Mitochondrial adrenodoxins (ADXs) are small iron–sulfur proteins encoded by nuclear genes that are ubiquitously found in plants, animals, and bacteria. They belong to the large family of [2Fe-2S]–type ferredoxins and have electron transfer properties. In animals, ADXs function as mobile shuttles that transfer electrons between an adrenodoxin reductase (ADXr) and mitochondrial P450s, which is crucial for steroidogenesis. Here we show that a plant mitochondrial steroidogenic pathway, dependent on an ADXR–ADX–P450 shuttle, is essential for female gametogenesis and early embryogenesis through a maternal effect. The steroid profile of maternal and gametophytic tissues of wild-type (WT) and adxr mutants revealed that homocastasterone is the main steroid present in WT gametophytes and that its levels are reduced in the mutant ovules. The application of exogenous homocastasterone partially rescued adxr and P450 mutant phenotypes, indicating that gametophytic homocastasterone biosynthesis is affected in the mutants and that a deficiency of this hormone causes the phenotypic alterations observed. These findings also suggest not only a remarkable similarity between steroid biosynthetic pathways in plants and animals but also a common function during sexual reproduction.

Plant reproduction | gametophyte | homocastasterone | embryogenesis | P450

A mitochondrial ADXR–ADX–P450 electron transport chain is essential for maternal gametophytic control of embryogenesis in Arabidopsis

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Edited by Ravishankar Palanivelu, The University of Arizona, Tucson, AZ; received January 9, 2020; accepted December 11, 2021 by Editorial Board Member Krishna K. Niyogi

Mitochondrial ferredoxins, also known as adrenodoxins (ADXs), are small iron–sulfur proteins encoded by nuclear genes that are ubiquitously found in plants, animals, and bacteria. They belong to the large family of [2Fe-2S]–type ferredoxins and have electron transfer properties. In animals, ADXs function as mobile shuttles that transfer electrons between an adrenodoxin reductase (ADXr) and mitochondrial P450s (1–4). The reduction of the mitochondrial cytochrome P450 catalyzed by ADXR is a crucial step that leads to steroidogenesis (5). The name “adrenodoxin” is in fact related to the tissue from which ADX was first isolated: the adrenal glands. In adrenal mitochondria, ADX transfers electrons from the NADPH-dependent ferredoxin reductase ADXR to the P450 CYP11A1 and the enzymes of the CYP11B family. CYP11A1, also known as P450scc, catalyzes three consecutive hydroxylation steps that result in cholesterol side-chain cleavage producing pregnenolone, which is the precursor of all mammalian steroid hormones, including glucocorticoids, mineralocorticoids, and sex steroids (3). The system is also functional in invertebrates. In Drosophila melanogaster, ADXR is encoded by the nuclear gene dare, and it also plays an essential role inecdysteroid biosynthesis. Ecdysteroids are steroid hormones that function both in male and female arthropods that are stored in oocytes for use during embryogenesis (4). Null mutants of dare undergo developmental arrest that is rescued by feeding mutant larvae with the insect steroid hormone 20-hydroxyecdysone (4). In addition, reduction of dare expression affects olfactory-driven behavior, and causes degeneration of adult neurons.

Although two genes encoding for ADX (ADX1 and ADX2) and one gene encoding for ADXR (ADXR) are present in the Arabidopsis thaliana genome (5), very little is known about the potential roles for these proteins in plants. Recombinant ADX1 and ADXR proteins are sufficient to transfer electrons from NADPH to cytochrome c in vitro (5) and ADXR exhibits kinetics properties and electron source preferences that are similar to those reported for mammalian proteins (6). The only role attributed to the plant ADX–ADXR system so far is the synthesis of biotin. In association with BIO2, an iron–sulfur (Fe-S) cluster enzyme, ADXR and ADX1 form an efficient plant biotin synthase complex in vitro (6). However, no functional/physiological studies have been performed for these proteins in planta and there are no in vivo data about possible reduction partners. One possibility is that the ADX–ADXR system reduces a cytochrome P450 in plant mitochondria, as reported in animal systems. However, even when A. thaliana cytochrome P450 proteins are encoded by a divergent superfamily of genes that includes 244 genes and 28 pseudogenes, no mitochondrial P450s were found or at least reported so far.

Significance

Mitochondrial adrenodoxins (ADXs) are small iron–sulfur proteins that function as mobile shuttles transferring electrons. Their function has been largely known in animals, as they transfer electrons between an adrenodoxin reductase (ADXr) and mitochondrial P450s, which is a crucial step that leads to steroidogenesis. Here we show that a functional mitochondrial ADX–ADXR–P450 pathway is essential for steroid biosynthesis and that its function is required for plant sexual reproduction.

Author contributions: J.O., O.N., E.I.Z., D.F.F., and G.C.P. designed research; A.M.B., A.M.D., N.S., M.M.C., I.O., and O.N. performed research; N.S. and I.A.R. contributed new reagents/analytic tools; A.M.B., A.M.D., N.S., I.O., O.N., D.F.F., and G.C.P. analyzed data; and A.M.B., A.M.D., E.I.Z., D.F.F., and G.C.P. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission. R.P. is a guest editor invited by the Editorial Board.

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2000482119/-/DCSupplemental.

Published January 19, 2022.
Here we show that a functional mitochondrial ADX–ADXR–P450 pathway is essential for homocastasterone biosynthesis and that its function is required for plant sexual reproduction. The study of adxr mutants and double mutants for ADX1 and ADX2 revealed that this electron shuttle is critical for female gametophyte development and for early embryogenesis through a maternal effect. Three mitochondrial cytochrome P450 proteins were identified as ADX–ADXR–P450 interactors and triple-mutant plants mimic adx and adxr mutant phenotypes, suggesting overlapping physiological roles. The study of the steroid profile of maternal and gametophytic tissues of wild-type (WT) and adx ovules revealed that homocastasterone is mostly found in gametophytes and that its levels are reduced in mutant ovules. Moreover, application of exogenous homocastasterone partially rescued the phenotypes of ADXR and cytochrome P450 mutants. Altogether, our results indicate that the mitochondrial shuttle ADX–ADXR–P450 is essential for Arabidopsis reproduction and required for homocastasterone biosynthesis in the female gametophyte.

