The Plant Cell, Vol. 20: 2631–2642, October 2008, www.plantcell.org © 2008 American Society of Plant Biologists

NAC Family Proteins NARS1/NAC2 and NARS2/NAM in the Outer Integument Regulate Embryogenesis in *Arabidopsis* [W][OA]

Tadashi Kunieda,[a,b] Nobutaka Mitsuda,[b,c] Masaru Ohme-Takagi,[b,c] Seiji Takeda,[d] Mitsuhiro Aida,[d] Masao Tasaka,[d] Maki Kondo,[e] Mikio Nishimura,[e] and Ikuko Hara-Nishimura[a,b,1]

[a] Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
[b] Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan
[c] Research Institute of Genome-Based Biofactory, National Institute of Advanced Industrial Science and Technology, Tsukuba 305-8562, Japan
[d] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma 630-0192, Japan
[e] Department of Cell Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan

Seed morphogenesis consists of embryogenesis and the development of maternal tissues such as the inner and outer integuments, both of which give rise to seed coats. We show that expression of chimeric repressors derived from NAC-REGULATED SEED MORPHOLOGY1 and -2 (NARS1 and NARS2, also known as NAC2 and NAM, respectively) caused aberrant seed shapes in *Arabidopsis thaliana*. Double knockout mutant *nars1 nars2* exhibited abnormally shaped seeds; moreover, neither *nars1* nor *nars2* produced abnormal seeds, indicating that NARS1 and NARS2 redundantly regulate seed morphogenesis. Degeneration of the integuments in *nars1 nars2* was markedly delayed, while that of the wild type occurred around the torpedo-shaped embryo stage. Additionally, *nars1 nars2* showed a defect in embryogenesis: some *nars1 nars2* embryos were developmentally arrested at the torpedo-shaped embryo stage. Unexpectedly, however, neither NARS1 nor NARS2 was expressed in the embryo at this stage, although they were found to be expressed in the outer integument. Wild-type pistils pollinated with *nars1 nars2* pollen generated normal seeds, while the reverse crossing generated abnormal seeds. Taken together, these results indicate that NARS1 and NARS2 regulate embryogenesis by regulating the development and degeneration of ovule integuments. Our findings suggest that there is an intertissue communication between the embryo and the maternal integument.

INTRODUCTION

In angiosperm seeds, the embryo and endosperm are surrounded by the seed coat, which protects the embryo from mechanical stress and pathogen infections. The seed coat consists of two integuments (the outer and inner integuments) of maternal tissues, and multiple cell layers of these integuments develop after fertilization, resulting in specialized structures of the seed coat. The epidermal cells (oi2) of *Arabidopsis thaliana* integuments are characterized by a deposition of pectic polysaccharide, called mucilage, around a columella, which is a volcano-shaped structure of secondary cell wall (Beeckman et al., 2000; Windsor et al., 2000; Western et al., 2001; Debeaujon et al., 2003), while the endothelial cells (ii1) are characterized by the production of flavonoids such as proanthocyanidin (Beeckman et al., 2000; Windsor et al., 2000; Western et al., 2001; Debeaujon et al., 2003). These substances of the seed coat are considered to play important roles in protecting the embryo from influences of UV radiation or toxic chemicals.

Morphological analysis of developing seeds revealed that formation of columellae in oi2 is associated with the progress of embryogenesis (Beeckman et al., 2000; Windsor et al., 2000). In the ii1 cell layer, proanthocyanidin starts to accumulate in a few cells located at the micropylar end (i.e., around the embryo), and later, proanthocyanidin accumulation spreads to all ii1 cells during early seed development (Debeaujon et al., 2003). These histochemical features in both oi2 and ii1 suggest that integument development is coordinated with embryogenesis. However, little is known about communication between the embryo and the surrounding integument tissues. By contrast, the intertissue communication between the endosperm and integuments is well characterized. Mutants of *fertilization-independent seed development1* to *-3* (*fis1* to *fis3*) develop endosperm and integuments without fertilization (Chaudhury et al., 1997), suggesting that the endosperm regulates the initiation of seed coat development. Cell elongation in the integuments is abolished in *haiku2* (Garcia et al., 2003), although HAIKU2 is a Leu-rich repeat kinase expressed specifically in the endosperm during early seed development (Luo et al., 2005).

Each cell layer of the integuments undergoes cell death at different times of development following maturation, suggesting that cell death is programmed as a part of seed coat

[1] Address correspondence to ihnishi@gr.bot.kyoto-u.ac.jp.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the instructions for authors (www.plantcell.org) is: Ikuko Hara-Nishimura (ihnishi@gr.bot.kyoto-u.ac.jp).
[W]Online version contains Web-only data.
[OA]Open Access articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.108.060160

development. Previously, we reported that two of the three cell layers of the inner integuments (ii2 and ii3) undergo programmed cell death (PCD) around the torpedo-shaped embryo stage, which leads to the formation of hard seed coats to protect the next generation (Nakaune et al., 2005). PCD is mediated by the action of VACUOLAR PROCESSING ENZYME (δVPE), which is a Cys proteinase with caspase 1 (i.e., YVADase) activity. δVPE is expressed in ii2 and ii3 and is transiently expressed around the torpedo-shaped embryo stage (Nakaune et al., 2005). However, the molecular mechanism of PCD in the integuments remains unknown. The integuments function as the maternal conduit to the embryo, supplying nutrients to the embryo (Weber et al., 1995; King et al., 1997; Sheen et al., 1999; Wobus and Weber, 1999). Disruption of the normal timing of integument PCD could be detrimental to embryogenesis. Therefore, the coordination of development between embryo and integuments is an important aspect of proper seed development.

To better understand the molecular mechanism underlying coordinated seed development, we applied a forward genetic approach with chimeric repressor gene–silencing technology (CRES-T) in *Arabidopsis*. CRES-T, which dominantly suppresses the transcription of genes regulated by the transcription factor of interest, is a useful technique for functional analysis of transcription factors (Hiratsu et al., 2003). We generated a CRES-T mutant library for 178 transcription factors in families of AP2, ARF, HD, MADS, MYB, NAC, and TCP and used the library to identify transcription factors responsible for embryogenesis and/or morphogenesis of seeds. We found that two NAC transcription factors, which we designated NAC-REGULATED SEED MORPHOLOGY1 and -2 (NARS1 and NARS2, previously known as NAC2 [Takada et al., 2001] and NAM [Duval et al., 2002], respectively), act redundantly to regulate seed morphogenesis. We have also provided evidence that NARS1 and NARS2 control embryogenesis by regulating the development of the integument around the torpedo-shaped embryo stage.

