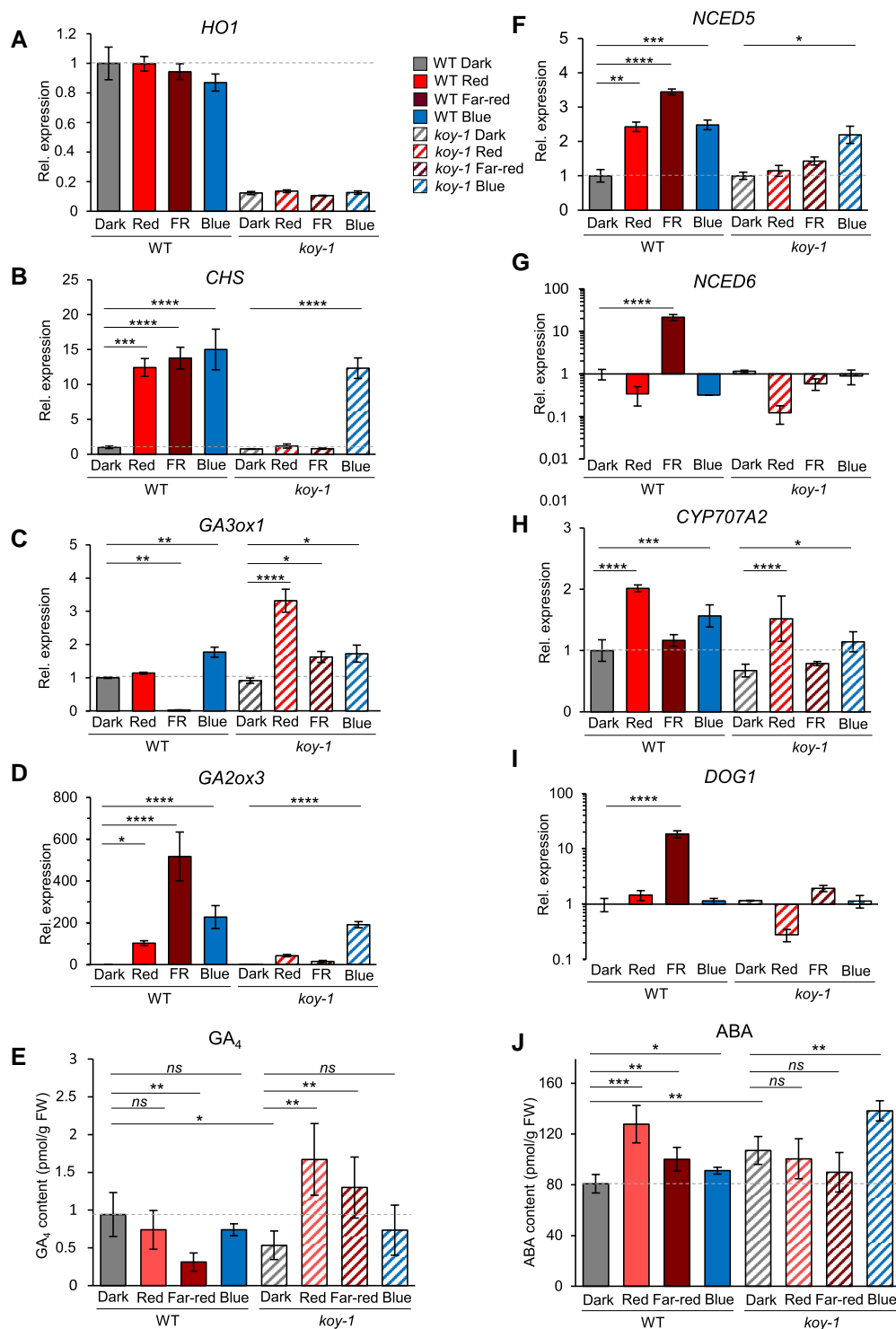
Figure 4. Light-induced gene expression and hormonal changes. **A–D)** and **F–I)** RT-qPCR for selected genes after 24-h light exposure with 80 μmol m^{-2} s^{-1} red light, 1 μmol m^{-2} s^{-1} far-red light, 56 μmol m^{-2} s^{-1} blue light, or maintenance in darkness. The gene expression levels in all light-exposed samples are presented as fold change relative to the average expression level in the darkness of the WT, which is set to 1 and indicated by gray dashed lines. Asterisks indicate significant differences between the light-induced and dark expression level of a gene, based on the Tukey test: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$. Error bars represent the standard deviation of 3 biological replicates. Hormone concentrations [in pmol g^{-1} fresh weight (FW)] of bioactive GA$_4$ (E) and ABA (J) are shown for samples grown in dark, red, far-red, or blue light as in **A–D** and **F–I**. Levels in the dark in WT are indicated by gray dashed lines. Error bars represent the standard deviation of 5 biological replicates. Asterisks indicate significant differences tested by Welch's t-test between the light-induced and dark values of one genotype or between the dark levels of WT and *koy-1*. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, or *ns* if $P > 0.05$.

(Supplemental Fig. S5). The addition of 100 μM GA$_{4+7}$ also rescued the germination under red light, although the seeds needed a longer time to overcome the inhibitory effect (Supplemental Fig. S5). Taken together, the transcriptional regulation of enzymes and the resulting amount and ratio between major hormonal determinants of germination are all concordant with the germination response of WT and *koy-1* mutant seeds to specific monochromatic light detected by phytochromes.

