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A role for auxin signaling in the acquisition of longevity during seed maturation

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Abstract

1. Seed longevity, the maintenance of viability during dry storage, is a crucial factor to preserve plant genetic resources and seed vigor. Inference of a temporal gene-regulatory network of seed maturation identified auxin signaling as a putative mechanism to induce longevity-related genes.

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2. Using auxin-response sensors and tryptophan-dependent auxin biosynthesis mutants of *Arabidopsis thaliana* L., the role of auxin signaling in longevity was studied during seed maturation.
3. DII and DR5 sensors demonstrated that concomitant with the acquisition of longevity, auxin signaling input and output increased and underwent a spatio-temporal redistribution, spreading throughout the embryo. Longevity of seeds of single auxin biosynthesis mutants with altered auxin signaling activity was affected in a dose-response manner depending on the level of auxin activity. Longevity-associated genes with promoters enriched in auxin response elements and the master regulator *ABSCISIC ACID INSENSITIVE 3* were induced by auxin in developing embryos and deregulated in auxin biosynthesis mutants. The beneficial effect of exogenous auxin during seed maturation on seed longevity was abolished in *abi3-1* mutants.
4. These data suggest a role for auxin signaling activity in the acquisition of longevity during seed maturation.

Introduction

Seed longevity, defined as the ability to remain alive during storage under dry conditions, is an important agronomic factor in the preservation of seed fitness after harvest. In addition, longevity is pivotal to ensure the preservation of our genetic resources through dry seeds of crops and wild species. Seeds with elevated longevity will deteriorate only slowly during conservation, and will retain high germination vigor. Seed vigor is an important trait that provides homogeneous seedling establishment, thereby ensuring high yield. Longevity is acquired during seed maturation, concomitant with the loss of chlorophyll and accumulation of protective molecules such as non-reducing sugars, late embryogenesis abundant (LEA) proteins and heat shock proteins (HSP) (Prieto-Dapena *et al.*, 2006; Hundertmark *et al.*, 2011; Chatelain *et al.*, 2012; Verdier *et al.*, 2013). Furthermore, antioxidants also play a role in longevity, such as glutathione (Kranter *et al.*, 2006), tocopherols (Sattler *et al.*, 2004), and flavonoids present in the testa (Debeaujon *et al.*, 2000).

One of the hormones regulating the acquisition of longevity is abscisic acid (ABA) (Leprince *et al.*, 2017), with *ABA-INSENSITIVE3 (ABI3)* (Sugliani *et al.*, 2009), and *ABA-INSENSITIVE5 (ABI5)* (Zinsmeister *et al.*, 2016) being the main regulatory genes known so far. Interacting with these regulators to affect longevity are *LEAFY COTYLEDON 1 (LEC1)* (Sugliani *et al.*, 2009) and *DELAY OF GERMINATION1 (DOG1)*, whose precise functioning

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remains unknown (Dekkers *et al.*, 2016). In addition, the seed specific *HEAT SHOCK FACTOR A9 (HSFA9)*, acting downstream of *ABI3* and controlling the developmentally regulated expression of *sHSP* (Kotak *et al.*, 2007), is part of the genetic program controlling resistance against accelerated deterioration during deleterious storage (Prieto-Dapena *et al.*, 2006; Carranco *et al.*, 2010). These regulators form an elaborate network that is necessary for the expression of LEA and HSP proteins, the accumulation of raffinose family oligosaccharides (RFO), chlorophyll breakdown and chloroplast dismantling during late maturation (Sugliani *et al.*, 2009; Dekkers *et al.*, 2016; Leprince *et al.*, 2017; Zinsmeister *et al.*, 2016). A second hormone involved in longevity is gibberellin (Bueso *et al.*, 2014). Mutants of the transcription factor *HOMEODOMAIN-BOX 25 (ATHB25)* are more sensitive to accelerated deterioration. *ATHB25* positively regulates GA synthesis and reinforces the seed coat (Bueso *et al.*, 2014). Recent indirect evidence suggests that auxin might be a third hormone regulating longevity (Carranco *et al.*, 2010; Righetti *et al.*, 2015). A gene coexpression network analysis of seed maturation of both the legume *Medicago truncatula* and *Arabidopsis thaliana* identified a conserved gene module related to seed longevity that contains genes that are significantly enriched in the *cis*-regulatory element ARFAT, an auxin response factor (ARF) binding site, in their promoter region (Righetti *et al.*, 2015). In addition, this module contains *CYP79B2*, a gene involved in a minor auxin biosynthesis pathway (*i.e.* indole-3-acetaldoxime pathway, IAOx) (Hull *et al.*, 2000; Zhao *et al.*, 2002). A putative role of auxin signaling in longevity also comes from the observation that the sunflower AUXIN-RESPONSIVE PROTEIN 27 (*HaIAA27*), a short-lived transcriptional repressor of early auxin response genes, represses *HaHSFA9*, and the subsequent accumulation of *sHSP*, via direct interaction (Carranco *et al.*, 2010). An auxin-resistant form of the sunflower *HaIAA27* was able to repress activation of the *sHSP* promoter by *HaHSFA9* in mature bombarded tobacco embryos and ectopic overexpression resulted in poor germination after artificial aging, comparable to *HSFA9* loss of function lines (Carranco *et al.*, 2010).

As a key hormone regulating plant growth and development, auxin is known to play a multitude of roles early during seed development, acting as a signal molecule during embryogenesis (Jenik and Barton, 2005) as well as the differentiation of the seed coat (Figueiredo *et al.*, 2016), endosperm cellularization (Batista *et al.*, 2019) and regulating seed size (Schruff *et al.*, 2006; Liu *et al.*, 2010). Auxin signaling is also regulating seed dormancy in *Arabidopsis* (Liu *et al.*, 2010; Bai *et al.*, 2018). Next to auxin synthesis, differential auxin distribution acts as key signal triggering developmental processes. For example, early during

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embryogenesis, differential auxin accumulation establishes the specification of the apical cells and later establishment of the root pole and cotyledons (Friml *et al.*, 2003) whereas at the torpedo stage, increased auxin activity was detected in the cotyledon primordia and radicle tip (Liao *et al.*, 2015), consistent with its role in the formation of vascular tissues and maintenance of the pattern of root meristem. Auxin signaling activity depends also on the properties of the core signaling network consisting of TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX proteins (TIR1/AFBs) acting as co-receptor, the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressors and the ARF transcription factors (Salehin *et al.*, 2015).

Here, we demonstrate a role for auxin signaling in the acquisition of seed longevity. Auxin-responsive reporters DR5 and DII show that auxin signaling activity increases during final seed maturation of Arabidopsis and spreads throughout the embryo, concomitant with an increase in longevity. Seeds of single mutants deficient in the Trp-dependent auxin biosynthesis enzymes were affected in longevity and auxin signaling activity in a dose-response manner. The master regulator of longevity *ABI3*, its target *EMI* and longevity-correlated genes were induced by auxin in developing embryos and deregulated in the auxin biosynthesis mutants, and the beneficial effect of exogenous auxin during seed maturation on seed longevity was abolished in *abi3-1* mutants. These data point to a role for auxin signaling activity in the acquisition of longevity during seed maturation.

Materials and Methods

Plant Material

Arabidopsis thaliana homozygous T-DNA insertion mutants *cyp79b2-1*, *cyp79b2-2* (17), *ami1-1*, *ami1-2*, *taa1-1* (*ckrc1-1-DR5::GUS*; N66988) (28), *taa1-1-DR5::GUS* (*ckrc1-1-DR5::GUS*; N16703), *taa1-2* (*wei8-1*), *tar1* (*tar1-1*), *tar1-DR5::GUS* (N16703), *tar2* (*tar2-1*) (Stepanova *et al.*, 2008), *yuc1* and *yuc2* (Panoli *et al.*, 2015) and the heterozygous mutants *ao1-1*, *ao1-2* (all in Col-0 background) and *abi3-5* (in Ler-0) were obtained from the Nottingham Arabidopsis Stock Centre (Table S1). Seeds of *abi3-1* were a generous gift from H. Roschttardt. Homozygous seeds were confirmed by PCR (for primer details, see Table S2). Homozygous *DR5::GUS*, *DR5::VENUS* and *DII::VENUS* were obtained from Dr. P. Nacry (Montpellier, France). Three independent homozygous lines of *cyp79b2-1-DR5::GUS*, *ami1-2-DR5::GUS*, *tar2-DR5::GUS* and *yuc2-DR5::GUS* were obtained by crossing the mutant lines with pollen of the *DR5::GUS* line. First the T1 generation was screened for each

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mutant allele. Next, *DR5::GUS* insertion was examined in the T1 generation for a 3:1 segregation using GUS primers (Table S2). Homozygous *cyp79b2-1*, *ami1-2*, *tar2*, and *yuc2* lines with *DR5::GUS* heterozygous or homozygous were selected to obtain final homozygous T2 generation.

