Dual and opposing roles of EIN3 reveal a generation conflict during seed growth

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ABSTRACT

Seed size critically affects grain yield of crops and hence represents a key breeding target. The development of embryo-nourishing endosperm is a key driver of seed expansion. We here report unexpected dual roles of the transcription factor EIN3 in regulating seed size. These EIN3 functions have remained largely undiscovered because they oppose each other. Capitalizing on the analysis of multiple ethylene biosynthesis mutants, we demonstrate that EIN3 represses endosperm and seed development in a pathway regulated by ethylene. We, in addition, provide evidence that EIN3-mediated synergid nucleus disintegration promotes endosperm expansion. Interestingly, synergid nucleus disintegration is not affected in various ethylene biosynthesis mutants, suggesting that this promoting function of EIN3 is independent of ethylene. Whereas the growth-inhibitory ethylene-dependent EIN3 action appears to be encoded by sporophytic tissue, the growth-promoting role of EIN3 is induced by fertilization, revealing a generation conflict that converges toward the key signaling component EIN3.

Key words: seed size, EIN3, ethylene biosynthesis, fertilization, generation conflict, synergid disintegration

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INTRODUCTION

Seeds are a key food source for humans and animals and an important basis for biofuel production, making them an important target of breeding programs. In flowering plants, the main components of the seed, the embryo and the embryo-nourishing endosperm, result from fertilization of an egg and an adjoining central cell. The two sperm necessary for this so-called double fertilization are delivered by a single pollen tube, which finds its way to the female gametes due to a sophisticated guidance system (Johnson et al., 2019; Hater et al., 2020; Hafidh and Honya, 2021). Short-range pollen tube attraction is mediated by two egg-cell-adjoining synergid cells, which secrete cysteine-rich peptides (Higashiyama et al., 2001; Márton et al., 2005; Okuda et al., 2009; Takeuchi and Higashiyama, 2012; Meng et al., 2019; Zhong et al., 2019) and mediate the discharge of two sperm from the pollen tubes arriving in the female gametophyte (Huck et al., 2003; Rotman et al., 2003; Amien et al., 2019). Pollen tube arrival is accompanied by programmed cell death of the first synergid. The disintegration of the second synergid and concomitant termination of pollen tube attraction require gamete fusion. This is evidenced by the work of Beale et al. and Kasahara et al., who have shown that incomplete fertilization or the delivery of gamete-fusion-defective sperm suppresses disintegration of the second synergid, resulting in the attraction of supernumerary pollen tubes (Beale et al., 2012; Kasahara et al., 2012).

Synergid disintegration and the establishment of a pollen tube block is a multiphasic process. It involves (i) fertilization-induced cleavage of LURE1 by the egg-secreted endopeptidases ECS1 and ECS2 (Yu et al., 2021), (ii) dilution of LURE by fusion of the synergid with the central cell in a process that requires central cell fertilization (Maruyama et al., 2015), and (iii) synergid nucleus disintegration (Völz et al., 2013; Maruyama et al., 2015). The last step is regulated by fertilization-independent seed–Polycomb Repressive Complex 2 as well as the transcription factors EIN3 and EIL1, which are components of the ethylene response pathway (Maruyama et al., 2013, 2015; Völz et al., 2013). In plants defective for any of these factors, the nucleus of the
second synergid remains intact. We, in addition, have shown that the synergid-derived nucleus (sdn) in ein3eil1 mutants adopts the fate and division regime of the endosperm, which we also detected in single ein3 mutant (Figures 1A, 1B, and Supplemental Figures 1A–1C). To investigate the developmental implications of the resulting parental heterogeneity on endosperm development, we aimed to analyze early seed development at the four-nucleate stage, when the difference in the parental architecture and the concomitant formal shift in maternal-to-paternal genome ratio between ein3 and wild-type endosperm is particularly pronounced (Figure 1C and 1D). In a first step, we asked whether young wild-type seeds at the four-nucleate endosperm stage are already susceptible to changes in maternal-to-paternal ratio. We therefore performed an interploidy cross of diploid wild-type plants with a tetraploid wild-type pollen donor and determined the dorsoventral endosperm diameter in the four-nucleate stage (Figure 1E).

We detected a significantly increased endosperm diameter in seeds having inherited twice as many paternal copies (2n x 4n) (Figure 1F). While we cannot exclude the possibility that

### RESULTS AND DISCUSSION

**Ploidy differences in the seed can be detected in the zygote stage**

We have previously shown that the synergid-derived nucleus (sdn) in ein3eil1 mutants adopts the fate and division regime of the endosperm to the asexual, maternal nuclei (Völz et al., 2013) (Supplemental Figure 1A–1C). To investigate the developmental implications of the resulting parental heterogeneity on endosperm development, we aimed to analyze early seed development at the four-nucleate stage, when the difference in the parental architecture and the concomitant formal shift in maternal-to-paternal genome ratio between ein3 and wild-type endosperm is particularly pronounced (Figure 1C and 1D). In a first step, we asked whether young wild-type seeds at the four-nucleate endosperm stage are already susceptible to changes in maternal-to-paternal ratio. We therefore performed an interploidy cross of diploid wild-type plants with a tetraploid wild-type pollen donor and determined the dorsoventral endosperm diameter in the four-nucleate stage (Figure 1E).

We detected a significantly increased endosperm diameter in seeds having inherited twice as many paternal copies (2n x 4n) (Figure 1F). While we cannot exclude the possibility that
increased pollen tube content of 4n plants contributed to this effect (Kasahara et al., 2016; Zhong et al., 2017), these data suggest that even young seeds in the zygotic stage are responsive to ploidy changes.