Dual action of phytochromes on Aethionema seed germination

Our findings so far indicated that (i) the *koy-1* mutant lacks the red- and far-red-light-induced ABA accumulation and (ii) the GA$_4$ hormone level is not only unchanged in the mutant, but significantly increased under red and far-red. The latter seems to be in contrast to a lack of phytochrome activity. However, the residual (~10%) expression of *AearHO1* detected in seeds might allow the production of small amounts of photoreversible phytochrome in the *koy-1* mutant, which is also suggested by reduced but measurable chlorophyll and anthocyanin amounts under red light. In *Arabidopsis*, the positive photoblastic germination can be induced by the very low-fluence response (VLFR) through phyA, which requires as low as 0.1% of total phytochrome to be in the P$_{fr}$ form, or by, the low-fluence response (LFR) through phyB (Botto et al. 1996; Shinomura et al. 1996). Dormant Arabidopsis seeds contain only phyB protein, while phyA is newly synthesized upon imbibition (Konomi et al. 1987; Shinomura et al. 1994, 1996). Shortly upon imbibition, phyB mediates the classical red/far-red photoreversible germination induction. Forty-eight hours later, the induction is photo-irreversible, as both red and far-red pulses trigger germination through phyA (Shinomura et al. 1996). Therefore, we aimed to elucidate which of the phytochrome action modes plays a role in the germination of Aethionema, which is strongly inhibited by light in a dosage-dependent way (Fig. 1) (Mérai et al. 2019). Knowing that the germination of Aethionema CYP WT seeds is neither inhibited by a 5 min red or far-red-light pulse nor a 24-h red or far-red illumination (Mérai et al. 2019) clearly indicates that the inhibition is not a VLFR response. As both red and far-red light inhibits germination, it is also unlikely that the inhibition is a LFR, which is characterized by red/far-red reversibility (Casal et al. 1998). Furthermore, seeds fully germinate after a far-red pulse, regardless of this are applied at 0, 3, 6, or 18 h after seed imbibition, while continuous far-red light strongly inhibits (Supplemental Fig. S6). This points to the third type of phytochrome response, high irradiance response (HIR), in which light pulses interrupted by dark cannot induce the same degree of response as continuous illumination, despite the same total fluence over time (Casal et al. 1998). To test whether the germination inhibition has HIR features, we compared WT seed germination either in constant far-red or red light or with hourly light pulses of 15 min followed by 45 min darkness. Importantly, the intermitted far-red or red light did not inhibit the germination, even at 3 to 4 orders of magnitude higher total fluence (Fig. 5A). This confirms that germination inhibition by light via phytochrome is an HIR response.

The increasing GA$_4$ hormone level in the *koy-1* mutant under red and far-red light (Fig. 4, E and J) indicated that, under certain circumstances, light exposure might support rather than inhibit seed germination. As the residual expression of *AearHO1* in *koy-1*, as discussed, is possibly sufficient for a VLFR response, we hypothesized that VLFR might induce germination, similar to *Arabidopsis*. As non-dormant Aethionema seeds germinate in darkness without any light, we tested 8-wk-old semi-dormant WT and *koy-1* seed batches with a limited germination percentage of around 25% in darkness, allowing us to see a positive effect of light exposure. We performed germination assays in complete darkness, with or without a 5 min far-red pulse 24 h after imbibition (Fig. 5B). Indeed, the light pulse increased the germination to 84% and 97% in WT and *koy-1* seeds, respectively, indicating that the short light exposure triggers VLFR in both lines (Fig. 5B). Concomitantly, seeds that received the 5-min far-red pulse had enhanced expression of *AearGA3ox1* (Fig. 5C). All data taken together indicate that phytochromes have 2, opposite effects on seed germination in the CYP accession of *A. arabicum*: continuous red and far-red light strongly inhibits germination in a dosage-dependent manner, while a short far-red pulse can induce germination (Fig. 5D). Mature seeds that have lost the primary dormancy germinate in darkness without needing any light, but the seeds retain the innate module of VLFR induction.