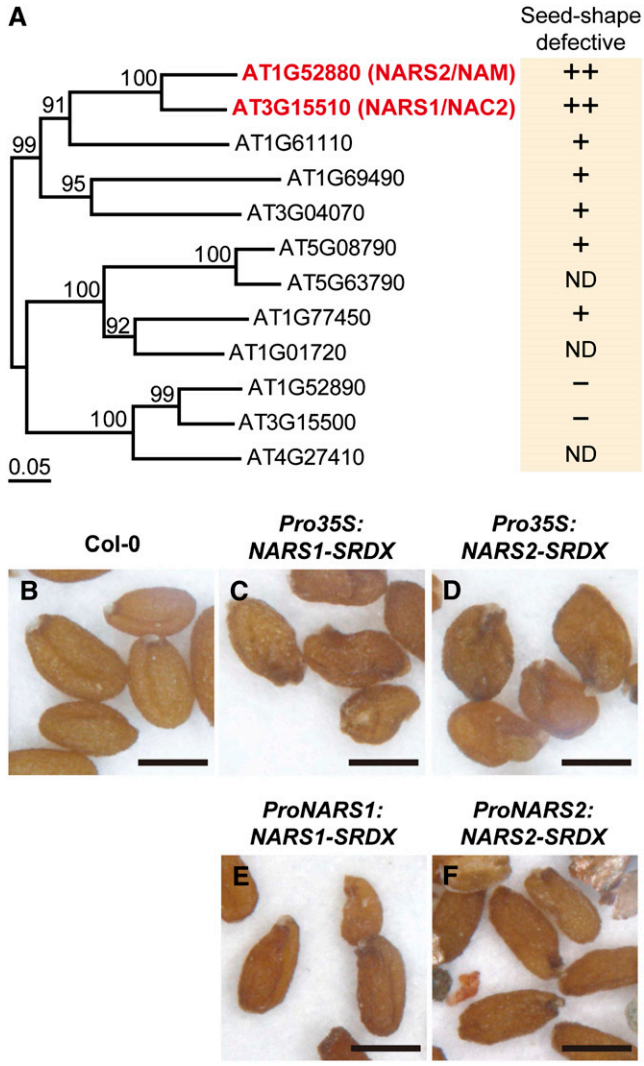


Figure 1. Forward Genetic Approach with the CRES-T Library Showed That NAC III Subfamily Genes Are Responsible for Seed Morphology of *Arabidopsis*.

(A) A phylogenetic tree of the NAC III subfamily. Some members of the family produced abnormally shaped seeds. The abnormality was evaluated by the frequency of seeds with defective shapes, which is shown in decreasing order of the abnormality as ++, +, and – (ND, not determined). Photographs of these seeds are shown in Supplemental Figure 1 online, and the alignment used to generate the tree is available as Supplemental Data Set 1 online. The CRES-T lines of two closely related members (At1g52880 and At3g15510) exhibited high frequencies of seed shape defects. These were designated NARS1 (At3g15510) and NARS2 (At1g52880).

(B) Dry seeds of the wild type. Bar = 300 μm.

(C) to **(F)** Abnormal morphology of seeds of CRES-T lines (*Pro35S:NARS1-SRDX* **[C]**, *Pro35S:NARS2-SRDX* **[D]**, *ProNARS1:NARS1-SRDX* **[E]**, and *ProNARS2:NARS2-SRDX* **[F]**). Bars = 300 μm.

RESULTS

Expression of Chimeric Repressors Derived from the NAC III Subfamily Causes Aberrant Morphogenesis of Seeds

We examined dry seeds of the CRES-T library using a stereoscopic microscope and isolated 32 mutant lines that had abnormally shaped seeds. The transcription factors responsible for these mutants belonged to the NAC family (10 mutants), the MYB family (7 mutants), the TCP family (5 mutants), the AP2 family (3 mutants), the MADS family (2 mutants), the HD family (2 mutants), the DOF family (2 mutants), and the GRAS family (1 mutant). Previously, we reported that members of the NAC family were classified into seven subgroups (Mitsuda et al., 2005). Abnormally shaped seeds were frequently found in the CRES-T lines of subgroup III of the NAC family (NAC III subfamily), especially in the lines of two closely related NAC III members (NAC2/At3g15510 and NAM/At1g52880) (Figure 1A; see Supplemental Figure 1 online). Here, we renamed these two genes *NARS1* and *NARS2* because of their functional redundancy for seed development (see below). Seeds of *Pro35S:NARS1-SRDX* (Figure 1C)

and *Pro35S:NARS2-SRDX* (Figure 1D) had rough shapes, whereas seeds of the wild type were more round (Figure 1B). NARS1 and NARS2 were localized in the nucleus and exhibited transactivation activity (see Supplemental Figure 2 online), indicating that NARS1 and NARS2 function as transcription factors.

To determine whether the seed shape abnormality of the two CRES-T mutants was caused by ectopic expression of *Pro35S:NARS1-SRDX* and *Pro35S:NARS2-SRDX*, we generated transgenic plants that expressed either *NARS1-SRDX* or *NARS2-SRDX* under the control of the predicted promoter region of *NARS1* (*ProNARS1:NARS1-SRDX*) or *NARS2* (*ProNARS2:NARS2-SRDX*), respectively. These transgenic plants produced aberrantly shaped seeds, as did the plants with *Pro35S:NARS1-SRDX* and *Pro35S:NARS2-SRDX* (Figures 1E and 1F). This result indicates that expression of chimeric NARS1 and NARS2 repressors causes aberrant morphogenesis of seeds.

NARS1 and NARS2 Regulate Seed Morphogenesis Redundantly

Previously, we reported that CRES-T suppresses not only the function of the respective transcription factor of interest but also the function of closely related transcription factors (Hiratsu et al., 2003). Since NARS1 and NARS2 are closely related, this raised the possibility that NARS1 and NARS2 chimeric repressors coincidentally suppressed the expression of genes regulated by NARS2 and NARS1, respectively. To determine which gene was responsible for aberrant seed morphogenesis, we isolated *nars1* knockout mutants (SM line, SM_3_28017) and *nars2* knockout mutants (WiscDs-Lox line, WiscDsLox364F11) (Figure 2A). RT-PCR revealed that *nars1* and *nars2* did not express transcripts of *NARS1* and *NARS2*, respectively (Figure 2B). Unexpectedly, however, neither *nars1* nor *nars2* exhibited any abnormality in seed shapes (Figures 2C and 2D).

To explore redundant regulation of seed morphology by *NARS1* and *NARS2*, we generated the double knockout mutant *nars1 nars2* by crossing *nars1* and *nars2*. *nars1 nars2* produced abnormally shaped, shrunken seeds (Figure 2E). The phenotype was similar to those of transgenic plants with *ProNARS1:NARS1-SRDX* and *ProNARS2:NARS2-SRDX* (Figures 1E and 1F). These results, together with the coexpression profiles of *NARS1* and *NARS2* (see Supplemental Figure 3 online) (Obayashi et al., 2007) and a high degree of amino acid sequence similarity between NARS1 and NARS2 in the NAC domain (Figure 1A), indicate that NARS1 and NARS2 redundantly regulate seed morphogenesis.

nars1 nars2 Delays Seed Development and Causes Partial Embryonic Lethality

Abnormal seed morphology appears to be determined during seed development. To understand how abnormal seeds of *nars1 nars2* developed, we observed the inner tissues of seeds at each seed development stage. Figures 3A to 3J show the developing embryos of both wild type and *nars1 nars2* at 6, 9, 12, and 15 d after pollination (DAP). Compared with the wild type, *nars1 nars2* showed two types of defects in embryogenesis. In one type, *nars1 nars2* embryo development was arrested at the torpedo-shaped embryo stage (Figures 3G and 3H), resulting in shrunken seeds (Figure 3H). In the other defect, *nars1 nars2* embryos underwent irregular bending without arrest at the torpedo-shaped embryo stage, resulting in deformed seeds (Figure 3J). These *nars1 nars2* morphological abnormalities were indistinguishable during early embryogenesis prior to the torpedo-shaped embryo stage.