Physiological Analyses

Plants were grown at 20°C/18°C with a 16-h photoperiod. Flowers were tagged and developing seeds were harvested at different time intervals after flowering until siliques opened (corresponding to 20 DAF). Dry weight was assessed gravimetrically for triplicate samples of 25-50 seeds after drying at 96°C for 2 d. For all physiological analyses, seeds at different developmental stages were dried for 2 d under an airflow at 43% RH. A biological replicate consisted of a pool of 150-200 seeds harvested from 3-6 plants. Three biological replicates were used for all physiological analyses.

For germination assays, seeds were stratified for 72h at 4 °C, followed by imbibition on wet filter paper at 20°C, 16h light. Germination was scored after 10d of imbibition when the radicle protruded the endosperm after 10d of imbibition. Longevity was determined as the storage time that was needed to decrease the germination percentage of the seed population to 50% (P50) (Righetti *et al.*, 2015) and was derived from survival curves of germination percentage over storage time (75% RH, 35°C). The effect of IAA and ABA on germination was determined on mature dried seeds that were imbibed on filter paper in a range of IAA concentrations (mixed isomers, Sigma, St Louis, MO, USA) or ABA (Sigma, St Louis, MO, USA) at 20°C, 16h light. IAA and ABA were dissolved in MeOH prior to dilution in water. Control seeds were imbibed in diluted MeOH corresponding to that of the highest IAA/ABA concentration (0.05% MeOH). To determine the effect of IAA on the acquisition of seed longevity during development, Col-0, Ler, or *abi3-1* siliques were dipped for 30 sec in 0.1 or 10 µM IAA or water twice with 24-h interval between 10 and 12 DAF and harvested at final maturation, after which they were aged at 35°C, 75% RH. To determine the effect of IAA during imbibition on seed longevity, mature Col-0 seeds were imbibed on 1 µM IAA or water and aged for 21d at 75% RH, 35°C, after which final germination was determined. Permeability of the seed layers was assessed by incubation of mature, dried seeds of the different IAA mutants in 1µM 2,3,5 triphenyltetrazolium for 24h, as previously described (Righetti *et al.*, 2015). Dormancy was determined by counting the percentage on germinated seeds of freshly harvested seeds and at different time intervals during after-ripening at 20°C

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in the dark. DSDS50 was calculated as the time to obtain 50% germination of the seed lots (Bentsink and Koornneef, 2008).

Fluorescence quantification and confocal microscopy

For the GUS and VENUS reporter quantification assays, seeds of *DR5::VENUS*, *DR5::GUS* and *DII::VENUS* lines were harvested every two days between 10 and 20 DAF and at 18 and 20 DAF for the auxin biosynthesis mutants containing *DR5::GUS* and rapidly frozen in liquid nitrogen. For activity in root tissues, seeds were sterilized and 5 replicates of 80-100 were imbibed on vertically placed 12 cm squared Petri dishes with agar (0.9%) for 10d at 16h light at 20°C after which roots were harvested and frozen directly in liquid nitrogen. Total soluble proteins were extracted from tissues in 25 mM Tris-HCl (pH 7.6), with 1 mM MgCl₂, 1 mM EDTA and 0.1% β-mercaptoethanol (v/v). The resulting homogenate was centrifuged (14,000g for 30 min at 4°C). Protein content of the soluble fractions was determined using the Bradford reagent and bovine serum albumin as a standard. The fluorometric GUS assay was based on 4-methylumbelliferone (4-MU, Sigma) fluorescence measured by a FLUOstar Omega (Halder and Kombrink, 2015). GUS substrate was 4-methylumbelliferyl-β-D-glucuronide (Rose Scientific). 4-MU was excited with 365 +/-15 nm width and detected with emission of 460 +/-20 nm. VENUS fluorescence was directly measured after seed protein extraction, similarly to GFP (Richards *et al.*, 2003). VENUS was excited with 510 nm and detected with emission band of 530 nm. GUS and VENUS activities were calculated in relative units of fluorescence produced per μg protein. Each measurement was carried out with three biological replicates of 150-200 seeds each for measurements during seed maturation, and four biological replicates of 50-100 seeds for the IAA biosynthesis mutants, or five replicates of 80-100 roots, using a duplicate colorimetric or fluorometric reaction.

For confocal microscopy observations, up to 20 seeds or embryos containing *DR5::VENUS* and *DII::VENUS* constructs were analyzed immediately after harvest. Seeds were stained by 1% propidium iodide (Sigma-Aldrich) during 5 min under vacuum. Observations were performed under a Nikon A1S1 confocal laser microscope (Nikon Instruments, Melville, NY, USA) equipped with argon-ion (488 nm) and diode (561 nm) lasers and NIS element software (Nikon). VENUS and propidium iodide were excited with 488 nm and 561 nm, respectively, with an emission band of 500 to 550 nm for GFP detection, 580 to 720 nm for propidium iodide detection. For each experiment, fluorimeter or confocal microscope parameters were kept unchanged between each biological sample for comparison.

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RNA extraction, reverse transcription and real-time quantitative PCR

For total RNA extraction, seeds were freshly harvested at different developmental stages and directly frozen into liquid nitrogen. At final maturation, seeds were dried an additional 2d at 43% RH (noted as 20 DAF). For IAA inducibility, freshly harvested *DR5::GUS* seeds at 18 DAF were imbibed on 1 μ M IAA or water for 4h and rapidly frozen in liquid nitrogen. Total RNA was purified using NucleoSpin® RNA Plus extraction kit (Macherey-Nagel), following the manufacturer's instructions. Reverse transcription was performed using iScript™ Reverse Transcription Supermix (Bio-rad) on 0.5-1 μ g of total RNA. Quantification of transcript levels were performed by RT-qPCR in CFX96 (Bio-rad) qPCR using SsoAdvanced Universal SYBR Green Supermix (Bio-rad) as described by the manufacturer and using primers described in Table S3. Efficiencies of each primer pair were checked and primers were retained with efficiency between 90-110%. Experiments were performed on at least two independent biological replicates from pools of 200 seeds harvested on three plants and on three technical replicates. Relative expressions were calculated using Bio-rad CFX manager 3.1 after normalization by four endogenous reference genes, *MAP2B* (At3g59990), *ARP6* (At3g33520), *TIP41-like* (At4g34270) and *UBC21* (At5g25760).

Sugar extraction and quantification

For sugar extraction, 10 mg of mature Arabidopsis seeds from three independent pools of three plants were extracted from the Col-0 and different mutant lines. Soluble sugar analysis was performed using a DIONEX equipped with a Carbopac PA-1 column (Dionex Corp., Sunnyvale, CA, USA) as previously described (Rosnoblet *et al.*, 2007).

IAA and ABA quantification

Extraction and purification of IAA and ABA was performed with 20 mg dry seeds and Oasis HLB, MCX and WAX cartridge columns (30 mg, 1 cc; Waters) as described previously (Kanno *et al.*, 2010). Quantification of IAA and ABA by liquid chromatography-tandem mass spectrometry was performed as described previously (Kanno *et al.*, 2016).