Manipulation of EIN3 signaling suggests an inhibitory role of synergid-derived nuclei in endosperm expansion

In contrast to interploidy crosses, which shift the ploidy of both endosperm and embryo, the overall maternal-to-paternal ratio in ein3 mutants is affected only in the endosperm. In addition, ein3 mutants exhibit a parental internuclear heterogeneity that contrasts with the homogeneous parental ratio characteristic of endosperm nuclei resulting from wild-type or interploidy crosses. To understand whether this idiosyncratic endosperm composition affects endosperm development, we measured dorsoventral endosperm diameter of ein3 seeds in the four-nucleate stage. We capitalized on our previous finding that the defect of synergid nucleus inheritance is not fully penetrant in ein3 mutants (Figures 1C and 2A) (Völz et al., 2013), i.e., we were able to compare seeds from the same flowers that either had or had not inherited ein3. We found that endosperm expansion was significantly reduced in ein3 seeds containing both biparental endosperm and sdn compared with ein3 seeds with biparental endosperm only (Figure 2B). While this result suggests that sdn negatively affect endosperm expansion, we could not rule out the possibility that development in sdn-segregating ovules was retarded. In fact, interploidy crosses performed by Scott et al. showed that increased maternal copies correlated with delayed mitotic progression and premature celluarization (Scott et al., 1998). To test this hypothesis, we assessed endosperm size dynamics on the basis of live-cell imaging. We introduced a combinatorial multicolor marker, FGR 7.0, into ein3 plants. FGR 7.0 confers fluorescence to synergids, zygotes, and endosperm (Völz et al., 2013). In addition, we established a protocol for visualization of nuclear dynamics in early endosperm. This allowed us to trace fertilized ovules over a period of 24 h, during which the endosperm underwent up to three mitotic divisions (Figure 1A and 1B; Supplemental Videos 1 and 2). To determine whether early seed development is slowed down in sdn-containing seeds, we used the decondensation of nuclei as a molecular timer. The durations of nuclear disintegration between the two-nucleate endosperm stage and the four-nucleate endosperm stage were comparable, independent of the segregation of sdn (Figure 2C). Together, these results indicate that the size differences are not an artifact introduced by retarded development, but that instead the segregation of sdn correlates with reduced early endosperm expansion.

To test whether this effect was indeed causally linked to the ein3 locus, we generated a CRISPR-induced EIN3 allele (ein3-c1). This allele contains a frameshift insertion at the position of the 495th base, resulting in a premature stop codon after 165 amino acids (Supplemental Figure 1D and 1E). The ein3-c1 allele exhibits a stronger phenotype than the ein3 allele with respect to the...
frequency of sdn-segregating seeds (Supplemental Figure 1F), whereas the overall effect of sdn on endosperm size was comparable (Figure 2D).

The correlation between sdn inheritance and endosperm size is compatible with two conceptually different scenarios: either endosperm expansion and the degeneration of the synergid nucleus are different effects of EIN3-dependent processes operating in the female gametophyte, or the persistence of the maternal haploid synergid nucleus is causally linked with reduced endosperm expansion. To discriminate between the two scenarios, we confined the EIN3-dependent defect to synergids only making use of the SRDX repressor motif (Hiratsu et al., 2003). We have previously shown that expression of the dominant negative EIN3_SRDX fusion under the control of the synergid-specific pDD2 promoter phenocopies the synergid nuclear disintegration defect of ein3 mutants (Volz et al., 2013). When analyzing the endosperm diameter of different pDD2::EIN3_SRDX transgenic lines, we observed substantial phenotypic variations. However, as a common denominator, we found that endosperm size is reduced in the presence of sdn compared with seeds without sdn (Figure 2E). As a control, we expressed the construct after fertilization only in the endosperm using the AtrBohD promoter (Volz et al., 2013). This approach did not affect synergid disintegration (n = 136/146/203 for wild type/L1/L2), nor did we observe reduced seed size (Supplemental Figure 1G), suggesting that the endosperm does not contribute to the effect in sdn-containing pDD2::EIN3_SRDX.

Together, our results indicate that internuclear heterogeneity caused by sdn and reduced endosperm size are causally linked. This also implies that fertilization-dependent synergid nuclei disintegration mediated by EIN3 promotes endosperm expansion. This finding is also in line with and in support of the parental conflict theory, which holds that both parents have different interests in the allocation of resources to a single seed of the same mother plant (Haig and Westoby, 1989).

**Sporophytic EIN3-dependent signaling represses endosperm and seed expansion**

While young sdn-segregating ein3 seeds have a reduced endosperm diameter, previous results have reported an increased mature seed size of ein3 mutants resulting from inhibitory effects of EIN3 on embryo development (Meng et al., 2018). We similarly observed an effect on seed size in both ein3 and ein3-c1 alleles (Supplemental Figure 2A–2C). Notably, this effect was evident only when we introduced the mutation through the female (Supplemental Figure 2B). We next asked whether and to what extent a growth-inhibiting effect of EIN3 is observable at an early developmental stage. In fact, we detected an EIN3-dependent growth-inhibiting effect also in early developmental stages, where it is masked by the opposing sdn-dependent effect (Figure 3A). Combining the all, sdn-segregating, and no sdn-segregating ein3 categories yields an endosperm diameter comparable to that of wild type, while the endosperm diameter in ein3 seeds without sdn is significantly bigger compared with wild type (Figure 3A). Since there is no significant developmental shift between wild type and ein3 (Figure 3B), we can exclude that size deviations are caused by different developmental stages, which was further substantiated by live-cell imaging and by analyzing the ein3-c1 allele (Supplemental Figure 2D–2F).
Figure 4. Ethylene reduction affects the growth-inhibitory but not the growth-promoting function of EIN3.

