# Suspensor Length Determines Developmental Progression of the Embryo in Arabidopsis[1][W][OA]

Yashodar Babu, Thomas Musielak, Agnes Henschen, and Martin Bayer*

Department of Cell Biology, Max Planck Institute for Developmental Biology, Spemannstrasse 35, 72076 Tuebingen, Germany

ORCID ID: 0000-0001-5806-2253 (M.B).

The first structure that differentiates during plant embryogenesis is the extra-embryonic suspensor that positions the embryo in the lumen of the seed. A central role in nutrient transport has been ascribed to the suspensor in species with prominent suspensor structures. Little is known, however, about what impact the size of the rather simple Arabidopsis (*Arabidopsis thaliana*) suspensor has on embryogenesis. Here, we describe mutations in the predicted *exo*-polygalacturonase gene *NIMNA* (*NMA*) that lead to cell elongation defects in the early embryo and markedly reduced suspensor length. Mutant *nma* embryos develop slower than wild-type embryos, and we could observe a similar developmental delay in another mutant with shorter suspensors. Interestingly, for both genes, the paternal allele has a stronger influence on the embryonic phenotype. We conclude that the length of the suspensor is crucial for fast developmental progression of the embryo in Arabidopsis.

Annual, self-pollinating weeds such as Arabidopsis (*Arabidopsis thaliana*) benefit from a short life cycle in their natural ephemeral habitats (Snell and Aarssen, 2005). A rapid progression through embryogenesis is a prerequisite for early seed maturation, and it is therefore not surprising that Arabidopsis sets up its body plan already after a very limited number of cell divisions (Aarssen, 2000; Lau et al., 2012).

Arabidopsis embryogenesis starts with the fertilized egg cell, the zygote, which elongates about 3-fold before it divides asymmetrically. The smaller apical cell is the founder of the embryo proper that contributes to most of the later seedling, while the larger basal cell develops into a support structure called the suspensor (Jeong et al., 2011a). The suspensor is formed by a series of transverse cell divisions followed by longitudinal cell expansion forming a stalk-like structure. Only the upper-most suspensor cell, the hypophysis, will contribute to parts of the root meristem, while the rest of the suspensor remains extraembryonic and will cease its growth at the heart stage of the embryo (Yeung and Meinke, 1993). The suspensor is thought to be important for pushing the embryo into the lumen of the seed, where the embryo is surrounded by the nourishing endosperm. In addition, a key function in nutrient and hormone transport to the embryo is assigned to the suspensor (Kawashima and Goldberg, 2010).

The Arabidopsis suspensor achieves its maximum length with a minimum number of cells by having a rod-shaped structure built by a single cell file. Although several mutants with distorted or shorter suspensors have been described in Arabidopsis, little is known about what impact suspensor length has on embryo development (Schwartz et al., 1994; Vernon and Meinke, 1994; Lukowitz et al., 2004; Breuninger et al., 2008; Bayer et al., 2009; Jeong et al., 2011b).

As in all plant cells, the size and shape of suspensor cells is primarily determined by the elasticity of the cell wall. While rigid cellulose microfibrils determine the direction of cell expansion, it is the pectin matrix that plays a major role in determining the stiffness of the cell wall (Peaucelle et al., 2012). Several pectin-modifying enzymes have been described that modulate the elasticity of the cell wall. Among these, pectin methyl esterases that determine the degree of homo-galacturonan methylation seem to be major players (Palin and Geitmann, 2012). Pectin-degrading enzymes such as polygalacturonases (PGs) have been implicated with cell expansion, but their role in this process is much less clear (Hadfield and Bennett, 1998).

The pectin-rich middle lamella serves as cement to aid cell adhesion, and it is therefore not surprising that several PG genes are involved in dehiscence and abscission events (Rhee et al., 2003; González-Carranza et al., 2007, 2012; Ogawa et al., 2009). The expression profile of many PG genes correlates temporally and spatially with cell elongation and therefore implies a role in cell expansion (Sitrit et al., 1999). The exact function of PGs during cell elongation is still unclear, and the lack of elongation defects in loss-of-function mutants further complicates matters.

Here, we describe mutations in a PG gene, which we called *NIMNA* (*NMA*), that severely affect cell elongation in the embryo and suspensor.

We demonstrate that embryos with shorter suspensors show a significant delay in development during embryogenesis, possibly caused by reduced access to nutrients from the endosperm. Interestingly, reciprocal crosses reveal an unequal parental contribution to *NMA* function in cell elongation of the suspensor.

## RESULTS

In a forward genetic screen for embryonic mutants, we isolated a candidate with abnormal suspensor development. In this mutant, suspensors were severely reduced in length and embryos were positioned in the micropylar niche, instead of being pushed into the lumen of the seed (Fig. 1). We therefore called the mutant *nimna* (*nma*) after the Sanskrit word for "sunken."

Differences between mutant and wild-type embryos become apparent directly after fertilization. In *nma* mutants, the anisotropic cell elongation of the zygote is reduced, resulting in shorter but wider daughter cells (Fig. 1A). Initially, cells of the embryo proper and the suspensor are affected in cell elongation, but cells in the proembryo recover by early heart stage, while the cells of the suspensor remain short and wide (Fig. 1).

The short-suspensor phenotype is primarily caused by shorter cells and not by reduced cell number in the suspensor, pointing toward a function of *NMA* in cell elongation (Fig. 1, C and D).

We were able to recover homozygous seedlings, and adult plants show no apparent differences from the wild type (Supplemental Fig. S1).

### Embryos with Shorter Suspensor Show a Lag in Developmental Progression

When studying the *nma* phenotype in detail, we noticed an apparent developmental delay in *nma* embryos compared with the wild type.

To determine the developmental stages of embryos at a defined time point, we emasculated and self pollinated *nma-1*–/− mutants and wild-type plants and analyzed the resulting embryos 4 d after pollination (dap). To ensure that fertilization of the analyzed embryos took place in a narrow time window, we only analyzed 10 to 20 neighboring ovules from the upper half of each silique. In our analysis, embryos were classified into six developmental stages: midglobular, late globular, triangular, early heart, late heart, and early torpedo (Fig. 2A).

Compared with the wild type, *nma* embryos consistently appeared to be at earlier developmental stages. At 4 dap, the majority of wild-type embryos reached early heart stage, while most *nma* embryos belonged to late globular or triangular stage. By using numerical values for the different developmental stages, the average for *nma* embryos (2.47 ± 0.35; *n* = 385) was significantly

lower than the average for the wild type (3.79 ± 0.33; *n* = 322; Student's *t* test, *P* < 0.01; Fig. 2B). This indicates that the *nma* mutation affects developmental progression of the embryo in addition to cell elongation. This might be a consequence of lower nutrient allocation to the embryo due to reduced suspensor or embryo surface in contact with the endosperm.

To test if this developmental delay is caused by the reduced suspensor size or depends on a *NMA* function in the embryo proper, we analyzed the developmental progression of embryos in another mutant with shorter suspensors.

The *SHORT SUSPENSOR* (*SSP*) gene functions in the YODA (YDA) mitogen-activated protein kinase pathway to determine aspects of suspensor identity (Lukowitz et al., 2004; Bayer et al., 2009). Although *ssp* embryos have shorter suspensors like *nma* embryos, the cause of the reduced size is different. The majority of *ssp* mutant suspensors are shorter than the wild type because they consist of fewer cells, while the average cell size is unaffected (Bayer et al., 2009). We therefore considered *ssp* mutants as good candidates to test if suspensor size affects the timing of embryonic development.