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Results

Auxin signaling activity correlates with the acquisition of longevity during seed maturation

A possible role of auxin signaling in the acquisition of seed longevity in *Arabidopsis* was investigated using seeds that were produced from two lines containing the auxin-inducible *DR5* promoter fused with reporters (*DR5::GUS* and *DR5::VENUS*) and from the *DII-VENUS* line. Both promoters are used as auxin activity signals and have an antagonistic response, *DR5* activity increases with increased auxin signalling output, whereas *DII* activity decreases with increased auxin signalling input (Liao et al., 2015). During maturation, from 10 to 14 days after flowering (DAF), water content decreased sharply (Fig. S1a) and DT, i.e. the ability to survive rapid drying, was acquired (Fig. 1b; Fig. S1a). From 14 DAF onwards, water content decreased more gradually and was accompanied by the loss of chlorophyll (Fig. 1a). Concomitantly, longevity, expressed as the time of storage necessary to obtain 50% viability (P50), in developing seeds from the *DR5::GUS* line increased from 4 to 24 d until 20 DAF (Fig. 1b, Fig. S1a). For both *GUS* and *VENUS* reporter lines, *DR5* promoter activity increased steadily from 10-12 DAF onwards until the seeds were completely matured, at 20 DAF (Fig. 1b). To verify that the *DR5* promoter acted as an auxin signaling reporter in our system, we checked the efficiency of an external IAA treatment on 18 d-old siliques, which induced both *DR5::GUS* and *TIR1* expression in the seeds (Fig. 1c,d). We confirmed that the increased *DR5* promoter activity was linked to increased auxin signaling input. The profile of *DII::VENUS* fluorescence during maturation was opposite to that of *DR5::GUS* and *DR5::VENUS* (Fig. 1b, S1c), reflecting a progressive increase in the degradation of the *DII* fusion protein and therefore an increased auxin signaling input during the later stages of maturation. Finally, considering that *DII-VENUS* activity is not only linked to auxin accumulation but also depends on the *TIR1/ABF* co-receptor concentration (Brunoud *et al.*, 2012), we measured the transcript level of these genes as a proxy. *TIR1* transcript levels decreased during seed maturation from 10 to 16 DAF (Fig. 1e). However, from 16 DAF onwards, transcript levels increased again significantly until seeds were mature (inset Fig. 1e). *AFB* transcript levels decreased gradually from 12 DAF onwards (SI Appendix, Fig. S2a-c).

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To test whether the increased auxin signaling activity plays a role in longevity acquisition during maturation, 10-12 d old siliques were dipped in a 10 μ M IAA solution for two consecutive days and the resulting mature seeds were aged for 21 d, after which the percentage of remaining viable seeds was determined. Exogenously applied IAA significantly increased the percentage of viable seeds after storage compared to seeds from water-sprayed siliques, both in the Col-0 and Ler background (Fig. 1f, g). Lower concentration of IAA (100 nM) was not effective in increasing longevity. To exclude the possibility that the added IAA had an effect during imbibition, mature seeds from non-treated plants were aged for 21 d and subsequently imbibed in IAA or water. The percentage of viable seeds was not different between IAA- and water-imbibed seeds (Fig. 1g). While IAA exogenously supplied during imbibition did not affect the survival of stored seeds, we did observe a dose-dependent delay in germination (Fig. S3a), as previously reported (Liu *et al.* 2013). Determination of the *DR5* promoter activity during imbibition showed that auxin signaling activity decreased immediately upon imbibition, and this was corroborated by the opposite response of the *DII::VENUS* reporter (Fig. S3b).

Functional analysis of auxin biosynthesis mutants provides a genetic link between auxin signaling and the acquisition of seed longevity

Using a trait-based gene significance analysis, we previously identified *CYP72B2* (IAOx pathway) to be expressed in parallel to the acquisition of longevity in developing seeds of *M. truncatula* (Righetti *et al.*, 2015). Here, the role of this gene and those underlying the major IAA synthesis pathways were further investigated in relation to longevity. We first confirmed that in Arabidopsis Col-0 seeds, *CYP79B2* transcript levels also increased at the onset of acquisition of longevity (Fig. S4b). Besides *CYP79B2*, which is part of the indole-3-acetaldoxime (IAOx) pathway leading to auxin biosynthesis, three other tryptophan (Trp)-dependent pathways for auxin biosynthesis have been identified, namely the indole-3-acetamide (IAM) pathway, the tryptamine (TAM) pathway, and the indole-3-pyruvic acid (IPA) pathway. Study of expression profiles of genes encoding enzymes from the other three pathways leading to IAA biosynthesis (Fig. S4a,b) also revealed variations during seed maturation, with *AAOI* and *YUC2* transcript levels increasing from 10 and 14 DAF onwards, respectively (Fig. S4d-f). To determine the genetic link between auxin and longevity, mutants of each Trp-dependent pathway (Fig. S4a) were identified and seeds were produced under the same growth conditions together with the wild type (Col-0). To avoid embryonic defects that

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might indirectly influence longevity due to impaired embryogenesis, only single mutants were used. Freshly harvested seeds of all single mutants germinated for 98-100% after stratification without defects (Fig. S5, 0d of storage). Longevity was determined on seeds of the different mutants and Col-0 by assessing the P50 values from survival curves obtained during storage (Fig. S5, Fig. 2). For the IAOx pathway, seeds from both *cyp79b2* alleles exhibited shorter longevity (-20%) compared to Col-0 seeds (Fig. 2a). For the IAM pathway, seeds of the *ami1-2* allele showed a significant increase in longevity compared to Col-0 (Fig. 2b). Likewise, seeds of all mutant lines from the IPA pathway showed a significant increase in longevity (ca. 25%) compared to Col-0 seeds (Fig. 2c). The longevity of seeds of two *aaol* alleles was comparable to Col-0 seeds (Fig. S5c) and these lines were not studied further.

To investigate how the auxin signaling activity was modified in these mutants, the *DR5* reporter system was introgressed by crossing *DR5::GUS* lines with the auxin biosynthesis mutants of interest. For *tar1* and *taa1-1*, these lines already existed (Zhou *et al.*, 2011). After selection of homozygous mutants, *DR5::GUS* activity was determined in seeds harvested at 18 DAF and final maturation (Fig.2d-f, Fig. S6). In the *cyp79b2-1* seeds, *DR5::GUS* activity was significantly lower compared to Col-0, whereas higher *DR5::GUS* activity was detected in the *ami1-2* and the *taa1-1*, *tar1*, and *yuc2* seeds compared to Col-0 (Fig.2d-f, Fig. S6), and increased concomitantly with longevity in these mutants. When auxin signaling activity was expressed as function of P50 obtained during seed development of both WT and different mutants, this revealed the existence of a dose-response relationship (Fig. 3g). Longevity of the mature seeds of the double mutant *wei8,tar1 (taa1,tar1)* was reduced (Fig. 2h, Fig. S5m).

Considering the surprising increase in activity of the IPA pathway mutants, we verified that the mutants showed reduced auxin activity in roots, as reported previously (Biswas *et al.*, 2007) (Fig. S7). All IPA mutants except *tar1* showed a significant decrease in *DR5::GUS* fluorescence, confirming the reduced auxin activity phenotype in the roots of these lines. One explanation of the increased *DR5::GUS* activity in the seeds of the IPA mutants might be the activation of an overcompensation mechanism (Biswas *et al.*, 2007). . To investigate whether the expression of the genes involved in the other Trp-dependent IAA pathways increased in the single IPA mutants, RT-qPCR was performed on seeds harvested at 18 DAF. Although there were slight variations in gene expression, it is unlikely that these differences in transcript levels between the different IAA biosynthesis mutants and Col-0 could explain overcompensation (Fig. S8). Determination of the IAA content in the different

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mutants showed no statistical difference between the mutants and Col-0, indicating that the increased auxin signaling activity in the IPA mutant seeds was not due to increased IAA content (Fig. S9a). Analysis of seeds from another culture of the *taal* and *tar1* mutants showed a reproducible increase in longevity (Fig. S9d), whereas for this seed batch, also no significant differences in IAA levels were found compared to Col-0 (Fig. S9c). Thus, if there is overcompensation in the single mutant seeds, this does not occur at the level of IAA biosynthesis, but further downstream in the IAA signaling cascade.

Next, we verified whether mutant seeds affected in longevity exhibited pleiotropic effects that could be explained by impairment of auxin signaling early during seed embryogenesis that in turn would indirectly influence the acquisition of longevity during maturation. In none of the single mutants, seed abortion was observed in mature siliques and seed size was comparable to that of Col-0 plants. There was no difference in the morphology of mature embryo between mutants and Col-0. Seed filling and water content during maturation were also identical between the single mutants and Col-0 seeds (Fig. S10a-g). For all mutant lines, mature seeds showed typical complete degradation of chlorophyll compared to Col-0 (Fig. S10h). Mutant seeds germinated without any abnormalities in germination speed and seedling morphology compared to Col-0.