(A) Dry seed size of different ethylene biosynthetic mutants and wild type (WT) after selfing (n = 658/5322/4751/4736/5390/2911/3453/4966/5597 for WT/mat3-1/sam1sam2-1/sam1sam3-1/sam2sam3-1/acs6-1/-/acs octuple/-/aco2/-/aco2aco3/-). The area of each seed collected from 15 individual plants sown at three different times was measured in ImageJ. A boxplot was generated based on individual seed area calculated in pixels. The blue line shows the WT median.

(B) Dry seeds of WT and acs octuple/- plants. Scale bars, 1 mm.

(C) Dorsoventral endosperm diameter of WT × WT and acs octuple/- × WT seeds in the four-nucleate endosperm stage. Seeds showing abnormal development at the micropylar end (see also Supplemental Figure 4B and 4C) are excluded (n = 53/79 for WT/acs octuple/-).

(legend continued on next page)
We next asked whether the repressive effect of EIN3 originated from the gametophyte or the sporophyte. To discriminate between sporophytic and gametophytic function we compared seeds from wild-type, ein3 homozygous, or ein3 heterozygous plants pollinated with wild type. While ein3 heterozygous seeds were smaller than ein3 homozygous seeds, they were still significantly bigger than wild-type seeds (Figure 3C). This result is compatible with two different scenarios: either EIN3 has a dose-dependent sporophytic effect, which would affect the size of all seeds, or the intermediate seed size results from a mixed filial generation containing smaller wild type segregating and bigger ein3/+ segregating seeds. To distinguish between the two scenarios, we followed individual seeds to the seedling stage, where we genotyped them. Our results revealed that all seeds from ein3 heterozygous plants are significantly bigger than wild type but similar in size independent of the genotype of their embryo (Figure 3D). This result indicates that the EIN3 growth-inhibiting effect is attributable to the sporophytic tissue.

Together, these data indicate that EIN3 has opposing and spatially distinct roles in seed expansion: the EIN3 growth-inhibiting function is regulated by the sporophytic tissue, and this effect is counteracted in early endosperm stages by a growth-promoting EIN3 function, which is mediated by disintegration of the non-receptive synergid nucleus after fertilization. In mature seeds, the effect of sdn appears to become dominated by the growth-inhibiting effect of EIN3, potentially due to the fact that the endosperm is degraded during seed development.

Ethylene reduction affects the growth-inhibitory but not the growth-promoting function of EIN3

EIN3 is stabilized in the presence of ethylene (Guo and Ecker, 2003; Potuschak et al., 2003). In addition to its fundamental role in many developmental processes and stress responses, the plant hormone has a regulatory role in cell division, cell expansion, and growth (Abeles et al., 1992; Dubois et al., 2018). In light of the opposing effects of EIN3, we next asked whether the growth-promoting and the inhibitory roles of EIN3 equally respond to ethylene.

Ethylene biosynthesis is initiated by the production of S-adenosyl-methionine from methionine by S-adenosylmethionine synthase (SAM), which is followed by two additional steps: first, S-adenosyl-L-methionine is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by aminocyclopropane-1-carboxylic acid synthase (ACS), and second, ACC is converted to ethylene by aminocyclopropane-1-carboxylic acid oxidase (ACO) (Adams and Yang, 1979; Pattyn et al., 2020).

To trace ethylene biosynthesis in time and space, we generated transcriptional and translational reporter lines and analyzed 15 members of the SAM, ACS, and ACO enzyme families involved in ethylene biosynthesis. Twenty-four hours after fertilization, when the endosperm effect of ein3 was already evident, we detected SAM1 and SAM2 in the sporophyte, endosperm, and zygote; SAM3 in the endosperm and zygote; pACS2 in the endosperm only; pACS6 and pACO2 in the sporophyte only; and pACO5 in the endosperm of young seeds (Supplemental Figure 3A–3C). This result is also supported by a recent study revealing that ethylene production as well as mRNA abundance of some ACS genes gradually increases during seed development (Sun et al., 2020).

We next capitalized on ethylene biosynthesis mutants to address the functional relevance of ethylene production during seed development. It was previously reported that the level of ethylene production in Arabidopsis is directly regulated by ACSs (Tsulisaka et al., 2009) and SAMS (Mao et al., 2015). When analyzing dry seed size of various mutants and mutant combinations targeting SAM, ACS, and/or ACO genes, we detected an increase in seed size compared with wild type (Figure 4A). The effect was particularly pronounced in acs octuple seeds in which ethylene production has previously been shown to be strongly reduced (Tsulisaka et al., 2009) (Figure 4A and 4B). Except for acs octuple mutants, which show 49% non-developing/sterile ovules in mature siliques (n = 910), all mutants show fertile siliques, indicating that the larger seed size is not due to additional resources freed up by the formation of fewer seeds (Supplemental Figure 4A). On closer inspection of acs octuple mutants, we found that a substantial fraction exhibited integument abnormalities and early defects in female gametophyte development (Supplemental Figure 4D), which potentially contribute to a previously described defect of acs octuple mutants in pollen tube attraction (Mou et al., 2020). In addition, we observed an increased dorsoventral endosperm diameter in the four-nucleate stage, indicating that seed size deviation initiates early in acs seed development (Figure 4C).

