Arabidopsis *COGWHEEL1* links light perception and gibberellins with seed longevity

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SUMMARY

Light is a major regulator of plant growth and development by antagonizing gibberellins (GA) and we provide evidence for a role of light perception and GA in seed coat formation and seed longevity. We have identified two activation-tagging mutants of *Arabidopsis thaliana* (*cog1-2D* and *cdf4-1D*) with improved seed longevity linked to increased expression of *COG1/DOF1.5* and *CDF4/DOF2.3* respectively. These encode two highly homologous DOF ("DNA-binding with One Finger") transcription factors, with *COG1* most expressed in seeds. Resistance to seed deterioration was reproduced in transgenic plants over-expressing these genes, and loss-of-function by RNA interference resulted in opposite phenotypes. Over-expressions of *COG1* and *CDF4* has been described to attenuate various light responses mediated by phytochromes and we found that *phyA* and *phyB* mutants exhibit increased seed longevity. The additional seed longevity conferred by gain-of-function of *COG1* and by loss-of-function of phytochromes is of maternal origin and correlates with a seed coat with increased suberin and reduced permeability. In developing siliques of the *cog1-2D* mutant expression of the GA biosynthetic gene *GA3OX3* and levels of GA_1 are higher than in wild type. These results underscore the important role of GA in the reinforcement of the seed coat and explain the antagonism between phytochromes and *COG1* in terms of inhibition and activation, respectively, of GA action.

SIGNIFICANCE STATEMENT

Seed longevity depends on anti-aging defenses, morphological and biochemical, only partially understood. *COG1* encodes a transcription factor previously described to attenuate phytochrome responses to light and we found that it is a positive regulator of seed longevity while light perception by phytochromes is negative. The proposed mechanism is that *COG1* increases GA levels, leading to a seed coat containing more suberin and less permeable to oxygen. Light is known to inhibit GA action.

INTRODUCTION

Seeds constitute the main system for plant propagation but a major limitation is their gradual loss of viability during storage (aging), a phenomenon mostly caused by oxidative damage and influenced by environmental and genetic factors (Rajjou and Debeaujon, 2008; He *et al*., 2014; Sano *et al.*, 2015). Knowledge regarding the molecular determinants of seed development controlling seed longevity is important for providing reliable crops to farmers and for conservation of genetic diversity in seed banks.

Seed development is orchestrated by a network of seed-specific transcription factors (LEC1, LEC2 and FUS3 in *Arabidopsis thaliana,* in the following Arabidopsis) and mediated by the growth hormones auxin (IAA) and gibberellins (GA) during the first morphogenesis phase and by the stress hormone abscisic acid (ABA) and a seed-specific, ABA-activated transcription factor (ABI3 in Arabidopsis) during the second maturation phase (Parcy *et al*., 1997; Holdswoth *et al*., 1999; Gutierrez *et al*., 2007; Braybrook and Harada, 2008; Suzuki and McCarty, 2008). Loss-of-function mutants in these transcription factors displays considerably reduced seed longevity in Arabidopsis (Clerkx *et al*., 2004).

The formation of the seed coat during morphogenesis and the accumulation of cellular stress defenses and repair mechanisms during maturation are crucial for seed longevity (Rajjou and Debeaujon, 2008; Sano *et al.,* 2015). We have a reasonable knowledge about the induction of defenses and repair (Gutierrez *et al*., 2007; Kotak *et al.,* 2007) and their protecting mechanisms against desiccation (Hoekstra *et al*., 2001; Farrant and Moore, 2011) and oxidative stress (Bailly, 2004). Mutant analysis has demonstrated the important role of vitamin E (Sattler et al., 2004), methionine sulfoxide reductase (Châtelain *et al*., 2013) lipoxygenase (Xu *et al*., 2015), DNA glycosylase/ Apurinic/Apyrimidinic lyase (Chen *et al.*, 2012) and protein-L-isoaspartyl methyltransferase (Ogé *et al*., 2008).

The formation of the seed coat and related mechanisms involved in protecting seeds during aging are less well known. The seed coat is of maternal origin and in Arabidopsis differentiates primarily from cells of the ovule integuments into specialized cell types, including endothelium, palisade and epidermis. This process is almost complete 10 days after fertilization. Cells of the innermost layer (endothelium) synthesize proanthocyanidin (PA), flavonoid compounds (condensed tannins) which act

as antioxidants to protect seeds during aging (Debeaujon *et al.*, 2000; Rajjou and Debeaujon, 2008; Sano *et al*., 2015). Several genes encoding enzymes involved in the biosynthesis of flavonoids, such as *BAN* (Albert *et al*., 1997) or different transcription factors such as *TT16/AGL32* (Nesi *et al.*, 2002) have been implicated in this pathway. The epidermal layer is mainly characterized by the extrusion of mucilage, a pectinaceous carbohydrate. As in the case of endothelial differentiation, the analysis of mutants defective in epidermal formation define genes that fall into two general classes: those apparently required for mucilage biosynthesis, such as the *MUM* genes (Western *et al*., 2004; Haughn and Chaudhury, 2005; Arsovski *et al*., 2010) and those regulating differentiation of the epidermis, such as *AP2* (Jofuku *et al*., 1994). Finally, in the subepidermal (palisade) layer, a thickened wall on the inner tangential side of the cell is produced, characterized by a deposition of suberin that controls seed permeability (Molina *et al*., 2008). In this layer FAR1 (a fatty acyl-CoA reductase) and GPAT5 (an acyl transferase) are important enzymes for the biosynthesis of suberin (Domergue *et al*., 2010; Beisson *et al*., 2007). The role of mucilage and suberin on seed longevity, however, has not been investigated. Although several genetic studies have addressed seed coat development, only a few have established relationships with hormones or different environmental conditions. It is known, however, that GA contribute to the formation of the seed coat through induction of starch degradation (Kim *et al*., 2005).

Knowledge on genes related to seed longevity described above was the result of reverse genetics, testing phenotypes of mutants in genes identified by its role in seed development. Some time ago we started a forward genetic approach to directly identify seed longevity mutants (Bueso *et al*. 2014a and b). It was based on screening an "activation-tagging" mutant collection of Arabidopsis (Weigel *et al*., 2000) for tolerance to "accelerated aging" of seeds. This method mimics natural aging conditions and it was utilized to isolate five dominant mutants with improved seed longevity. A gain-of-function approach overcomes the problem of redundancy within plant gene families and possible artefacts can be discarded by showing opposite phenotypes by loss-of-function of candidate genes. From this screening, two mutants were characterized: *isl1-1D* (increased seed longevity, gene 1, allele 1 dominant) that over-expresses the transcription factor gene *ATHB25* that up-regulatess GA biosynthesis (Bueso *et al*., 2014a), and *isl2-1D* that over-expresses the *RSL1* gene, encoding an E3 ubiquitin ligase involved in seed longevity (Bueso *et al*., 2014b).