Since auxin is involved in the formation of the seed coat (Figueiredo *et al.*, 2016), whose properties can affect longevity (Debeaujon *et al.*, 2000), we also tested whether seed coat permeability was affected using tetrazolium staining. Seeds from *taal*, *tar1* and *yuc1* showed a significant increase in permeability for tetrazolium, evident from the increased number of red seeds, ranging between 15-30% (Fig. S11), consistent with previous observations on the *wei8/tar* double mutants producing 30% of seeds with defects in cell expansion of the seed coat (Figueiredo *et al.*, 2016). However, this increased permeability cannot explain the longevity phenotype of these mutant seeds, since P50 in these lines is higher than in Col-0 seeds. In addition, we also measured the contents of sucrose and RFO, since in Arabidopsis, these sugars increase during maturation in parallel to the acquisition of longevity (Righetti *et al.*, 2015). Compared to Col-0 seeds, only slight differences in sugar levels were found that were not correlated with either longevity or auxin signaling activity (Fig. S12). These results suggest that the embryogenesis and maturation programs were not affected or truncated by defects in auxin synthesis in the mutant seeds.

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IPA pathway mutants link dormancy to auxin and ABA

Another trait that we investigated was dormancy since a link with auxin has recently been proposed (Liu *et al.*, 2013, Bai *et al.*, 2018). Just after harvest, all seed lots showed dormancy, which was gradually released during after-ripening for one month (Fig. S13a). Seeds of *taal*, *tar1* and *yuc1* showed a faster dormancy release (SI Appendix, Fig. S13a). The decreased dormancy phenotype of these mutants was consistent with a decreased ABA sensitivity compared to Col-0 (Fig. S13b), and coincided with decreased ABA and IAA contents in the *taal* and *tar1* mutants (Fig. S9b). Comparison of all the different phenotypes of the IAA biosynthesis mutant showed that auxin activity is linked with longevity, whereas dormancy is linked to ABA sensitivity and seed coat permeability (Table S4).

The pattern of auxin signaling distribution changes between embryogenesis and late maturation

In addition to auxin synthesis, differential auxin distribution can also act as a key signal triggering developmental processes. Therefore the distribution of auxin signaling activity during seed maturation was investigated on isolated embryos containing the *DR5::VENUS* and *DII::VENUS* construct by confocal microscopy at two developmental stages (Fig. 3). Fluorescence was detected in the nucleus as previously described (Brunoud *et al.*, 2012). At 10-12 DAF, corresponding to beginning of seed filling, auxin signaling activity was detected in the radicle tip, the provascular tissue and primordia of the cotyledons (Fig. 3a-f), as previously reported (Friml *et al.*, 2003; Liao *et al.*, 2015). *DII* activity was opposite of that of *DR5* in the cortex and epiderm cells, and high around the provascular tissue (cf Fig. 3b vs c and e vs f), indicating increased auxin input signaling in these specific locations. At 16-18 DAF, the distribution of the auxin signaling activity had changed, becoming visible throughout the embryo, both in the epidermal and cortical cells of the cotyledons and radicle (Fig. 3g,i,k). Again, *DII* activity was opposite of that of *DR5*, being completely absent from the cortex and epiderm cells (cf Fig. 3g vs h and i vs j). In the radicle tip, fluorescence remained high throughout seed maturation (Fig. 3k). These data show that there exists a distinct transition in the distribution of the auxin signaling activity from a highly localized distribution to a general spread throughout the embryo during the final stages of seed maturation concomitant with an increase in auxin signaling activity.

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Downstream regulation of genes involved in longevity by auxin signaling

Previous identification of a longevity-related gene module revealed a strong enrichment of ARF elements in the promoters, certain of them being first neighbors of CYP79b2 (Righetti *et al.*, 2015, Fig. 4a). We used the auxin biosynthesis mutants to investigate if two of these direct neighbors, *CHX18* and *HSP21*, are regulated by auxin signaling during maturation. Incubation of freshly harvested 18d-old *DR5::GUS* seeds in IAA for 4h resulted in an increase in *CHK18* transcripts showing that *CHX18* was inducible by exogenous IAA (Fig. 4b). In the *cyp79b2* mutants, *CHX18* transcript levels were significantly lower at 15 and 18 DAF compared to Col-0 (Fig. 4c), whereas in the mutants of the IAM (Fig. 4d) and IPA pathway (Fig. 4e), they were significantly higher than Col-0 at 18 DAF. For *HSP21*, transcript levels strongly increased during the final stage of maturation in Col-0 seeds (Fig. 4f). *HSP21* transcript levels were affected in mutant seeds defective in the IAOx and IPA pathway, consistent with their differences in auxin activity compared to Col-0 (Fig. 4f-h). Levels of *HSP21* transcript were lower in mature *cyp79b2* seeds whereas they were higher in the *taa1*, *tar1* and *yuc1* mutants. No difference was found in the *ami1* mutants compared to Col-0. Transcript levels of *WRKY3*, a transcription factor regulating longevity (Righetti *et al.*, 2015) were significantly down-regulated in the *cyp79b2* mutants at 18 DAF, and up-regulated in the *ami1* and *taa1* mutants (Fig. 4i). However, they were not affected in *tar1* and down-regulated in *yuc1* mutant seeds. Overall, these results suggest that auxin signaling activity is implicated in the transcriptional regulation of specific sets of longevity-correlated genes in a temporal manner during seed maturation.

Role of ABI3 in downstream of auxin signaling to affect longevity

To further investigate which downstream pathways leading to the acquisition of longevity are affected by auxin signaling, we assessed if key regulators of longevity such as *ABI3* (Ooms *et al.*, (1993), *DOG1* and *ABI5* (Dekkers *et al.*, 2016) and *HSFA9* (Prieto-Dapena *et al.*, 2006) could be induced by IAA. Out of these three regulators, only *ABI3* transcripts were induced by exogenous IAA in 18d-old seeds (Fig. 5a, Fig. S14a and S15). In developing Col-0 seeds, the expression profile of *ABI3* decreased gradually between 15 and 20 DAF (Fig. 4b), concomitant with the increase in *DR5::GUS* increase and *DII-VENUS* decrease (cf Fig. 1b vs 5b). In the *cyp79b2-1* mutant, *ABI3* transcripts were significantly lower at 18 DAF compared to Col-0 (Fig. 5b). In contrast, in seeds of mutants of the IAM (Fig. 5c) and IPA pathway (Fig. 5d), *ABI3* transcripts were significantly higher at 18 DAF, concomitant with the increase

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in auxin signaling activity in these mutants (Fig. S6). To investigate if the transcriptional cascade downstream of ABI3 was also affected in the biosynthesis mutants, we assessed the transcript levels of *EMI*, a LEA gene that is strongly impaired in *abi3-5* seeds (Fig. 5e) and whose expression peaks during the late stage of seed maturation in Col-0, around 18 DAF (Fig. 5f). At 18 DAF, *EMI* transcript levels were lower in seeds of the *cyp79b2* mutant (Fig. 5f), and higher in the mutants of the IAM (Fig. 5g) and IPA pathway (Fig. 5h), consistent with the auxin signaling activity and *ABI3* expression profile in these mutants. To further investigate the role of ABI3 downstream of auxin signaling, the effect of exogenous IAA applied during seed maturation affected longevity in the *abi3-1* mutant. Whereas exogenous IAA application on the siliques of the wild type Ler during maturation increased longevity (Fig. 5i), this increase was completely abolished in the *abi3-1* mutants (Fig. 5j).

Another downstream target of ABI3 is HSF9, a transcription factor implicated in resistance against ageing during deleterious storage (Prieto-Dapena *et al.*, 2006; Kotak *et al.*, 2007, Carranco *et al.*, 2010). Here, transcript levels of *HSFA9* were not inducible by IAA (Fig. S14a). They were significantly lower in the *cyp79b2* mutants, but instead of being up-regulated in the IPA pathway mutants concomitant with the increase in auxin signaling activity, transcripts were down-regulated at 18 DAF compared to Col-0 (Fig. S14b-d). Thus, the relation of ABI3 by auxin does not activate the transcriptional cascade of HSF9. Considering that *HSFA9* is also under the direct control of AUX/IAA proteins (Prieto-Dapena *et al.*, 2006; Carranco *et al.*, 2010), this can explain that transcript levels do not correlate with auxin signaling output measured by *DR5::GUS*. In conclusion, these results suggest that auxin signaling activity activates not only genes from the longevity module (Fig. 4) but also certain downstream targets regulated by ABI3, a main regulator of seed longevity.

Discussion

Longevity permits seeds to remain in suspended animation in the dry state until water and favourable conditions are available for germination and seedling establishment. Whereas it is well established that this trait is acquired during late seed maturation, the upstream signaling cascade activating this acquisition remains largely unknown (Leprince *et al.*, 2017). Here we demonstrated that the auxin signaling pathway is activated in parallel to the acquisition of longevity in a dose-response manner. Whether this correlation is causal remains to be ascertained, but addition of exogenous IAA induced higher longevity that was abolished in the *abi3-1* mutant, suggesting that the activation of the TIR1/ABF-ARF pathway is involved

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in the regulation of seed longevity through the activation of genes involved in the longevity signaling cascade.