Fruit and seed formation in WT and *koy-1* plants

The reduced level of HO1 and photoreversible phytochrome let us expect additional effects besides altered regulation of seed germination, seedling development and pigment synthesis. Double and triple mutants of the *HO1* gene family in Arabidopsis display severe growth abnormalities, small chlorotic leaves, and early flowering (Emborg et al. 2006). Aethionema *koy-1* mutant plants grow well but resemble a shade-avoidance response (Keuskamp et al. 2010), including a tall, elongated stem, long hypocotyls and internodes, and pale green color (Fig. 6, A and B). Unlike in many species, we did not observe earlier flowering or modified branching patterns (Fig. 6B). A specific feature of *A. arabicum* is the formation of heteromorphic fruits: the same individual plant generates both, indehiscent (IND) and dehiscent (DEH) fruit morphs enclosing either a single or up to 6 seeds, respectively (Fig. 7A) and (Lenser et al. 2016). Both fruit types can appear also with aborted seeds (Fig. 7A). Previous experiments mainly made with the Aethionema accession originating from Turkey had shown that the ratio of IND to DEH fruit morphs is a plastic phenotype influenced by various factors (Lenser et al. 2016; Bhattacharya et al. 2019), in response to auxin treatment, defoliation, and shading. Given the shade-
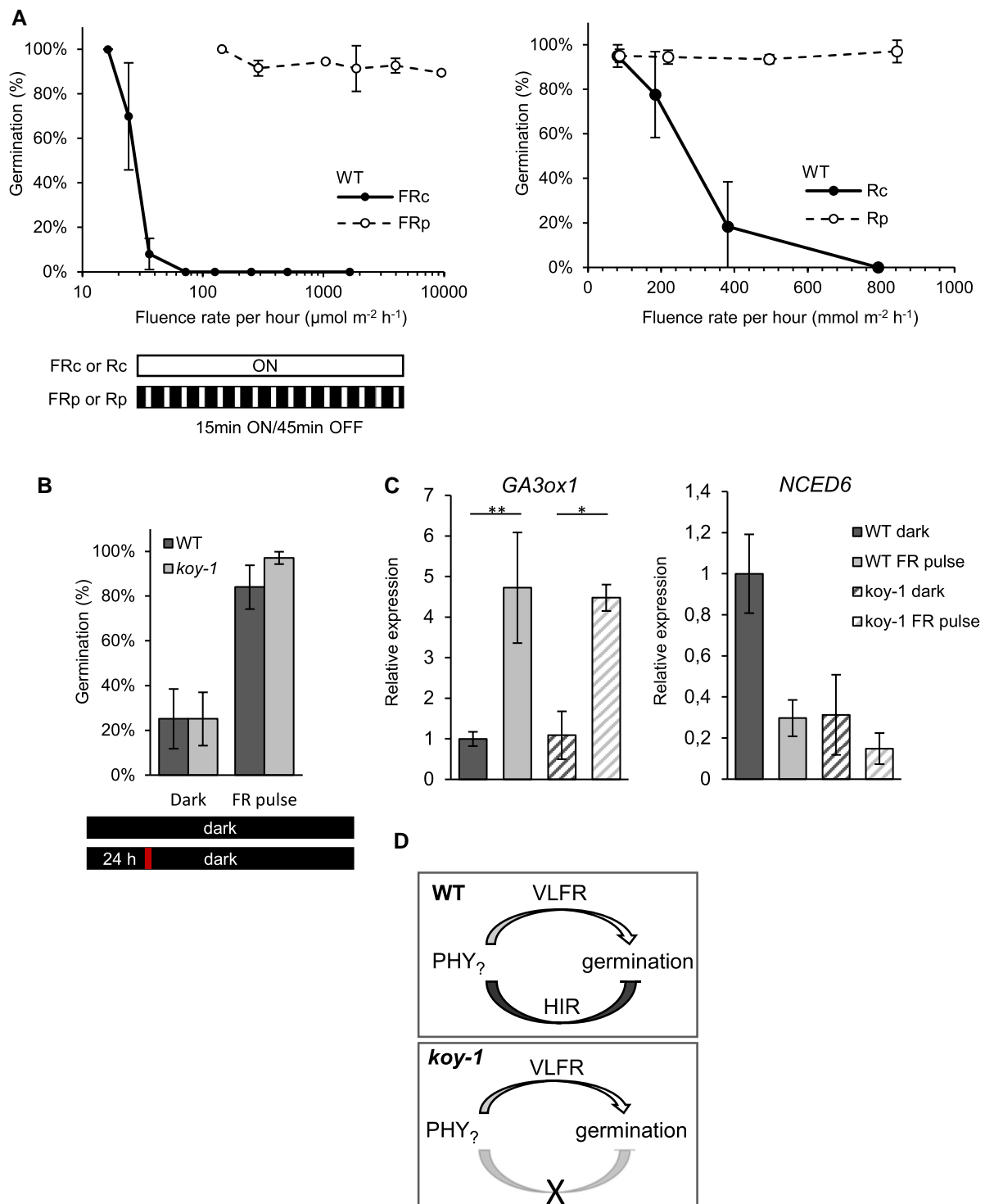
Figure 5. Opposite effect on germination in high irradiance and very LFRs. **A)** Total germination of WT seeds under constant far-red light (FRc, black line) or 15 min far-red pulses (FRp, dashed line) intermitted with 45-min dark periods. **B)** Total germination of 8-wk-old semi-dormant WT and *koy-1* seed batches in darkness with or without a 5 min far-red pulse 24 h after imbibition. **C)** RT-qPCR of *GA3ox1* and *NCED6* from seeds described in **B)**. Samples were collected 30 h after imbibition; 6 h after the far-red pulse. Asterisks indicate significant differences based on the Tukey test: *$P < 0.05$, ** $P < 0.01$. Error bars represent standard deviation of 3 biological replicates. **D)** Schematic model of dual action of phytochromes. The germination induction by VLFR is present in both WT and *koy-1* mutant seeds, while the germination-inhibiting HIR only operates in WT seeds.
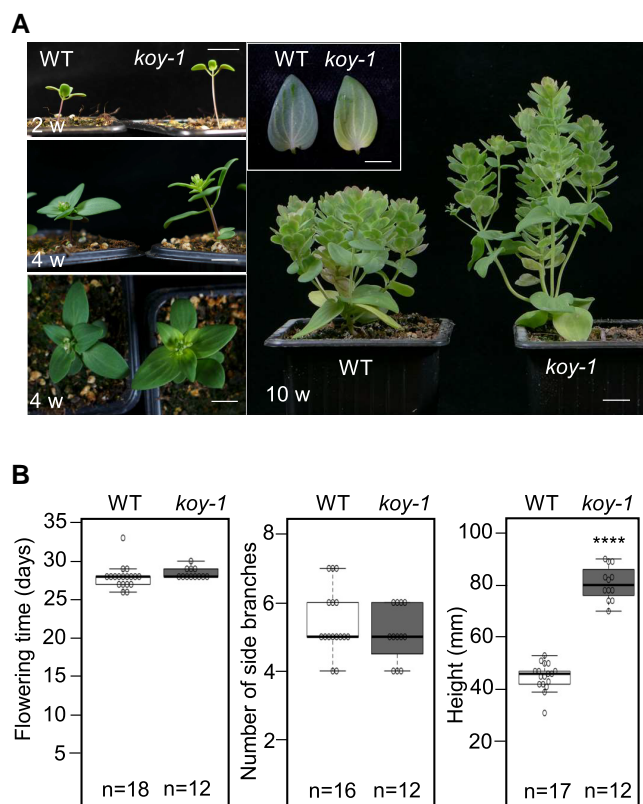
A

B

Figure 6. Phenotype of WT and *koy-1* mutant plants: **A)** Photos of 2-, 4-, or 10-wk-old plants. Scale bar is 1 cm. **B)** Flowering time, number of primary side branches, and plant height was recorded for 12 to 18 plants per box plot. Center lines show the medians; box limits indicate the 25th and 75th percentiles, and whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. Asterisks indicate significant differences at **** $P < 0.0001$ tested with Welch's *t*-test.

avoidance phenotype of *koy-1* plants, we tested if fruit and seed production or the fruit-morph ratio in the mutant would be different compared to the CYP WT. Plants were grown under the same spectral, diurnal, and temperature conditions but either under 300 μmol m^{-2} s^{-1} light intensity, the standard laboratory growth condition for *A. arabicum*, or at lower, suboptimal 200 μmol m^{-2} s^{-1} light intensity. The WT plants were highly sensitive to the light condition: although they produced more fruits under the limiting light, these had less viable seeds than under regular light (Fig. 7, B and C) and lower seed numbers in the seeded DEH fruits (Fig. 7D). In contrast, *koy-1* plants tolerated the lower light intensity much better, with even slightly higher fruit numbers, comparable total seed numbers, and only minor reduction of seeded DEH fruits (Fig. 7, B–D).