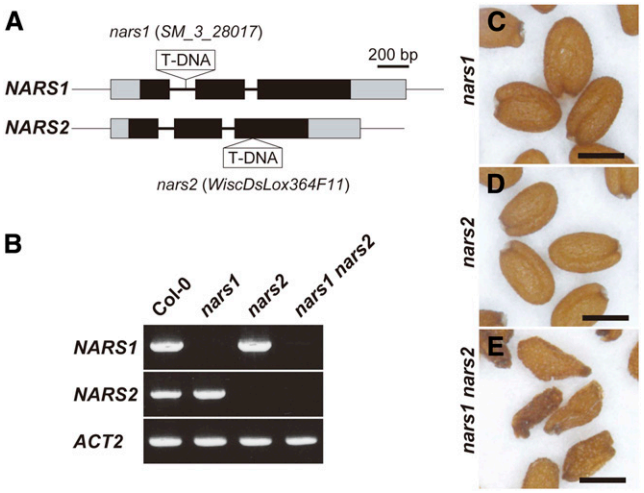


Figure 2. Double Knockout Mutation of *NARS1* and *NARS2* Causes Abnormally Shaped Seeds.

(A) Schematic representation of the *NARS1* and *NARS2* genes, both of which consist of three exons (black boxes) and two introns (thick lines) with untranslated regions (gray boxes). T-DNA insertion sites are shown in the *NARS1* (SM_3_28017) and *NARS2* (WiscDxLox364F11) genes.
(B) Double mutant *nars1 nars2* was generated by crossing T-DNA–inserted mutants (*nars1* and *nars2*). RT-PCR of 35 cycles showed that single mutants did not express their respective genes, while the double mutant expressed neither *NARS1* nor *NARS2*. *ACT2* was used as a loading control.
(C) to **(E)** Double mutant (*nars1 nars2* **[E]**) exhibited abnormal seed morphology, although single mutants (*nars1* **[C]** and *nars2* **[D]**) did not. Bars = 300 μm.

In addition to the morphological abnormalities described above, all of the *nars1 nars2* embryos tended to be delayed in development. The pollen tubes of *nars1 nars2* grew normally and succeeded in fertilization, showing that the delay of embryogenesis was caused some time following fertilization. To evaluate the delay of embryogenesis in *nars1 nars2*, we examined embryo development stages in siliques of the wild type and the mutant at 6 and 9 d following manual pollination (Figure 3K). Almost 75% of the seeds in wild-type siliques had torpedo-shaped embryos at 6 DAP, and ~93% of the seeds showed completed embryogenesis with full-sized embryos at 9 DAP (Figure 3K, dark gray bars). By contrast, in *nars1 nars2* siliques, ~89% of the seeds had heart-shaped embryos at 6 DAP, and ~75% of the seeds showed the torpedo-shaped embryo stage at 9 DAP (Figure 3K, light gray bars). Shrunken seeds resulting from a failure in embryogenesis were observed in 44% (n = 191) of *nars1 nars2* seeds (Figure 3L), and these seeds could not germinate, indicating that absence of *NARS1* and *NARS2* caused partial embryonic lethality.

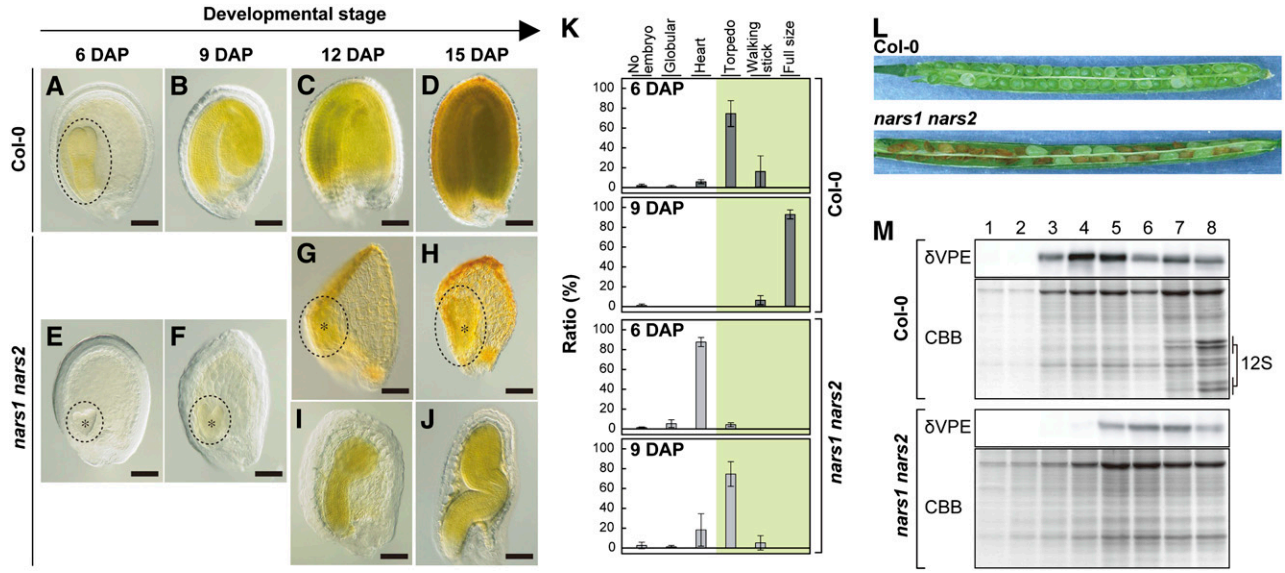


Figure 3. Delay of Seed Development and Embryonic Lethality of *nars1 nars2*.

(A) to **(J)** Developing seeds of the wild type (Col-0) and *nars1 nars2* were inspected at various stages after pollination using a differential interference contrast microscope. Asterisks and dotted circles show embryos. Bars = 100 μm.

(A) and **(E)** Seeds at 6 DAP. The wild type had a torpedo-shaped embryo **(A)**, while *nars1 nars2* had a heart-shaped embryo **(E)**.

(B) and **(F)** Seeds at 9 DAP. The wild type had an almost full-sized embryo **(B)**, while *nars1 nars2* had a heart-shaped or torpedo-shaped embryo **(F)**.

(C), **(G)**, and **(I)** Seeds at 12 DAP. The wild type had a full-sized embryo **(C)**. By contrast, *nars1 nars2* exhibited two types of seeds: one type was shrunken and brown **(G)** and the other type was at the torpedo-shaped embryo stage and apparently continued normal embryogenesis **(I)**.

(D), **(H)**, and **(J)** Seeds at 15 DAP. The wild type had a full-sized embryo with pigmented seed coats **(D)**. By contrast, *nars1 nars2* had two types of seeds, as at 12 DAP: in one type of seeds, embryogenesis was stopped at the torpedo-shaped embryo stage with browned seed coats and produced shrunken seeds **(H)**, while the other type of seeds had embryos with abnormal bending **(J)**.