In the present work we describe the characterization of the *isl3-1D* and *isl5-1D* mutants. These longevity mutations are caused by over-expression of two homologous genes encoding known transcription factors, *CDF4/DOF2.3* (Fornara *et al*., 2009) and *COG1/DOF1.5* (Park *et al*., 2003), respectively. Both factors have been described to attenuate light responses mediated by phytochromes and therefore our results establish a connection between light perception and seed longevity. Over-expression of COG1 increases GA in the mother plant and reduces the permeability of the seed coat with by increasing suberin. The antagonism between COG1 and phytochromes is explained by inhibition by the latter of GA synthesis and perception.

RESULTS

Molecular characterization of *isl3-1D* and *isl5-1D* mutants

The *isl3-1D* mutant (Bueso *et al*., 2014a and b) contained a single insertion of T-DNA by Southern blot analysis (Supplementary Figure S1A). In order to test whether the tolerance to aging of this mutant was linked to the T-DNA insertion, homozygous mutant plants were crossed to Columbia wild-type plants. From the segregating F2 generation, 29 plants from seeds tolerant to accelerated aging were selected, and the T-DNA was detected by PCR in 27 individuals. Accordingly, we conclude that the phenotype of this mutant is linked to the T-DNA ($|^2$=5.07 and P<0.05 for the hypothesis that it follows a 3:1 segregation, which must be rejected). The insertion was located by plasmid rescue and sequencing to a region between At2g34140 and At2g34150 (Figure 1A). At2g34140 encodes transcription factor CDF4/DOF2.3 (in the following CDF4; Fornara *et al*., 2009) and is over-expressed, whereas At2g34150 is repressed (Figure 1C). The latter locus encodes WAVE1/SCAR1, a member of the WAVE/SCAR family that activates nucleation of actin filaments (in the following WAVE1; Zhang *et al*., 2008).

The *isl5-1D* mutant was isolated in a new screening of 60,000 lines of the Arabidopsis activation-tagging mutant collection (Weigel *et al*., 2000; approximately 300,000 seeds) selecting for increased seed longevity as determined by an accelerated aging procedure of imbibed seeds (Bueso *et al.*, 2014a and b). Three dominant mutants were identified and named *isl5-1D*, *isl6-1D* and *isl7-1D*. *isl5-1D* presented only one T-DNA insertion, as shown by Southern blot (Supplementary Figure S1B). In order to test

whether the tolerance of this mutant to aging was linked to the T-DNA insertion, homozygous mutant plants were crossed to Columbia wild-type plants. From the segregating F2 generation 25 plants from seeds tolerant to accelerated aging were selected, and the T-DNA was detected by PCR in 24 individuals. Accordingly, we conclude that the phenotype of this mutant is linked to the T-DNA (χ^2=5.88 and P<0.05 for the hypothesis that it follows a 3:1 segregation, which must be rejected). This insertion was located by plasmid rescue and sequencing to a region between At1g29160 and At1g29170 (Figure 1B). At1g29160 encodes transcription factor COG1/DOF1.5 (in the following COG1; Park *et al*., 2003) and is over-expressed, whereas At1g29170 is repressed (Figure 1C). The latter locus contains the coding sequence for WAVE2, another member of the WAVE family (see above) highly homologous to WAVE1.

As CDF4 and COG1 are highly homologous transcription factors (Riechmann *et al.,* 2000; Yanagisawa, 2002), the T-DNA insertions in *isl3-1D* and *isl5-1D* occur in genomic regions that seem to originate from a duplication event including *WAVE* and *DOF* genes. The intergenic position of the T-DNA insertion is also similar in both regions. Finally, *isl6-1D* presented the same insertion than *isl5-1D,* demonstrating the robustness of the results. *isl7-1D* has not yet been characterized.

A note on nomenclature: the *COG1* gene name is ascribed by TAIR (The Arabidopsis Information Resource, www.arabidopsis.org) as a secondary name to *RECOGNITION OF PERONOSPORA PARASITICA1* (*RPP1*; At3g44480), not considering the priority of the *COGWHEEL1* (*COG1*) publication (Park *et al.*, 2003).

Over-expression of *COG1* and *CDF4* increases seed longevity in Arabidopsis and loss-of-function has the opposite phenotype

In order to discern whether the increase of seed longevity in *isl3-1D* and *isl5-1D* is a consequence of the over-expression of the transcription factor or due to the repression of the WAVE family member, we generated transgenic plants in which the complementary DNA (cDNA) of *COG1* and *CDF4* was cloned in sense orientation. We also generated plants containing vectors in which *WAVE1* was cloned in the antisense orientation. As indicated in Figure 1D, transgenic lines over-expressing *COG1* and *CDF4* exhibited improved germination after accelerated aging. On the other hand, the loss-of-function mutant *wave1-2* and antisense lines of *WAVE1* (with reduced expression of both *WAVE1* and *WAVE2*, Supplementary Figure S2) presented seed longevity responses similar to the wild-type control (Supplementary Figure S3).

Therefore the *isl3-1D* mutant was renamed as *cdf4-1D* and the *isl5-1D* mutant as *cog1-2D* (second dominant allele over-expressing *COG1* after the first one described by Park *et al*., 2003).

Loss-of-function mutants of *COG1* and *CDF4* could not be evaluated since *CDF4* mutants have not yet been identified, while NASC line N545465, a putative *COG1* loss-of-function mutant according to the database, was checked by sequencing and presented the T-DNA insertion outside of the open reading frame. Also, antisense lines showed no change in expression of *COG1* and *CDF4*. Therefore, we generated RNA interference lines by expression of specific amiRNAs (Schwab *et al*., 2006) that target both transcription factors. Partial silencing of *COG1* and *CDF4* (45% and 70% of wild type expression, respectively) was verified using quantitative RT-PCR analysis (Supplementary Figure S4) and these lines exhibited reduced seed longevity (Figure 1D).