Results

ADXs and ADXR Are Essential for Normal Female Gametophyte Development and Early Embryogenesis in A. thaliana. To analyze possible roles for the ADX–ADXR shuttle in A. thaliana, two independent insertional lines (Columbia [Col-0] ecotype) with T-DNA insertions located at exon 4 (adxr-1) and intron 4 (adxr-2) were characterized (SI Appendix, Fig. S1). After backcrossing these lines to the Col-0 ecotype, the progeny from self-crossed plants was examined by PCR-based genotyping. No homozygous mutant plants were recovered in the offspring from either adxr-1/ADXR or adxr-2/ADXR self-pollinated plants. The ratio of hemizygous to WT plants was 1.05 for adxr-1/ADXR (n = 449) and 1.23 for adxr-2/ADXR plants (n = 247). Such ratios, as previously reported, suggest failure to transmit the mutation to the next generation through either the male or female gametophyte (7). As no homozygous adxr plants were recovered, only hemizygous mutant plants were analyzed. No obvious sporophytic defects were observed in the hemizygous plants (SI Appendix, Fig. S1). However, siliques of adxr/ADXR plants showed a reduction in seed set of about 35% (Fig. 1 A and B), suggesting that the gene is essential for gametogenesis and/or seed development. To study the genetic basis of the defect in the seed set observed, we crossed hemizygous plants as either pollen or pistil donors to WT plants from the Columbia accession, and the transmission efficiency (TE; no. of progeny with T-DNA insertion/WT offspring) was calculated (SI Appendix, Table S1). Only ~19% of megagametophytes carrying adxr-1 and around 30% of megagametophytes carrying adxr-2 successfully transmitted the insertion to the next generation, showing that the mutation affects female gametophyte development or function. TE through the pollen was ~88% for adxr-1 and 83% for adxr-2, suggesting that male gametophytes are not severely affected by the mutation (SI Appendix, Table S1).

We then analyze how this mutation alters female gametophytes, the terminal phenotype of female gametophytes from emasculated adxr/ADXR flowers was studied (Fig. 1 C–F and SI Appendix, Fig. S2 and Table S2). In WT Arabidopsis, the female gametophyte is a seven-cell structure that includes two gametic cells (the egg cell and the central cell) and five accessory cells: three antipodal cells of unknown function at the chalazal end of the female gametophyte and two synergid cells that flank the egg cell at the micropylar end. Together, the egg cell and the two synergid cells (responsible for pollen tube attraction) form the egg apparatus (7). While WT pistils only showed 2% of abnormal female gametophytes, adxr/ADXR pistils showed that around 13% of the ovules were carrying anomalous gametophytes (n = 720 for adxr-1/ADXR and n = 801 for adxr-2/ADXR; SI Appendix, Table S2). Mutant adxr-1 gametophytes showed developmental delay, with female gametophytes displaying unfused polar nuclei (8.2%). Additionally, 4.3% of all female gametophytes observed showed an aberrant egg apparatus, with mispositioned synergid nuclei (Fig. 1). A small fraction of female gametophytes were found collapsed but in a proportion similar to the one observed for WT pistils (less than 1%). Similar phenotypes and distribution were observed when adxr-2/ADXR pistils were analyzed (SI Appendix, Fig. S2). In addition, we studied WT pollen tube attraction in adxr/ADXR pistils by aniline blue staining. When WT pistils were analyzed, 90.7 ± 3.06% of the ovules analyzed showed a pollen tube reaching the micropyle (n = 10 pistils, 231 ovules quantified). The percentage was 83.7 ± 4.72% for ovules in adxr-1/ADXR pistils (n = 5 pistils, 92 ovules quantified, P = 0.0006, t test) and 86.2 ± 3.77% in adxr-2/ADXR pistils (n = 5 pistils, 126 ovules quantified, P = 0.0032, t test). As the percentage of ovules targeted by pollen tubes was slightly but significantly different from WT in the mutants, these results indicate that a small fraction of the ovules carrying defective gametophytes were not attracting pollen tubes and therefore could not be fertilized (SI Appendix, Fig. S3).

The fraction of abnormal female gametophytes observed before pollination could not explain the high ratio of aborted seeds detected in mature siliques, the phenotype of ovules in adxr/ADXR pistils was studied after fertilization. Self-pollinated pistils of 7 to 8 mm, corresponding to 72 h after pollination (72 HAP), were analyzed. While 93.3% of the embryos analyzed in WT pistils were found at a preglobular to globular stage, adxr/ADXR pistils showed that only 60% of the embryos analyzed were able to reach that stage (59.7% for adxr-1/ADXR and 61.4% for adxr-2/ADXR; SI Appendix, Table S3). Embryogenesis was found arrested in 20 to 24% of the ovules analyzed, where development stopped at the zygote or one/two-celled proembryo (Fig. 1 G–R and SI Appendix, Table S3). In addition, 18.2 and 7.7% of the ovules were found collapsed in adxr-1/ADXR and adxr-2/ADXR, respectively. Notably, 7% of the embryos found arrested in adxr-2/ADXR pistils showed abnormal planes of cell division in the embryo proper (Fig. 1 H and N and SI Appendix, Table S3). In adxr-1/ADXR pistils, arrest in embryogenesis was due to a gametophytic maternal effect, we crossed both adxr/ADXR allelic mutants as females using WT plants as pollen donors. The resulting progeny was studied by scoring the number of ovules showing defects in embryogenesis 72 HAP. In both cases, the percentage of ovules showing abnormal embryos found was around 35% (SI Appendix, Table S3), indicating that the arrest in embryogenesis observed arises from the female gametophyte, as it is not rescued by WT pollen. Conversely, we crossed WT plants as females, using both adxr/ADXR allelic mutants as pollen donors. In this case, the percentage of ovules showing abnormal embryogenesis found 72 HAP was around 6%, a value comparable to the one found in hand pollination controls (WT x WT crosses; SI Appendix, Table S3). This result indicates that the arrest in embryogenesis observed originates from a defect in the female gametophyte. As the ratio obtained was similar to what is expected from WT crosses, the result also suggests that the slight decrease in TE observed from the male side in reciprocal crosses might be due to a defect in pollen development or pollen tube growth. However, we were not able to find obvious developmental defects when pollen grains were analyzed (SI Appendix, Fig. S3). This suggests that the decrease in TE observed might arise from defects in the fertilization process.