(K) Comparison of wild-type (Col-0) and *nars1 nars2* embryo development showing the ratio of embryo types in the siliques at different ages. Most seeds (91%) in a 6-DAP silique of the wild type had torpedo-shaped or walking stick–shaped embryos, which then grew to be full-sized embryos at 9 DAP. By contrast, most seeds (89%) in a 6-DAP silique of *nars1 nars2* had heart-shaped embryos, which remained as heart-shaped or torpedo-shaped embryos even at 9 DAP. Error bars indicate SD of three biological replicates. Green background shows the period after the torpedo-shaped embryo stage.

(L) Wild-type (Col-0) and *nars1 nars2* seeds in 12- to 15-DAP siliques. Most seeds of the wild type were green and produced viable seeds. By contrast, approximately half of *nars1 nars2* seeds were shrunken.

(M) Developmental changes in the levels of seed coat and embryo markers in the wild type (Col-0) and *nars1 nars2*. δVPE is a marker of inner integuments at the torpedo-shaped embryo stage; 12S globulin, a seed storage protein, is a marker of full-sized embryos. We harvested siliques of the wild type and *nars1 nars2* at sequentially different stages: from flowers just opened at the top of plants (lane 1) to siliques at the bottom of plants (lane 8). One-tenth of the total protein from one silique was subjected to SDS-PAGE followed by either immunoblot analysis with anti-δVPE antibody or Coomassie Brilliant Blue (CBB) staining. 12S, 12S globulin.

We investigated whether the delay of embryogenesis affected the biosynthesis of seed storage proteins in *nars1 nars2* embryos. Accumulated levels of a major storage protein, 12S globulin, were measured in *nars1 nars2* siliques of sequentially different stages, ranging from flowers just opened at the top of plants to siliques at the bottom of plants (Figure 3M). Protein profiles obtained from analysis of one-tenth total protein from each silique showed that *nars1 nars2* accumulated no storage protein, while levels in the wild type started to accumulate at the full-sized embryo stage (Figure 3M, lane 7). We also evaluated the seed coat formation defect in *nars1 nars2* using a marker (δVPE) that is transiently and specifically expressed in inner integuments during the early stage of seed development and whose level is maximized at the heart- to torpedo-shaped embryo stages (Nakaune et al., 2005). An immunoblot of one-tenth total protein from each silique probed with anti-δVPE antibody revealed that *nars1 nars2* had delayed expression of δVPE (Figure 3M, δVPE). These results suggest that the development of inner integuments is also delayed in *nars1 nars2*. Together, these results indicate that *nars1 nars2* has a defect in seed development leading to abnormally shaped seeds, despite having normally shaped flowers and siliques.

The Integuments of *nars1 nars2* Failed to Develop and to Degenerate

To better characterize seed development of *nars1 nars2*, we histologically examined developing seeds at 6, 9, 12, and 15 DAP (Figure 4). The development and degeneration of integuments were delayed in *nars1 nars2* compared with the wild type. This result is consistent with the observation of delayed δVPE expression (Figure 3M). Inner integument of the wild type collapsed

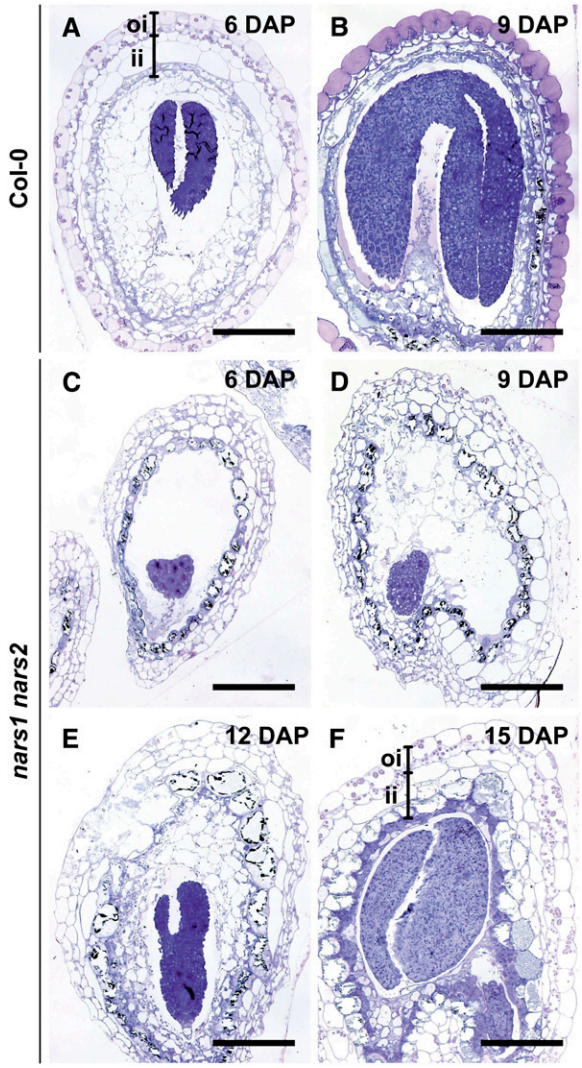


Figure 4. Structure and Development of Wild-Type and *nars1 nars2* Seeds.

Plastic sections (700 nm) were prepared and stained with toluidine blue.
(A) Wild-type (Col-0) seed at 6 DAP. Embryo developed to torpedo-shaped embryo stage. Cellularized endosperms were observed.
(B) Wild-type seed at 9 DAP. Embryo developed to full-sized embryo stage. Integument cells collapsed and became a thin layer of seed coat. The cells of the outermost integument accumulate mucilage, as stained in purple.
(C) *nars1 nars2* seed at 6 DAP. Embryo exhibited heart shape, and endosperm was not yet cellularized.
(D) *nars1 nars2* seed at 9 DAP. Endosperm started to become cellularized at the early torpedo-shaped embryo stage.
(E) *nars1 nars2* seed at 12 DAP. *nars1 nars2* seed had a middle torpedo-shaped embryo. Development of integument cells was disarranged.
(F) *nars1 nars2* seed at 15 DAP. Deformed embryo occupied the inner space of the seed, and integument cells did not collapse.
ii, inner integument; oi, outer integument. Bars = 50 μm.

immediately after the torpedo-shaped embryo stage (Figures 4A and 4B). By contrast, the inner integument of *nars1 nars2* did not collapse, even at the full-sized embryo stage (Figures 4C to 4F), suggesting that NARS1 and NARS2 are involved in PCD in the inner integument.