Quality light perception affects seed longevity

Over-expression of both *COG1* and *CDF4* delays flowering under long day conditions (Fornara *et al*., 2009). In addition, it has been shown that over-expression of *COG1* attenuates various responses to red (phytochrome B) and far-red (phytochrome A) light, while *COG1* antisense lines were hypersensitive to red and far red light (Park et al., 2003). Therefore, these two genes positively regulating seed longevity are negative regulators of phytochrome-mediated light responses by unknown mechanisms.

In order to discern whether the increased seed longevity presented by plants over-expressing these transcription factors is due to reduced light perception, we performed an accelerated aging experiment with seeds from different phytochrome and cryptochrome loss-of-function mutants. Both *phyB-9* and *phyA-211* mutants presented better germination after accelerated aging than wild type control, pointing to a negative role for red and far-red light perception in seed longevity. Interestingly, a double mutant in both *CRY1* and *CRY2* genes, encoding blue light receptors, was more sensitive to accelerated aging, suggesting a positive role for blue light perception by these receptors in seed longevity (Figure 2).

Seed longevity of *cog1-2D* and phytochrome mutants shows a maternal effect and correlates with suberin accumulation in the palisade layer of the seed coat

DOF transcription factors have key regulatory roles in many plant processes but were never connected with seed longevity. The complete Arabidopsis genome contains 36-37 putative *DOF* genes (Riechmann *et al*., 2000; Yanagisawa, 2002). COG1/DOF1.5 and the most closely related CDF4/DOF2.3 are classified in subgroup II of this family of transcription factors, showing 78% amino acid identity. However, *in silico* expression analysis in the Arabidopsis eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) shows that *COG1* is expressed quasi-specifically at the end of the morphogenesis phase of seed development, while expression of *CDF4* is mainly confined to the root (Supplementary Figure S5). Recently it has been reported that CDF4 promotes differentiation of root columella stem cells and that it is counteracted by WUSCHEL HOMEOBOX5 (WOX5), which represses *CDF4* expression (Pi *et al*., 2015). These data suggests that the most important role of *COG1* should be carried out in the seed and its over-expression could be responsible for the increase of seed longevity. On the other hand, ectopic over-expression of *CDF4* would have the same effects in seed longevity, because it would be mimicking the function of *COG1* in seeds. Consequently, over-expression of each gene driven from the 35S promoter in both lines lead to similar phenotypes but the physiological role in seed longevity regulation is expected to be carried out mainly by *COG1*. Thus, the following experiments are focused on discovering the molecular mechanisms underpinning *COG1*-meditated resistance to seed aging.

In order to discern whether the increase in seed longevity of mutants over-expressing *COG1* or with loss-of-function of phytochromes is provided by mechanisms involving the seed coat (maternal origin) or fertilization-derived tissues (embryo and endosperm), we performed reciprocal crosses between the wild type and the mutants and found that the F1 seeds are tolerant to accelerated aging if the mother plant is *cog1-2D* or phytochrome mutant, but not when the mother plant is wild type (Figures 3A and B).

To study suberin accumulation in the palisade layer, we stained seeds with Sudan Red. In the case of the aging-sensitive *cry1cry2* double mutant, suberin accumulation was not detected, in contrast to aging-resistant phytochrome mutants and *COG1* over-expression lines, where this layer appeared thicker than in the wild type (Figure 3C). These results suggest that resistance presented in *cog1-2D* and phyrochrome mutants may be the result of a modification of the seed coat that could protect the embryo from oxidative stress.

In order to quantify the seed coat permeability of the different mutants, we measures tetrazolium salt uptake using the triphenyltetrazolium chloride reduction method (Molina et al, 2008). Tetrazolium salts are reduced by active dehydrogenases in the embryo to red-colored, water-insoluble formazans (Berridge *et al.,* 2005). As shown in Figure 4A, phytochrome mutants and *COG1* over-expression produced less red formazans than the wild type. Formazan accumulation can be quantified by extraction with ethanol (Figure 4B) and determination of absorbance at 485 nm (Figure 4C). After 48 h of incubation the accumulation of formazan was 60% of wild type in *phyA-211*, 40% in *cog1-2D* and 30% in *phyB-9*. On the other hand, in the *cry1 cry2* mutant the accumulation of formazan was 140% of wild type.

Global Gene Expression of the *cog1-2D* mutant reveals a transcriptional profile similar to that of *athb25-1D* mutant.

In order to further characterize the *cog1-2D* mutant, we studied the differences in transcriptomic profiles with respect to wild type employing microarray experiments in seedlings. A 2-fold change threshold was used to identify 901 differentially expressed genes; 391 genes were upregulated and 510 genes were downregulated in *cog1-2D* as compared with wild type (see complete list of genes at Supplementary Table S1). As indicated in Supplementary Table S2, within both the up-regulated and down-regulated genes “response to stress” and “response to stimulus” were the most significant overrepresented Gene Ontology categories. Within

We carried out a “preferentially located motif” study (Bernard *et al.*, 2010) in the two sets of differentially regulated genes (Supplementary Table S3). This approach allows the identification of overrepresented motifs in a specific location relative to the transcription start site. Among the genes up-regulated in the *cog1-2D* samples, we observed, at position -100 to -300 from the transcription start site, the TATTAAT motif, that includes the sequence ATTA, the canonical binding site for homedomain proteins (Tan and Irish, 2006). Also enriched in the promoters of up-regulated genes were ACGTG motifs, that could be part of the G-box (CACGTG), an element present in both induced and repressed PHYA-responsive promoters (Hudson and Quail, 2003) and bound by PIF transcription factors (Leivar and Quail, 2011; de Lucas and Prat, 2014). The ACGTG motif is also part of the ABRE (YACGTGKC; International Union of Biochemistry coding: Y = T or C; K = G or T), a motif of ABA-induced genes (Yamaguchi-Shinozaki and Shinozaki, 2005). We also found enrichment of the TGACY

motif in the promoters of COG1 up-regulated genes. This is part of the WRKY box (TTGACY; Y = T or C; Ulker and Somssich, 2004) and of SORLREP5 (TTGCATGACT), another light and PHYA-repressed motif (Hudson and Quail, 2003).

The motif most represented for downregulated genes is identical to SORLIP1 (GCCAC) a motif overrepresented in PHYA-induced promoters genes (Hudson and Quail, 2003). The presence of SORLREP5 and SORLIP1 in the promoters of genes induced and repressed, respectively, by over-expression of COG1 is in agreement with the antagonism between COG1 and phytochrome responses (Park *et al*., 2003; Fornara *et al.* 2009).