Using auxin signaling input and output sensors, auxin signaling activity was found to increase in developing seeds from 14 DAF onwards, when cell division or growth are arrested and the principal goal for the seed is to prepare for desiccation and survival in the dry state (Leprince *et al.*, 2017). Its originates most likely from a complex interplay between synthesis, transport and cycling between active and inactive forms of auxin as well as changes in auxin sensitivity to regulate the auxin response in maturing seeds (Leyser, 2018). Several hypotheses can be suggested to explain how auxin is linked to longevity. Synthesis might take place via activation of *CYP79B2*, a gene that is part of the IAOx pathway, encoding a cytochrome P450 that catalyzes the conversion of tryptophan to indole-3-acetaldoxime (Mikkelsen *et al.*, 2000) and whose expression correlates with the acquisition of longevity both in *Arabidopsis* and *M. truncatula* (Righetti *et al.*, 2015). Indeed, seeds from *cyp79b2* mutants showed reduced auxin signaling output together with decreased transcript levels of genes related to longevity (Figs. 2, 4, 5). Several auxin input pathways can also modulate the final auxin activity, including those linked to conjugate hydrolases, the IBA response or methyl hydroxylase (Spiess *et al.*, 2014). Auxin transport from the radicle tip or surrounding maternal tissues into the embryo might also be partially responsible for the increased auxin signaling activity (Robert *et al.*, 2018). An altered spatial distribution of the auxin response taking place from 14 DAF throughout the embryo cells (Fig. 3) might explain the increased expression of the *TIR1* receptor (Fig. 1e) and activation of downstream signaling cascade via the perception of the newly arrived auxin by the TIR1/ABF proteins. Irrespective of its origin, one could argue that a uniform redistribution of auxin activity throughout the embryo is needed if it plays a role in the activation of the longevity program, considering that all the embryonic cells need to be protected in the dry state to allow for vigorous germination upon subsequent imbibition.

Using single mutants representative of the Trp-dependant auxin biosynthesis pathways, we established a genetic link between the auxin signaling output and longevity. The IPA pathway is considered the main auxin biosynthesis pathway that controls many developmental processes such as embryogenesis, endosperm and seed coat development and seedling growth (Figueiredo and Köhler, 2018; Batista *et al.*, 2019). Surprisingly, our data revealed that mutants of the IPA pathway displayed an enhanced auxin response in conjunction with a 20-30% increase in longevity (Fig. 3). This might be explained by the redistribution of auxin throughout the embryo at the later stages of maturation; even if auxin

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levels are slightly reduced (Fig. S9a). It could be hypothesized that transport of auxin from the radicle tip will be perceived by the TIR1/ABF proteins in all the cotyledon and radicle cells, leading to downstream activation of DR5 and auxin response (Fig. 2). Alternatively, because auxin homeostasis is regulated, at least in part, through negative feedback by auxin-inducible proteins such as Aux/IAA transcriptional repressors and GH3 auxin-conjugating enzymes (Weijers and Wagner, 2016; Leyser, 2018), the initial decrease in IAA could abolish this feedback loop and consequently lead to overcompensation further downstream of the auxin signaling pathway. So far, for most of the seed phenotypes, double or triple mutants defective in auxin synthesis were needed to demonstrate the role of auxin in Arabidopsis embryo development (Figueiredo *et al.*, 2016; Weijers and Wagner, 2016) and dormancy (Liu *et al.*, 2013). Here, the double mutant *wei8,tar1* showed indeed a decreased longevity compared to the increase in the single mutants, suggesting that the auxin decrease was enough to affect the redistribution and obtain a phenotype. Although it remains unclear why the single IPA pathway mutants show increased signaling output, this phenotype reinforces the link between auxin signaling activity and longevity already found for the *cyp79b* mutants, and enabled to link this signaling activity to the regulation of downstream genes involved in longevity such as *CHX18*, *HSP21*, *WRKY3* and the master regulator *ABI3* and its LEA protein target.

Whereas auxin signaling activity is linked with longevity, the IAA level in mature seeds appeared to be linked to dormancy, since those IPA pathway mutants that have reduced IAA contents also show a reduced dormancy phenotype (Fig. S9 and S13). Recent studies identified a role for auxin in seed dormancy, either showing that auxin is required for ABA-mediated inhibition of seed germination (Liu *et al.*, 2013) or via tryptophan-dependent auxin biosynthesis that controls DOG1-dependent seed dormancy (Bai *et al.*, 2018). We found that the reduced dormancy in *taa1*, *tar1* and *yuc1* is also linked to decreased ABA sensitivity, confirming the synergistic interaction of auxin with ABA (Brady *et al.*, 2003; Liu *et al.*, 2013).

Mutants with altered auxin signaling activity also displayed altered *ABI3* transcript levels, suggesting that auxin activity between 15 and 18 DAF is important to maintain the expression of *ABI3* and allow the induction of protective molecules such as the *EMI* proteins. Evidence that *ABI3* plays a role downstream of auxin signaling comes from the observation that the increase of longevity by IAA application during seed maturation was abolished in the *abi3-1* mutants (Fig. 5). *ABI3* is involved in the initiation and maintenance of the maturation phase, inducing several pathways not only leading to the acquisition of longevity, but also to

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desiccation tolerance, seed filling, dormancy and chlorophyll retention, all of them acting independently after being promoted by the ABA/ABI3 system (Sugliani *et al.*, 2009; Delmas *et al.*, 2013). The downstream signaling pathway linking auxin to ABI3 and dormancy in Arabidopsis has been attributed to the recruitment of ARF10/ARF16 to control the expression of *ABI3* (Liu *et al.*, 2013). It is unlikely this ARF couple is responsible for the downstream auxin signaling leading to longevity, since *cyp79b2* does not show an ABA sensitivity or dormancy phenotype, and *ABI3* transcription is activated rather than repressed in mutants with reduced dormancy level. The cellular response to auxin depends on the presence of different ARF complements in different cells and thus auxin signaling specificity, and different homo- and hetero-oligomerization of Aux/IAAs at the promoter may add an additional mechanism for auxin response diversity (Rademacher *et al.*, 2012). Inspection of the ARF gene profiles from public data points to a large number of putative candidates (Fig. S16), and further research is needed to identify the IAA/Aux/ARF combination that leads specifically to the activation of *ABI3* as well as the longevity-module genes containing the ARE in their promoters.

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Author contributions

AP designed research, performed research, analyzed the data and wrote the paper, BLV, DL, MN, YK and MS performed research, JB and OL designed research, analyzed the data and wrote the paper.

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Supporting Information

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

Fig. S1. Quantification of longevity and correlation between DR5::VENUS and DII::VENUS fluorescence during Arabidopsis seed maturation

Fig. S2. *AFB* transcript levels decrease during seed development in Col-0

Fig. S3. Auxin influences germination speed during seed imbibition

Fig. S4. Genes encoding enzymes of the tryptophan-dependent auxin biosynthesis pathways are differentially expressed during seed maturation

Fig. S5. Survival curves of mature seeds show a longevity phenotype in single mutants of auxin biosynthesis pathways

Fig. S6. Auxin activity is affected in 18-DAF seeds of single auxin biosynthesis mutants

Fig. S7. Auxin activity in roots of IAA biosynthesis mutants

Fig. S8. Transcript levels of IAA biosynthesis genes are not significantly affected in the single mutants of the different IAA pathways

Fig. S9. IAA and ABA content in auxin biosynthesis mutants

Fig. S10. Seed filling and water content are not affected during maturation in auxin biosynthesis mutants compared to Col-0

Fig. S11. Seed coat permeability is affected in mature seeds of *taa1*, *tar1* and *yuc1* mutants

Fig. S12. Soluble sugar content in mature seeds of Col-0 and auxin biosynthesis mutants

Fig. S13. Dormancy and ABA sensitivity analysis in IAA biosynthesis mutants

Fig. S14. Auxin signaling does not activate the HSFA9 program in Arabidopsis

Fig. S15. *DOG1* and *ABI5* are not regulated by auxin signaling activity

Fig. S16. Expression profiles of *ARF* genes during Arabidopsis seed development

Table S1. List of mutants and primers used for mutant screening

Table S2. Primer sequences used for T-DNA insertion screening of *A. thaliana* mutants