Electron microscopy showed that in the wild type, the dead cells of the ii2 layer had disrupted vacuolar and plasma membranes at the torpedo-shaped embryo stage (Figure 5A), and the cell walls were compressed until the full-sized embryo stage was reached (Figure 5B). In *nars1 nars2*, however, the dead cells of the ii2 layer did not compress cell walls even at the full-sized embryo stage (Figure 5D), although their PCD occurred at the torpedo-shaped embryo stage, as in the wild type (Figure 5C). This result suggests that NARS1 and NARS2 are involved in cell wall modification of ii2 after PCD.

nars1 nars2 exhibited some abnormal phenotypes in outer integument. The wild type accumulated a large quantity of mucilage in oi2 (Figure 4B), while *nars1 nars2* did not (Figure 4F). Scanning electron micrographs revealed that the outermost cells of wild-type integuments had distinct outlines and a columella (Figures 6A and 6B), while those of *nars1 nars2* were broken and failed to form a columella (Figures 6C and 6D). A similar deformed seed coat phenotype was observed in seeds of the transgenic plants of *Pro35S:NARS1-SRDX* and *Pro35S:NARS2-SRDX* (Figures 6E to 6H). These results indicate that NARS1 and NARS2 regulate the development of cells of the outer integument.

nars1 nars2 also exhibited a defect in the development of endosperm (Figure 4). Wild-type endosperm showed cellularization at 6 DAP (torpedo-shaped embryo stage; Figure 4A) and was degenerated at 9 DAP (full-sized embryo stage; Figure 4B). By contrast, *nars1 nars2* endosperm did not show cellularization at 6 DAP (heart-shaped embryo stage; Figure 4C). Instead, the cellularized endosperm was observed during the torpedo-shaped embryo stage (9 and 12 DAP; Figures 4D and 4E) and then degenerated at 15 DAP (full-sized embryo stage; Figure 4F). This result indicates that the development of endosperm is delayed in *nars1 nars2*; the developmental status of endosperm appears to correspond with embryogenesis rather than with the number of DAP.

NARS1 and *NARS2* Are Expressed in Outer Integuments of Seeds but Not in Embryos

Both *NARS1* and *NARS2* were expressed throughout seed development, and their expression levels were maximized around the torpedo-shaped embryo stage, as was observed for *δVPE* (Figure 7A). To clarify the tissue-specific expression of *NARS1* and *NARS2*, we performed high-resolution RT-PCR. We separated developing seeds at the torpedo-shaped embryo stage into two parts: the embryo and the other parts, including integuments and endosperms. cDNAs were then prepared from each part for RT-PCR. Neither *NARS1* nor *NARS2* was detected in the embryo. However, both *NARS1* and *NARS2* were detected in the other tissues (Figure 7B), and the distribution pattern corresponded to that of *δVPE*, which served as a marker of the inner integuments. *PROTODERMAL FACTOR1* (*PDF1*), which is expressed specifically in protodermal cells of embryo during embryogenesis

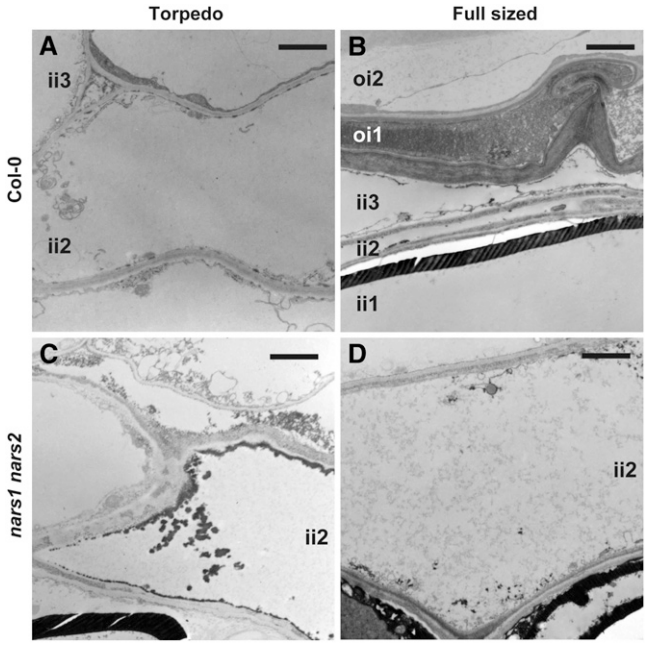


Figure 5. Ultrastructural Changes of Integument Cell Layers in Developing Wild-Type and *nars1 nars2* Seeds.

(A) Wild-type (Col-0) seed at torpedo-shaped embryo stage, which corresponds to 6 DAP. The vacuolar and plasma membranes of ii2 cells were disrupted, indicating dead cells.
(B) Wild-type seed at full-sized embryo stage, which corresponds to 9 DAP. The dead cells of the ii2 layer were completely crushed and compressed their cell walls.
(C) *nars1 nars2* seed at torpedo-shaped embryo stage, which corresponds to 12 DAP. The plasma membranes and cellular organelles were disrupted in cells of the ii2 layer, indicating dead cells.
(D) *nars1 nars2* seed at full-sized embryo stage, which corresponds to 15 DAP. The dead cells of the ii2 layer do not compress cell walls.
ii, inner integument; oi, outer integument. Bars = 2 μm.

(Abe et al., 1999), was used as a marker for embryo tissue and was not detected by RT-PCR in the nonembryo tissue sample (Figure 7B), showing that the tissue preparation was not contaminated by embryos. This result indicates that *NARS1* and *NARS2* are expressed in integuments and/or endosperms but not in the embryo.

To histologically explore the expression pattern of these two genes, in situ hybridization was performed with developing seeds. Since *NARS1* and *NARS2* are the most similar genes in the NAC family, *NARS1* and *NARS2* antisense probes were designed from divergent sequences that excluded the coding regions of the NAC domain. Signal stains showing the expression of *NARS1* and *NARS2* genes were not detected in embryos at the torpedo-shaped embryo stage, but they were detected in outer integuments of seeds (Figures 7C, 7D, 7G, 7H, 7K, and 7L). *δVPE* and *PDF1* antisense probes, which were used as controls, were detected in the inner integuments and epidermal cells of embryo, respectively (Figures 7E, 7F, 7I, 7J, 7M, and 7N), as reported previously (Abe et al., 1999; Nakaune et al., 2005). These detected signals emphasize that *NARS1* and *NARS2* antisense probes hybridize specifically to their respective genes. This tissue expression pattern is also consistent with the finding that the double mutant *nars1 nars2* had defects in the formation of integuments.

The Male Gametophyte of *nars1 nars2* Is Not Responsible for Aberrant Embryogenesis

nars1 nars2 exhibited defects not only in the formation of seed coats but also in embryogenesis. However, neither *NARS1* nor