Both the canonical binding site for homeodomain protein and SORLREP5 were motifs overrepresented in the promoters of upregulated genes in the *athb25-1D* mutant previously characterized in our laboratory. This mutant over-expresses the homeobox transcription factor ATHB25 and, like the *cog1-2D* mutant, exhibits improved seed longevity of maternal origin and a reinforced seed coat (Bueso *et al*., 2014a). This led us to compare the gene expression profile of *COG1* over-expression with that of *ATHB25* over-expression and we found a significant intersection (Supplementary Figure S6). For up- and down-regulated genes we found an overlap of 44 and 92 genes, respectively ($p < 0.01$). From the common up-regulated genes (listed at Supplementary Table S4) two genes highly expressed in the seed coat (Arabidopsis eFP Browser) are of special relevance: *LACS9*, encoding a long chain acyl-CoA synthetase which could participate in suberin synthesis (Pollard *et al.*, 2008) and *PRX25*, a cationic cell wall peroxidase involved in lignification (Shigeto *et al.*, 2013). The latter could also participate in suberin synthesis by oxidation of ferulic acid to form the lignin-like core.

We selected two genes up-regulated at different levels in the *cog1-2D* mutant for further analyses as putative direct targets of COG1: *ESP* (*EPITHIOSPECIFIER*; At1G54040; involved in response to bacteria and glucosinolate production; 23-fold induction) and *WAG2* (At3G14370; encoding a kinase involved in auxin polar transport; 2-fold induction). We tested the response of the corresponding promoters *in vivo* to COG1 presence, using the Luc/Ren system described by Hellens *et al.* (2005). For our experiment, approx. 2 kb of the ESP 5' genomic sequence and 3 kb of the WAG2 5' genomic sequence were cloned into the pGREENII 0800-Luc vector to generate the *ESP::Luc* and *WAG2::Luc* reporters respectively. Coinfiltration of the reporters with *35S::COG1* in *Nicotiana benthamiana* produced significant (> 2-fold) upregulation of

LUC activity in both cases (Figure 5). These results strongly suggested that COG1 could directly bind *ESP* and *WAG2* promoters to activate their expression in vivo.

Over-expression of COG1 and AtHB25 increases GA_1 in siliques through regulation of *GA3OX3*.

Seed development is essential for longevity and it is regulated by the hormones GA, auxin and ABA (Braybrook and Harada, 2008; Santos-Mendoza et al., 2008). Consequently, we measure the concentration of these hormones in siliques with mature-green-stage seeds (14-17 days after pollination; Le *et al*., 2010) the hormone concentration that is influencing the maturation of seed in control wild type and in the two mutant resistant to aging *cog1-2D* (present work) and *athb25-1D* (Bueso *et al.*, 2014a). Hormone concentration patterns presented by both mutants were similar hormone accumulation pattern (Figure 6.). More specifically, while the levels of auxin and GA_4 in both mutants were similar to those of wild type, both mutants presented a 11-15 fold increase in GA_1 and 3-6 fold more ABA. These data suggest that the higher accumulation of these hormones could trigger substantially different development program in seed coat which would confer appropriate features to better cope with theoxidative stress produced during future storage.

In order to determine the reason why these mutants accumulate more bioactive GA we performed an expression analysis in siliques (same growth stage as above) of *GA3OX3* and *GA3OX4*, the two gibberellin-3 oxidase genes expressed in seed coats (Hu *et al*., 2008; in sameArabidopsis eFP Browser). Gibberellin-3 oxidase seems to be the limiting enzyme for GA biosynthesis (Hedden and Thomas, 2012). *GA3OX4* was slightly (40%) decreased in the *cog1-2D* and *athb25-1D* mutants (Supplementary Figure S4) but, as its shown in Figure 7A, *GA3OX3* appeared more than 2 fold induced in the mutants, thus suggesting that this oxidase is the best candidate to produce the marked increase in bioactive GA in *cog1-2D* and *athb25-1D* mutants. As an additional approach, to determine whether COG1 and AtHB25 could directly regulate this oxidase, we tested the response of the *GA3OX3* promoter after coinfiltration of the Luc/Ren reporters with *35S::COG1* or *35S::ATHB25* in *Nicotiana benthamiana* (Hellens *et al.*, 2005) presences. The results suggest that ATHB25 could directly regulate *GA3OX3* because it increased LUC activity more than 6-fold. COG1, on the other hand, only increased LUC activity by 50% and although this was statistically significant, we

cannot discard that the effect of COG1 on *GA3OX3* expression could be indirect it seems that (Fig. 7B).

DISCUSSION

COG1 participates in an emerging pathway regulating seed coat development

Our genetic analysis of seed longevity (Bueso *et al*., 2014b) has uncovered two transcription factors expressed at the seed coat that regulate seed coat development: ATHB25 (zinc-finger homeodomain or homeobox; Bueso *et al*., 2014a) and COG1 (DOF, present work). Genetic modifications by gain and loss-of-function indicate that these factors are important for seed coat formation and seed longevity and increase the amount of both mucilage (Bueso *et al.*, 2014a for ATHB25 and Supplementary Figure S8 for COG1) and suberin (present work) at the epidermal and palisade layers, respectively.

ATHB25 and COG1 may belong to a seed coat developmental pathway unrelated to the classical embryo/endosperm pathway ruled by transcription factors LEC1, LEC2, FUS3 and ABI3. The latter factors are concerned with development of the embryo and endosperm, the seed components derived from fertilization, and with accumulation into them of nutrient stores and stress defenses (Gutierrez *et al*., 2007; Braybrook and Harada, 2008; Suzuki and McCarty, 2008). Accordingly, these transcription factors are expressed at both the embryo and endosperm but not at the seed coat (Arabidopsis eFP browser). ATHB25 and COG1 are expressed at the seed coat, an a tissue of maternal origin derived from the inner integuments of the ovule. Both pathways are triggered by fertilization and should be coordinated by unknown mechanisms (Haughn and Chaudhury, 2005).