We next asked whether synergid degeneration was also susceptible to a reduction of ethylene content. To characterize synergid disintegration in young acs octuple seeds, we analyzed cleared whole mounts 1 day after pollination. Interestingly, only 6% (n = 105) of the analyzed acs octuple seeds showed sdn in the four-nucleate endosperm stage, which is similar to the 4% observed in wild type (n = 53). Similar results were obtained when analyzing various combinations of acs and aco mutant lines, suggesting that synergid disintegration requires no or only a small amount of ethylene (Figure 4D). These results are consistent with recent work by Li et al., which shows that synergid disintegration is not affected in plants fully depleted of ACO function (Li et al., 2021). Our data suggest that synergid degeneration and the concomitant EIN3 growth-promoting function are not or are less responsive to ethylene, indicating that there might be a factor X activating EIN3 signaling after fertilization. Candidates include jasmonic acid (Zhu et al., 2011), salicylic acid (He et al., 2017), or salt (Peng et al., 2014), which have previously been implicated in the regulation of EIN3.
Dual and opposing roles of EIN3

In conclusion, our findings unravel an unexpected dual role of EIN3 during early seed development, a function that is masked in wild-type plants because the effects oppose each other. Intriguingly, the effects are exerted by different tissues representing different generations: while sporophytic tissue represses endosperm and seed expansion in an EIN3-dependent manner, fertilization-triggered synergid disintegration promotes endosperm expansion, thereby ensuring the biparental origin of all endosperm nuclei. Our results, in addition, suggest that the dual and conflicting processes exerted by the two genera- tions differ with respect to ethylene responsiveness: while the latter process is either fully ethylene independent or sensitive to small traces of ethylene (Figure 4E). Given that ethylene integrates various external and internal stresses, it will be an attractive challenge for the future to determine whether and to what extent endosperm development is amenable to adaptation and how the different functions of EIN3 are regulated.

METHODS

Plant materials and growth conditions

Seeds of Arabidopsis thaliana were sown and stratified at 4°C for 2 days. Stratified seeds were transferred to a Conviron MTPS growth chamber for germination and further growth under long-day conditions (16 h light/8 h dark) at 23°C. Plants were later transferred to 18°C after bolting.

The following plant lines are in the Ler background: ein3-1 (referred to as ein3 in this study), ein3-1eil1-2 (referred to as ein3eil1 in this study), ein3eil1 with pMEA:NLS_tdTomato or pRPSSa::NLS_GFP; pDD2::EIN3_SRDX (Völz et al., 2013). The ein3-1eil1-2 double mutant was kindly provided by Richard D. Vierstra. ein3-1 was crossed out with Ler wild type. For live-cell imaging, FGR 7.0 lines in the Ler and ein3-1 background were used (Völz et al., 2013). For interplorphy assay, 2n Ler and 4n Ler kindly provided by Prof. Dr. Tobias Würschum were used.

Ethylene biosynthetic mutants were obtained from the European Arabidopsis Stock Center (NASC) (Nottingham, UK): sam1 (N573599), sam2 (N676306), sam3 (N552289), mat3 (N519375), acs6-1 (N16569), acs heptape (N16650), acs octape (N16651), aco1 (N682904), aco2 (N527311), aco3 (N582132), aco4 (N514965), and aco5-2 (N411335). sam1sam2, sam1sam3, sam2sam3, and aco2aco3, aco quadruple (aco1/−; aco2/−; aco3/−; aco4/−), and aco pentuple (aco1/−; aco2/−; aco3/−; aco4/−; aco5-2/) were generated by crossings of the respective single mutants. Col-0 was used as a control.

Generation of ein3-CRISPR line (ein3-c1)

All constructs as well as cloning procedures were described previously by Fauser et al. (2014). The protospacer used as a recognition site for the Cas9 nuclease was localized in the exon at position 478–498 of the coding sequence (Supplemental Table 1) and was followed by an AGG protospacer-adjacent motif. The thereby induced mutation has a 1 nt insertion between positions 494 and 495 of the coding sequence and was named ein3-c1 (Supplemental Figure 1D).

This insertion disrupts an NlaII restriction site important for genotyping and induces a frameshift resulting in a premature stop codon after 165 of 628 amino acids. The ein3-c1 mutant was outcrossed several times to remove the CAS9 gene and to reduce off-target mutations. The homozygous mutant was then used for further analyses. Ler and ein3 were used as controls.

Molecular cloning

To generate pSAMX::gSAMX_tdTomato_tNOS plasmids, the promoter and genomic loci of SAM1 (AT1G20500), SAM2 (AT4G01850), and SAM3 (AT3G17390) were amplified from the Arabidopsis Col-0 DNA library by the respective primers listed in Supplemental Table 1. The promoters digested with Ascl and PacI and the genomic fragments digested with PacI and AvrII were subcloned into DR13 plasmid (pAt5g40260::NLS_tdTomato_tNOS) (Völz et al., 2013) followed by exchanging pAt5g40260 with pSAMX and NLS with gSAMX. In addition, to generate pACSX::NLS_GUS_tNOS plasmids, the promoters of ACS2 (AT1G01480), ACS4 (AT2G22810), ACS5 (AT5G65800), ACS6 (AT4G11280), ACS7 (AT4G26200), ACS8 (AT4G37770), ACS9 (AT3G49700), and ACS11 (AT4G08040) were amplified from the Arabidopsis Ler DNA library by the respective primers listed in Supplemental Table 1. The promoters pACS2, pACS6, and pACS11 were digested with Ascl and PacI, whereas the promoters pACS4, pACS5, pACS7, and pACS9 were digested with Ascl and Pvul, and the promoter pACS8 was digested with Ascl and Xhol. Afterward, they were subcloned into pLIS::NLS_GUS_tNOS (Groß-Hardt et al., 2007), followed by exchanging pLIS with pACXS. Last, to generate pACOX::NLS_tdTomato_tNOS plasmids, the promoters of ACO1 (AT2G19590), ACO2 (AT1G62380), ACO3 (AT1G12010), and ACO5 (AT1G77330) were amplified from the Arabidopsis Ler DNA library by the respective primers listed in Supplemental Table 1. The promoters digested with Ascl and PacI were subcloned into DR13 plasmid followed by exchanging pAt5g40260 with pACOX. All plasmids were then transformed into Col-0 plants by floral dip as previously described (Zhang et al., 2006).