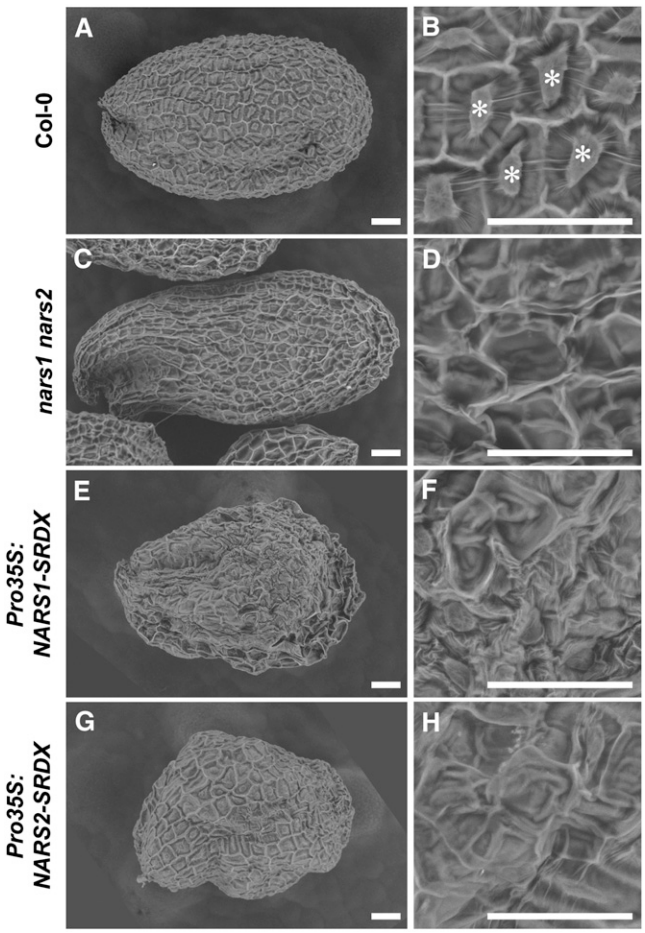


Figure 6. Double Knockout Mutation or Repression of *NARS1* and *NARS2* Disrupted Columellae on Outermost Cells of the Seed Coat.

The surface of dry seeds was examined using a scanning electron microscope. In *nars1 nars2* and *Pro35S:NARS1-SRDX* and *Pro35S: NARS2-SRDX* transgenic plants, the outermost integument cells did not construct columellae, which are specific structures of the seed coat surface.
(A) and **(B)** Wild type (Col-0).
(C) and **(D)** *nars1 nars2*.
(E) and **(F)** *Pro35S:NARS1-SRDX*.
(G) and **(H)** *Pro35S:NARS2-SRDX*.
(B), **(D)**, **(F)**, and **(H)** are higher magnifications of the seed coats shown in **(A)**, **(C)**, **(E)**, and **(G)**, respectively. Asterisks show columellae. Bars = 50 μm.

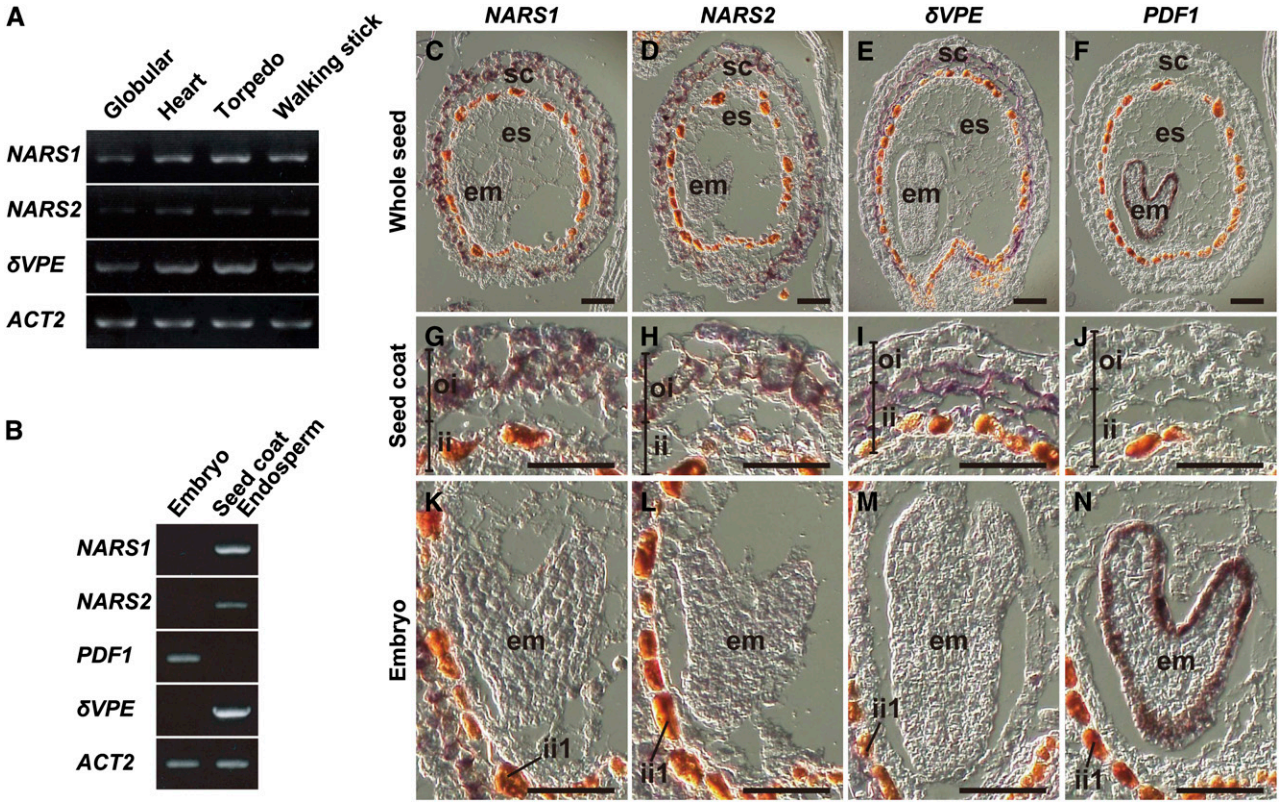


Figure 7. Expression of *NARS1* and *NARS2* in Integuments during Seed Development.

(A) Developmental changes in the expression levels of *NARS1* and *NARS2* during seed development. The developing seeds were harvested at various stages, and total RNA from developing seeds was subjected to RT-PCR of 30 cycles. *ACT2* was used as a loading control.

(B) Developing *Arabidopsis* seeds were separated into embryo and seed coats plus endosperm. These tissues were subjected to RT-PCR of 30 cycles. Both *NARS1* and *NARS2* were expressed in seed coats and/or endosperm but not in embryo. *PDF1* and *δVPE* were used as tissue-specific markers of embryo and seed coats, respectively. *ACT2* was used as a loading control.

(C) to **(N)** In situ hybridization of developing seeds at the torpedo-shaped embryo stage with *NARS1* (**[C]**, **[G]**, and **[K]**), *NARS2* (**[D]**, **[H]**, and **[L]**), *δVPE* (**[E]**, **[I]**, and **[M]**), and *PDF1* (**[F]**, **[J]**, and **[N]**) antisense probes. Dark purple stain indicates specific tissue staining by these probes, while light brown stain indicates nonspecific tissues of ii1.

(C) to **(F)** Whole seed.

(G) to **(J)** Higher magnification of seed coats.