As with *nma* mutants, we self pollinated *ssp-2*–/− mutants and compared the developmental stages of embryos 4 dap to those of wild-type plants pollinated at the same time. As in *nma* mutants, we can observe a significant lag in developmental progression in mutant embryos (Fig. 2B).

Our classification of embryonic developmental stages was based on the morphology of the embryo. We therefore had to avoid possibly misinterpreting an altered morphology in the mutant embryo as a younger developmental stage. To make sure that *ssp* and *nma* embryos do show a delay in development and not just a change in overall shape, we counted, for each genotype, the cells in a central optical section of embryos 3 dap (Supplemental Fig. S2). Both *nma* and *ssp* mutant embryos show significantly fewer cells in the proembryo than the corresponding wild-type embryos, indicating that they develop slower than their wild-type counterparts (*P* < 0.001 in Mann-Whitney *U* test).

This could indicate that the reduced suspensor length in these mutants is responsible for the delay in development of the embryo. Alternatively, delayed fertilization in these mutants could cause the observed difference in development. Because *NMA* functions in cell elongation and is expressed in pollen, slower pollen tube growth in the mutant would be a likely scenario. We therefore measured the pollen tube length of in vitro-germinated *nma-1* and wild-type pollen. After 6 h of inoculation in pollen tube growth medium, the average length of *nma* pollen tubes (134 ± 56 μm; *n* = 189) did not differ significantly from the average length of wild-type pollen tubes (129 ± 46 μm; *n* = 146; *P* > 0.79 in Mann-Whitney *U* test; Supplemental Fig. S3). In addition, we analyzed the segregation of the mutant allele in progeny of reciprocal crosses between wild-type plants and *nma* heterozygous plants. There was no
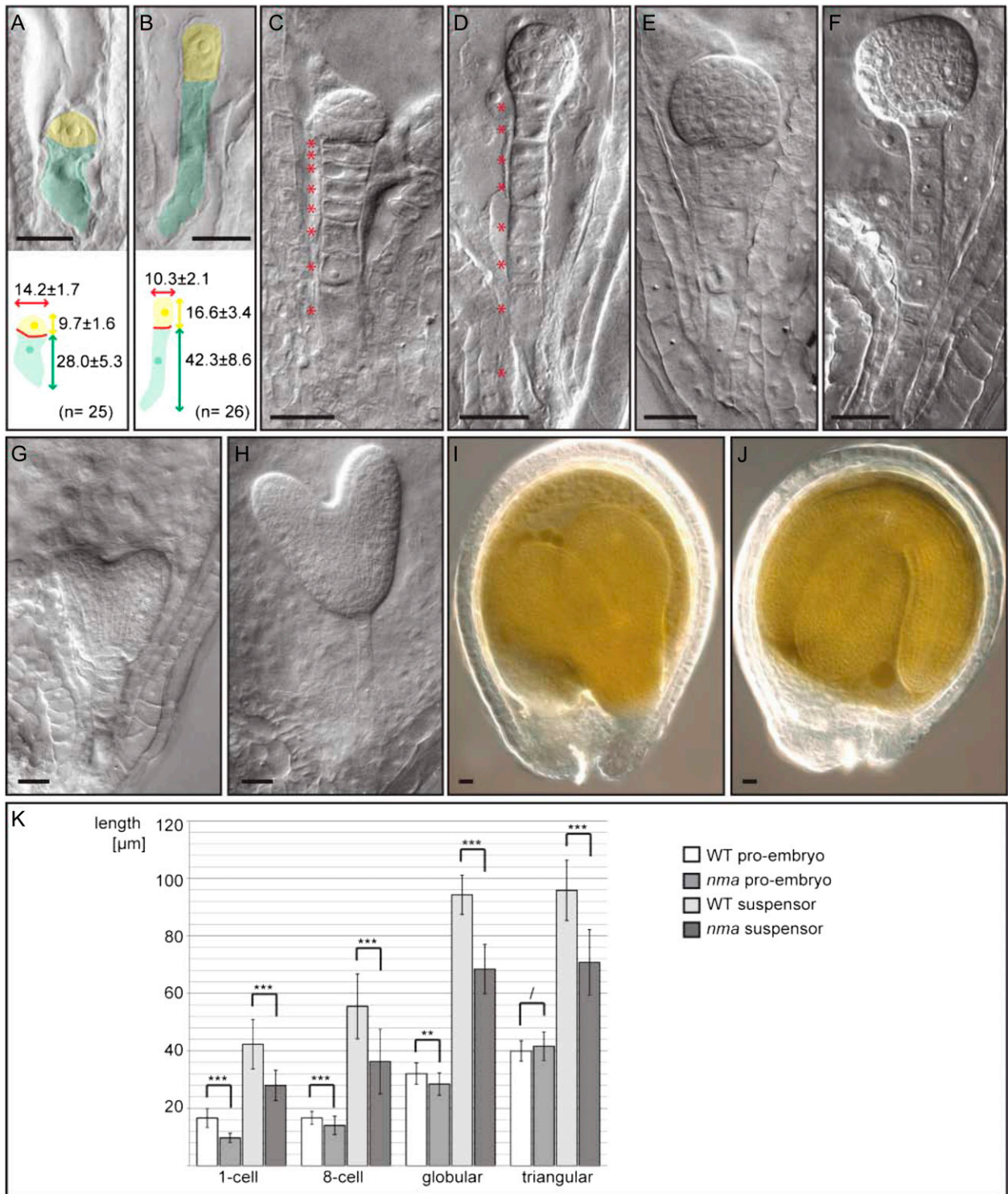
**Figure 1.** Embryonic phenotype of *nma* mutants compared with the wild type. A and B, Embryonic phenotype at one-cell stage of *nma* (A) and the wild type (B). Apical cells are false colored in yellow; basal cells are false colored in green. Measurements are given as average with sd in micrometers. C and D, Embryonic phenotype at 16-cell stage of *nma* (C) and the wild type (D). Suspensor cells are highlighted by accompanying asterisks. E and F, Late globular stage embryos of *nma* (E) and the wild type (F). G and H, Heart stage embryos of *nma* (G) and the wild type (H). I and J, Developing seed of *nma* (I) and the wild type (J) 7 dap. Bars = 20 $\mu$m. K, Measurements of embryo proper and suspensor length at different developmental stages. Number of analyzed embryos at one-cell stage: *nma*, $n = 26$ and the wild type, $n = 26$. Number at eight-cell stage: *nma*, $n = 101$ and the wild type, $n = 98$. Number at globular stage: *nma*, $n = 36$ and the wild type, $n = 29$. Number at

significant difference to the expected one-to-one segregation ratio of *nma+/−* and wild-type plants in the offspring of *nma+/− ×* wild-type crosses (90 *nma+/−* and 79 wild-type seedlings) and wild-type *× nma+/−* crosses (76 *nma+/−* and 97 wild-type seedlings; chi-square test showed no significant difference to expected segregation at 5% level), respectively. These results strongly suggest that the developmental delay we observe in *nma* embryos is not caused by slower pollen tube growth and delayed fertilization. For *ssp*, it was also shown that there is no significant divergence from Mendelian segregation in a F2 population, suggesting no effect of this mutation on pollen tube growth (Bayer et al., 2009). To test if the observed lag in development of *ssp* and *nma* mutants arises during embryogenesis and not during the fertilization process, we analyzed developmental stages of embryos 30 h after pollination in a similar manner as we have done before at 4 dap (Fig. 2C). Embryos were classified into three developmental stages with assigned numerical values: zygote stage (1), one-cell stage (2), and two-/four-cell stage (3). In differential interference contrast images of cleared ovules, it is not always easy to distinguish the two- and four-cell stage of the embryo proper. We therefore combined these in a single developmental stage to avoid possible mistakes.