ADXs are essential for ADXR activity, as they function as mobile shuttles that transfer electrons between ADXR and a physiological roles. The study of the steroid profile of maternal and gametophytic tissues of wild-type (WT) and adx ovules revealed that homocastasterone is mostly found in gametophytes and that its levels are reduced in mutant ovules. Moreover, application of exogenous homocastasterone partially rescued the phenotypes of ADXR and cytochrome P450 mutants. Altogether, our results indicate that the mitochondrial shuttle ADX–ADXR–P450 is essential for Arabidopsis reproduction and required for homocastasterone biosynthesis in the female gametophyte.
Fig. 1. ADXR and ADX1/2 are essential for normal female gametophyte development and embryogenesis in *A. thaliana*. (A) Boxplot showing the rate of seeds aborted in WT and mutant plants. Asterisks indicate statistically significant differences from WT, as determined by Welch's two-sample t test (*P* < 0.01). (B) Stereomicrographs of siliques (arrowheads) at 10 d post fertilization from WT and mutant plants. (C) Image of a properly developed female gametophyte found in *adxr-1/ADXR* pistils 48 h after emasculation. (D) Female gametophyte showing unfused polar nuclei (upn). (E) Female gametophyte showing abnormal polarity in one of the synergid cells (Asyn). (F) Female gametophyte showing both an abnormal synergid and unfused polar nuclei. (G–R) Ovules were dissected 72 HAP. (G–L) Ovules from *adxr-1/ADXR* pistils. (M–R) Ovules from *adxr-2/ADXR* pistils. (G) Ovule from an *adxr-1/ADXR* pistil showing an embryo at the preglobular stage. (H) Embryo at the preglobular stage showing asymmetric divisions in the embryo proper (Inset, arrowheads). (I) Embryogenesis is arrested at the four-cell stage. (J) Embryo showing an aberrant short suspensor (s). Endosperm is developed normally. (K) Embryogenesis is arrested at the one-cell stage. (L) Fertilized ovule showing embryogenesis arrested at the zygote stage (z). (M) Ovule from an *adxr-2/ADXR* pistil showing an embryo at the preglobular stage. (N) Embryo at the preglobular stage showing asymmetric divisions in the embryo proper (Inset, arrowheads). (O) Ovule showing embryogenesis arrested at the four-cell stage. (P) Embryo showing an aberrant short suspensor. (Q) Fertilized ovule showing embryogenesis arrested at the zygote stage and normal endosperm development. (R) Fertilized ovule showing embryogenesis arrested at the zygote stage and no endosperm development. (S) Seed abortion rate in WT, *adx1*, *adx2*, and double-mutant plants. Asterisks indicate statistically significant differences from WT, as determined by Welch's two-sample t test (*P* < 0.01). (T–Z) Ovules from *adx1,adx2/ADXR* pistils were analyzed 48 h after emasculation (T–W) or 48 h after pollination (X–Z). (T) Ovule showing a normal female gametophyte at stage FG7. (U) Female gametophyte showing unfused polar nuclei. (V) Female gametophyte showing abnormal polarity in one of the synergid cells. (W) Female gametophyte showing both an abnormal synergid and unfused polar nuclei. (X) Ovule from an *adx1,adx2/ADXR* pistil showing an embryo at the preglobular stage. (Y) Embryo showing an aberrant short suspensor. (Z) Fertilized ovule showing embryogenesis arrested at the one-cell stage. Ccn, central cell nucleus; Ecn, egg cell nucleus; Ep, embryo proper; syn, synergid cell. (Scale bars, 50 μm.) See also SI Appendix, Figs. S1–S4 and Tables S1–S3.
(SI Appendix, Fig. S4A). To determine whether similar defects to the ones observed in adxr/ADXr mutants could be observed in plants impaired in ADX function, single insertional knock-out mutants for each gene were examined (SI Appendix, Fig. S4 B–E). All mutants analyzed show siliques with full seed sets, and no obvious defects in vegetative or reproductive tissues were observed (SI Appendix, Fig. S4 C and D). The absence of phenotypes in adx1 and adx2 mutants together with their high sequence similarity suggests that ADX1 and ADX2 might be functionally redundant. To study this possibility, we crossed single adx mutants and analyzed the phenotype of plants carrying adx1,adx2/ADX2 and adx1/ADX1,adx2 mutant allele combinations, as we were not able to detect double-homozygous plants in the F2 progeny (n = 254 plants genotyped). The rate of ovule abortion observed was 16.9% in siliques from adx1/ADX1 adx2/ADX2 plants, 43.3% in adx1,adx2/ADX2 siliques, and 39.9% in adx1/ADX1,adx2 siliques (Fig. 15). As shown by adx1/ADX1 plants, this phenotype could not be rescued by pollination with WT pollen, suggesting a maternal effect (SI Appendix, Table S4). In addition, female gametophytes and embryos showed defects that were similar to the ones observed in adx1/ADX1 plants (Fig. 1 T–Z).

**ADXR Is Expressed in Gametophytic and Young Sporophytic Tissues.** To study the temporal and spatial expression pattern of ADXR during different stages of the plant life cycle, we analyzed transgenic plants carrying an ADXR promoter–GUS reporter gene fusion (pADXR-GUS). The sequence used as the ADXR putative promoter was the complete intergenic region located upstream of the ADXR ATG codon (640 bp). GUS expression was detected in regions of active cell division in the young seedlings in the hypocotyl, cotyledons, and leaf primordia (Fig. 2 A–D). In mature leaves as well as in sepal, expression was weaker and mainly confined to vascular tissues. High expression was also detected in the filaments of mature flowers (Fig. 2E). GUS expression was also detected in mature pollen grains (Fig. 2F) and mature female gametophytes, associated with the egg apparatus and central cell (Fig. 2G). After fertilization, GUS activity was detected in the zygote and until the one-cell embryo stage (Fig. 2 H and J). GUS activity was not detected at later stages of embryogenesis (Fig. 2I). To further know the basis of the maternal effect observed, we crossed WT plants with homozygous pADX-GUS plants and studied GUS staining after fertilization. As can be observed in SI Appendix, Fig. S5, while GUS staining was detected from the maternally inherited allele in the zygote, no signal was detected when GUS staining was assayed to look for expression of the paternally inherited allele. This result suggests that the paternal ADXR allele is probably not expressed in the embryo or endosperm, at least before the globular stage.

To analyze ADXR localization in the female gametophyte, the fusion protein ADXR-GFP (green fluorescent protein) was followed in transgenic WT plants carrying the construct pADX- R:ADX-GFP. This construct was proven to be fully functional as it was able to complement the phenotype observed in adxr/ADXR plants (SI Appendix, Table S5). In ovules, ADXR-GFP was detected both in the integuments of the ovule, in the nucellus, and inside the female gametophyte, mostly in the egg cell and the central cell, associated with mitochondria (Fig. 2 K and L).

**ADXr–ADXR Shuttle Dysfunction Does Not Affect Reactive Oxygen Species Levels or Mitochondrial Functional Status.** One possible consequence of a failure in electron transport is reactive oxygen species (ROS) accumulation, which might well result in developmental arrest as previously described (8). To explore that possibility, ROS levels were analyzed using the ROS-sensitive probe H$_2$DCFDA and quantifying the fluorescence found inside the female gametophytes of pistils from WT, adxr/ADXr, and adx1,adx2/ADX plants. No significant differences were found (SI Appendix, Fig. S6A), indicating that ROS production/accumulation was not altered in the mutant female gametophytes. In addition, we assessed whether ADXR–ADX shuttle dysfunction might affect mitochondrial membrane potential/Δψ. By using the membrane potential (Δψm) indicator JC-1. While in functional mitochondria with high Δψm, JC-1 spontaneously forms red fluorescent complexes, it remains as a green monomer in unhealthy mitochondria (8). As can be observed in SI Appendix, Fig. S6B, gametophytic mitochondria from each of the pistils analyzed showed similar distributions of red to green values, suggesting that mitochondrial Δψm was not affected by the mutations examined.