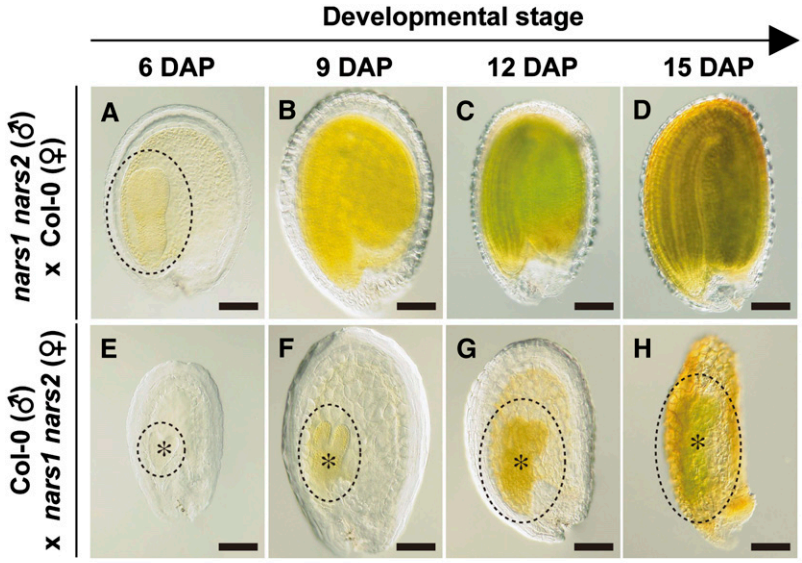
(K) to **(N)** Higher magnification of embryos.

em, embryo; es, endosperm; ii, inner integument; oi, outer integument; sc, seed coat. Bars = 50 μm.

NARS2 was expressed in the embryo. To determine whether the male gametophyte is responsible for these defects, we pollinated wild-type pistils with *nars1 nars2* pollen, and *nars1 nars2* pistils with wild-type pollen, to generate heterozygous embryos. Seeds produced from the crossing between wild-type pistils and *nars1 nars2* pollen developed normally (Figures 8A to 8D), indicating that the male gametophyte of *nars1 nars2* is not responsible for defects in embryogenesis or the formation of seed coats. This suggests that *NARS1* and *NARS2* do not affect the male gametophyte. However, seeds produced from the crossing between *nars1 nars2* pistils and wild-type pollen exhibited defects in both embryogenesis and the formation of seed coats (Figures 8E to 8H). These seed development defects were identical to those of homozygous *nars1 nars2* seeds.

To exclude the possibility that the *nars1 nars2* embryo affects seed morphogenesis, we analyzed the situation in which the genotype of the embryo was a double mutant and the genotype of the integuments and endosperm was heterozygous. We observed that genotypes of the second progeny (F2) from a crossing of the wild type and *nars1 nars2* segregated into double mutants and others at a 1:15 ratio, although the shapes of these seeds were indistinguishable from those of wild-type seeds (Figure 8I). This result indicates that the absence of both *NARS1* and *NARS2* genes in the embryo does not affect embryogenesis.

The finding that proper seed coat formation was abolished only in the seeds formed by a crossing between *nars1 nars2* pistils and wild-type pollen was expected, because the integuments represent parental tissues. Interestingly, embryogenesis



I

	Genotypes		P (x^2)
	nars1 nars2	Others	
Col-0 x *nars1 nars2* (F2 seeds)	11	161	0.937

Figure 8. Male Gametophytes of *nars1 nars2* Are Not Responsible for Abnormal Embryogenesis.

The wild type (Col-0) and *nars1 nars2* were crossed, and the resulting heterozygous embryos collected at different stages after pollination were inspected using a differential interference contrast microscope.

(A) to **(D)** Heterozygous seeds generated by crossing wild-type and *nars1 nars2* pollen developed normally.

(E) to **(H)** Heterozygous seeds generated by crossing *nars1 nars2* and wild-type pollen showed embryogenesis arrested at the torpedo-shaped embryo stage, as observed in homozygous seeds of *nars1 nars2*.

Asterisks and dotted circles show embryos. Bars = 100 μm.

(I) Segregation rate of second filial progeny (F2) generated by wild type and *nars1 nars2* crossing. The F2 seeds used for genotyping exhibited normally shaped seeds. P was calculated by χ^2 test with 15:1 segregation.

was abolished in seeds produced from *nars1 nars2* pistils and wild-type pollen but not in the seeds produced from the opposite cross. This indicates that the aberrant embryogenesis in *nars1 nars2* seeds is caused by some defect of the integuments. Together, these results imply that both NARS1 and NARS2 regulate embryogenesis by regulating the development of the integuments.

The Absence of both NARS1 and NARS2 Induced a Delay of Silique Senescence

The senescence of *nars1 nars2* siliques was clearly delayed (Figure 9). At a time when half of the wild-type siliques had changed to yellow and one-third of the siliques in the main stem were dehisced, the *nars1 nars2* siliques remained green and undehisced (Figure 9A). At 9 weeks after sowing, 75% of the wild-type siliques were senescent while only 22% of the *nars1 nars2* siliques were senescent (Figure 9B). Senescence of the *nars1* and *nars2* single mutants was not different from that of the wild type, and leaf senescence was not different between the wild type and *nars1 nars2* (Figure 9C). These results suggest that NARS1 and NARS2 redundantly regulate silique-specific senescence in addition to seed morphogenesis.

DISCUSSION

Maternal Transcription Factors Regulate Embryogenesis

An interesting finding from the wild-type and *nars1 nars2* crossing experiments (Figure 8) is the observation that maternal factors, NARS1 and NARS2, are responsible for regulating development of the embryo. Maternal regulation of embryo growth offers an advantage because it minimizes the amount of nutrients and energy expended on immature seeds with aberrant seed coats that will not survive. NARS1 and/or NARS2 may function as a checkpoint of this regulatory system.

It remains unclear how these maternal factors, which are located in the integuments and do not directly contact the embryo, regulate embryogenesis. One physiological role of the integuments is to supply nutrients for the developing embryo. In

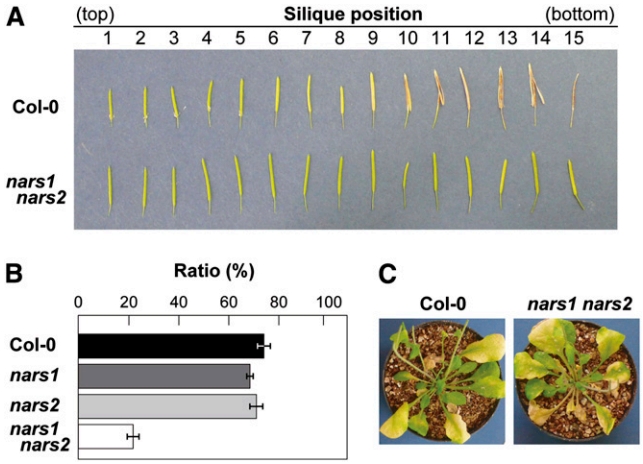


Figure 9. Double Mutant *nars1 nars2* Showed Delayed Senescence of Siliques.

(A) Developmental changes of senescing siliques of the wild type (Col-0) and *nars1 nars2*. The siliques were harvested from the top (position 1) to the bottom (position 15) of a stem. Wild-type siliques started senescence at position 8 and split open at position 10, whereas *nars1 nars2* siliques remained green without senescing, even at position 15. Eight-week-old plants were used.

(B) Ratios of senesced siliques on the *nars* plants. Only 22% of the *nars1 nars2* siliques senesced on 9-week-old plants, while ~75% of the siliques senesced on wild-type (Col-0) plants and single mutants (*nars1* and *nars2*). Error bars indicate SD of three biological replicates.