While DEH fruit morphs shed the valves upon maturity so that seeds fall off and remain close to the mother plant, IND fruit morphs keep enclosing a single seed and have a winglet-like structure with high dispersal ability (Fig. 7A) (Lenser et al. 2016; Bhattacharya et al. 2019). In the TUR accession, seeds within IND fruits have stronger dormancy (Lenser et al. 2016; Arshad et al. 2019; Bhattacharya et al. 2019).

Therefore, the IND fruit type provides spatiotemporal flexibility for colonization compared to seeds originating from DEH fruits (Bhattacharya et al. 2019). Interestingly, while the WT plants of the CYP accession did not produce seeded IND fruits under low light and only a few under 300 μmol m^{-2} s^{-1}, *koy-1* plants have significantly more seeded IND fruits at both light intensities (Fig. 7, E and F). At 300 μmol m^{-2} s^{-1}, the number of seeded IND fruits is the only significantly different parameter between *koy-1* and WT, while the number of DEH fruits and seeds per fruit are similar (Fig. 7, D and F). These results indicate that phytochromes affect fruit and seed production in response to light intensity and the fruit-morph ratio in *A. arabicum*.

Discussion

Although negative photoblasty is a common feature with multiple evolutionary origin, it contrasts with current textbook knowledge, and its molecular basis is only scarcely understood. Therefore, the natural variation of the trait in a *Brassicaceae* species, closely related to the classical model *A. thaliana*, and the highly improved gene annotation in a relatively small genome (Fernandez-Pozo et al. 2021) recommend *A. arabicum* as an attractive model to investigate the complex regulation of seed germination in response to light. We created a mutant collection for *A. arabicum* to perform a forward genetic screen for lack of white light-inhibited germination. In this study, we present the characterization of a mutant harboring a promoter deletion in the gene *HEME OXYGENASE 1*, encoding a key enzyme for chromophore biosynthesis. The difference between this mutant and the parental WT allowed us to explain differences in the light response compared to Arabidopsis and additional phenotypic changes mediated by light reception via phytochromes.

Phytochrome action in WT and *koy-1* seeds

As there is only one member of the HO group I in Aethionema, it was plausible that the deletion in the promoter of the *AearHO1* gene in the *koy-1* mutant resulted in a strongly reduced amount of photoconvertible phytochromes, confirmed by the resulting effects on hypocotyl growth, seed germination, the lack of *AearCHS* induction under red and far-red light, and the lack of anthocyanin accumulation under far-red light. Moreover, the *koy-1* plant phenotype resembles that of Arabidopsis *phyB* null mutants. However, residual chlorophyll and anthocyanin accumulation under red light indicated that a minor portion of phytochromes can still be activated in *koy-1*.

We demonstrated that the phytochromes play a dual, opposite role in the germination of Aethionema (CYP) seeds: they inhibit germination in an HIR action mode but induce the germination through the VLFR. In WT seeds, increasing light intensity and duration shift the positive effect of VLFR toward the inhibition by HIR. Importantly, the residual amount of photoreversible phytochrome in *koy-1* is sufficient
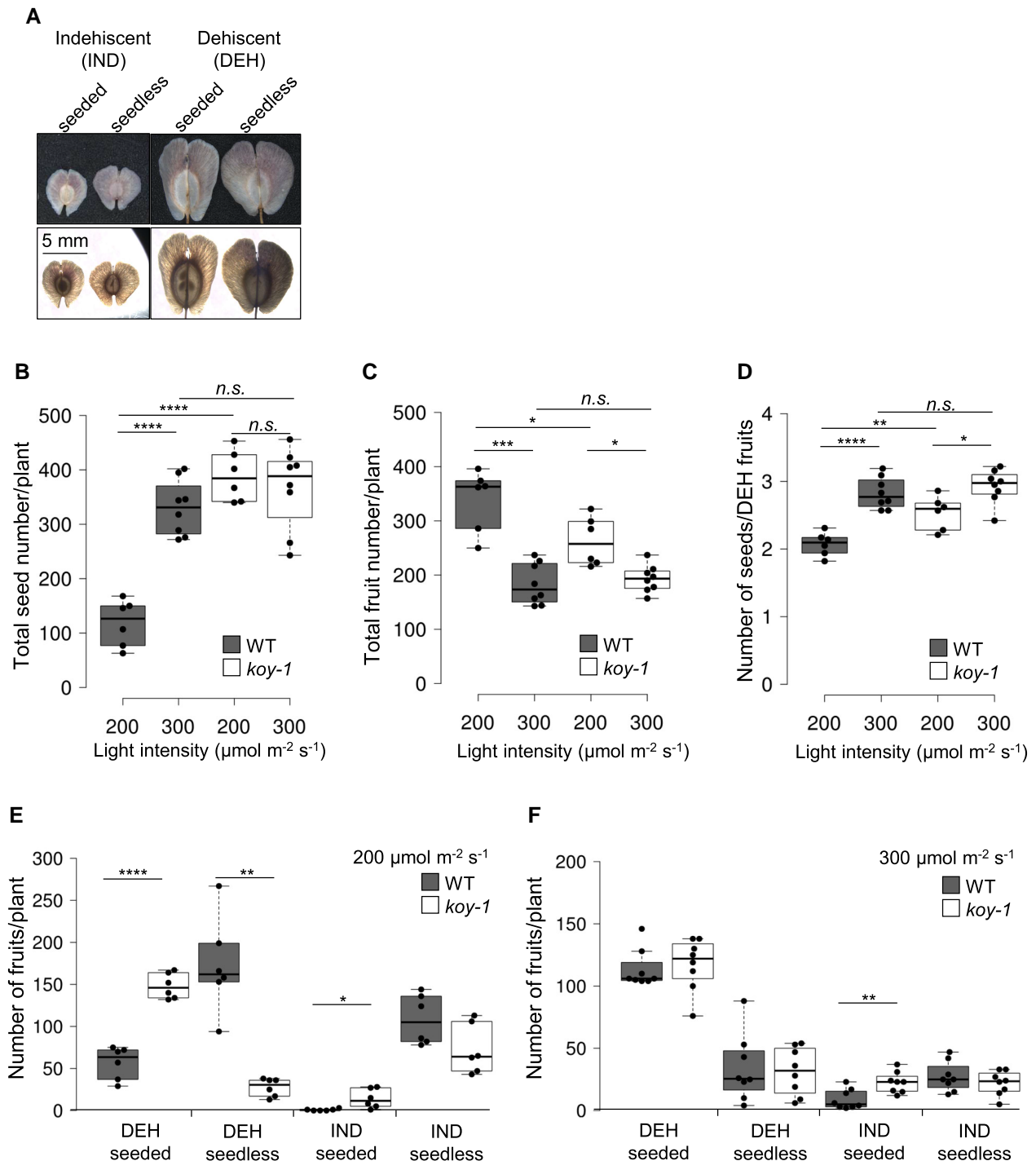
Figure 7. Fruit and seed production and fruit-morph ratio in WT and *koy-1* mutant plants. **A)** Appearance of IND and DEH fruit types with or without seeds. Note that the seed number in the DEH fruit type varies from 1 to 6. **B–F)** Seed and fruit numbers of fully matured plants kept under 200 or 300 μmol m^{-2} s^{-1} white light from germination on. Center lines show the medians; box limits indicate the 25th and 75th percentiles, and whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles. $N = 6$ or 8, for 200 and 300 μmol m^{-2} s^{-1} intensities, respectively. Asterisks indicate significant differences with *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, or *n.s.* as no significant difference, tested with Welch's *t*-test.