**ADXR Interacts with Mitochondrial Cytochrome P450s.** As no ROS accumulation nor mitochondrial dysfunction was found in ADXR and ADX mutant gametophytes, we decided to identify putative redox partners of the ADXR–ADX shuttle by means of a high-throughput yeast two-hybrid screening using ADX1 as a bait (HibriGenics Services). Among the putative interacting proteins identified, there were clones with a predicted or a reported localization that was associated either with the plasma membrane or with different organelles such as plastids, chloroplasts, or mitochondria (SI Appendix, Table S6). As one of the clones identified corresponded to the P450 cytochrome CYP711A1, we decided to investigate if ADX1 can indeed interact with a cytochrome P450 as occurs in animal systems. Thus, we first analyzed if CYP711A1 can be found in mitochondria by analyzing the localization of a CYP711A1-GFP fusion protein using a transient expression system in Nicotiana benthamiana and the mitochondrial selective dye MitoTracker red (Invitrogen). As can be observed in Fig. 3A, CYP711A1-GFP colocalizes with MitoTracker red, indicating its mitochondrial localization. In addition, to confirm ADX1–CYP711A1 interaction, we performed a bimolecular fluorescence complementation (BiFC) assay using transient expression in N. benthamiana. We fused CYP711A1 and ADX1 to N- and C-terminal yellow fluorescent protein (YFP) fragments to generate CYP711A1-nYFP and ADX1–cYFP fusion constructs under control of the UBQ10 promoter. After Agrobacterium infiltration of N. benthamiana leaves, YFP signal was strongly detected, indicating ADX1–CYP711A1 interaction (Fig. 3B).

The analysis of two allelic insertion mutant lines with a T-DNA insertion in CYP711A1 [lines CS9564 and CS717630 (9)], however, showed that homoyzogous cyp711a1 plants did not show any gametophytic defect, only a slight increase in lateral branching as already reported (10) (SI Appendix, Fig. S8 A–D). This suggests that other P450 cytochromes might function redundantly with CYP711A1 in Arabidopsis mitochondria, thus replacing the CYP711A1 role in loss-of-function mutants.

The Arabidopsis genome encodes for 244 P450 genes and 28 pseudogenes (11). However, apart from CYP711A1 shown here (Fig. 3A), there are no reports so far that locate any of the other 243 Arabidopsis cytochrome P450s in mitochondria. To identify putative mitochondrial P450 cytochromes, we applied 10 different bioinformatics approaches based on sequence characteristics (presence of an N-terminal mitochondrial targeting sequence and other features that direct proteins to mitochondria). The programs used were TargetP, Mitoprot2, SubLoc, iPSORT, Predotar, MitoPred, PeroxP, MultiLoc, WoLFPSORT, and LOCtree. We were able to identify eight candidates that showed different putative localizations according to the software applied (SI Appendix, Table S7). We selected the first four candidates shown in SI Appendix, Table S7 to evaluate if they were indeed localized in mitochondria. We designed C-terminal translational fusion constructs for each protein with GFP under the control of the CaMV 35S promoter and subcellular
Fig. 2. ADXR is expressed in gametophytic and young sporophytic tissues. (A–J) Histochemical localization of GUS activity in transgenic Arabidopsis plants harboring the pADXR-GUS construct. (A) GUS expression observed in a 21-d-old seedling showing expression in hydathodes (hy), vascular tissue, vegetative meristem, and young leaves. (B) Expression in a trichome. (C) GUS expression is observed in guard cells. (D) GUS expression is shown in a young inflorescence. (E) Open flower at stage 12 showing GUS staining in sepalts, petals, and pistil. (F) Anther showing GUS-stained pollen grains. (G) Female gametophyte showing GUS expression associated with the egg cell (Ec) and central cell (Cc). (H) Fertilized female gametophyte showing GUS staining in the zygote and in the developing endosperm. Arrowheads point to endosperm nuclei. (I) GUS staining is still detectable at the one-cell embryo stage, both in the embryo proper (Ep) and in the developing endosperm. (J) GUS staining is no longer detectable at the two-cell embryo stage. (K) ADXR-GFP is detected inside the female gametophyte, mostly associated with the egg cell and the central cell. FM4-64 staining was used to underline the cells composing the female gametophyte. The pictures shown correspond to the same single confocal slice. Egg apparatus cells are outlined. Controls of autofluorescence display a representative picture of an ovule from a plant that does not carry the GFP fusion construct. Both channels are shown. (L) Representative confocal images showing a female gametophyte expressing ADXR-GFP stained with MitoTracker red. The pictures shown correspond to the same single confocal slice. (M) Boxplots showing the results of the colocalization analyses of ADXR-GFP and MitoTracker red performed by using the correlation test (PSC) plug-in for ImageJ after background subtraction. As an example, the ROI used is delimited in L. Scale bars are 50 μm in F–J and 25 μm in K–L. See also SI Appendix, Figs. S5 and S7.
localization was studied using a transient expression system in *N. benthamiana* and MitoTracker red. Three of the four candidates analyzed were found to colocalize with MitoTracker red. CYP90A1 (CPD), CYP90D1, and CYP75B1 (TT7) localize in mitochondria while CYP89A2 does not (Fig. 4A). To test whether these cytochromes found in mitochondria are able to interact in vivo with ADX1, we performed a BiFC assay, as described for CYP711A1. After Agrobacterium infiltration of *N. benthamiana* leaves, a strong YFP signal was detected for CYP90A1 and CYP75B1, while control *N. benthamiana* leaves and leaves infiltrated with constructs to detect ADX1 interaction with CYP90D1 did not show BiFC signal (Fig. 4B). Altogether, these results indicate that ADX1 can interact in vivo with at least three mitochondrial P450s: CYP711A1, CYP90A1, and CYP75B1.

**Functional Characterization of Mitochondrial P450s Interacting with ADX1.** As we did for CYP711A1, single-knockout mutants for each of the genes encoding for CYP75B1 and hemizygous plants for CYP90A1 [as homozygous plants are not fertile (12)] were studied to analyze if they showed a defect similar to the ones described for adx or adxr mutants. However, seed sets were normal for CYP90A1 mutants and only slightly lower than WT for CYP75B1 mutants, suggesting functional redundancy (SI Appendix, Fig. S8). To study if that was the case, we constructed double mutants crossing each of the mutant lines available, obtaining three combinations of double-hemizygous mutants in F1 (cyp711a1/cyp711A1 cyp90a1/cYP90A1; cyp711a1/cyp711A1 cyp75b1/cYP75B1; cyp75b1/cYP75B1 cyp90a1/cYP90A1).