Table S3. Primer sequences used for RT-qPCR

Table S4. Summary of phenotypes of IAA biosynthesis mutants

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Figure 1. Auxin activity correlates with the acquisition of longevity during late seed maturation of *Arabidopsis thaliana*. (a) Representative images of Col-0 seeds harvested at 12, 14, 16, 18 and 20 days after flowering (DAF). (b) Evolution of desiccation tolerance (DT), longevity (P50) and DR5::GUS, DR5::VENUS and DII::VENUS activity in *Arabidopsis* seeds during maturation. Desiccation tolerance was determined by rapidly drying seeds under an airflow at 43% RH and counting the seeds that germinated after imbibition. Longevity was determined from the survival curves obtained during storage at 75% RH and 35°C (Supporting Information Fig. S1b) and quantified as P50, the storage time needed for half of the seed population to lose viability. (c, d) Activation of *DR5* (c) and *TIR1* (d) expression in 18-d old seeds by incubation in 1 μM IAA for 4h. (e) Transcript levels of *TIR1* during seed maturation. The inset shows the significant increase in the transcript level at 18 and 20 DAP compared to 16 DAP. (f, g) Viability of mature Col-0 (f) and Ler (g) seeds after 21d of aging at 75% RH and 35°C from 10-12 d old siliques that were dipped in 10 μM IAA or mock solution. (h) Viability of mature seeds after 21d of aging at 75% RH and 35°C that were imbibed with or without 1 μM IAA. Significance (student t-test) is indicated by asterisks. * P<0.05, **P<0.01, ***P<0.001. Data are means ±S.E.M of 150-200 seeds of three independent biological replicates.

Figure 2. Auxin biosynthesis mutants of *A. thaliana* provide a link between auxin signaling activity and longevity. (a-c) Longevity (expressed as P50) of mature Col-0 and mutant lines, determined from survival curves obtained from storage at 75% RH and 35°C (Supporting Information Fig. S5). (a) *cyp79b2-1* and *cyp79b2-2*, (b) *ami1-1* and *ami1-2*, (c) *taa1-1*, *taa1-2*, *tar1*, *tar2*, *yuc1* and *yuc2*. Data are means ±S.E.M of 150-200 seeds of three independent biological replicates. Asterisks indicate a significant difference from Col-0 using ANOVA followed by Multiple Comparisons versus Control (Col-0) Group analysis (Fisher LSD), * P<0.05, **P<0.01, ***P<0.001. (d-f) DR5::GUS activity in mature seeds of Col-0 and (d) *cyp79b2-2*, (e) *ami1-2*, (f) *taa1-1*, *tar1*, *tar2* and *yuc2*. Data are the mean ± S.E.M of four biological replicates of 100 seeds. (g) Relation between of DR5::GUS activity and longevity (P50) of developing Col-0 seeds (black symbols) and auxin biosynthesis mutant lines (colored symbols). The curve follows a third-order sigmoidal fit. (h) Longevity (expressed as P50) of mature Col-0 and double mutant *wei8,tar1*. (d-h) Significance (student t-test) is indicated by asterisks. * P<0.05, **P<0.01, ***P<0.001.

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Figure 3. Dynamic changes in auxin signaling distribution during seed maturation of *Arabidopsis thaliana*. (a-f) DR5::VENUS or DII-VENUS activity in embryos 10-12 days after flowering (DAF), (g-k) DR5::VENUS or DII-VENUS activity in embryos 14-18 DAF. (a, b, d, e, g, i) Seeds with DR5::VENUS construct; (c, f, h, j) Seeds with DII::VENUS construct. (a) embryo (b, c) cortical cells, provascular tissue and primordia of cotyledons, (d,j) provascular tissue and tip of radicle; (e, f, g, h) epidermal and cortical cotyledons, (k) cortical cells of radicle. All embryos were stained with propidium iodide. The scale bars indicate 100µm. The arrow indicates the radicle tip.

Figure 4. Genes of the conserved longevity module are deregulated in auxin biosynthesis mutants. (a) Network of longevity-module genes of *Arabidopsis thaliana* with auxin response elements in their promoters. First neighbours of CYP79b2 are in yellow. Asterisks indicate the two genes screened in detail. (b) Relative expression of *CHX18* in 18 days after flowering (DAF) seeds with or without 4h incubation in 1 µM IAA. (c-e) Relative expression of *CHX18* during seed maturation in (c) *cyp79b2*, (d) *ami1-2* and (e) *taa1-1*, *tar1* and *yuc1*. (f-h) Transcript level of *HSP21* during seed maturation in (f) *cyp79b2*, (g) *ami1-2* and (h) *taa1-1*, *tar1* and *yuc1*. Data represent the average ± S.E.M of three technical replicates from two biological pools of 200 seeds. (i) Relative expression of *WRKY3* in the auxin biosynthesis mutants at 18 DAF. Significance (student t-test) of mutants compared to Col-0 determined for each time point is indicated by asterisks, *P<0.05, **P<0.01, ***P<0.001.

Figure 5. *ABI3* and its downstream target *EM1* are deregulated in auxin biosynthesis mutants of *Arabidopsis thaliana*. (a) Relative expression of *ABI3* in 18 days after flowering (DAF) seeds with or without 4h incubation in 1 µM IAA. (b-d) Relative expression of *ABI3* during seed maturation in (b) *cyp79b2*, (c) *ami1-2* and (d) *taa1-1*, *tar1* and *yuc1*. (e) Transcript level of the *ABI3* target *EM1* in mature *abi3-5* seeds. (f-h) Relative expression of *EM1* during seed maturation in (f) *cyp79b2*, (g) *ami1-2* and (h) *taa1-1*, *tar1* and *yuc1*. Data represent the average ± S.E.M of three technical replicates from two biological pools of 200 seeds. (i-j) Survival curves of mature seeds of Ler (i) and *abi3-1* (j) during aging at 75% RH and 35°C from 10-12d-old siliques that were dipped in 10 µM IAA or mock solution. NS: non-significant. Data are means ±S.E.M of 150-200 seeds of three independent biological replicates. Significance (student t-test) of mutants compared to Col-0 determined for each time point is indicated by asterisks, * P<0.05, **P<0.01, ***P<0.001.