for the VLFR induction, but not for the inhibition through the HIR. The *koy-1* mutant allows the genetic dissection of the 2 response types. For example, VLFR and HIR both control the GA$_4$ level, as the *AearGA3ox1* expression and the

GA$_4$ hormone accumulation show contrasting response between WT and mutant. In contrast, the degradation of GA$_4$ through GA2ox3 is only an HIR response, as there was no significant induction in *koy-1*. The ABA synthesis through

NCED5 and NCED6 is an HIR response, while the ABA degradation via CYP707A2 might be mainly due to VLFR, as the expression profile is similar in WT and *koy-1* seeds. Similar phenomena were found in *Datura ferox* seeds, where the far-red HIR antagonized the VLFR induction of the *DfGA3ox* gene (Arana et al. 2007).

As VLFR and HIR upon far-red exposure are mediated by the PhyA photoreceptor, it is very likely that PhyA has a major role in the control of seed germination in Aethionema. However, the presented data do not exclude the role of other phytochromes and the LFR type in the process, particularly in the dosage-dependent red-light inhibition. The PhyA-mediated germination inhibition by far-red HIR is well known from tomatoes (Appenroth et al. 2006; Auge et al. 2009). A limited number of studies report germination inhibition by continuous red light in California poppy seeds (*Eschscholzia californica*) and *Phacelia tanacetifolia*, or by red light regimes in *Bromus sterilis*, but the specifically responsible phytochrome is unknown (Schulz and Klein 1963; Goldthwaite et al. 1971; Hilton 1982). The mutant population established in *A. arabicum* could likely provide a valuable resource to screen for individual phytochrome mutants to study their role in the inhibition of seed germination.

Two distinct pathways for light-inhibited germination in Aethionema

Loss of seed germination inhibition in the *koy-1* mutant in red and far-red light, but its persistence under blue light, indicates the existence of at least 2 pathways for the light response in Aethionema: a phytochrome-mediated red and far-red inhibition, and a blue light-induced, phytochrome-independent pathway. This also explains the inhibition of *koy-1* seeds under high-intensity white light. The role of blue light in seed dormancy and germination depends strongly on the taxon. In monocots, blue light inhibition of seed germination is reported for barley (*Hordeum vulgare*), ryegrass (*Lolium rigidum*), and *Brachypodium* (Goggin et al. 2008; Gubler et al. 2008; Barrero et al. 2012). RNAi application in barley revealed that this is mediated through the CRY1 photoreceptor, which induces ABA synthesis (Barrero et al. 2014). In dicots, inhibitory effects of blue light on seed germination are described for watermelon, *Laportea bulbifera*, and *Trifolium subterraneum* (Tanno 1983; Thanos and Mitrakos 1992; Costa et al. 2016). The lack of genetic tools in these species so far does not allow concluding which photoreceptor/s mediate the inhibition. In *Arabidopsis*, blue light has a positive effect on dormancy alleviation through the PHYB photoreceptor (Stawska and Oracz 2019). Interestingly, our results indicate a phytochrome-independent blue light inhibition in a dicot plant.

The inhibition of germination under blue light, stronger in the *koy-1* mutant than in the WT, might indicate an interplay between the 2 pathways. The significant increase of the CRY1 expression in the *koy-1* mutant under blue light corresponds to the stronger response (Supplemental Fig. S4). Interactions between the phytochromes and the blue light photoreceptor CRY1 have been demonstrated in many aspects, including hypocotyl growth, cotyledon unfolding and expansion, chlorophyll and anthocyanin accumulation (Casal and Boccalandro 1995; Ahmad and Cashmore 1997; Neff and Chory 1998). However, in contrast to *Arabidopsis* phytochrome mutants (Neff and Chory 1998), we did not observe differences in hypocotyl length, chlorophyll or anthocyanin content between WT and *koy-1* seedlings under blue light. Alternatively, the stronger inhibition by blue light in *koy-1* might be a consequence of lower GA_4 and higher ABA levels in the seed before imbibition. Even if the ABA induction upon blue light is equal in WT and *koy-1* seeds, it could result in higher absolute ABA levels if the initial ABA level was already higher in the mutant. This explanation is supported by the similar expression patterns of key regulator genes under blue light in both genotypes and the higher ABA level in *koy-1* in the dark samples.