PCR-based genotyping

Genotyping primers are listed in Supplemental Table 2.

Histology and microscopy

For the analysis of early seed development, the oldest closed flower bud of a given inflorescence was emasculated. One day after emasculation, the flowers were pollinated with wild-type pollen and harvested 24 h later.

For whole-mount clearings flowers were vacuum infiltrated in an ethanol:acetic acid solution (9:1) for 30 min, kept at 4°C overnight, washed for 1 h each with 80% and 70% ethanol, and mounted in chloral hydrate:glycerol:water solution (8:2:1; v:v:v). Cytochemical staining of GUS activity was performed on samples as described previously (Vielle-Calzada et al., 2000). GUS-stained samples as well as cleared whole mounts were then visualized under a Zeiss Axioscope (Zeiss, Oberkochen, Germany) and images were captured by a Canon PowerShot G10 camera. Fluorescence signals were detected by a Leica DMi6000B microscope (Leica Microsystems, Wetzlar, Germany).
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Live-cell imaging

Flowers were used 20 h after pollination to perform live-cell imaging. Pistils were harvested and the two septa were separated by an apical–basal incision alongside the transmitting tract by using an insulin syringe (BD MicroFine). The septum halves with the attached ovules were transferred to an ovule medium modified after Palanivelu et al. (2003): 1 mM MgSO₄, 4 mM CaCl₂, 0.01% H₂BO₃, 3% PEG 4000, 14.5% sucrose (pH 5.9 adjusted with KOH), and 1.5% NuSieve GTG agarose (Lonza Bioscience). Subsequently the ovules were covered with 200 μl halocarbon oil 700 (Sigma-Aldrich). Live-cell imaging was performed using a Leica DMi6000B microscope (Leica Microsystems, Wetzlar, Germany) equipped with LAS AF version 2.2.1. Ovules in the four-nucleate endosperm stages.

Seed size measurement

Mature seeds were harvested and dried. Dry seeds were scanned by a CanoScan 9000F Mark II in black/white mode with transmitting light and 1200 dpi resolution. Seed area measurement was performed as described previously by using ImageJ (Herridge et al., 2011).

Data analysis

Datasets were analyzed using Microsoft Excel 2007. Bar charts were created in Microsoft Excel and modified in Adobe Illustrator. Boxplot graphs were generated with BoxPlotR (Spitzer et al., 2014) and modified in Adobe Illustrator. Center lines show the medians; box limits indicate the 25th and 75th percentiles, and outliers are represented by dots. Crosses indicate the mean. Whiskers extend 1.5 times the interquartile range (IQR) from the 25th and 75th percentiles, and medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the IQR. The notches are defined as ±1.58 * IQR/sqrt(n) and represent the 95% confidence interval for each median. Statistical analyses were performed in the Analysis ToolPak of Microsoft Excel 2007.

SUPPLEMENTAL INFORMATION

Supplemental information is available at Molecular Plant Online.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.H., I.E.S., R.V., and R.G.; methodology, J.H., I.E.S., and R.G.; investigation, J.H., I.E.S., T.H., D.V., Y.M., S.G., and T.N; visualization, J.H., I.E.S., Y.M., and R.G.; writing – original draft, J.H., I.E.S., and R.G.; writing – review & editing, J.H., I.E.S., and R.G.; resources and funding acquisition, R.G.

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Supplemental information

Dual and opposing roles of EIN3 reveal a generation conflict during seed growth

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SUPPLEMENTAL INFORMATION

Dual and opposing roles of EIN3 reveal a generation conflict during seed growth

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Supplemental Figure 1-4

Supplemental Table 1 and 2

Supplemental movie 1 and 2 legends
**SUPPLEMENTAL FIGURES**

(A-C) Incorporation of paternally introduced GFP into sdn. *ein3eil1* seeds carrying both a maternally introduced endosperm marker *pMEA::NLS_tdTomato* expressed in endosperm and sdn, and a paternally introduced ubiquitous marker *pRPS5A::NLS_GFP* expressed in zygote, endosperm and sdn (A), *ein3eil1* seeds carrying only the maternally introduced *pMEA::NLS_tdTomato* marker (B) and wild type (WT) seeds carrying only the paternally introduced *pRPS5A::NLS_GFP* (C). Endosperm nuclei, yellow arrowhead; sdn, white arrowhead; zygote, asterisk. Paternal GFP signal was observed in 90% (n=48) of all tdTomato positive sdn (negative control (B), 6% (n=51)). (D) Frameshift CRISPR/Cas9 generated *EIN3* allele *ein3-c1*. * indicates premature stop codon. (E) PCR and NlaII based detection of the *ein3-c1* allele. WT indicates wild type. (F) Frequency of one, two or four sdn in wild type and *ein3-c1* x WT seeds in the four- (4nES) and eight-nucleate endosperm stage (8nES) (n for 4nES: 261/335 and n for 8nES=292/397 for WT and *ein3-c1/+* respectively). (G) Dorso-ventral endosperm diameter of wild type and of *pAtRbohD::EIN3_SRDX* x wild type young seeds at the four-nucleate endosperm stage. L1 and L2 indicate independent *pAtRbohD::EIN3_SRDX* lines (n=289/146/203 for WT/L1/L2 respectively). Two-tailed Student’s t-test did not detect significant difference between WT and L1 for endosperm diameter. Scale bar, 20 µm. Data indicate mean ± SEM. Two-tailed student’s t-test: *** p < 0.001 and **** p < 0.0001.