The seed coat development pathway has been mostly investigated with mutants affected on either proanthocyanidin accumulation at the endothelium (Debeaujon *et al*., 2000; Haughn and Chaudhury, 2005; Rajjou and Debeaujon, 2008) or mucilage secretion by the epidermal cells (Western *et al*., 2004; Haughn and Chaudhury, 2005; Arsovski et al., 2010). Although many identified genes corresponded to biosynthetic enzymes of either proanthocyanidins or mucilage, some encode transcription factors that regulate seed coat development. These include *APETALA2* (*AP2*), *TRANSPARENT TESTA2* (*TT2*), *TRANSPARENT TESTA8* (*TT8*), *TRANSPARENT TESTA16*

(*TT16/AGL32*), *GLABRA2* (*GL2*), *TRANSPARENT TESTA GLABRA1* (*TTG1*), *TRANSPARENT TESTA GLABRA2* (*TTG2*), *ENHANCER OF GLABRA3* (*EGL3*), *MYB5* and *MYB61*. TT3, TTG2, GL2 and MYB61 induce expression of genes encoding biosynthetic enzymes of mucilage or proanthocyanidins. On the other hand, TT16, AP2, TT2, TT8 and TTG1 regulate earlier stages of seed coat development. Some of these genes have functions outside seeds, for example *AP2* is best known for being involved in the specification of floral organ identity (Jofuku *et al.*, 1994). This correlates with two major sites of *AP2* expression: shoot meristems and seed coat (Arabidopsis eFP Browser).

Haughn and Chaudhury (2005) have proposed the existence of two separate seed coat development pathways. One would be initiated by transcription factor TT16 followed by TTG1, TT8, TT2 and TG2 to differentiate the proanthocyanidin-producing endothelium. The other would be initiated by AP2 followed by TTG1 and TT8 to differentiate the epidermal (mucilage-producing) and subepidermal (palisade) layers. ATHB25 and COG1 belong to the epidermal-subepidermal pathway because they regulate the formation of both the mucilage (epidermis) and suberin (subepidermis or palisade) layers.

The relationship between all these transcription factors, their mechanisms of activation and their position within the seed coat regulatory pathway is mostly unknown. One clue is the role of GA in this pathway (see below). As indicated by Sparks *et al.* (2013): "The signalling mechanisms that direct plant development include long-range effectors, such as phytohormones, and molecules with a local intra-organ range, such as peptides, transcription factors and some small RNAs". After many years of intense research these mechanisms are starting to be elucidated in the development of flowers (Ó'Maoiléidigh *et al*., 2014), roots (Wachsman *et al*., 2015) and shoots (Puig *et al*., 2012; Sparks *et al*., 2013). The seed coat development pathway will need an equivalent effort and the important role of this structure for seed longevity deserves the effort.

COG1 increases the level of GA and improves the suberin layer of seeds

GA are important for the normal formation of the Arabidopsis seed coat by promoting starch degradation at the epidermis and palisade and increasing mucilage synthesis at the epidermis (Kim *et al*., 2005). Also, we have shown that seed longevity in

Arabidopsis is regulated by this growth-promoting hormone (Bueso *et al*., 2014a). Now we report that over-expression of *COG1* improves seed longevity and increases the level of GA_1 in siliques (Figure 6), probably through up-regulation of the biosynthetic gene *GIBBERELLIN 3-OXIDASE 3* (*GA3OX3*; Figure 7). Over-expression of *ATHB25* exhibited the same behavior. Therefore, one mechanism of the seed coat development pathway directly connected to seed longevity is to activate biosynthesis of GA by increasing expression of gibberellin 3-oxidases, enzymes known to be rate-limiting for biosynthesis of the hormone (Hedden and Thomas, 2012). The connection of this phytohormone, and therefore ATHB25 and COG1, with known transcription factors of the epidermal-subepidermal development pathway (see above) should be investigated.

As accumulation of stress defenses is not of maternal origin and is regulated by the classical embryo-endosperm pathway (see above), our hypothesis for the improved seed longevity of the *cog1-2D* (and *athb25-1*) mutant is that the seed coat should present a modification that hinders the contact between oxygen and the embryo. Mucilage is unlikely to form a permeability barrier but suberin in roots and cutin in stems and leaves form such a barrier (Pollard *et al.*, 2008). The cork industry is based on this property of suberin and it has been shown that the formation of suberized barriers in roots restricts permeability to oxygen (De Simone *et al.*, 2003), the major cause of seed aging (Bailly, 2004). Therefore our working hypothesis is that a suberin layer at the seed coat is responsible for the increased seed longevity of the *cog1-2D* (and *athb25-1D*) mutant. *cog1-2D* (and *athb25-D1*) seeds present an over-accumulation of suberin in the subepidermal layer (Figure 3C) and exhibit reduced permeability to tetrazolium (Figure 4). In the Arabidopsis seed coat, suberin is preferentially associated with the outer integument or palisade layer (Molina *et al*., 2008) and regulates seed permeability measured with tetrazolium (Beisson *et al.*, 2007). Taken together, these data suggest that suberin could regulate seed longevity by restricting oxygen access to the embryo. COG1 (and ATHB25) probably regulates suberin (and mucilage) biosynthesis in the seed coat by two complementary mechanisms: by increasing GA (for starch degradation to provide energy and carbon skeletons) and by up-regulating expression of peroxidases and acyltransferases that are good candidates to catalyze steps of suberin biosynthesis (Pollard *et al*., 2008).

COG1 links light percerption to seed longevity

Light, in addition to powering photosynthesis, is a major regulator of plant growth and development by activating phytochromes (red and far-red) or phototropin and cryptochrome (blue) light receptors (de Lucas and Prat, 2014). One important aspect of plant development is the production of mature seeds with enough longevity to secure the propagation of species. However, the effect of light perception independent of photosynthesis, in this process was mostly unknown (de Lucas and Prat, 2014; He *et al*., 2014).

COG1 encodes a DOF transcriprion factor that was identified in an Arabidopsis screening searching for mutants with altered photo-responses. That study concluded that COG1 functions as a negative regulator in both PHYA and PHYB signaling pathways. In addition, the *COG1* gene is induced by light in a phytochrome-dependent manner and therefore it provides fine-tuning of phytochrome-signaling pathways by feed-back inhibition, explaining the *COGWHEEL1* name (Park *et al*., 2003). Our identification of COG1 as an important determinant of seed longevity prompted an investigation on the effect of light perception on seed longevity by testing phytochrome and cryptochrome mutants. Our results indicate that red (phytochrome B/PHYB) and far-red (phytochrome A/PHYA) lights are negative for seed longetivity because *phyA* and *phyB* mutants produce seeds with increased longevity, reduced permeability to tetrazolium and increased suberin content. On the other hand, blue light seems to be positive because a cryptochrome mutant (*cry1 cry2*) produced seeds very sensitive to aging, with increased permeability to tetrazolium and reduced suberin content. The opposite behaviour of phytochrome and cryptochrome mutants is observed in other aspects of plant physiology such as flowering time, accelerated in the first case and delayed in the second (Liu *et al*., 2008; Hajdu *et al.*, 2015).