Ecological role of phytochrome-mediated germination control

Several data presented before and here suggest that the ecological role of light-inhibited germination is likely a day-length sensing mechanism to ensure the appropriate timing of germination in the original habitat of the CYP accession (Mérai et al. 2019). Besides moisture, a temperature range of around 14 °C is necessary for *A. arabicum* seed germination (Arshad et al. 2019), but these conditions can occur in other seasons. Combining these requirements with that for a short day length restricts the germination period to a narrow time-window in early spring. Consequently, the plants finish their life cycle of ~4 mo before the dry and warm summer (Bhattacharya et al. 2019; Mérai et al. 2019). Other examples of light-inhibited germination can be found among desert or Mediterranean maritime plants, which are also challenged by drought, heat, and high light exposure (Thanos et al. 1991; Lai et al. 2016; Carta et al. 2017). We demonstrated that the diurnal regulation of germination is mediated by phytochromes, as *koy-1* mutant germinates under all diurnal regimes. On the other hand, *A. arabicum* accessions from Turkey and the closely related species *Aethionema heterocarpum* originating from Israel have light-neutral seeds that germinate equally well in darkness and light; therefore, their germination does not depend on the day-length (Mérai et al. 2019). Why other closely related Aethionema accessions from similar climate conditions did not acquire, or have lost, the potential advantages of the day-length sensing mechanism remains an open question. The evolution of germination strategies is not independent of other traits, for example, the formation of soil seed bank, alternative risk-reducing traits like stress-tolerant morphology, seed size, or bet-hedging strategies (Venable and Brown 1988; Saatkamp et al. 2019). *A. arabicum* is a fascinating model for a double bet-hedging strategy with a spatial and temporal

dispersal dimorphism. The multi-seeded DEH fruits release seeds with low dormancy close to the parental plant, while the single-seeded IND fruits with winglet-like pericarps are dispersed by wind or water currents and have pronounced dormancy due to much more ABA compared to the DEH seeds (Lenser et al. 2016; Arshad et al. 2019). In most other dimorphic systems described, high dispersal probability is combined with less dormancy, and *vice versa* (Venable and Lawlor 1980; Baskin et al. 2014). The opposite association, the high dormancy of far-distributed seeds in Aethionema, makes the appearance and amount of IND fruits a key factor for the bet-hedging strategy (Arshad et al. 2019). Interestingly, the ratio between the 2 fruit morphs strongly varies among the accessions. In field data collected in the original habitat of the TUR accession, the IND:DEH ratio was between 0.52 and 0.58:1 (Bhattacharya et al. 2019); data for CYP in its original habitat are not available. In controlled conditions in growth chambers, the ratio in TUR plants was shifted toward more IND fruit (2.5:1), whereas the CYP accession produced only a few IND fruits (0.08:1, IND:DEH). *koy-1* Mutants produce 2.5 times more IND fruits, resulting in a significant increase in the absolute number of IND fruits and a higher (0.19:1) IND:DEH fruit-morph ratio(Supplemental Fig. S7). Whether and how the loss of light sensitivity of the *koy-1* mutant is functionally connected with the appearance of more IND fruits remains to be explored, but it is not unlikely, as the TUR accession with fully light-insensitive seeds has substantially more IND fruits. Such a connection might reflect alternative strategies: Aethionema accessions originating from semi-arid habitats have either a precise timing of germination controlled by multiple factors including daylength sensitivity or a bet-hedging strategy with more IND fruits and spatiotemporal dispersal at the cost of the loss of light sensitivity and seasonal control (Supplemental Fig. S7B). Future research with more accessions can address if there is a negative correlation between light sensitivity and dimorphic dispersal of seeds. Independent of this potentially adaptive aspect, the already available range of natural diversity within the Aethionemeae and growing genetic and genomic resources in one of its members open avenues to study several traits of ecological and physiological relevance that are not accessible in other model plants. The *koy-1* mutant presented in this study demonstrates that the generated mutant seed collection allows us to perform additional forward genetic screens that may result in stable and mappable mutants for phenotypes that cannot be observed in Arabidopsis.

Materials and methods

Plant material
Experiments were conducted with *A. arabicum* (L.) Andrz. ex DC. accessions TUR ES1020 and CYP (obtained from Eric Schranz, Wageningen). WT and *koy-1* plants were propagated for seed material under 16 h light/19 °C and 8 h dark/16 °C diurnal cycles, under ~300 μmol m^{-2} s^{-1} light intensity. Fruits and seeds were counted manually after complete maturation from 6 or 8 plants, under 200 or 300 μmol m^{-2} s^{-1} light intensity, respectively. WT and *koy-1* plants were randomly distributed on the shelves.

Plant chambers and light source
The mutant screen and white light germination assays were carried out in a Percival plant growth chamber equipped with fluorescent white light tubes (Philips). Red, far-red, and blue light treatments were performed using customer-designed LED light sources, using LEDs from OptoSupply (www.optosupply.cn). The spectral properties and the LED types are added in Supplemental Fig. S8. For experiments under higher light intensities (Fig. 1, A and F), a Percival growth chamber was equipped with a Valoya LightDNA-8 LED light source (https://www.valoya.com/lightdna/). Light spectra and intensity were measured using LED Meter MK350S (UPRtek).

A. arabicum genome and annotations
A. arabicum genome version 3.0, gene models, cDNA, and protein annotations (version 3.1) were obtained from *A. arabicum* database (https://plantcode.cup.uni-freiburg.de/aetar_db/) (Haudry et al. 2013; Fernandez-Pozo et al. 2021).