At this early time point of embryogenesis, there is no significant difference in development between *nma*, *ssp*, and the corresponding wild-type embryos, respectively (*nma-1*/ecotype Landsberg *erecta* [Ler], *P* > 0.05; *ssp-2*/ecotype Columbia [Col-0], *P* > 0.05 in Mann-Whitney *U* test; Fig. 2C).

These results clearly indicate that the observed differences in developmental progression are not caused by delayed fertilization but arise during the course of embryogenesis.

## NMA Function Has a Stronger Paternal Contribution

Mutations in *ssp* show a paternal effect on embryogenesis. This unusual parent-of-origin effect was discussed in the light of a parental conflict regarding nutrient allocation to the embryo (Bayer et al., 2009). Because *nma* embryos are also affected in developmental progression, we wondered if *NMA* function might also be under paternal control. To test for any parent-of-origin effects, we performed reciprocal crosses between *nma-1* and wild-type plants. We could observe significantly shorter suspensors in crosses where *nma* was introduced from the paternal side (wild type *× nma*) compared with crosses from the maternal side (*nma ×* wild type) or crosses of wild-type plants (wild type *×* wild type; *P* < 0.001 in Mann-Whitney *U* test; Supplemental Fig. S4).

Homozygous *nma* embryos exhibit even shorter suspensors than the paternal cross. This argues for a dosage dependency of the suspensor phenotype with an unequal parental contribution (Supplemental Fig. S4).

## NMA Codes for a Predicted *Exo*-PG

We identified the affected gene in *nma-1* by map-based cloning (Lukowitz et al., 2000). Bulk segregant analysis as well as rough mapping positioned *NMA* on chromosome 2 in a 380-kb interval between marker T32F6 and F4P9. Screening 4,002 chromosomes for recombinations in this interval, we were able to recover 17 F2 plants that defined a 153-kb interval between markers 255-X (At2g33255) and 5B2-1 (At2g32870) with no further recombination (Supplemental Fig. S5).

Sequencing of coding regions in this interval revealed a G-to-A substitution at nucleotide position +836 in the protein coding sequence of At2g33160. This point mutation leads to an amino acid substitution of a conserved Gly to Asp at position 279 in the predicted PG protein sequence (Supplemental Fig. S6). We were able to confirm this candidate gene by analyzing additional transfer DNA (T-DNA) insertion alleles. SALK_126968 (*nma-2*) and SALK_015991 (*nma-3*) exhibit embryonic phenotypes that are basically indistinguishable from *nma-1* (Supplemental Fig. S7). We could not detect transcripts downstream of the insertion site in *nma-3* by reverse transcription (RT)-PCR, which indicated either low expression or that this line is possibly a null allele (data not shown). To confirm the predicted *NMA* gene model, we performed RT-PCR and 3′ RACE, which revealed an incorrectly annotated splice donor site in the TAIR10 release. The corrected gene model contains a single intron and codes for a protein with a predicted N-terminal signal peptide (SP) followed by a single PG domain (Supplemental Fig. S6). The corrected gene model will be submitted to The Arabidopsis Information Resource (TAIR).

We tested the mapping result by genetic complementation with a genomic fragment covering the coding region of At2g33160, including 2.5 kb upstream of the translational start codon and 1.6 kb downstream of the stop codon. In addition, we generated a variant of the above construct that carries a yellow fluorescent protein (YFP) at the N terminus of the mature protein after cleavage of the predicted SP. Both constructs complemented the embryonic phenotype of *nma-1*, further confirming that At2g33160 is the affected gene (all 17 transgenic lines carrying *pNMA::NMA*, and 27 out of 30 transgenic lines carrying *pNMA::YFP-NMA* complemented the phenotype; Supplemental Fig. S8).

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**Figure 1.** (*Continued.*)
triangular stage: *nma*, *n* = 54 and the wild type, *n* = 33. Mean values with sd are shown. Significant differences were determined in pairwise comparison by Mann-Whitney *U* test (* = *P* < 0.05, ** = *P* < 0.01, *** = *P* < 0.001, and / = *P* > 0.05). WT, Wild type.
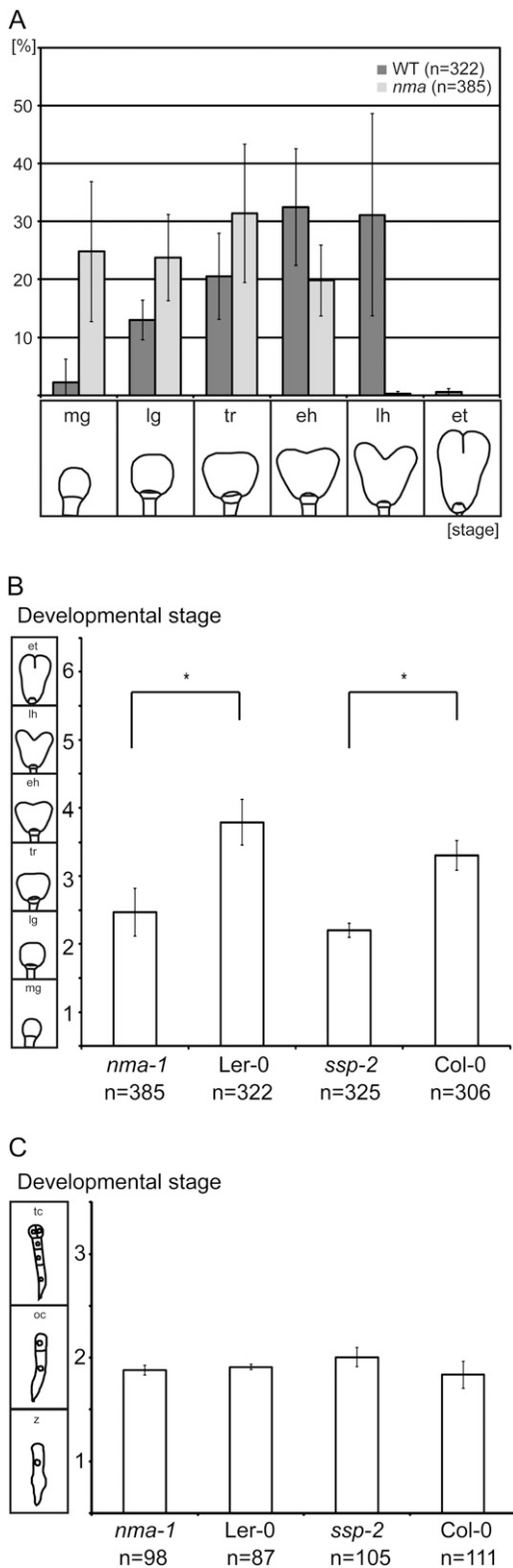
**Figure 2.** Developmental stages of embryos. A, Wild-type and *nma* embryos 4 dap were classified into six developmental stages: mid-globular (mg), late globular (lg), triangular (tr), early heart (eh), late heart (lh), and early torpedo (et). Schematic depictions of the developmental stage are given below the graphs. Mean values of three independent

## NMA Is Predominantly Expressed in Reproductive Tissue

Mutant *nma* plants show obvious aberrant phenotypes only during embryogenesis. We therefore wondered if this is due to a very restricted expression pattern of *NMA*. Expression of redundant genes outside the embryo could also account for the temporal and spatial restriction of the phenotype. To monitor *NMA* expression with cellular resolution, we transcriptionally fused a 2.5-kb promoter fragment to nuclear-localized triple YFP (Fig. 3).