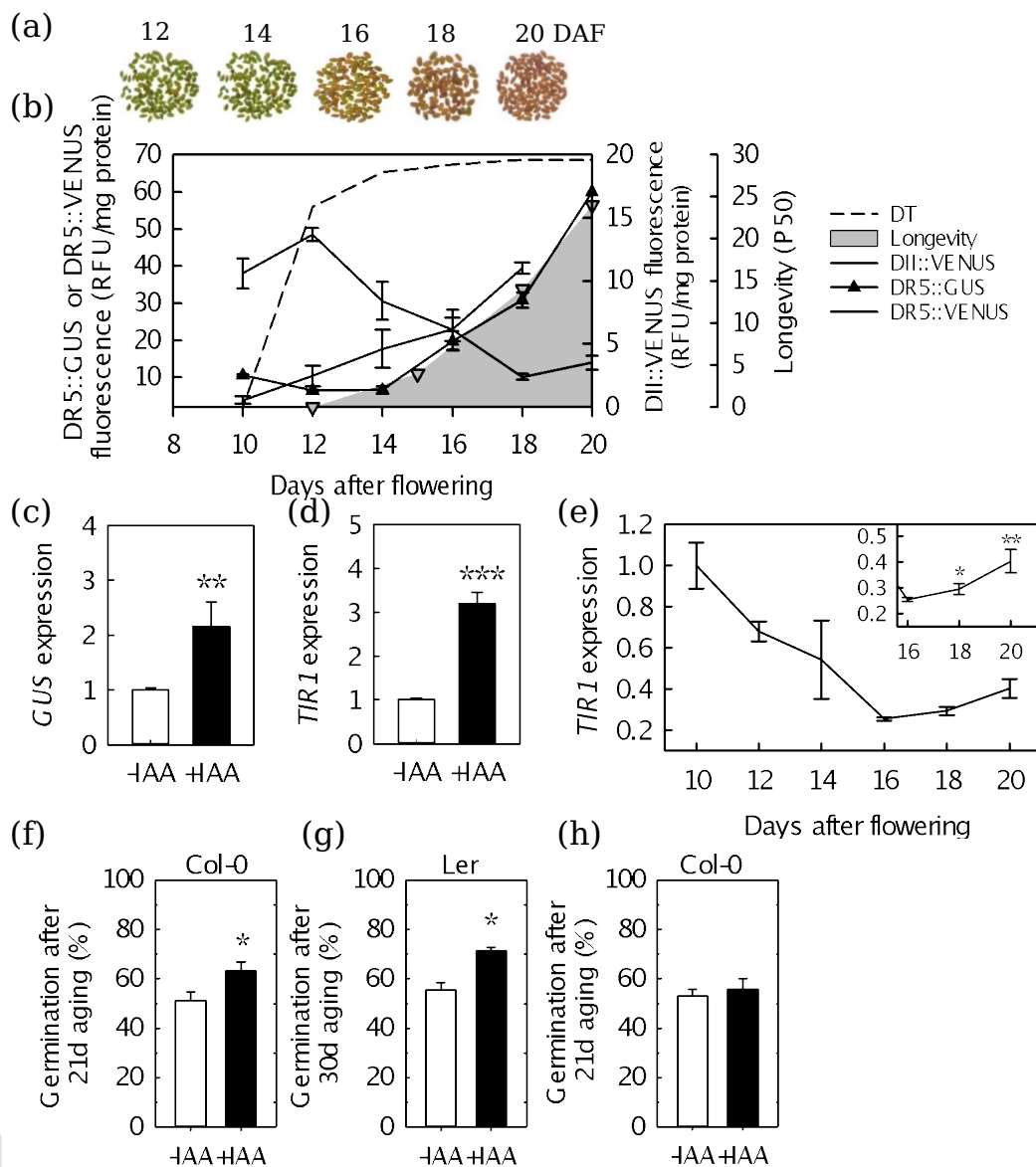
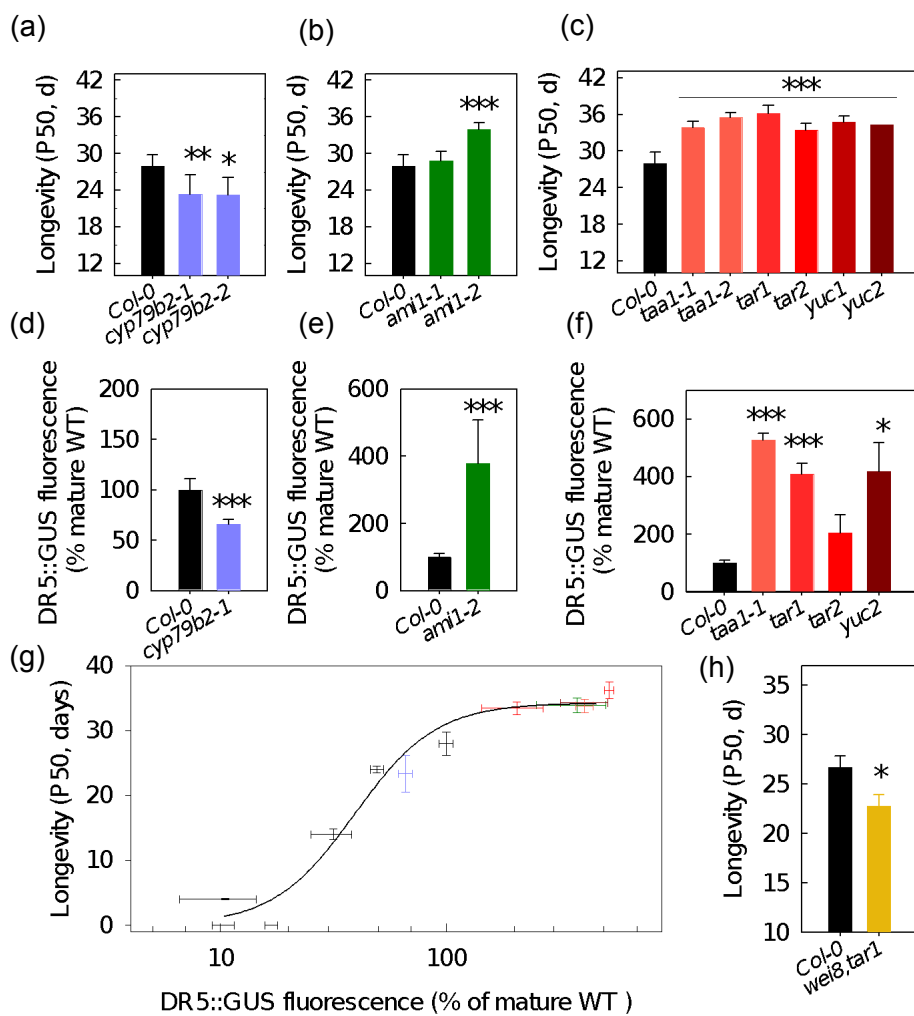
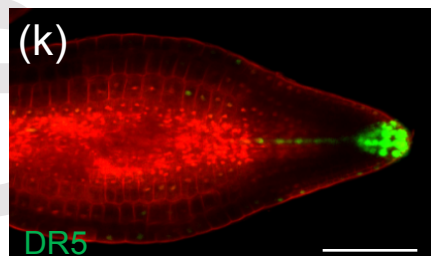
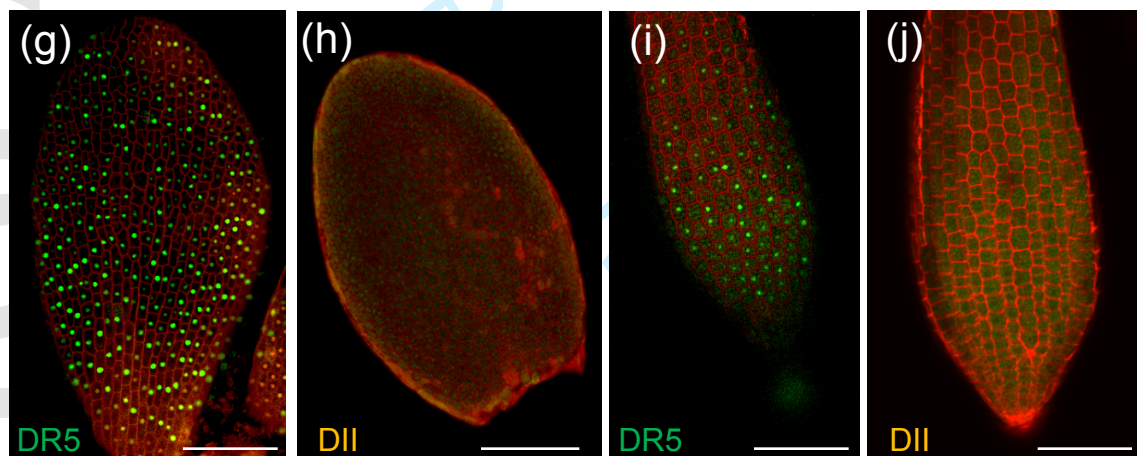
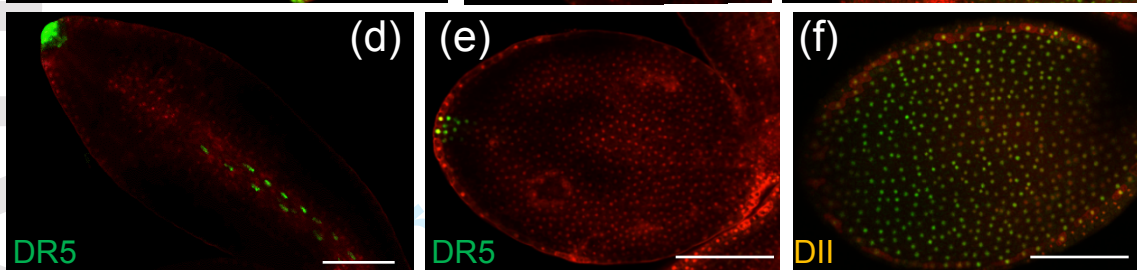
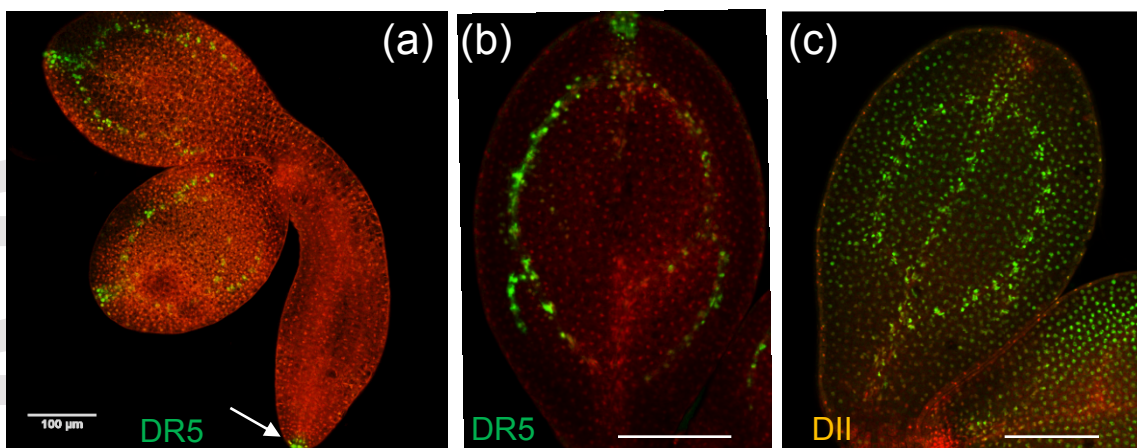