Phylogenetic analysis
About 45 HO protein sequences from 22 species were selected for the analysis, listed in Supplemental Data Set S1. Aethionema HO genes were called by nucleotide blast using the Arabidopsis HO genes as query and translated protein sequences were used. Alignment and phylogenetic tree was created with CLC Main Workbentch 7.7.2 software. Tree was created with the Neighbor Joining method with 1,000 bootstrapping replication. Protein distance was measured with the Jukes–Cantor method.

Fast neutron mutagenesis and screening
Seeds of *A. arabicum* (CYP accession) were irradiated in a TRIGA Mark-II research reactor at the Atomic Institute of the Technical University, Vienna (https://ati.tuwien.ac.at/startpage/EN/). Two thousand seeds were irradiated with either 1 or 5 kW for 1 min, corresponding to 8×10^9 and 4×10^{10} cm^{-2} s^{-1} thermal neutron flux densities, respectively. Seeds were plated for germination on the day of irradiation on wet filter paper and kept in darkness at 14 °C for 6 d. Approximately 2,000 individually bar-coded M1 seedlings were planted in a glasshouse under 19 °C and 8 h dark/16 °C diurnal cycles. One thousand three hundred twenty plants produced seeds representing the M2 mutant seed bank. Lines with seed amounts insufficient for direct screening were further propagated to M3 seed lots by pooling the seeds from 5 M2 plants. Seeds were subjected to the forward genetic screening not earlier than 6 mo after harvest. Germination was scored after 7 d under continuous white light illumination with 160 μmol m^{-2} s^{-1} light intensity at 14 °C in a growth chamber.

Identification of the causative mutation in *koy-1*

koy-1 Originates from a single seedling that germinated under light from one of the M2 seed batches originating from the lower, 1 kW radiation. From the same plate, 8 other non-germinating seeds were placed on a new plate in darkness with the addition of 100 μM GA$_4$ to induce germination and plants were grown in parallel with *koy-1*. A WT phenotype in all their progeny and the mutant phenotype of *koy-1* progeny were confirmed for the seeds of the 2 following generations. These seed pools originated from the same M1 mother plant and were expected to share many of the non-causative mutations, except the causative mutation of *koy-1*. Eighteen koy-1 seedlings versus 64 WT seedlings were pooled for DNA isolation and sequenced on an Illumina H2500 platform with 100 bp single-end mode by the Next Generation Sequencing Facility of the Vienna BioCenter Core Facilities (VBCF), member of the Vienna BioCenter (VBC), Austria. Sequencing reads were processed by the CLC Genomics Workbench 9.5.1 software (Qiagen). Reads were mapped to the *A. arabicum* PacBio contigs originating from the CYP accession (see below). After the removal of duplicated reads, 55.8 and 48.1 million reads resulted in 21.98 and 18.97× coverage in the *koy-1* and WT pool, respectively. We expected the *koy-*1 pool to be homozygous for the mutation and the WT pool homozygous for the reference genome sequence; therefore, variants were called with 90% minimum frequency in the *koy-*1 pool and filtered against the WT reads. Seventeen SNPs were uniquely found in the *koy-*1 pool, but none of them overlapped with genic regions. InDel variants were called if evidence for insertion or deletion was detected in a minimum of 6 reads and if they appeared as homozygous variants (variant ratio >0.8) in the *koy-*1 pool but not in the WT pool. Out of the 63 variants that fulfilled these criteria, 2 overlapped with genic regions. One of them was sorted out due to mapping errors, while the remaining one corresponded to the promoter deletion of the HEME OXYGENASE 1 (*Aa31LG4G11055*). The deletion was confirmed by PCR with the primers 5′CCTGGTGGT GGTAATGAACTC and 5′CGGTGGGGCAAAAGCGAATCC. The promoter sequence was analyzed using the PlantCARE tool (Lescot et al. 2002).

Germination test

After seed harvest, seed stocks were kept in darkness at 50% humidity and 24 °C for 6 mo, except for the experiments in Fig. 7, B and C where semi-dormant seed batches 8 wk after harvest were used to test the germination induction of light pulses. One seed batch consists of the harvest from at least 6 plants; replicates represent different seed batches. For each experiment, only seeds of the DEH fruit morph were used, as CYP plants produce only a few seeds in IND fruits. Except that in Supplemental Fig. S1, all germination tests were conducted at the optimal temperature of 14 °C for 6 d in Petri dishes on 2-layer filter paper wetted with distilled H$_2$O and supplemented with 0.1% v/v Plant Preservative

Mixture—PPM (Plant Cell Technology) containing 0.135% w/v 5-Chloro-2-methyl-3(2H)-isothiazilone and 0.0412% w/ v 2-methyl-3(2H)-isothiazolone. For dark treatments, seeds were placed on wet filter paper under complete darkness. Segregation assays were performed under 120 μmol m^{-2} s^{-1} white light. If all 3 replicates of a certain treatment germinated 100%, we assumed 1 non-germinating seed in 1 replicate to obtain variation that is necessary to apply statistical tests.

Hypocotyl elongation test

Seeds were plated on Petri dishes with 2-layer filter paper wetted with distilled H$_2$O and supplemented with 0.1% plant preservative PPM. To induce germination, plates were kept in dark at 14 °C for two and a half days, then transferred to red, far-red, or blue light, or kept in darkness as a control. After 5 d, seedlings were transferred to 1% (v/w) agarose plates, and images were captured. Images were analyzed using Fiji software (https://fiji.sc/).

Chlorophyll and anthocyanin measurement

As described for the hypocotyl elongation test, seeds were induced for germination in darkness followed by a 5-d-long light treatment or kept in darkness. Chlorophyll and anthocyanin measurements were performed as described (Chory et al. 1989; Holm et al. 2002), using 100 mg seedlings harvested in darkness.