After fertilization, *NMA* promoter activity can be observed in all cells of the embryo proper and the suspensor with similar fluorescence intensity (Fig. 3A). At late globular stage, the expression in the proembryo is reduced, while it stays strong in the suspensor (Fig. 3B). From heart stage onwards, *NMA* promoter activity is restricted to cells of the cotyledons, stele, quiescent center, and columella, as well as suspensor cells (Fig. 3C).

In the endosperm, p*NMA::n3xYFP* is initially expressed throughout at a very high level but becomes restricted to the chalazal endosperm from late heart stage onwards (Fig. 3D).

Because the expression of *NMA* can be detected already in the zygote, we also analyzed expression of the reporter gene in male and female gametophytes. *YFP* expression can be detected in all three cells of the mature pollen, with highest levels in the sperm cells (Fig. 3E). In the female gametophyte, *YFP* expression can be observed after meiosis in the functional megaspore and the nuclei of the developing gametophyte (Fig. 3, F and G). After cellularization, the central cell shows very strong signal, while egg cell, synergids, and antipodal remainder exhibit much weaker expression (Fig. 3H).

To observe *NMA* expression in seedlings and adult plants, we made a *pNMA::GUS* transcriptional fusion. Strong GUS expression can be detected as expected in pollen and young ovules (Fig. 3I). Additionally, GUS activity is also present in expanding leaf margins and root vasculature (Fig. 3J).

We corroborated this data by measuring *NMA* expression in various tissue types by quantitative RT-PCR (Supplemental Fig. S9). *NMA* transcripts accumulate to high levels in reproductive tissue, such as flowers, pollen, and siliques. Very low level of expression can be detected throughout the plant. This data are also in agreement with publicly available microarray data (Supplemental Fig. S10; Schmid et al., 2005; Winter et al., 2007).

biological replicates with sd are shown. B, Average developmental stage of embryos 4 dap in *nma-1*, Ler, *ssp-2*, and Col-0. Embryos were classified in six developmental stages as in Figure 2A. Numerical values were assigned to each stage. Averages and sds of three biological replicates are shown as bar graphs. Asterisks indicate sds in pairwise comparison (Mann-Whitney *U* test; *P* < 0.001). C, Average developmental stage of embryos 30 h after pollination in *nma-1*, Ler, *ssp-2*, and Col-0. Embryos were classified in three developmental stages: zygote (Z), one-cell (oc), and two- and four-cell (tc). Numerical values were assigned to each stage. Averages and sds of three biological replicates are shown as bar graphs.
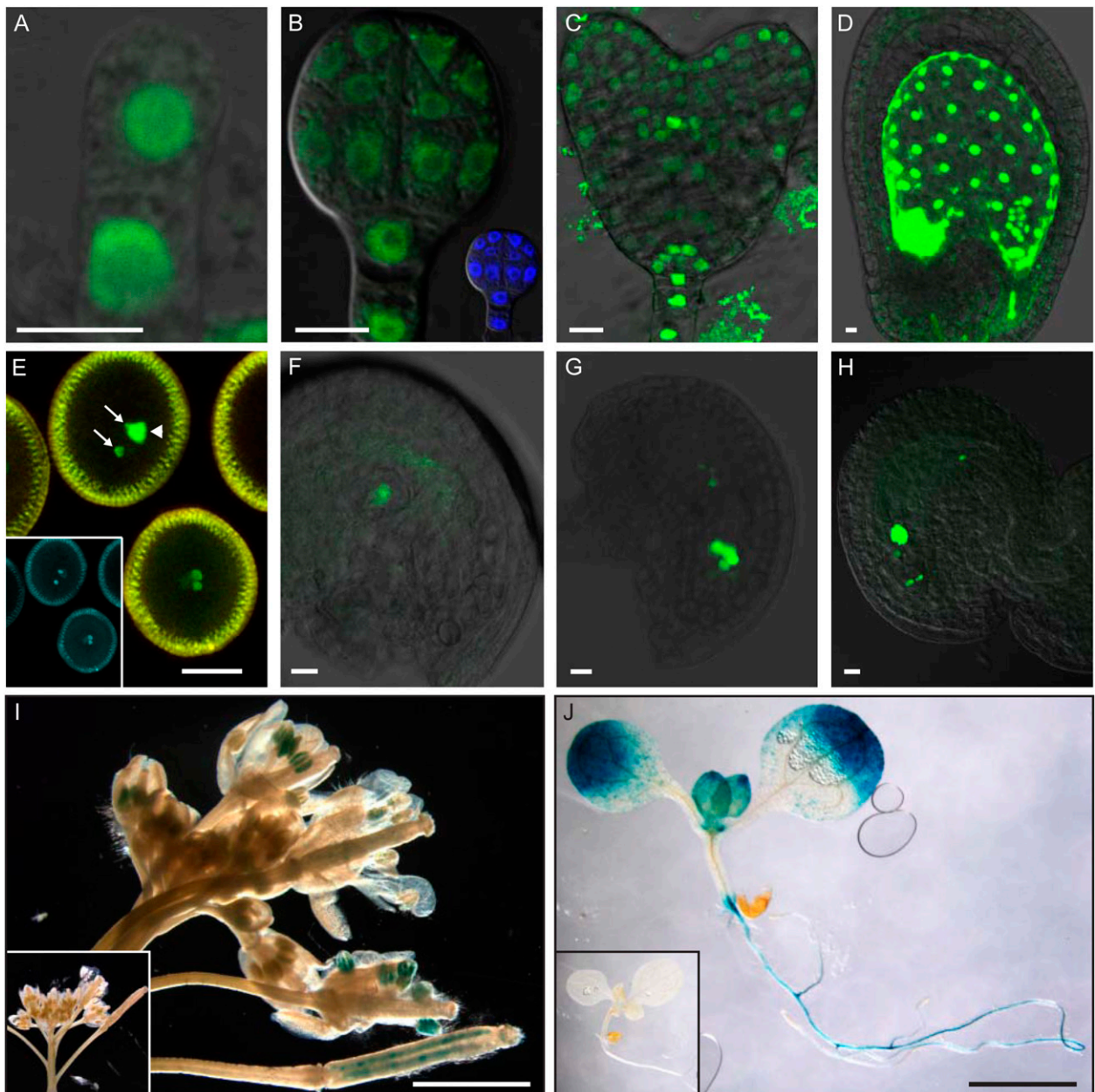
**Figure 3.** Expression analysis of *NMA* promoter::reporter gene constructs. A to H, Fluorescence micrographs of transgenic plants carrying a transcriptional fusion of a *NMA* promoter fragment to nuclear-localized triple Venus-YFP. A, Embryo after the first zygotic division. B, Globular-stage embryo, inset shows 4′,6-diamidino-2-phenylindole (DAPI) staining. C, Heart-stage embryo. D, Developing seed 2 dap. E, Pollen, arrows mark sperm cell nuclei and arrowhead indicates vegetative cell nucleus; inset shows DAPI staining. F, Female gametophyte after meiosis. G, Female gametophyte at eight-nuclei stage. H, Mature female gametophyte. Bars = 10 $\mu$m. I and J, GUS activity staining in transgenic plants carrying a transcriptional *pNMA::GUS* fusion construct. I, Inflorescence. J, Seedling. Insets in I and J show transgenic control plants without *GUS* gene. Bars = 2 mm.