**Fig. 3.** CYP711A1 localizes to mitochondria and interacts with ADX1. (A) Representative confocal images showing *N. benthamiana* epidermal cells transiently expressing CYP711A1-GFP and stained with MitoTracker red. (B) Boxplots showing the results of the colocalization analyses of CYP711A1-GFP and MitoTracker red performed by using the correlation test (PSC plug-in for ImageJ) after background subtraction. Quantification was performed in at least four ROIs per photo. Three photos were taken per assay. (C) BiFC assay: representative microphotographs showing reconstituted YFP fluorescence or DIC images of *N. benthamiana* epidermal cells transformed with plasmids harboring the indicated constructs (CYP711A1-nYFP, ADX1-cYFP, or both). (Scale bars, 50 μm.) (D) Boxplots showing YFP fluorescence intensity values obtained in the BiFC assays. Quantification was performed in at least four ROIs per photo. Three photos were taken per assay. The BiFC assays were independently repeated at least three times. A.U., arbitrary units.
Plants. P450 cytochromes, we decided to address if the ADX–ADXR
mutant plants were also present in these triple mutants, we analyzed unfertilized and fertilized ovules (SI Appendix, Fig. S8 Q–V). When ovules were studied 48 h after emasculation, 58.33% of the female gametophytes were found at FG7 stage (normal female gametophyte), 25% were observed with unfused polar nuclei, 5.55% presented abnormal egg apparatuses, and 11.11% were found collapsed (n = 36). At 72 HAP with WT pollen, embryogenesis showed significant abnormalities. While 62.68% of the ovules presented an embryo at early globular stage, 9.52% of the fertilized ovules were found arrested at the zygote stage, 0.79% were found at a two-four-cell stage with an abnormal short suspensor, and 13.48% presented abnormal planes of cell division (SI Appendix, Table S9). Additionally, we studied WT pollen tube attraction in WT and triple-mutant plants cyp711a1 cyp90a1/cYP90A1 cyp75b1 by aniline blue staining (SI Appendix, Table S9). Although most cyp triple-mutant ovules are able to attract pollen tubes, there is a slight but significant difference when compared with WT ovules (88 ± 5.77 vs. 94.7 ± 6.06%, P = 0.0399, t test). However, 35.43 ± 9.16% of targeted ovules looked smaller/arrested in comparison with ovules in the same pistil (SI Appendix, Table S9), suggesting that even when most ovules are able to attract pollen tubes, seed development is arrested in the triple mutant, as previously shown by differential interference contrast (DIC) microscopy and silique analyses. Altogether, these results suggest that ADXR, ADX, and the P450 cytochromes studied were involved in the same physiological/developmental pathway during female gametophyte development/function and early embryogenesis.

Steroid Profile of Arabidopsis Ovules in WT and adxr/ADXR Mutant Plants. Since ADX1 was found to interact with mitochondrial P450 cytochromes, we decided to address if the ADX–ADXR shuttle was involved in steroid biosynthesis, as described in animal systems. As a first approach, we harvested pistils at stage 14 (13) from WT and adxr/ADXR mutant plants and attempted to detect brassinosteroids (BRs) in pistil extracts using an enzyme-linked immunosorbsent assay. However, we were not able to detect steroids in these extracts by this method, suggesting that steroids, if present, might occur at very low concentrations. As steroids present in ovules might be diluted if we used pistil extracts, we decided to directly collect Arabidopsis ovules to identify and measure, if possible, the BRs present.

Because SPOROCYTELESS (SPL) is required for megasporogenesis initiation, spl ovules lack a female gametophyte (14). Thus, by comparing WT and spl ovules, it would be possible to differentiate steroids present in the sporophytic maternal tissues and the ones that are specific to the female gametophyte. To perform this experiment, we harvested ovules from pistils at stage 14 (13) using custom-designed equipment that suctioned and immediately freezes the ovules inside a collecting tube (Materials and Methods). Around 40 to 90 mg of ovules was extracted from plants of the indicated genotypes and BRs were analyzed using ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). As the spl mutant is in the Landsberg erecta (Ler) background, WT plants of the corresponding ecotype were used to compare the steroid content. While BRs like brassinolide, 24-epibrassinolide, 24-epi-castasterone, and 28-norcastasterone were not detected, castasterone and principally homocastasterone were found in ovules from both WT and spl plants. Homocastasterone content in spl ovules was lower than the levels found in WT ovules (Table 2). This result indicates that homocastasterone contribution in WT ovules was mainly from the female gametophyte. Specifically, the contribution of homocastasterone from the female gametophyte seems to be around 75%, while the sporophytic tissues of the ovule account for the remaining 25% (Table 2). To study if homocastasterone levels in the female gametophyte are dependent on ADXR, we measured the steroid content in ovules from adxr-1/ADXR and WT plants (ecotype Col-0, adxr-1/ADXR background). In this case, we expect only half of the ovules collected from adxr-1/ADXR plants to have ADXR activity in their female gametophyses, while the other half would not, assuming that the mutation is fully penetrant. The contribution of homocastasterone from the sporophytic tissues should be maintained. The value obtained (0.42 ± 0.09 pmol per gram fresh weight [gFW]) is similar to the expected value if ADXR is required for homocastasterone biosynthesis, assuming full penetrance of the mutation in a mix of 50:50 of adxr and ADXR female gametophytes (0.455 ± 0.085 pmol/gFW; P = 0.7914, t test; Table 2). These results suggest that ADXR activity is required for homocastasterone biosynthesis in the female gametophyte.

Exogenous Homocastasterone Treatment Partially Rescues the Phenotype of ADXR and Cytochrome P450 Mutants. To investigate whether the gametophytic phenotypes observed were due to low levels of BRs, pistils from WT and adxr/ADXR and cyp711a1 cyp90a1/cYP90A1 cyp75b1 mutant flowers were treated every 24 h with homocastasterone, homobrassinolide, 24-epibrassinolide, or a mock solution from stage 9 to stage 14, allowing self-pollination. When mature siliques were scored for abortions, a significant reduction in the rate of ovule abortion was observed in siliques from mutant flowers treated with homocastasterone in comparison with mock-treated plants (Fig. 5). No significant differences were observed when pistils were treated with homobrassinolide or 24-epibrassinolide. Although the rescue obtained after homocastasterone treatment was partial, this is likely a reflection of an inefficient uptake of the hormone by the female gametophytes. While BRs were administered to the pistils, they are required inside the female gametophytes, which are enclosed by the maternal tissues of the ovule.

Collectively, our results suggest that homocastasterone might be the bioactive BR required for normal development and function of the female gametophyte in A. thaliana and that the ADX–ADXR–P450 shuttle is involved in its biosynthetic pathway inside the gametophytic mitochondria.