RT-qPCR

Imbibed WT and *koy-1* seeds were illuminated at 14 °C with 80 μmol m^{-2} s^{-1} red light, 1 μmol m^{-2} s^{-1} far-red light, under the condition that the WT seed germination is fully inhibited. The intensity of blue illumination was chosen to be 56 μmol m^{-2} s^{-1}, where the germination is fully inhibited for *koy-1* mutant but not for WT seeds. As a control, seeds were kept in darkness. After 24-h exposure, seeds with intact seed coats were collected for RNA extraction, with 3 biological replicates for each sample. RNA extraction, cDNA synthesis, and RT-qPCR were performed as described (Mérai et al. 2019), using the primer pairs listed in Supplemental Table S2. The geometric mean of Aethionema putative orthologues of POLYUBIQUITIN10 (*AearUBQ10*, *Aa3LG9G835*) and ANAPHASE-PROMOTING COMPLEX2 (*AearAPC2*, *Aa31LG10G13720*) was used for normalization (Mérai et al. 2019). For each gene, the expression levels are presented as fold change relative to the level of the dark samples in WT seeds, where the average expression was set to one. Statistical analysis was done using the SATQPCR tool (Rancurel et al. 2019). Error bars represent standard deviation. Asterisks indicate significant differences from the WT or *koy-1* dark level with *P*-values as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ calculated with the Tukey test.

Measurement of hormone levels

For ABA and GA analyses, seed samples were collected as described for RNA extraction, except that 5 biological replicates

were prepared per sample. Sample preparation and targeted profiling of ABA (Turecková et al. 2009) and the members of GA biosynthetic as well as metabolic pathways (Urbanová et al. 2013) were performed as described earlier (Mérai et al. 2019).

Extraction of high molecular weight DNA

The high molecular weight DNA was extracted as described by (Hofmeister et al. 2020; Barragan et al. 2021) with minor modifications. Twenty grams of leaf tissue was harvested from 4-wk-old plants grown under standard conditions and ground in liquid nitrogen. The fine powder was resuspended in 200 ml cold nuclear isolation buffer (10 mM Tris–HCl, 100 mM KCl, 10 mM EDTA pH 8.0, 0.5 M sucrose, 4 mM spermidine, and 1 mM spermine) and filtered through 2 layers of miracloth. After adding Triton X-100 to 1% v/v final concentration, the nuclei were pelleted at $2{,}200{\times}g$ at 4 °C for 15 min and resuspended in 40 ml nuclear isolation buffer with 1% v/v Triton X-100. Nuclei were lysed by the addition of G2 lysis buffer (Qiagen) supplemented with 50 μg ml^{-1} RNaseA and 200 μg ml^{-1} Proteinase K. Samples were incubated first at 37 °C for 30 min and then at 50 °C for 2 h. Nuclear debris was pelleted by centrifugation at $10{,}000{\times}g$ at 4 °C for 15 min. The supernatant was further purified using the Blood&Cell Culture DNA kit (Qiagen) according to the manufacturer's protocol. After elution, the DNA was precipitated overnight at 4 °C by adding 0.7 volume of isopropanol. The visible DNA pellet was spooled out using a glass rod, transferred to 300 μl 1xTE buffer and completely dissolved. The DNA size and integrity were assayed using the Femto Pulse System (Agilent) and confirmed as a single 165 kb-large intact band.

PacBio sequencing

Library preparation and PacBio sequencing were performed by the Next Generation Sequencing Facility at Vienna BioCenter Core Facilities (VBCF), a member of the Vienna BioCenter (VBC), Austria. The DNA was sheared to 34 to 165 kb and size-selected by a BluePippin instrument (sage science) with a lower cutoff at 25 kb. The library was prepared using the SMRTBell express Kit (PacBio) and sequenced on a PacBio Sequel platform (PacBio) with a yield of 22.04 Gb. This acquired 816,659 subreads with 14.7 kb mean length, 4.28 kb lowest quartile length, and 22.5 kb highest quartile length, resulting in 12,063,792,222 total base pairs.

Assembly and annotation of PacBio contigs

The genome was assembled from 22 Gb of Sequel long read data using *Canu* (version 1.8; genomeSize = 235 m; corMaxEvidenceErate = 0.15, other parameters in default settings) (Koren et al. 2017). Raw contigs were polished with *Arrow* in 2 rounds using the default parameters and Pacbio reads. Mapping and alignment of cDNAs generated with GMAP (Wu and Watanabe 2005), using the *A. arabicum* cDNA annotations version 3.1 (Fernandez-Pozo et al. 2021).

Assembled PacBio contigs are deposited at https://plantcode.cup.uni-freiburg.de/aetar_db/downloads.php as CYP_Pacbio_v2.fasta.gz.

In vivo spectroscopy

The total amount of photoreversible phytochrome was measured by dual-wavelength ratio spectrophotometer (ratiospect) as described (Klose 2019), using seedlings of WT and *koy-1* that had been germinated on wet filter paper at 14 °C in darkness for 6 d and collected under safety green light.

Accession numbers

Accession numbers of Aethionema genes used in this study are listed in Supplemental Table S1. Protein sequence data used for phylogenetic analysis can be found in the NCBI GenBank under accession numbers listed in Supplemental Data Set S1.

Author contributions

Z.M. planned and designed the research; Z.M., F.X., A.M., C.K., F.A., S.K., D.T., and V.T. performed the experiments; Z.M., F.X., L.M.S.-J., K.L., C.K., D.T., V.T., M.S., and O.M.S. analyzed and interpreted the data; Z.M. and O.M.S. wrote the paper. All authors approved the submitted version.

Supplemental data

The following materials are available in the online version of this article.

Supplemental Figure S1. Temperature-dependent seed germination of WT and *koy-1* mutant seeds.

Supplemental Figure S2. Phylogram of HOs in seed plants, related to Fig. 2C.

Supplemental Figure S3. Light-induced expressional changes of phytochromes.

Supplemental Figure S4. Light-induced expressional changes of blue light receptors.

Supplemental Figure S5. Germination rescue with norflurazon and gibberellin.

Funding

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