## *NMA* Acts Cell Autonomously in Suspensor Cell Elongation

The strong expression of *NMA* in the endosperm raises the question of whether the cell elongation defect in the embryo is caused in a cell-autonomous matter or if it is possibly an indirect effect of the central cell restricting the growth of the embryo.

We therefore expressed a translational *YFP*-fusion of *NMA* under the control of tissue-specific promoters in *nma-1* background and tested for complementation of the mutant phenotype. The *OBF BINDING PROTEIN1* (*OBP1*) promoter is active in the embryo proper and suspensor but not in the endosperm during early embryogenesis (Supplemental Fig. S11; Skirycz et al.,

2008), and *pOBP1::YFP-NMA* expression rescued the cell elongation defects in the suspensor in *nma-1* (Supplemental Fig. S8). On the other hand, *YFP-NMA* expression under the control of the *SUCROSE PROTON SYMPORTER5* (*SUC5*) promoter, which is active in the endosperm but not the embryo during early stages of embryogenesis (Baud et al., 2005; Pommerrenig et al., 2013), could not complement the *nma-1* suspensor phenotype. These results argue in favor of a cell-autonomous function of *NMA* in the embryo. We then expressed *YFP-NMA* under the control of the *J10* promoter, which is exclusively active in the suspensor during early stages of development (Supplemental Fig. S11; J. Kong and G. Jürgens, personal communication). *pJ10::YFP-NMA* expression was sufficient to rescue the short-suspensor phenotype of *nma-1* embryos (Supplemental Fig. S8). Again, this strongly suggests a cell-autonomous function of *NMA* in cell elongation of the suspensor.

### Suspensor-Specific Expression of *NMA* Is Sufficient to Rescue the Developmental Delay of *nma* Embryos

Our results indicate that NMA acts cell autonomously in cell elongation. Expressing *YFP-NMA* suspensor specifically under the control of the J10 promoter in *nma–/–* mutants leads to wild-type-looking suspensors. The cells of the embryo proper, on the other hand, still display cell elongation defects because *YFP-NMA* is not expressed in this domain in the transgenic *pJ10::YFP-NMA* plants. We therefore considered the *pJ10::YFP-NMA* transgenic line as a good background to test our hypothesis that reduced suspensor length causes slower development of the embryo.

We analyzed the developmental stage of embryos in *pJ10::YFP-NMA* transgenic *nma–/–* plants compared with *nma* and wild-type plants 4 dap in a similar way as we had before. Expressing *YFP-NMA* only in the suspensors of *nma–/–* mutants rescues the developmental delay of the embryo proper (Supplemental Figure S12).

By using numerical values for the different developmental stages, the average for *pJ10::YFP-NMA* embryos (3.01 ± 0.09; n = 374) was significantly higher than for *nma* embryos (2.48 ± 0.29; n = 500; Student's *t* test $P <$ 0.05) but did not completely reach the wild-type level (3.61 ± 0.27; n = 1102).

This result indicates that in the context of developmental progression, NMA activity in the suspensor affects noncell autonomously the development of the embryo proper. We therefore conclude that the length of the suspensor critically influences the development of the embryo.

### NMA Protein Localizes to the Cell Wall

The predicted pectin-modifying function of NMA would suggest an apoplastic localization of the protein.

SignalP4.0 predicts a SP with cleavage site between amino acids 21 and 22 at the N terminus of the NMA protein sequence (discrimination score D = 0.798), which would target the protein to the secretory pathway (Petersen et al., 2011). To test the functional requirement of this predicted SP, we made two YFP-tagged versions of NMA under the control of the gene's own regulatory sequences. In one case, we fused the YFP directly at the N terminus (*pNMA::YFP-NMA dSP*), which displaces the SP from the N terminus and therefore disrupts its presumed function (Rapoport, 2007). In the other case, we introduced YFP after the predicted cleavage site (*pNMA::YFP-NMA*).

Only the latter version was able to complement the embryonic mutant phenotype when introduced in *nma-1* plants (27 out of 30 transgenic lines). The former version did not show any complementation in 12 independent transgenic lines. To test whether this sequence is responsible for targeting the protein to the apoplast, we replaced the *NMA* promoter in these two constructs with the strong constitutive *Cauliflower mosaic virus* 35S promoter for transient expression in protoplasts (Fig. 4).

The version with intact SP was only detectable as tiny dots on the surface of the protoplasts, while the variant with disrupted SP gave strong cytoplasmic YFP fluorescence 24 h after transformation. Seventy-two hours after transformation, when the protoplasts were reforming cell walls, we could detect strong YFP fluorescence on the surface of the protoplasts in patches of newly formed call walls (Fig. 4) with the construct carrying an intact SP. This signal could not be detected if the SP was disrupted or in untransformed protoplasts. The variant with disrupted SP still showed cytoplasmic YFP localization at this time point (Fig. 4).

We were not able to detect the protein in vivo using the functional YFP-tagged version under the control of the native promoter, probably due to low steady-state protein levels. The cell wall localization of NMA is supported by a proteome study of cell wall proteins in etiolated hypocotyls. The authors of this study detected fragments of the NMA protein in cell wall extracts (Irshad et al., 2008).

### DISCUSSION

Embryonic suspensors of flowering plants exist in a wide range of sizes and shapes. Historically, the suspensor was thought to just anchor the embryo within the seed, although some reports already speculated about a function as temporary embryonic root (for review, see Yeung and Meinke, 1993).

The suspensor is the first tissue that differentiates during embryogenesis, and biochemical, physiological, and anatomical studies suggest a role in nutrient supply to the embryo (for review, see Kawashima and Goldberg, 2010). Studies in *Phaseolus vulgaris* and *Phaseolus coccineus* clearly demonstrated active transport
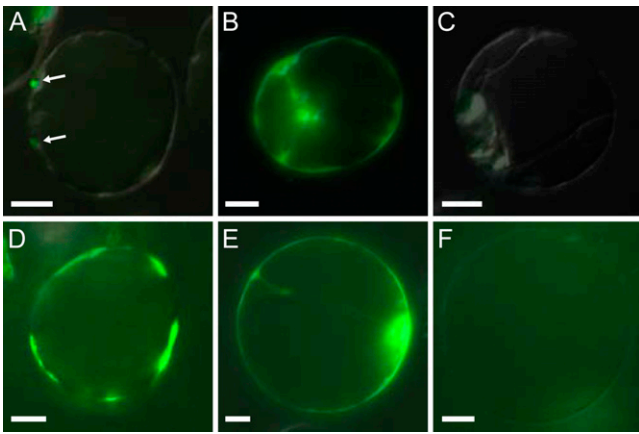
**Figure 4.** Transient expression of YFP-NMA fusion proteins in protoplasts. A to C, 24 h after transformation. D to F, 72 h after transformation. A and D, *p35S::YFP-NMA* fusion construct with intact SP sequence. Arrows in A point to dotted signals on protoplast surface. B and E, *p35S::YFP-NMA dSP* fusion construct with disrupted SP sequence. C and F, Untransformed control. Bars = 10 µm.

of nutrients and a growth-promoting function of the suspensor (Cionini et al., 1976; Yeung and Sussex, 1979; Yeung, 1980; Nagl, 1990). In cases where the endosperm supplies few nutrients to the embryo, the suspensor can be large and branched and form haustorium-like structures into the maternal tissue (Wardlaw, 1955); however, these are specialized cases with seeds that contain little endosperm. It is therefore questionable if the physiology and function of the relatively simple Arabidopsis suspensor can be compared to these large suspensors. Although molecular transporters for nutrients and hormones are also expressed in the Arabidopsis suspensor, it is still a matter of debate if, in this case, the suspensor also functions in allocating nutrients to the embryo (Friml et al., 2003; Meyer et al., 2004; Hruz et al., 2008).