Discussion

Our results show that the ADX–ADXR shuttle is essential for normal development and function of the female gametophyte in A. thaliana. The only role attributed so far to ADXs and ADXR is related to biotin biosynthesis. In association with BIO2, ADX and ADXR constituted a reduction system that allowed the formation of an in vitro plant biotin synthase complex (6). However, mutants deficient in biotin are impaired during late embryogenesis [gibberellin to early cotyledon stages (15, 16)] while mutants impaired in ADXR or ADX show phenotypes related to female gametophyte development and function, indicating that these proteins might be also involved in other pathways that are required earlier during development. This is actually not surprising, as ferredoxin reductase–ferredoxin electron transfer systems are considered to be functionally promiscuous
Fig. 4. Mitochondrial cytochrome P450s CYP90A1 and CYP75B1 interact with ADX1. (A) Representative images showing *N. benthamiana* epidermal cells stained with MitoTracker red transiently expressing the indicated P450-GFP fusions. Boxplots show the results of the colocalization analyses of CYP-GFP fusion proteins and MitoTracker red performed by using the correlation test (PSC) plug-in for ImageJ after background subtraction. Quantification was performed in at least four ROIs per photo. Three photos were taken per assay. (B) BiFC analysis of ADX1 interaction with candidate mitochondrial cytochrome P450s. ADX1 and the P450 candidates were fused to the C- and N-terminal YFP fragments to generate ADX1-cYFP and P450-nYFP fusion constructs. The figure shows representative microphotographs displaying reconstituted YFP fluorescence in *N. benthamiana* epidermal cells transformed with plasmids harboring the P450-nYFP constructs alone or together with the ADX1-cYFP construct. Boxplots show YFP fluorescence intensity values obtained in the BiFC assays. Quantification was performed in at least four ROIs per photo. Three photos were taken per assay. The BiFC assays were independently repeated at least three times. (Scale bars, 40 μm.)
Table 1. Quantification of abortions observed in simple and multiple mutants for genes encoding mitochondrial P450s that interact with ADX1

| Genotype                      | N (pistils analyzed) | % ovule abortion* |
|-------------------------------|----------------------|-------------------|
| WT                            | 25                   | 0.99*             |
| cyp711a1                      | 25                   | 1.38*             |
| cyp90a1/CYP90A1               | 25                   | 1.41*             |
| cyp75b1                       | 30                   | 2.08ab            |
| cyp711a1/CYP711A1cyp90a1/CYP90A1 | 25             | 1.12*             |
| cyp711a1/CYP711A1cyp75b1/CYP75B1 | 25             | 3.02b             |
| cyp90a1/CYP90A1cyp75b1/cYP75B1 | 25                   | 3.44b             |
| cyp711a1/cyp90a1/CYP90A1cyp75b1 | 15                   | 33.51c            |

*Different letters indicate significant differences (P < 0.05) shown by the Kruskal-Wallis test with Dunn's post hoc test. Multiple testing was corrected by using the Bonferroni method.

Table 2. Brassinosteroid levels in ovules extracted from the indicated genotypes, pmol/gFW

| Genotype       | Castasterone, mean ± SEM | Homocastasterone, mean ± SEM |
|----------------|--------------------------|-----------------------------|
| WT Ler         | <LOD                     | 1.22 ± 0.12                 |
| Spl            | <LOD                     | 0.32 ± 0.06*                |
| WT Col         | 0.96 ± 0.13              | 0.74 ± 0.19                 |
| adxr/ADXr      | 0.73 ± 0.12              | 0.42 ± 0.09†                |

Numbers represent the mean of three replicates. For each experiment, 40 to 90 mg of ovules was harvested from plants of the indicated genotypes and BRs were analyzed using UHPLC-MS/MS. <LOD indicates concentrations below the calculated limit of detection.

*Significantly different from WT Ler values (t test, P = 0.0204).

†Similar to the expected value (0.455 ± 0.085) if homocastasterone content in female gametophytes is dependent on ADXR activity, assuming full penetrance of the mutation in a mix of 50:50 of adxr and ADXR female gametophytes (t test, P = 0.7914).
reported to promote cell expansion in Arabidopsis cell cultures (35), which is a crucial process during female gametogenesis and is rapidly induced after fertilization. Taking into account BR biological activities, we propose that homocastasterone biosynthesis through the ADXR–ADX–P450 mitochondrial shuttle might be required in the female gametophyte to sustain early embryo development, mediating both cell division and expansion and cell-division orientation. Altogether, our findings reveal not only a remarkable similarity between steroid biosynthetic pathways in plants and animals but also a common function during sexual reproduction. Although sexual reproduction appeared in eukaryotes before the divergence of plant and animal lineages, it is too early to dissect if these common molecular pathways represent ancestral conserved sexual features or if they are a consequence of convergent evolution.

Materials and Methods

Plant Material and Growth Conditions. All seeds used in this work were obtained from the Arabidopsis Biological Resource Center, Ohio State University (9) except for those donated as specified. Plant material and growth conditions are described in detail in SI Appendix, Materials and Methods.
Morphological and Histological Analyses. Pistils from different floral developmental stages were dissected and cleared overnight in Hoyzer’s solution. For GUS staining, developing carpels and siliques were dissected and incubated in GUS staining buffer as already described (36). Ovules were observed on a Zeiss Axio Imager A2 microscope using DIC optics. Images were captured on an Axioscan HRC charge-coupled device camera (Zeiss) using the Axiosview program (version 4.2). Mitochondrial distribution was studied by using MitoTracker red (Invitrogen) and confocal microscopy. Stock solutions were prepared fresh for each use at 1 mM in dimethyl sulfoxide (DMSO). The working solution was prepared by diluting the stock in phosphate-buffered saline (pH 7.2) to a final concentration of 10 μM. To detect mitochondria in the female gametophytes, the pistils were dissected and incubated in the working solution for 30 min on a microscope slide at room temperature protected from light. After incubation, pistils were observed using confocal microscopy (Eclipse C1 Plus confocal microscope; Nikon). Images were obtained with EZ-C1 3.80 imaging software and Ti Control. Laser power was set at 10.5% in all studies. The objective used was Super Fluor 40×/1.30/0.22 oil at a working distance of 0.22 μm with Immersol 518F oil (Carl Zeiss). The excitation for GFP was 488 nm; the excitation for FM4-64 red fluorescent protein was 561 nm. The pinhole was set at 33.3 μm.

Aniline Blue Staining. Details of aniline blue staining can be found in SI Appendix, Materials and Methods.

Yeast Two-Hybrid Screen and Analysis. Yeast two-hybrid screening was performed by Hybrigenics Services (http://www.hybrigenics-services.com). Details of the yeast two-hybrid screen can be found in SI Appendix, Materials and Methods.