Figure S1: Manipulation of EIN3 signaling suggests an inhibitory role of synergid-derived nuclei in endosperm expansion
Figure S2: Sporophytic EIN3-dependent signaling represses endosperm and seed expansion.

(A) Size of wild type (WT) x WT and ein3/- x WT mature seeds (n=1392/297 for WT/ein3/+). (B) Size of WT x WT and WT x ein3/- mature seeds (n=198/192 for WT/ein3/+). (C) Size of WT x WT and ein3-c1/- x WT mature seeds (n=454/481 for WT/ ein3-c1+/+). (D) Endosperm diameter of WT x WT and ein3/- x WT seeds with no sdn extracted from live-cell imaging videos. We used nuclear disorganization as initiation points for mitotic divisions and defined the last picture frame, in which the GFP signal in the two- and four-nucleate endosperm stage was still nuclear localized as t1 and t3, respectively, while the first picture frame in the four-nucleated endosperm stage with condensed GFP signal is defined as t2. (t1; WT, n=64; ein3/+, n=49, t2; WT, n=66; ein3/+ n=51 and t3, WT, n=65; ein3/+ n=51). (E) Frequency of WT x WT and ein3-c1/- x WT seeds in different developmental stages 25 hours after pollination: two-, four- and eight-nucleate endosperm stage (2nES, 4nES and 8nES, respectively). (n=691/838 for WT/ ein3-c1+/+). (F) Dorso-ventral endosperm diameter of WT x WT and ein3-c1/- x WT seeds in the four-nucleate endosperm stage. (n=253/45 for WT/ ein3-c1+/+ with no sdn). Two-tailed student’s t-test: * p < 0.05; *** p < 0.001.
Figure S3: Expression pattern of ethylene biosynthesis genes in young seeds of wild type plants.

(A-C) Expression of pSAMx::gSAMX_tdTomato in young seeds of Col-0 plants (A), pACOX::N-LS_tdTomato (B) and pACSX::NLS_GUS (C) in young seeds of Ler plants two day after emasculation and one day after pollination. White arrow, persistent synergid nucleus; yellow arrow, degenerating synergid nucleus; asterisk, zygote; arrow head, endosperm nucleus; empty triangle, antipodal nuclei. Scale bar, 20μm.
Figure S4: Female gametophyte development and integument formation are impaired in acs octuple mutants.

(A) Fertility of wild type (WT) and different ethylene synthetic mutants after selfing. (B-C) Cleared whole mounts of wild type (B) and acs octuple embryo sacs (C) at the four-nucleated endosperm stage. 23% of the acs octuple seeds at the four-nucleate endosperm stage showed abnormal embryo sac at the micropylar end (n=103). (D) Cleared whole mounts of acs octuple ovules in 2DAE carpels indicate severe developmental abnormalities. Categories are as following. (I): Early arrest. This category indicates the ovules in a developmental stage between around 2-IV to 3-II (Schneitz et al., 1995). The figure represents 2-IV staged ovule. (II): Ovules containing swollen integuments and abnormally developed female gametophyte (III): Ovules containing undeveloped integuments and expelled female gametophyte (IV): Ovules containing small female gametophyte. (V): Wild type like ovules. This category indicates fully developed ovules with mature female gametophyte. The figure represents WT ovule. Synergid nucleus, arrow; egg cell nucleus, asterisk; central cell nucleus, triangle; nucellus, n; white line, estimated female gametophyte border; black line, estimated outer integument border; yellow line, inner integument border. Scale bars, 20μm. Error bars, mean ± SEM. Statistical significance was calculated by two-tailed Student’s t-test (** p < 0.01, *** p < 0.001, **** p < 0.0001).

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**Supplemental Table 1**: List of primers used for molecular cloning in this study