We demonstrated that *nma* and *ssp* mutant embryos with shorter suspensors show an obvious developmental delay. The two genes have fundamentally different functions, and the mutant phenotypes do not resemble each other. The reduced size of the suspensor is the result of different developmental defects in the two mutants. While in *nma*, the reduced size is a consequence of failed cell elongation, *ssp* embryos possess suspensors with reduced cell number (Bayer et al., 2009). The observed developmental delay can therefore not be caused by a common function in the embryo proper. The one thing both mutations have in common is the diminished size of the suspensor. Furthermore, we could show that there is no obvious delay in fertilization in these mutants and that early embryos at 30 h after pollination show no difference in their development compared with wild-type embryos.

We therefore conclude that the developmental delay observed in both mutants is likely caused by the reduced suspensor length. This is supported by the fact that suspensor-specific expression of *NMA* in *nma*–/–

background is sufficient to partially rescue the developmental delay of the *nma* embryo proper. This strongly argues in favor of the suspensor length influencing the developmental progression of the embryo. The *J10* promoter becomes active only from the eight-cell stage of the embryo onwards (approximately 2 dap). This might explain why we observe only a partial rescue in this experiment.

An important role of the suspensor in nutrient and hormone transport to the embryo has been shown in *Phaseolus* spp. (Yeung, 1980). It is therefore possible that the reduced size of these mutant Arabidopsis suspensors impairs nutrient allocation to the embryo. In this scenario, a lack of nutrient availability could be the cause for slower growth of the embryo. It is unclear though if the lack of nutrients is a consequence of the embryo not being sufficiently pushed into the nourishing endosperm or because there is less suspensor surface to take up nutrient and transport them to the embryo.

Reciprocal crosses demonstrate that the paternal allele of *NMA* has a stronger contribution to suspensor length than the maternal allele. Although this effect is rather mild, this unequal parental contribution is unmasked by a dosage dependency. *NMA* is expressed at higher levels in sperm cells and much lower levels in the egg cell, based on the fluorescence intensity of promoter-reporter gene fusions. Unequal amounts of RNA and/or protein carried over from the gametes during fertilization might be an easy explanation for the different phenotypic strengths seen in reciprocal crosses. It is interesting to observe that the *NMA* gene that affects suspensor length also shows a paternal effect that seems to rely on a similar mechanism as the paternal effect of the *ssp* mutant (Bayer et al., 2009). Although this is far from conclusive evidence, it is tempting to speculate if there might be an evolutionary incentive to bring suspensor size and therefore efficient nutrient transport to the embryo under paternal control. It would be an elegant way to balance the maternal control over endosperm proliferation (Spillane et al., 2007). Further studies will be needed to address this question in the light of the parental conflict theory.

*NMA* displays highest expression levels in reproductive tissue and only low level in vegetative tissue. This expression pattern is common for many plant PGs that have been classified as clade C PGs (Torki et al., 2000). Based on the relative high *exo*-PG activity of pollen protein extracts, it was assumed that class C PGs are mainly *exo*-PGs (Pressey, 1991; Barakate et al., 1993). NMA carries all known conserved residues that characterize the protein as a putative PG. These are namely the NTD motif at position 201, GDD at position 223, GHG at position 246, and RIK at position 281, which are conserved residues found in all plant PGs and are diverged in other family 28 hydrolases, such as rhamnogalacturonases or xyloglucan hydrolases (Markovic and Janecek, 2001).

So far, all Arabidopsis PG genes for which a loss-of-function phenotype has been described have been

classified as *endo*-PG and are involved in cell separation and abscission events (Rhee and Somerville, 1998; Rhee et al., 2003; González-Carranza et al., 2007; Ogawa et al., 2009). The lack-of-mutant phenotypes for *exo*-PG genes in Arabidopsis was thought to be the consequence of high genetic redundancy in this multigene family (Hadfield and Bennett, 1998; Kim et al., 2006).

Notably, *nma* loss-of-function mutations lead to a strong embryonic phenotype, although there seems to be a large number of PG genes that have generally overlapping expression patterns with *NMA* (Kim et al., 2006). This could indicate that in the embryo proper, for a short time period after fertilization and in the suspensor in general, there are no other active PGs present. Alternatively, this could also indicate different substrate preferences and/or functions for coexpressed PG family proteins. A more detailed study of expression patterns for these genes with cellular resolution would be necessary to address this question. The embryonic phenotype of *nma* makes it possible to study specificity of predicted *exo*-PG genes in the future in complementation assays by expressing various PG-coding regions under the control of *NMA* regulatory sequences in a *nma* mutant background.

## CONCLUSION

*NMA* codes for a predicted *exo*- PG that is involved in cell elongation during early embryogenesis. Loss-of-function mutations in *NMA* lead to shorter suspensors and a developmental delay in the embryo. This lag in embryonic development can be seen in other embryonic mutants with shorter suspensors. We therefore conclude that the size of the suspensor is critical for efficient developmental progression during Arabidopsis embryogenesis. This study further suggests that there might be an incentive to bring development of the suspensor under paternal control.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana* Col-0 and L*er*) were used in this study. Plants were grown under long-day conditions (16 h at 3-kilolux illumination, 8-h-dark period) in walk-in chambers at 23°C and 65% relative humidity on commercial potting mix (Topferde CL T, Einheitserde) containing systemic insecticide added with the first watering (Confidor WG70, 200 $\mu$g L$^{-1}$; Bayer CropScience).

T-DNA alleles of *nma* and *ssp* (*nma-2*, SALK_126968; *nma-3*, SALK_015991; *ssp-2*, SALK_051462; Alonso et al., 2003) were obtained from the Nottingham Arabidopsis Stock Center. T-DNA insertion lines were genotyped by PCR using primers LBb1.3 and 160-3 R for *nma-3*, LBb1.3 and SSP1-R for *ssp-2*, and SSP1-R and SSP1-I for the *SSP* allele. A derived cleaved amplified polymorphic sequences marker was designed to genotype *nma-1* using primers NMA dC-F and NMA dC-R. The PCR product of the mutant allele carries a restriction site for *Swa*I. Sequences of primers can be found in the supplementary material (Supplemental Table S1).

### Genetic Screen for Paternal Effect Mutations

*nma-1* was found in a forward genetic screen for paternal effect mutations. Ethyl methanesulfonate-mutagenized M2 L*er* plants (Lehle Seeds) were used

individually as pollen donors to manually pollinate conditionally sterile *oxophytodienoate-reductase3* plants (Stintzi and Browse, 2000). Seed set was monitored, and dissected immature seeds were cleared in Hoyer's solution for microscopic analysis of embryonic phenotypes 3 dap as described previously (Bayer et al., 2009). A comprehensive summary of the screen will be published elsewhere.