Colocalization Experiments in N. benthamiana. The complementary DNA (cDNA) of each P450 cytochrome was PCR-amplified using the primers listed in SI Appendix, Table S19. Each cDNA was cloned into pENTR TOPO (Gateway technology; Invitrogen) and then recombined into the destination plasmid pMDCM83 (37), resulting in a p3SS-P450-GFP fusion. Agrobacterium tumefaciens strain GV3101 carrying p3SS-P450-GFP was infiltrated together with A. tumefaciens carrying the p19 helper plasmid (which contains a gene-silencing suppressor) into leaf epidermal cells of 5-wk-old N. benthamiana plants; 48 to 72 h after infiltration, sections of infiltrated leaves were incubated with 200 nM MitoTracker red (Invitrogen) for 30 min. Finally, subcellular localization studies were performed on a confocal microscope (Nikon Eclipse C1 Plus). Laser power was set at 5 to 15%. The objective used was Super Fluor 40×/1.30/0.22 oil at a working distance of 0.22 μm with Immersol 518F oil (Carl Zeiss). The excitation for YFP was 515 nm. The pinhole was set at 33.3 μm. Fluorescence intensities were measured using ImageJ imaging software. Quantification analysis was performed using Leica (Eclipse C1 Plus confocal microscope; Nikon). Images were obtained with EZ-C1 3.80 and ImageJ imaging software. Laser power was set at 5 to 15%. The objective used was Super Fluor 40×/1.30/0.22 oil at a working distance of 0.22 μm with Immersol 518F oil (Carl Zeiss). The excitation to detect YFP was 515 nm. The pinhole was set at 33.3 μm. Fluorescence intensities were measured using ImageJ imaging software. Quantification analysis was performed using Leica (Eclipse C1 Plus confocal microscope; Nikon). Images were obtained with EZ-C1 3.80 and ImageJ imaging software. Laser power was set at 10.5%. The objective used was Super Fluor 40×/1.30/0.22 oil at a working distance of 0.22 μm with Immersol 518F oil (Carl Zeiss). The excitation for GFP was 488 nm; the excitation for MitoTracker red was 561 nm. The pinhole was set at 33.3 μm. Linear (Pearson’s) and nonlinear (Spearman’s) correlation coefficients for colocalization analysis were obtained by analyzing the images with the PSC colocalization plug-in (ImageJ). The threshold level was set at 10. At least 10 Regions of Interest (ROIs) outlining the embryo sac of each embryo sac were analyzed in each case as indicated.

BIFC Analysis. In vivo analysis of protein interaction was performed by a BIFC assay. The complete cDNAs corresponding to ADX1 and the P450 cytochromes (see primers in SI Appendix, Table S10) were cloned into pENTR TOPO and then recombined into BIFC destination plasmids pUBN-CyFP and pUBC-ncYFP, respectively (38). The binary plasmids were then introduced into A. tumefaciens strain GV3101. Split nYFP- and cYFP-tagged protein pairs were coexpressed in N. benthamiana leaves by A. tumefaciens-mediated infiltration. Leaves were examined 48 to 72 h postinfiltration using confocal microscopy (Nikon Eclipse C1 Plus) and EZ-C1 3.80 and ImageJ imaging software. Laser power was set at 5 to 15%. The objective used was Super Fluor 40×/1.30/0.22 oil at a working distance of 0.22 μm with Immersol 518F oil (Carl Zeiss). The excitation to detect YFP was 515 nm. The pinhole was set at 33.3 μm. Fluorescence intensities were measured using ImageJ imaging software. Quantification analysis was performed using at least four round ROIs per photo (50 μm in diameter) of N. benthamiana epidermal cells transformed with plasmids harboring both constructs (CYP711A1-ncYFP and ADX1-cYFP) or in cells transformed with plasmids carrying only one of the constructs (CYP711A1-ncYFP or ADX1-cYFP) as negative controls (shown in Figs. 3 and 4). Three photos were taken per assay. The BIFC assay was independently repeated at least three times.

Extraction and Quantification of Endogenous BRs. Material and quantification of endogenous BRs are described in SI Appendix, Materials and Methods.

Mitochondrial Membrane Potential Studies. These studies are described in detail in SI Appendix, Materials and Methods. In addition, we published a tutorial of this procedure in Bio-protocol (39).

Synthesis of Steroids. Synthesis of steroids is described in detail in SI Appendix, Materials and Methods.

Rescue Experiments. Petals and sepals of flowers from stages 9 to 14 (13) were gently opened and pistils were immersed in a working solution containing either 24-epibrassinolide (Sigma), homobrassinolide, or homocastasterone at 1 μM or a mock solution containing 1:1,000 DMSO for 5 s every 24 h until stage 14. Mature siliques were then analyzed to score the number of ovule aborts present in each one.

Quantification and Statistical Analysis. For each assay, presented values are the mean ± SD of the mean. For each experiment, n represents the number of pistils analyzed from at least three different plants. Statistical tests performed are detailed in each experiment (40). Numbers of repetitions and replicates are mentioned for each experiment.

Data Availability. All study data are included in the article and/or SI Appendix.

ACKNOWLEDGMENTS. We thank Dr. Rita Gross-Hardt’s lab for the marker lines pAt::NILS.GUS, pCKX::NLS.GUS, pMEA::NILS.GUS, and pEC1::NILS.GUS; Dr. Ueli Grossniklaus for the marker lines ET1119 and ET884; Dr. Venkatesan Sundaresan for the antipodal marker pAt1g36340::GUS and spf line; Dr. Gary Drews for the central cell marker DDB5; and Dr. Miklos Szekeres for the cdp line. We thank Dr. Mario Artega Vazquez, who was very generous sharing his father design for the “Kobulienz,” the ovule aspirator. We also thank Daniel Villamonte for technical assistance with confocal microscopy and lab members for helpful discussions. This research was funded by grants to G.C.P. from Agencia Nacional de Promocion Cientifica y Tecnica Argentina (PICT-2016-0382), and a grant to D.F.F. from Agencia Nacional de Promocion Cientifica y Tecnica Argentina (PICT-2017-0323). A.M.B. is a postdoctoral fellow of Consejo Nacional de Investigaciones Cientificas y Tecnicas (CONICET); N.S. and M.M.C. are doctoral fellows of CONICET, and D.F.F., E.J.Z., A.M.D., and G.C.P. are CONICET researchers. The work was also supported by the Ministry of Education, Youth and Sports of the Czech Republic (European Regional Development Fund Project “Plants as a Tool for Sustainable Global Development,” CZ.02.1.01/0.0/0.0/16_019/0000827).
1. E. L. Vickery, Molecular recognition and electron transfer in mitochondrial steroid hydrolyase systems. Steroids 62, 124–127 (1997).

2. K. M. Even, M. Kleser, R. Bernhardt, Adrenodoxin: The archetype of vertebra-type (2Fe-2S) cluster ferredoxins. Biochim. Biophys. Acta 1814, 111–125 (2011).

3. W. L. Miller, Minireview: Regulation of steroidogenesis by electron transfer. Endocrinology 146, 2544–2550 (2005).

4. M. R. Freeman, A. Dobritsa, P. Gaines, W. A. Segraves, J. R. Carlson, The dare gene: Steroid hormone production, olfactory behavior, and neural degeneration in Drosophila. Development 126, 4591–4602 (1999).

5. K. Takubo et al., Identification and molecular characterization of mitochondrial ferredoxins and ferredoxin reductase from Arabidopsis. Plant Mol. Biol. 52, 817–830 (2003).