| Primer name | Primer Sequence (5’-3’) | Details |
|-------------|--------------------------|---------|
| IE216s      | AGGCCGCGCCTGGGCCTATTGGGCTATGT (Ascl) | Amplification of promoter SAM1 |
| IE216as     | CTTAATTAATCTGCTACAAGAAGAAACAAA (PacI) | |
| IE199s      | CTTAATTAAATGGAGACTTTTCTATCTGAGTCT (PacI) | Amplification of SAM1 |
| IE218as     | ATTTCTAGGAGCTTGGAGTTTGCACCATG (PacI) | |
| IE217s      | AGGCCGCGCCTGGGCCTATTGGCCTATGT (Ascl) | Amplification of promoter SAM2 |
| IE217as     | CTTAATTAATCTGCTACAAGAAGAAACAAA (PacI) | |
| IE203s      | CTTAATTAAATGGAGACTTTTCTATCTGAGTCT (PacI) | Amplification of SAM2 |
| IE219as     | ATTTCTAGGAGCTTGGAGTTTGCACCATG (PacI) | |
| IE209s      | AGGCCGCGCCTGGGCCTATTGGCCTATGT (Ascl) | Amplification of promoter SAM3 |
| IE209as     | CTTAATTAATCTGCTACAAGAAGAAACAAA (PacI) | |
| IE211s      | CTTAATTAAATGGAGACTTTTCTATCTGAGTCT (PacI) | Amplification of SAM3 |
| IE220as     | ATTTCTAGGAGCTTGGAGTTTGCACCATG (PacI) | |
| SG12s       | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS2 |
| SG12as      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| RV283s      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS4 |
| RV283as     | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| RV284s      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS5 |
| RV284as     | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| SG37s       | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS6 |
| RV274as     | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| SG38s       | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS7 |
| SG41as      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| SG15s       | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS8 |
| SG15as      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| SG17s       | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS9 |
| SG17as      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| JH119s      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACS11 |
| JH119as     | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| JH120s      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACO1 |
| JH120as     | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
| JH119s      | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | Amplification of promoter ACO2 |
| JH119as     | ATGGGCCGCGCTGGGCCTATTGGCCTATGT (Ascl-Xhol) | |
|    | Sequence                                                                 | Restriction Enzyme | Function                  |
|----|--------------------------------------------------------------------------|--------------------|---------------------------|
| JH123s | AT\text{GGCGCGCC}GTGAATATATATCTCTGACCCCAAGGG (Ascl)                        |                    | Amplification of promoter ACO3 |
| JH123as | GCTTAATTAACCTCTCTCTCTCTCTCTTAACTAGC (PacI)                                |                    |                           |
| JH124s | AT\text{GGCGCGCC}GATCATTTCCTTATGGGGTTCTG (Ascl)                           |                    | Amplification of promoter ACO5 |
| JH124as | GCTTAATTAATTCAGATCCGCAAAGAGAGAG (PacI)                                    |                    |                           |
| YM13-F  | AT\text{TGGAATATCCCGGGGATTCATGA (BbsI)}                                  |                    | sgRNA of ein3-c1          |
| YM14-R  | AAACCTATCGATCCGCGGTACGATTT (BbsI)                                        |                    |                           |
Supplemental Table 2: List of primers used for PCR based genotyping in this study.