### Mapping of *nma-1*

Map-based cloning of nma-1 was performed as described by Lukowitz et al. (2000). Rough mapping positioned *nma-1* on chromosome 2 in a 380-kb interval between marker T32F6 and F4P9. Screening 4,002 chromosomes for recombinations in this interval, we were able to recover 17 F2 plants that defined a 153,640-bp interval between markers 255-X (At2g33255) and 5B2-1 (At2g32870) with no further recombination. Coding regions in this interval were amplified by PCR and sequenced.

### Microscopic Analysis of Embryos, Measurements, and Reporter Gene Analysis

Immature seeds were dissected and cleared in Hoyer's solution as described previously (Bayer et al., 2009) and analyzed using a Zeiss Axio Imager.Z1 with an AxioCam HRc camera. Size measurements were performed using measurement tools of the Axiovision software. Fluorescence microscopy on Olympus FV1000 and Zeiss LSM510 and LSM780 NLO confocal microscopes and GUS activity staining was performed as described previously (Nawy et al., 2010). GUS staining patterns were analyzed using a Zeiss SteREO Discovery.V12 with the Axiovision software.

### In Vitro Germination of Pollen and Measurements of Pollen Tubes

Pollen was germinated as described (Boavida and McCormick, 2007), except that the agarose concentration was reduced to 1%. Pollen germination was performed on microscope slides incubated in moist chambers at 23°C for 6 h. Measurements were performed as described above for immature seeds.

### Transcript and Expression Analysis

RNA was extracted from various plant tissues using the RNeasy Plant Mini Kit (Qiagen). Synthesis of complementary DNA and quantitative real-time PCR was conducted as described previously (Lau et al., 2011) using primers qRT-F and qRT-R (Supplemental Table S1). For expression analysis in the wild type, *nma-2*, and *nma-3*, primers RT-5F and RT-5R, NRT-F3 and NRT-R4, and RT 160.2 F and RT 160.2 R were used (Supplemental Table S1). Three-prime RACE was conducted by using the ExactSTART Eukaryotic mRNA 5'- and 3'-RACE Kit (Epicentre).

### Molecular Complementation and Plasmid Constructs

For molecular complementation, a 5.7-kb genomic fragment harboring At2g33160 was PCR-amplified using primers At2g33160P-FC and At2g33160-RC and subsequently cloned into pGreen II 0229 (Hellens et al., 2000). To be able to distinguish transgenes from the endogenous locus, in all other *NMA* constructs, the intron was removed by site-directed PCR mutagenesis using primers NMAi1-F and NMAi1-R. To obtain translational fusions of citrine YFP with NMA, *Asc*I and *Pac*I restriction sites were introduced either before or right after the predicted SP sequence by site-directed mutagenesis using primers NMAi1 AP-F and NMAi1 AP-R or NMAi1 ASP-F and NMAi1 ASP-R, respectively. Subsequently, an YFP fragment was cloned into these previously introduced restriction sites, generating *pNMA::YFP-NMA dSP* and *pNMA::YFP-NMA*. To express *NMA* in a tissue-specific manner, we replaced the endogenous promoter sequence in *pNMA::YFP-NMA* with PCR-amplified promoter fragments of *OBP1* (Skirycz et al., 2008), *SUC5* (Baud et al., 2005), and *J10* (At5g42200; J. Kong and G. Jürgens, personal communication; Supplemental Table S1). For transient expression in rotoplasts, we replaced the endogenous promoter sequence in pNMA::YFP-NMA and pNMA::YFP-NMA dSP by a PCR-amplified *Cauliflower mosaic virus* 35S promoter (Table S1x).

The *NMA* promoter used in the complementation constructs was PCR-amplified with primers At2g33160P-FC and At2g33160P-RC and inserted in a pGreen variant carrying nuclear-localized 3xVenus-YFP or GUS, respectively.

## Transient Expression in Protoplasts

Transient expression of p35S::YFP-NMA and p35S::YFP-NMA dSP was carried out in protoplasts derived from an Arabidopsis Col-0 dark-grown cell suspension culture, which were transfected as previously described (Schütze et al., 2009). YFP fluorescence was observed 24 and 72 h after transfection by confocal microscopy as described above.

## Supplemental Data

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

**Supplemental Figure S1.** Adult phenotype of *nma-1* (L*er* background) and *nma-3* (Col-0 background).

**Supplemental Figure S2.** Number of cells in a central optical section of the embryo proper 3 dap.

**Supplemental Figure S3.** Pollen tube length of in vitro-germinated pollen.

**Supplemental Figure S4.** Measurements of suspensor length after reciprocal crosses of wild-type (L*er*) and *nma-1* mutant plants.

**Supplemental Figure S5.** Fine mapping of *nma-1*.

**Supplemental Figure S6.** Graphic representation of the *NMA* gene and protein.

**Supplemental Figure S7.** Embryonic phenotype of T-DNA insertion alleles of *nma*.

**Supplemental Figure S8.** Complementation assay based on suspensor length.

**Supplemental Figure S9.** Quantitative RT-PCR data of *NMA* expression in various tissue types.

**Supplemental Figure S10.** Graphic representation of NMA expression data in publicly available microarray data.

**Supplemental Figure S11.** Expression of promoter::reporter gene fusions.

**Supplemental Figure S12.** Average developmental stage of embryos 4 dap in *pJ10::YFP-NMA*, *nma-1*, and L*er*-0.

**Supplemental Table S1.** Primer sequences.

## ACKNOWLEDGMENTS

## LITERATURE CITED

Aarssen LW (2000) Why are most selfers annuals? A new hypothesis for the fitness benefit of selfing. Oikos **89:** 606–612

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science **301:** 653–657

Barakate A, Martin W, Quigley F, Mache R (1993) Characterization of a multigene family encoding an exopolygalacturonase in maize. J Mol Biol **229:** 797–801

Baud S, Wuillème S, Lemoine R, Kronenberger J, Caboche M, Lepiniec L, Rochat C (2005) The AtSUC5 sucrose transporter specifically expressed in the endosperm is involved in early seed development in Arabidopsis. Plant J **43:** 824–836

Bayer M, Nawy T, Giglione C, Galli M, Meinnel T, Lukowitz W (2009) Paternal control of embryonic patterning in *Arabidopsis thaliana*. Science **323:** 1485–1488

Boavida LC, McCormick S (2007) Temperature as a determinant factor for increased and reproducible in vitro pollen germination in *Arabidopsis thaliana*. Plant J **52:** 570–582

Breuninger H, Rikirsch E, Hermann M, Ueda M, Laux T (2008) Differential expression of WOX genes mediates apical-basal axis formation in the Arabidopsis embryo. Dev Cell **14:** 867–876

Cionini PG, Bennici A, Alpi A, Damato F (1976) Suspensor, gibberellin and invitro development of *Phaseolus coccineus* embryos. Planta **131:** 115–117

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G (2003) Efflux-dependent auxin gradients establish the apical-basal axis of Arabidopsis. Nature **426:** 147–153

González-Carranza ZH, Elliott KA, Roberts JA (2007) Expression of polygalacturonases and evidence to support their role during cell separation processes in *Arabidopsis thaliana*. J Exp Bot **58:** 3719–3730