Our observation that COG1 increases GA levels may explain its antagonism with phytochrome signaling because light, acting through phytochromes, inhibits GA biosynthesis (Hedden and Thomas, 2012) and perception (Hudson and Quail, 2003; Leivar and Quail, 2011; de Lucas and Prat, 2014). One possible explanation for the negative effect of light on seed longevity could be that plants grown under high illumination produce seeds that will germinate very fast after maturation due to the positive effect of light on seed germination (Leivar and Quail, 2011). Reinforcement of the seed coat to increase longevity would not be convenient for fast germination and, accordingly, is inhibited by light signaling.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown and germinated on Murashige and Skoog (MS) plates with sucrose as described (Alejandro *et al.,* 2007). The screening was performed using the Scheible and Somerville activation-tagging collection (NASC lines N31100). We used the following strains: wild-type Columbia, activation tagging mutant over-expressing *COG1/DOF1.5* (*cog1-2D*), activation tagging mutant over-expressing *CDF4/DOF2.3* (*cdf4-1D*), activation tagging mutant over-expressing *AtHB25* (*athb25-1D*), transgenic lines over-expressing *COG1* under the control of the 35S promoter (*35S:COG1*), transgenic lines over-expressing *CDF4* under the control of the 35S promoter (*35S:CDF4*), lines over-expressing antisense of At2g34150 (*ASWAVE1*), loss-of-function of At2g34150 (*wave1-2*, NASC line N442867), DOF RNAi lines (amiRNA DOF), loss-of-function of *PHYA* (*phyA-211*), loss-of-function of *PHYB* (*phyB-9*), double mutant in *CRY1* and *CRY2* (*cry1cry2*).

Accelerated Aging

This procedure was a modification of the "basal thermotolerance assay" (Tejedor-Cano *et al*., 2010). Seeds were imbibed in water, stratified for 2 days at 4 ºC, and incubated at 42 ºC for 1 to 2 days (relative humidity of 100%). In the initial screening for mutants, the seeds were exposed to 42 ºC for 48 h because, after this time, the germination of wild type seeds was completely inhibited. In other experiments, incubation at 42 ºC was for 24 h.

Molecular Techniques

Plasmid rescue from mutant plants, RNA extraction, qRT-PCR, PCR to detect T-DNA, and Southern-blot analysis to determine the number of T-DNA insertions were performed as described (Alejandro *et al.*, 2007). The constructs for over-expression of *COG1* (At1g29160), *CDF4* (At2g34140) genes under the control of the cauliflower mosaic virus 35S promoter were made as follows. The cDNA containing the complete open reading frame was obtained by reverse transcription of RNA prepared from wild type plants. The primers utilized were for *COG1* 5´-ATGGCGACCCAAGATTCTCA-3' and 5´-TTAACAAGATTGACCATCGGTGTAAC-3´ and for *CDF4* 5´-ATGGCGACTCAAGATTCTCAAGGG-3´and 5´-

TCAGCACGATTGACCGTCGGAGT-3′. The amplified cDNA was cloned into the pCR8/GW/TOPO plasmid (Invitrogen) and after determining the orientation of the insert, a LR-Gateway reaction was performed to transfer cDNA by homologus recombination into the binary destination vector Alligator2. The construct for antisense *ASWAVE1* (At2g34150) lines was obtained with same strategy using primers 5′-AATGTCTCCGAGTCTCTCTTTTC-3′ and 5′-CAGACGTAACATATTCTATAA-3'. The amplified cDNA was cloned into pCR8/GW/TOPO plasmid in the antisense orientation, a LR-Gateway reaction was performed to transfer cDNA by homologus recombination into the binary destination vector pMDC32. DOF RNAi lines (amiRNA DOF) were designed to knock down At1g29160 (*COG1*) and At2g34140 (*CDF4*), using the microRNA designer webpage http://wmd3.weigelworld.org/cgibin/webapp.cgi?page=Designer;project=stdwmd. The amiRNAs were obtained according to the protocol described in http://wmd3.weigelworld.org/downloads/Cloning_of_artificial_microRNAs.pdf. PCR products were cloned in the pCR8/GW/TOPO plasmid (Invitrogen, www.lifetechnologies.com) and then where recombined in the plasmid pMDC32 (Curtis and Grossniklaus, 2003) using the Gateway technology and LR Clonase reaction (Invitrogen). All PCR-derived constructs were verified by DNA sequencing. Agrobacterium mediated transformation of Arabidopsis to generate *35S:COG1* and *35S:CDF4*, *ASWAVE1* and amiRNA DOF transgenic plants were performed as described (Alejandro *et al*., 2007).

Global Gene Expression using Long Oligonucleotide Microarrays

Transcriptome analysis was done using the Agilent Arabidopsis (V4) Gene Expression 4344K Microarray, which contained 43,803 probes (60-mer oligonucleotides) and was used in a two colour experimental design according to Minimum Information About a Microarray Experiment guidelines (Brazma *et al.*, 2001). Four biological replicas for each genotype, *cog1-1D* and Col wild-type 7-day-old seedlings, were analyzed, and each mutant line was compared with the wild type with dye swap. Total RNA integrity was assessed using the 2100 Bioanalyzer (Agilent). Sample RNA (0.5 μg) was amplified and labelled with the Agilent Low Input Quick Amp Labelling Kit. An Agilent Spike-In Kit was used to assess the labelling and hybridization efficiencies. Hybridization and slide washing were performed with the Gene Expression Hybridization Kit and Gene Expression Wash Buffers, respectively. After washing and drying, slides were scanned in an Agilent G2565AA microarray scanner, at 5 μm

resolution and using the double scanning, as recommended. Image files were analyzed with the Feature Extraction software 9.5.1. Interarray analyses were performed with the GeneSpring 11.5 software. To ensure a high-quality data set, control features were removed, and only features for which the 'IsWellAboveBG' parameter was 1 in at least three out of four replicas were selected. To identify significantly expressed genes in each comparison, a Student's t test analysis was performed with false discovery rate adjustment (Benjamini and Hochberg, 1995). Features were selected only if the P value was below 0.05 after correction for multiple testing and the difference in the expression ratio was greater than two-fold.