| Primer name | Primer Sequence (5'-3’) | Details |
|-------------|-----------------------|---------|
| IE53s       | GAACTAGTGTTAATGAGATGGGAATGTG (SpeI) TTAGCAATATCAGGAAACATATGC | ein3" line confirmation (PCR product was digested by NlaIII) |
| RV130as     |                       |         |
| IE222s      | CCACCTCAAGTGGGACAAACCTCAAGC | Genomic fragment of SAM1 (IE222s+IE222as) |
| IE222as     | CAATTTCGCAAAGATCACATTGCCCTAACTC | T-DNA verification for sam1 (IE222as+Lba1) - (SALK-073599) |
| IE223s      | GAATCGAATCTCTTTGGATGAGATGCGTC | Genomic fragment of SAM2 (IE223s+IE223as) |
| IE223as     | GGTCACCAGCTCAATGTCTTTCTGG | T-DNA verification for sam1 (IE223as+Lba1) - (SALK-097197) |
| IE210s      | CTGTCAGATTGTACTCGCACGCAGG | T-DNA verification for sam3 (IE210s+Lba1) - (SALK-052289) |
| IE209as     | CCTAATTAAATTTCGATCTGATTCCGAAGAAACC (PacI) | Genomic fragment of SAM3 (IE210s+IE209as) |
| IE266s      | TGAGGCCTGTAGGTAAGACAC | Genomic fragment of MAT3 (IE266s+IE266as) |
| IE266as     | TAAAGGGACATCGACAAAGGCC | T-DNA verification for mat3 (IE266as+Lbb1.3) - (SALK-019375) |
| IE38s       | GCAATGAGACGATCATGTTCTG | Genomic fragment of ACS1 (IE38s+IE38as) |
| IE38as      | CGACGAGCCAGGAGGAC | T-DNA verification for acs1-1 (IE38as+NN186as) |
| NN186as     | CCATATTGACCATCATACTTACTTTC | GABI-Kat T-DNA LB primer |
| IE39s       | GGTGCGCCAGCACTGTCAG | T-DNA verification for acs2-1 (IE39s+Lba1) |
| IE39as      | CCATCAGGTTTCGTGCAACGGAAG | Genomic fragment of ACS2 (IE39s+IE39as) |
| IE41s       | CGAAGAAGCCTACGAGCAAGCCAAG | T-DNA verification for acs4-1 (IE41s+Lba1) |
| IE41as      | CGTCTTTCTTCTCGAAACCGTTAGTC | Genomic fragment of ACS4 (IE41s+IE41as) |
|   | Genomic fragment of ACS5 (IE378s+IE378as) |   |
|---|---|---|
| RV378s | CCAGCTATGTTCGATCTAATCAGTGTAGTTAAC |   |
| RV378as | TCCATGAAACCAGGAAAACCCAGTTAGAGACTGTC | T-DNA verification for acs5-2 (IE378as+LB1) |
| IE42s | GGTGGCTTTTGCAACAGAAAGCAAGATC | T-DNA verification for acs6-1 (IE42s+LB1) |
| IE42as | CGATCTCTCGATTTCCGCAACAC | Genomic fragment of ACS6 (IE42s+IE42as) |
| IE43s | CTACAATTGCCTTTTCTTATCGAC | FLAG T-DNA LB primer |
| IE44s | CGTTTGATCTTCTTGGAAACTAC | T-DNA verification for acs7-1 (IE44s+IE43s) |
| IE44as | GGTATCGTACCGGTCTTAAG | Genomic fragment of ACS7 (IE44s+IE44as) |
| RV381s | GGATGGGAGAAGATAACAGAGAAGACCC | T-DNA verification for acs9-1 (RV381s+LBa1) |
| IE45as | GGAGACTTTCGCTTTCTCGAGG | Genomic fragment of ACS9 (RV381s+IE45as) |
| RV350s | GACACCCCCGGCTAAGGAGACTTTCACACGTCGATATG | Verification of miRNA319a targeting ACS8 and ACS11 |
| CK138as | GCCTTTTCAGAAATGGATAAATAGCCTTTGCTCC | SAIL T-DNA LB primer |
| LB1 | GCGGATAACAATTTCACACAGGAAACAG | SALK T-DNA LB primer |
| LBa1 | TGGTCACGATGAGGCCATCG | SALK T-DNA LB primer |
| LBb1.3 | ATTTTCGCCATTTGCGGAAC | SALK T-DNA LB primer |
| JH125s | CCTCAACAGCTCAGAACTAAACG | T-DNA verification for aco1 (JH125s+Lbb1.3) - (SALK-127963) |
| JH119as | GCCTTTATCACCTTTTTTTATTTACTTTTTCACACACAG (PacI) | Genomic fragment of ACO1 (JH125s+JH119as) |
| AH13s | CAAAGGATCATATTACAAGACATG | T-DNA verification for aco2 (AH13s+Lbb1.3) - (SALK-027311) |
| AH13as | CTCTTTACAAACGTTCACCAGGG | Genomic fragment of ACO2 (AH13s+AH13as) |
| AH10s | CGACGATGCTTTGCAAACCTGG | Genomic fragment of ACO3 (AH10s+AH10as) |
| AH10as | CATTTGACATATCGGGATGTCG | T-DNA verification for aco3 (AH10as+LBa1) - (SALK-082132) |
| Primer ID | Sequence | Description |
|-----------|----------|-------------|
| AH32s     | GATCTCGACGACGATTACAG | T-DNA verification for aco4 (AH32s+LBb1.3) - (SALK-014965) |
| AH32as    | GAAAAATAACAGAGTCGCTTCCC | Genomic fragment of ACO4 (AH32s+AH32as) |
| AH12s     | GTCAGAAGATGGACTAACTG | Genomic fragment of ACO5 (AH12s+AH14as) |
| AH14as    | CTAGCTTCTCGCCAGAGTTC | T-DNA verification for aco5-2 (AH14as+AH16) - (GK-119A07) |
| AH16      | TAACGCTGCGACATCTACAT | GABI-Kat T-DNA LB primer |
| JH235as   | TCTTTGATGACCTCTCGGCC | Verification primer for C-terminally tagged tdTomato |
| IE214s    | AGGCCGCGCCGAAAACGTTTTCCCTATCTCACTTG (Ascl) | Verification primer for pSAM1::gSAM1_tdTomato fragment (IE214s+JH235as) |
| IE207s    | GATCTGCAGATGTGTGACTATTTG | Verification primer for pSAM2::gSAM2_tdTomato fragment (IE207s+JH235as) |
| IE210s    | CTGTCAAGTGTACTCGACGCGG | Verification primer for pSAM3::gSAM3_tdTomato fragment (IE210s+JH235as) |
| IE343s    | CTTTGATCATCATGTTGTATGG | Verification primer for pACO1::NLS_tdTomato fragment (IE343s+JH235as) |
| IE344s    | CTATGACTCGCACATATTTTCAG | Verification primer for pACO2::NLS_tdTomato fragment (IE344s+JH235as) |
| IE345s    | GCACACACACAAAATAACACTTCC | Verification primer for pACO3::NLS_tdTomato fragment (IE345s+JH235as) |
| IE347s    | GGTTGTACTTATGACATGCTTAGC | Verification primer for pACO5::NLS_tdTomato fragment (IE347s+JH235as) |
| IE300as   | CGAACTGATCGTTAAAACCTGCC | Verification primer for C-terminally tagged GUS |
| IE324s    | GGGAGATTGATGCTAGCAAAC | Verification primer for pACS2::NLS_GUS fragment (IE324s+IE300as) |
| IE326s    | CCATGTTGGTTAACCGGATAATG | Verification primer for pACS4::NLS_GUS fragment (IE326s+IE300as) |
| IE327s    | GATTTGCTGTATGTTGAATGC | Verification primer for pACS5::NLS_GUS fragment (IE327s+IE300as) |
| IE328s    | CAAATCTGAAATTTGGACTTTATG | Verification primer for pACS6::NLS_GUS fragment (IE328s+IE300as) |
| IE329s    | CGATCTCCTAAGATGAACCAC | Verification primer for pACS7::NLS_GUS fragment (IE329s+IE300as) |
| Primer   | Sequence                        | Description                                      |
|----------|---------------------------------|--------------------------------------------------|
| IE330s   | GCTCAATTTTGAGTGTGTTTCAG         | Verification primer for \(pACS8::NLS\_GUS\) fragment (IE330s+IE300as) |
| IE331s   | GAGATAGGAAGAGAGATTTTCTTTTAG     | Verification primer for \(pACS9::NLS\_GUS\) fragment (IE331s+IE300as) |
| IE333s   | CACTATAGCTAATAATGTCGGGA         | Verification primer for \(pACS11::NLS\_GUS\) fragment (IE333s+IE300as) |
SUPPLEMENTARY MOVIE LEGENDS

Movie S1, related to Fig. 1A: Live cell imaging of early endosperm nuclei divisions in ein3/+ seeds without sdn.
Pictures were taken every 10 minutes. The last frame before nuclear decondensation in the two-nucleate endosperm stage was set to 0. Frame-numbers are indicated in the video.

Movie S2, related to Fig. 1B: Live cell imaging of early endosperm nuclei divisions in ein3/+ seeds with sdn.
Pictures were taken every 10 minutes. The last frame before nuclear decondensation in the two-nucleate endosperm stage was set to 0. Frame-numbers are indicated in the video.