6. A. Picciocchi, R. Douce, C. Alban, The plant biotin synthase reaction: Identification and characterization of essential mitochondrial accessory protein components. J. Biol. Chem. 278, 24966–24975 (2003).

7. G. C. Pagnussat et al., Genetic and molecular identification of genes required for female gametophyte development and function in Arabidopsis. Development 132, 603–614 (2005).

8. M. V. Martín, D. F. Fiol, V. Sundaresan, E. J. Zabaleta, G. C. Pagnussat, ovula, a female gametophytic mutant impaired in a mitochondrial manganese-superoxide dismutase, reveals crucial roles for reactive oxygen species during embryo sac development and fertilization in Arabidopsis. Plant Cell 25, 1573–1591 (2013).

9. J. M. Alonso et al., Genome-wide insertion mutagenesis of Arabidopsis thaliana. Science 301, 653–657 (2003).

10. G. Lazar, H. M. Goodman, MAX1, a regulator of the plant cell death response. Proc. Natl. Acad. Sci. U.S.A. 103, 472–476 (2006).

11. S. Bak et al., Cytochromes P450. Arabidopsis Book 9, e0144 (2011).

12. J. Mathur et al., Transcription of the Arabidopsis CYP gene, encoding a cytochrome P450, is negatively controlled by brassinosteroids. Plant J. 14, 593–602 (1998).

13. J. R. Bowman, G. N. Drews, E. M. Meyerowitz, Expression of the Arabidopsis floral homeotic gene AGAMOUS is restricted to specific cell types late in flower development. Plant Cell 3, 749–758 (1991).

14. W. C. Yang, D. Ye, J. Xu, V. Sundaresan, The SPOROCYTELESS gene of Arabidopsis is required for initiation of sporogenesis and encodes a novel nuclear protein. Genes Dev. 13, 2108–2117 (1999).

15. T. Schneider, R. Dinkins, K. Robinson, J. Shellhammer, D. W. Weinke, An embryo-lethal mutant of Arabidopsis thaliana is a biotin auxotroph. Dev. Biol. 131, 161–167 (1989).

16. R. Muraïa et al., A bifunctional locus (BIO3-BIO1) required for biotin biosynthesis in Arabidopsis. Plant Physiol. 146, 60–73 (2008).

17. P. J. Bakkes et al., Design and improvement of artificial redox modules by molecular fusion of flavodoxin and flavodoxin reductase from Escherichia coli. Sci. Rep. 5, 12158 (2015).

18. D. L. Catalano-Dupuy, M. A. Musumeci, A. Lopez-Rivero, E. A. Ceccarelli, A highly stable plastidic-type ferredoxin-NAD(P)H reductase in the pathogenic bacterium Leptospira interrogans. PLoS One 6, e26736 (2011).

19. M. F. Portereiko et al., NUCLEAR FUSION DEFECTIVE1 encodes the Arabidopsis RPL21M protein and is required for karyogamy during female gametophyte development and fertilization. Plant Physiol. 141, 957–965 (2006).

20. C. Kagi, N. Baumann, N. Nielsen, Y.-D. Stierhof, R. Gross-Hardt, The gametic central cell of Arabidopsis determines the lifespan of adjacent accessory cells. Proc. Natl. Acad. Sci. U.S.A. 107, 22350–22355 (2010).

21. C. A. Christensen et al., Mitochondrial GFA2 is required for synergized cell death in Arabidopsis. Plant Cell 14, 2215–2222 (2002).

22. Y. Bi et al., Production of reactive oxygen species, impairment of photosynthetic function and dynamic changes in cadmium-induced cell death in Arabidopsis thaliana. Biol. Cell 101, 629–643 (2009).

23. P. Lindemann et al., A novel Arabidopsis thaliana protein is a functional peripheral-type benzodiazepine receptor. Plant Cell Physiol. 45, 723–733 (2004).

24. L. S. Clouse, Brassinosteroids. Arabidopsis Book 9, e0151 (2011).

25. S. H. Joo, M. S. Jang, M. K. Kim, J. E. Lee, S. K. Kim, Biosynthetic relationship between C29-brassinosteroids and C29-brassinosteroids in rice (Oryza sativa) seedlings. Phytochemistry 111, 84–90 (2015).

26. T. W. Kim et al., Arabidopsis CYP95A2, a cytochrome P450, mediates the Baeyer-Villiger oxidation of castasterone to brassinoide in brassinosteroid biosynthesis. Plant Cell 17, 2397–2412 (2005).

27. J. L. Nemhauser, T. C. Mockler, J. Chory, Interdependency of brassinosteroid and auxin signaling in Arabidopsis. PLoS Biol. 2, e258 (2004).

28. K. Tanaka et al., Physiological roles of brassinosteroids in early growth of Arabidopsis: Brassinosteroids have a synergistic relationship with gibberelins as well as auxin in light-grown hypocotyl elongation. J. Plant Growth Regul. 22, 259–271 (2003).

29. F. Lozano-Elena, A. Planas-Riverola, J. Vilarasa-Blasi, R. Schwab, A. J. Cano-Delgado, Paracrine brassinosteroid signaling at the stem cell niche controls cellular regeneration. J. Cell Sci. 131, jcs204065 (2018).

30. W. B. Jiang, W. H. Lin, Brassinosteroid functions in Arabidopsis seed development. Plant Signal. Behav. 8, e25928 (2013).

31. M. K. Chizhov et al., Brassinosteroid production and signaling differentially control cell division and expansion in the leaf. New Phytol. 197, 490–502 (2013).

32. M. Catterou et al., Brassinosteroids, microtubules and cell elongation in Arabidopsis thaliana. II. Effects of brassinosteroids on microtubules and cell elongation in the bul1 mutant. Planta 212, 673–683 (2001).

33. X. Liu et al., Brassinosteroid regulate pavement cell growth by mediating BIN2-induced microtubule stabilization. J. Exp. Bot. 69, 1037–1049 (2018).

34. Z. Zhang et al., Brassinosteroids regulate plasma membrane anion channels in addition to proton pumps during expansion of Arabidopsis thaliana cells. Plant Cell Physiol. 46, 1594–1594 (2005).

35. G. C. Pagnussat, H. J. Yu, V. Sundaresan, Cell-fate switch of synergid to egg cell in Arabidopsis eostre mutant embryo sac arises from misexpression of the BEL1-like homeodomain gene BLH1. Plant Cell 19, 3578–3592 (2007).

36. M. D. Curtis, U. Grossniklaus, A Gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol. 133, 462–469 (2003).

37. C. Grefer et al., A novel motif essential for SNARE interaction with the K(+) channel KC1 and channel gating in Arabidopsis. Plant Cell 22, 3076–3092 (2010).

38. M. V. Martin, D. F. Fiol, E. J. Zabaleta, G. C. Pagnussat, Arabidopsis thaliana embryo sac mitochondrial membrane potential stain. Bio Protoc. 4, e1128 (2014).

39. R Core Team, R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria, 2019). https://www.R-project.org/.