Suberin staining

Mature seeds were incubated for 24 h at room temperature in water containing 0.01% (w/v) Triton X-100 and 10% (v/v) commercial bleach to fade the seed coat pigments. After rinsing successively with distilled water and 100% ethanol, seeds were incubated for 30 min with chloroform:methanol (2:1, v/v), rinsed with 100% ethanol, and air-dried. Seeds were finally incubated at room temperature for 1 to 4 h with a solution of Sudan red 7B in polyethylene glycol 400:glycerol:water prepared as described by Brundrett *et al*. (1991), rinsed in water, mounted between slide and cover slip, and observed with a Leica MZ16 F microscope.

Seed Coat Permeability Test

Tetrazolium red assays were used for seed coat permeability tests (Debeaujon *et al*., 2000; Beisson *et al.,* 2007). Dried Arabidopsis seeds were incubated in the dark in an aqueous solution of 1% (w/v) tetrazolium red (2,3,5-triphenyltetrazolium chloride, Sigma-Aldrich) at 30°C for 4 to 48 h. The seeds were observed for change in color and imaged using a stereomicroscope. The quantitative formazan assay was carried out as described by Molina *et al.* (2008). If the tetrazolium salts penetrate into the seed, they are reduced by the embryo to a red precipitate called formazans. 50 mg of Arabidopsis seed wild type and mutants were incubated in 500 mL of 1% (w/v) aqueous solution of tetrazolium red at 30°C for 4, 48 and 96h in darkness. After incubation, the samples were washed twice with water, resuspended in 1 mL 95% (v/v) ethanol, and finely ground with a mortar and pestle to extract formazans. The final volume was adjusted to 2 mL with 95% (v/v) ethanol and immediately centrifuged for 3 min at 15,000g, and the

supernatant was recovered. Formazan concentration was determined by measuring the absorbance at 485 nm (Candler *et al.,* 1997). Each sample was assayed in triplicates.

Quantification of hormones

Plant material was suspended in 80% methanol-1% acetic acid containing internal standards and mixed by shaking during one hour at 4ºC. The extract was kept a -20ºC overnight and then centrifuged and the supernatant dried in a vacuum evaporator. The dry residue was dissolved in 1 % acetic acid and passed through an Oasis HLB (reverse phase) column as described in Seo *et al*. (2011). The dried eluate was dissolved in 5% acetonitrile-1% acetic acid, and the hormones were separated using an autosampler and reverse phase UPHL chromatography (2.6 µm Accucore RP-MS column, 50 mm length x 2.1 mm i.d.; ThermoFisher Scientific) with a 5 to 50% acetonitrile gradient containing 0.05% acetic acid, at 400 µL/min over 14 min. The hormones were analyzed with a Q-Exactive mass spectrometer (Orbitrap detector; ThermoFisher Scientific) by targeted Selected Ion Monitoring (SIM). The concentrations of hormones in the extracts were determined using embedded calibration curves and the Xcalibur 2.2 SP1 build 48 and TraceFinder programs. The internal standards for quantification of each of the different plant hormones were the deuterium-labelled hormones.

Luciferase assays

To generate promoter:LUC fusions for transient expression assays in Nicotiana benthamiana leaves, promoter fragments for GA3OX3 (-1974 to ATG), ESP (-2001 to ATG) and WAG2 (-3034 to ATG) were amplified from Arabidopsis Col-0 genomic DNA with primers promGA3OX3for (5´-GGACTAGTCCGGTTTTCAATGCAATAGTTATTAGTTATAATTG-3´)/promGA3OX3rev (5´-CATGCCATGGCATGGGATTTGCTTTTGTTGTTTCTCTTTC-3´), promESPfor (5´-CCGCTCGAGGCAGTACTTTCAAAGAAATTTCAAAAC-3´)/promESPrev (5´-CCGGAATTCGGCTGCAATATAAGTATAAAAGTGTTTTCG-3´) or oPBF77 (5'-CCCGGGACCTTCTGTTATGTTTTG-3)/oPBF78b (5'-CCATGGTGAGATCAAGATCGGTGTCA-3') respectively, cloned into pCRII vector (invitrogen), digested with SpeI, blunted, and NcoI (GA3OX3), EcoRI, blunted, and XhoI (ESP) or SmaI and NcoI (WAG2) restriction enzymes, and ligated into pGREEN_LUC (Hellens *et al*, 2005). The construct also contains a Renilla (REN) gene

under the control of the CaMV35S promoter to estimate the extent of the transient expression, which therefore serves as an internal standard when the activity of the promoter is represented as the ratio of LUC to REN activities (Luc/Ren). The transient expression assays were performed by *Agrobacterium*-infiltrated transient transformation of *N. benthamiana*, that was carried out following the protocol of Espley *et al.*, (2009) with minor modifications. Briefly, 300 µl of *Agrobacterium* containing the reporter or/and effector plasmids were infiltrated into a young leaf of *N. benthamiana* at three points. Firefly Luciferase and Renilla Luciferase were assayed 3 d after infiltration using the Dual-Luciferase Reporter Assay System (Promega, USA). Data were represented as the ratio of LUC/REN. Background controls were obtained by infiltrating only the reporter construct. At least three plants were used for each treatment, and the experiment was repeated three times.

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SHORT LEGENDS FOR SUPPORTING INFORMATION

Supplementary Figures

Supplementary Figure S1. Southern analysis of *isl3-1D* and *isl5-1D* mutants using the T-DNA 35S probe

Supplementary Figure S2. qRT-PCR analysis of expression of *WAVE1 and WAVE2* in wild type and antisense lines of *WAVE1*.

Supplementary Figure S3. *WAVE1* (At2g34150) does not play an important role in the seed longevity of Arabidopsis.

Supplementary Figure S4. qRT-PCR analysis of expression of *ACT8*, *COG1* and *CDF4* in wild type and *amiRNA DOF* lines.

Supplementary Figure S5. eFP-Browser gene expression developmental map of *COG*1 and *CDF4.*

Supplementary Figure S6. Global Gene Expression of the *cog1-2D* mutant reveals a similar transcriptional response than in *athb25-1D* mutant.