González-Carranza ZH, Shahid AA, Zhang L, Liu Y, Ninsuwan U, Roberts JA (2012) A novel approach to dissect the abscission process in Arabidopsis. Plant Physiol **160:** 1342–1356

Hadfield KA, Bennett AB (1998) Polygalacturonases: many genes in search of a function. Plant Physiol **117:** 337–343

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000) pGreen: a versatile and flexible binary Ti vector for *Agrobacterium*-mediated plant transformation. Plant Mol Biol **42:** 819–832

Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. Adv Bioinforma **2008:** 420747

Irshad M, Canut H, Borderies G, Pont-Lezica R, Jamet E (2008) A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: confirmed actors and newcomers. BMC Plant Biol **8:** 94

Jeong S, Bayer M, Lukowitz W (2011a) Taking the very first steps: from polarity to axial domains in the early Arabidopsis embryo. J Exp Bot **62:** 1687–1697

Jeong S, Palmer TM, Lukowitz W (2011b) The RWP-RK factor GROUNDED promotes embryonic polarity by facilitating YODA MAP kinase signaling. Curr Biol **21:** 1268–1276

Kawashima T, Goldberg RB (2010) The suspensor: not just suspending the embryo. Trends Plant Sci **15:** 23–30

Kim J, Shiu SH, Thoma S, Li WH, Patterson SE (2006) Patterns of expansion and expression divergence in the plant polygalacturonase gene family. Genome Biol **7:** R87

Lau S, De Smet I, Kolb M, Meinhardt H, Jürgens G (2011) Auxin triggers a genetic switch. Nat Cell Biol **13:** 611–615

Lau S, Slane D, Herud O, Kong J, Jürgens G (2012) Early embryogenesis in flowering plants: setting up the basic body pattern. Annu Rev Plant Biol **63:** 483–506

Lukowitz W, Gillmor CS, Scheible WR (2000) Positional cloning in Arabidopsis. Why it feels good to have a genome initiative working for you. Plant Physiol **123:** 795–805

Lukowitz W, Roeder A, Parmenter D, Somerville C (2004) A MAPKK kinase gene regulates extra-embryonic cell fate in Arabidopsis. Cell **116:** 109–119

Markovic O, Janecek S (2001) Pectin degrading glycoside hydrolases of family 28: sequence-structural features, specificities and evolution. Protein Eng **14:** 615–631

Meyer S, Lauterbach C, Niedermeier M, Barth I, Sjolund RD, Sauer N (2004) Wounding enhances expression of AtSUC3, a sucrose transporter from Arabidopsis sieve elements and sink tissues. Plant Physiol **134:** 684–693

Nagl W (1990) Translocation of putrescine in the ovule, suspensor and embryo of *Phaseolus coccineus*. J Plant Physiol **136:** 587–591

Nawy T, Bayer M, Mravec J, Friml J, Birnbaum KD, Lukowitz W (2010) The GATA factor HANABA TARANU is required to position the pro-embryo boundary in the early Arabidopsis embryo. Dev Cell **19:** 103–113

Ogawa M, Kay P, Wilson S, Swain SM (2009) ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and

QUARTET2 are polygalacturonases required for cell separation during reproductive development in Arabidopsis. Plant Cell **21**: 216–233

**Palin R, Geitmann A** (2012) The role of pectin in plant morphogenesis. Biosystems **109**: 397–402

**Peaucelle A, Braybrook S, Höfte H** (2012) Cell wall mechanics and growth control in plants: the role of pectins revisited. Front Plant Sci **3**: 121

**Petersen TN, Brunak S, von Heijne G, Nielsen H** (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods **8**: 785–786

**Pommerrenig B, Popko J, Heilmann M, Schulmeister S, Dietel K, Schmitt B, Stadler R, Feussner I, Sauer N** (2013) SUCROSE TRANSPORTER 5 supplies Arabidopsis embryos with biotin and affects triacylglycerol accumulation. Plant J **73**: 392–404

**Pressey R** (1991) Polygalacturonase in tree pollens. Phytochemistry **30**: 1753–1755

**Rapoport TA** (2007) Protein translocation across the eukaryotic endoplasmic reticulum and bacterial plasma membranes. Nature **450**: 663–669

**Rhee SY, Osborne E, Poindexter PD, Somerville CR** (2003) Microspore separation in the *quartet 3* mutants of Arabidopsis is impaired by a defect in a developmentally regulated polygalacturonase required for pollen mother cell wall degradation. Plant Physiol **133**: 1170–1180

**Rhee SY, Somerville CR** (1998) Tetrad pollen formation in quartet mutants of *Arabidopsis thaliana* is associated with persistence of pectic polysaccharides of the pollen mother cell wall. Plant J **15**: 79–88

**Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU** (2005) A gene expression map of *Arabidopsis thaliana* development. Nat Genet **37**: 501–506

**Schütze K, Harter K, Chaban C** (2009) Bimolecular fluorescence complementation (BiFC) to study protein-protein interactions in living plant cells. Methods Mol Biol **479**: 189–202

**Schwartz BW, Yeung EC, Meinke DW** (1994) Disruption of morphogenesis and transformation of the suspensor in abnormal suspensor mutants of Arabidopsis. Development **120**: 3235–3245

**Sitrit Y, Hadfield KA, Bennett AB, Bradford KJ, Downie AB** (1999) Expression of a polygalacturonase associated with tomato seed germination. Plant Physiol **121**: 419–428

**Skirycz A, Radziejwoski A, Busch W, Hannah MA, Czeszejko J, Kwaśniewski M, Zanor MI, Lohmann JU, De Veylder L, Witt I, et al** (2008) The DOF transcription factor OBP1 is involved in cell cycle regulation in *Arabidopsis thaliana*. Plant J **56**: 779–792

**Snell R, Aarssen LW** (2005) Life history traits in selfing versus outcrossing annuals: exploring the 'time-limitation' hypothesis for the fitness benefit of self-pollination. BMC Ecol **5**: 2

**Spillane C, Schmid KJ, Laoueillé-Duprat S, Pien S, Escobar-Restrepo JM, Baroux C, Gagliardini V, Page DR, Wolfe KH, Grossniklaus U** (2007) Positive Darwinian selection at the imprinted MEDEA locus in plants. Nature **448**: 349–352

**Stintzi A, Browse J** (2000) The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA **97**: 10625–10630

**Torki M, Mandaron P, Mache R, Falconet D** (2000) Characterization of a ubiquitous expressed gene family encoding polygalacturonase in *Arabidopsis thaliana*. Gene **242**: 427–436

**Vernon DM, Meinke DW** (1994) Embryogenic transformation of the suspensor in twin, a polyembryonic mutant of Arabidopsis. Dev Biol **165**: 566–573

**Wardlaw CW** (1955) Embryogenesis in Plants. Wiley, London.

**Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ** (2007) An "Electronic Fluorescent Pictograph" browser for exploring and analyzing large-scale biological data sets. PLoS ONE **2**: e718

**Yeung EC** (1980) Embryogeny of *Phaseolus*: the role of the suspensor. Zeitschrift Fur Pflanzenphysiologie **96**: 17–28

**Yeung EC, Meinke DW** (1993) Embryogenesis in angiosperms: development of the suspensor. Plant Cell **5**: 1371–1381

**Yeung EC, Sussex IM** (1979) Embryogeny of *Phaseolus coccineus*: the suspensor and the growth of the embryo-proper in vitro. Zeitschrift Fur Pflanzenphysiologie **91**: 423–433