Figure 1

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10-12 DAF



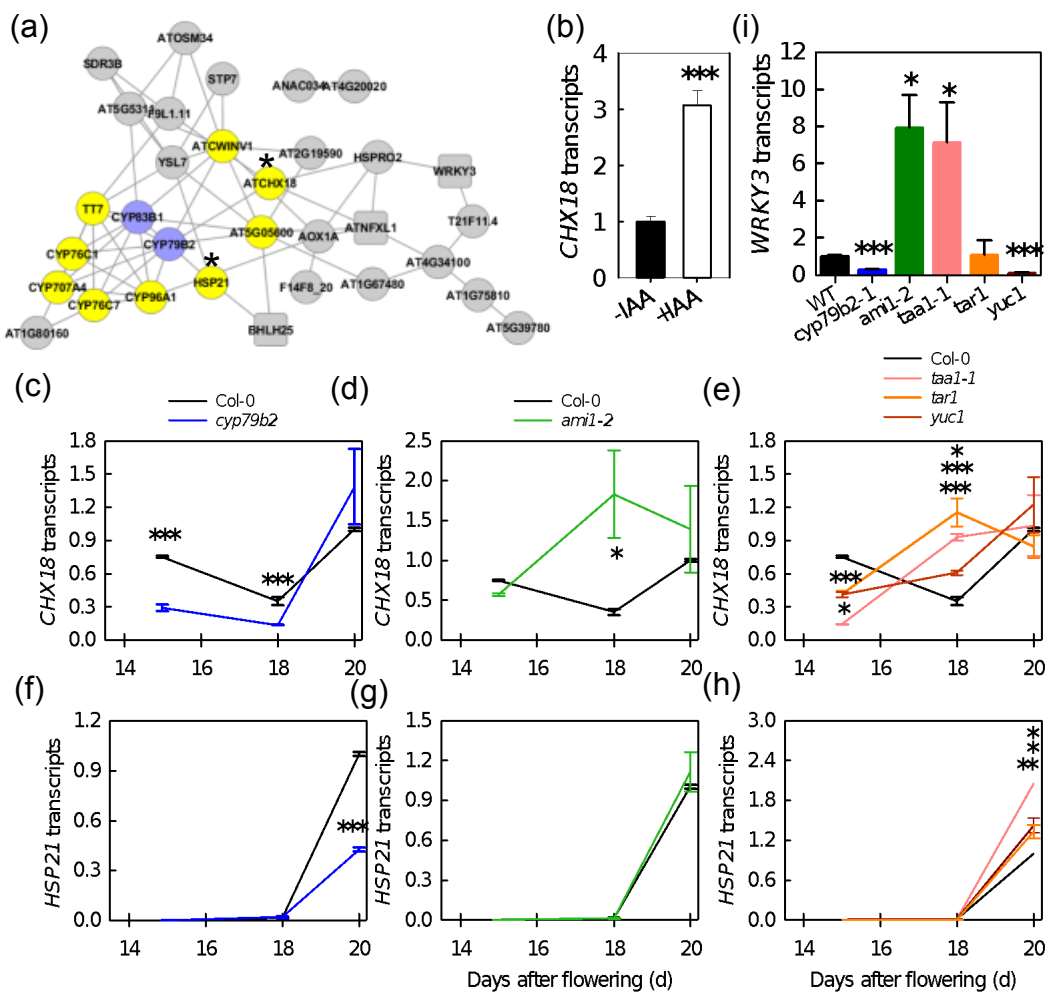
14-18 DAF

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Figure 3

Pellizaro, A., Neveu, M., Lalanne, D., Ly Vu, H., Seo, Leprince, O., Buitink, J. (2019). A role for auxin signaling in the acquisition of longevity during seed maturation. *New Phytologist*, 224, 150-160.

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Figure 4

Pellizaro, A., Neveu, M., Lalanne, D., Ly Vu, B., ... , Leo, Leprince, O., Buitink, J. (2019). A role for auxin signaling in the acquisition of longevity during seed maturation. *New*

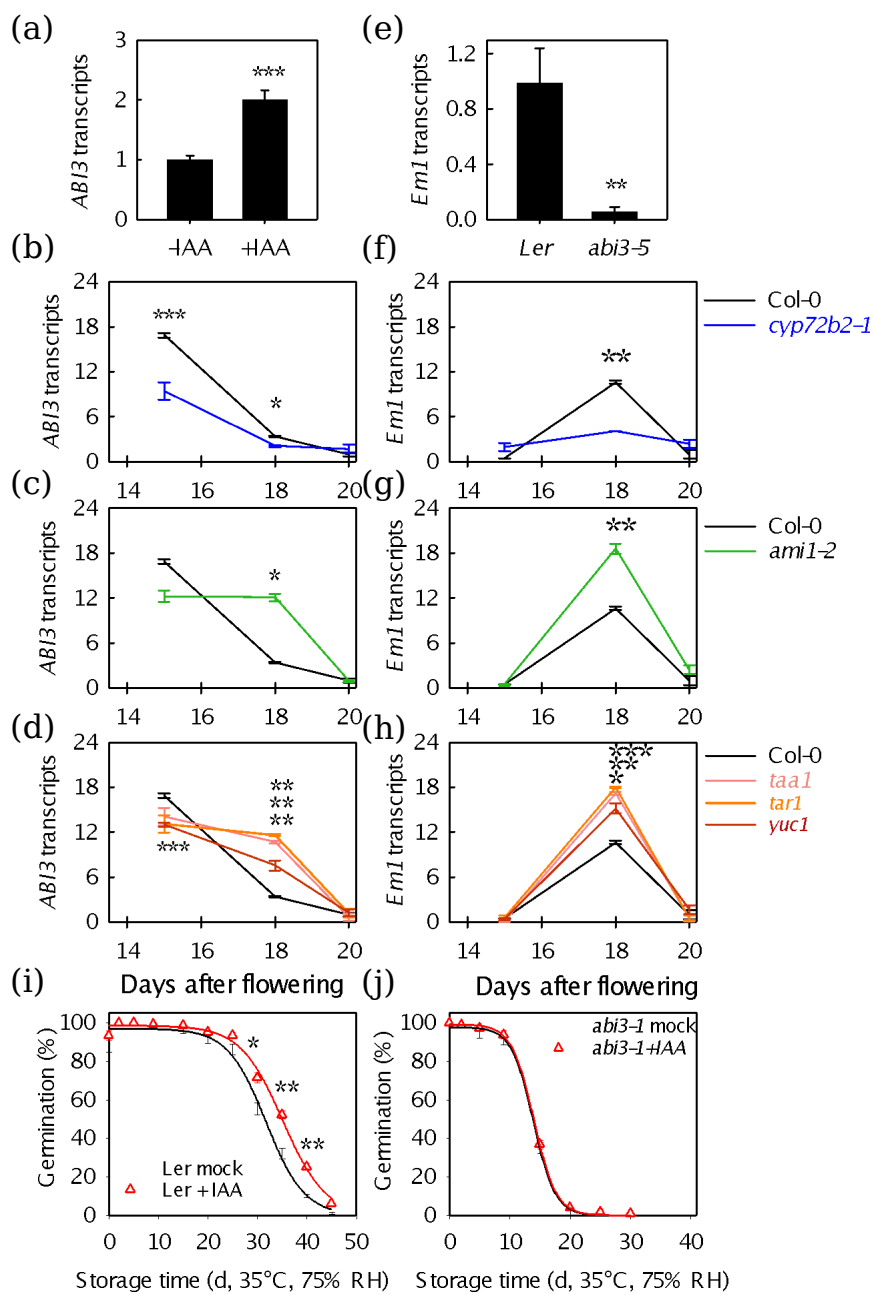


Figure 5