Supplementary Figure S7. qRT-PCR analysis of expression of *GA3OX4* in wild type, *cog1-2D* and *athb25-1D* lines.

Supplementary Figure S8. S. Mucilage staining in *cog1-2D* mutant (left panel) and WT (right panel) seeds

Supplementary Tables

Supplementary Table S1. List of genes regulated by over-expression of COG1

Supplementary Table S2. Functional categories of genes regulated by over-expression of COG1

Supplementary Table S3. Summary of microarray data of genes differentially expressed in the *cog1-2D* mutant with respect to the wild type

Supplementary Table S4. Genes induced both in the *athb25-1D* and in the *cog1-2D* mutants (> 2 fold)

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Figure Legends

Figure 1. Molecular characterization of the *isl3-1D* and *isl5-1D* mutants. (A) Location of T-DNA in the intergenic region between At2g34140 and At2g34150 as determined by plasmid rescue and sequencing in the *isl3-1D* mutant. (B) Location of T-DNA in the intergenic region between At1g29160 and At1g29170 as determined by plasmid rescue and sequencing in the *isl5-1D* mutant. (C) qRT-PCR analysis of expression of At2g34140 and At2g34150 in wild type (WT) and *isl3-1D* mutant (left) and qRT-PCR analysis of expression of At1g29150, At1g29160 and At1g29170 in wild type (WT) and *isl5-1D* mutant (right). Expression values are relative to *ACT8* and the resulting ratios are normalized to wild type. RNA was extracted from 2-week old seedlings grown in MS plates and results are the average of three determinations with bars corresponding to the standard error. (D) Seeds from activation tagging mutant *isl3-1D* (referred to as *cdf4-1D*), activation tagging mutant *isl5-1D* (referred to as *cog1-2D*), *35S:COG1*, *35S:CDF4*, wild type (WT) and *amiRNA* DOF mutant were subjected to accelerated aging for 24 h and sown on MS plates. The percentage of germination was recorded at the indicated times. The results are the average of 4 experiments with 100 seeds per line, and bars correspond to standard error. Untreated lines germinated more than 99% after 3 days. In the case of *35S:COG1*, *35S:CDF4* and *amiRNA DOF*, three independent transgenic lines were tested with similar results.

Figure 2. Seed longevity is regulated by perception of light. Seeds from wild type (WT), phytochrome B mutant (*phyB-9*), phytochrome A mutant (*phyA-211*) and double mutant in cryptochromes 1 and 2 (*cry1cry2*) were subjected to accelerated aging treatment for 24 h and sown on MS plates. The percentage of germination was recorded after 8 d. The results are the average of four experiments with 100 seeds per line, and bars indicate standard error. Not aged lines germinated more than 99% after 3 d.

Figure 3. Maternal effect and seed coat formation in seed longevity mutants. (A) Seeds from wild type (WT), mutant *cog1-2D* and F1 seeds from reciprocal crosses of wt as female parent and *cog1-2D* as male parent [WT (♀) x *cog1-2D* (♂)] and WT as male parent and *cog1-2D* as female parent [WT (♂) x *cog1-2D* (♀)] were subjected to accelerated aging for 24 h and sown in MS plates. The percentage of germination was

recorded after 8 d. The results are the average of four experiments with 100 seeds per line, and bars indicate standard error. Non-aged lines germinated more than 99% after 3 d. (B) Seeds from WT, mutant *phyA-211*, mutant *phyB-9* and F1 seeds from reciprocal crosses of WT and phytochrome mutants as female and male parents as indicated. Other conditions as in (A). (C) Sudan red 7B staining of the entire seed of wild-type (WT), *cry1cry2*, *phyA*, *phyB* and *cog1-2D* mutants. Lower panels show a detail of the staining of the subepidermal layer of seed coat (arrows indicate suberin layer).

Figure 4. Seed permeabilty of *phyA-211*, *phyB-9*, *cog1-2D* and *cry1 cry2* mutants. (A) Staining pattern in seeds of WT (wild type) and mutants incubated in 1% tetrazolium red at 30 ºC and imaged at the end of 48 h. Red color reflects formazan accumulation after penetration of tetrazolium red into the embryo and metabolic reduction. (B) Qualitative view of formazan extracted from seeds of different genotypes after 96 hincubation with tetrazolium red. (C) Quantitative time course of formazan extracted from seeds of different genotypes. The data (absorbance at 485 nm) are mean values of three replicates and bars indicate standard error.

Figure 5. Interaction of COG1 with putative target promoters in transient expression assays on N*icotiana* benthamiana leaves. WAG2:*LUC-35S*:REN and *ESP:LUC-35S*:REN reporter constructs were transiently expressed in N. benthamiana leaves together with mock or *35S*:COG1. The expression of *Renilla* luciferase (REN) was used as an internal control. LUC/REN values represent means and standard errors from at least six independent biological replicates. Asterisks above bars indicate a significant difference according to Student's t-test (*, $P < 0.05$; ***, $P<0.005$) from the values obtained when the corresponding promoter:*LUC-35S*:REN reporters were co-infiltrated with mock or *35S*:COG1.

Figure 6. Over-expression of *ATHB25* and COG1 produce similar hormonal changes in siliques with mature-green seeds. (A) Endogenous GA4 and GA1 levels in *cog1-2D*, wild type and *athb25-1D*. (B) ABA and IAA levels in *cog1-2D*, wild type and *athb25-1D*. Means were calculated from four independents experiments and bars indicate standard errors.

Figure 7. AtHB25 and COG1 regulate GA3OX3 expression. (A) qRT-PCR analysis of expression of GA3OX3 from the wild type (WT), *cog1-2D* and *athb25-1D* mutants. Expression values are relative to ACT8 expression values and the resulting ratios are normalized to wild type taken as 1. RNA was extracted from siliques with mature-green seeds and results are the average of three determinations with bars corresponding to the standard error. (B) Plasmid construct GA3OX3:*LUC-35S*:REN with two reporters (*GA3OX3* promoter driving LUC expression) was transiently expressed in Nicotiana benthamiana leaves together with mock, *35S*:COG1 or UBI1:AtHB25. The expression of *Renilla* luciferase (REN) was used as internal control. LUC/REN values represent means and standard errors from at least six independent biological replicates. Asterisk above bars indicate a significant difference according to Student´s t-test (*, $P < 0.05$; ***, $P<0.005$) from the values obtained when the GA3OX3:*LUC-35S*:REN reporters were infiltrated with mock.