(C) No difference in leaf senescence was observed between wild-type and *nars1 nars2* 8-week-old plants.

view of this, the delay of embryogenesis in *nars1 nars2* may be caused by a lack of nutrients for embryo growth at the early stage. Namely, *nars1 nars2* may prevent the transport of nutrients from the integuments to the developing endosperm and embryo, resulting in a delay of early development of the embryo. Similarly, downregulation of the integument-specific MADS box genes (FLORAL BINDING PROTEIN7 [FBP7] and FBP11) of petunia (*Petunia hybrida*) induced a defect of seed development by blocking the transport of nutrients through seed coats (Colombo et al., 1997). However, there is a critical difference between *nars1 nars2* and downregulation of the MADS box genes: in *nars1 nars2*, embryogenesis was arrested at the torpedo-shaped embryo stage or progressed with abnormally shaped embryos, while in petunia, the downregulation of the MADS box genes caused defects of embryogenesis in a wide range of developmental stages. Thus, other regulatory systems may act at the torpedo-shaped embryo and later developmental stages.

Maternal Regulation of Embryogenesis and Endosperm Development

The results in Figure 3 suggest that NARS1 and/or NARS2 exert regulatory functions at the torpedo-shaped embryo stage, which is supported by the corresponding temporal expression of these genes around the torpedo-shaped embryo stage (Figure 7A). One possible regulatory mechanism is that NARS1 and/or NARS2 generate a regulatory substance, such as a phytohormone, and deliver it to the embryo to enhance its growth. Previously, we reported that *Arabidopsis* KATAMARI2 (KAM2), which is required for the proper formation of endosomes and protein trafficking, is involved in determining the growth axis of the embryo prior to the walking stick–shaped embryo stage, possibly by way of a phytohormone (Tamura et al., 2007). The deformed shape of *kam2* embryos is similar to that of *nars1 nars2* embryos that escape developmental difficulty at the torpedo-shaped embryo stage (Figure 3J). It is likely that NARS1 and/or NARS2 play a role in the secretion of regulatory factors from the integuments to the developing embryo.

TRANSPARENT TESTA GRABRA2 (TTG2), which is a transcription factor of the WRKY family, is involved in proanthocyanidin synthesis and mucilage deposition in the seed coat (Johnson et al., 2002). The defects of mucilage deposition that were found in *ttg2* were also observed in *nars1 nars2* (Figure 4B). Garcia et al. (2005) reported that the *ttg2* mutant had interrupted cell elongation in the integument and restricted endosperm growth, suggesting that crosstalk occurs between the integument and the endosperm. They found that integument-dependent endosperm development was abolished in *ttg2* as in *nars1 nars2*. The molecular mechanism underlying the regulation of seed development by NARS1 and NARS2 may overlap partially with that of TTG2.

NARS1 and NARS2 Are Involved in PCD/Senescence in Reproductive Tissues

Previously, we reported that inner integuments of *Arabidopsis* undergo PCD after the torpedo-shaped embryo stage, an event that is mediated by δVPE (Nakaune et al., 2005). *nars1 nars2* exhibited delayed expression of δVPE (Figure 3M) and subsequent delayed PCD in the integuments. We also demonstrated that the cells of the ii2 layer showed cell death at the torpedo-shaped embryo stage in *nars1 nars2*, similar to the wild type at the same developmental stage (Figure 5). These results suggest that NARS1 and/or NARS2 function to facilitate an intracellular environment primed for PCD rather than to trigger PCD directly.

We found that NARS1 and/or NARS2 were responsible for senescence in the other tissues. Silique senescence of *nars1 nars2* was clearly delayed when compared with the wild type, although *nars1 nars2* leaves senesced like those of the wild type (Figure 9). Considering that senescence is a form of PCD, these results indicate that NARS1 and/or NARS2 are involved in PCD of siliques as well as in PCD of integuments.

Arabidopsis NAC-LIKE, ACTIVATED BY AP3/PI (NAP) (At1g69490), which is closely related to NARS1 and NARS2 (Figure 1A), is required for leaf senescence (Guo and Gan, 2006). Together, our results suggest that NARS1 and/or NARS2 regulate transcription of certain factors to promote senescence in reproductive tissues, including integuments and siliques.

Functional Redundancy between *NARS1* and *NARS2*

NARS1 and *NARS2* correspond to *NAC2* and *NAM*, respectively, although their biological functions are unknown (Takada et al., 2001; Duval et al., 2002). Using a CRES-T library, we

identified NARS1 and NARS2 as regulatory factors for embryogenesis and integument development. If ethyl methanesulfonate–mutagenized lines or T-DNA–tagged lines had been screened, NARS1 and NARS2 would have missed detection by the criterion of aberrant seed morphology, because neither of the single *nars1* or *nars2* knockout mutants exhibits an aberrant seed morphology phenotype (Figures 1B to 1F and 2C to 2E). This demonstrates the utility of a CRES-T library as a tool for forward genetic analysis of transcription factors, many of which have many homologs that function redundantly.

The genome of *Arabidopsis* comprises over 20 duplicated segments of 100 kb or larger (Arabidopsis Genome Initiative, 2000). *NARS1* and *NARS2* genes are located in the duplicated regions of chromosome 3 and chromosome 1, respectively, suggesting that *NARS1* and *NARS2* originated from a single gene. Two other NAC genes, *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *NST2*, are also located in the duplicated regions of chromosome 2 and chromosome 3, respectively. NST1 and NST2 share a high degree of sequence similarity compared with other members of the NAC family and redundantly regulate secondary wall thickening of anthers (Mitsuda et al., 2005). We found that seven other pairs of NAC genes that exhibit high similarity and a similar expression profile are located in duplicated genome regions. These results suggest that the functional redundancy of each pair of these NAC members, including NARS1 and NARS2, is a result of genome duplications.

METHODS

Plant Materials and Growth Conditions

We used *Arabidopsis thaliana* ecotype Columbia (Col-0) as the wild type. We obtained two T-DNA insertion mutants from the ABRC at Ohio State University: SM_3_28017 (*nars1*) and WiscDsLox364F11 (*nars2*). Surface-sterilized seeds were sown onto 0.8% agar (Wako) in Murashige and Skoog medium (Wako) that contained B5 vitamins and 1% sucrose and then were grown at 22°C under continuous light. Three-week-old plants were transferred onto soil and grown at 22°C under a 16-h-light day.

To generate heterozygous embryos, we pollinated wild-type pistils with *nars1 nars2* pollen and *nars1 nars2* pistils with wild-type pollen.

Generation of a Mutant Library of Transcription Factors Using CRES-T

Coding regions of possible transcription factor genes were amplified from an *Arabidopsis* cDNA library (Mitsuda et al., 2006). Specifically, the protein-coding regions of *NARS1* and *NARS2* were amplified using the following primers: At3g15510N and At3g15510C for *NARS1* and At1g52880N and At1g52880C for *NARS2* (see Supplemental Table 1 online). Each amplified region was introduced into the *Sma*I site of p35SSRDXG (Mitsuda et al., 2006), which was used to express the respective transcription factor fused with the EAR motif repression domain, SRDX (Hiratsu et al., 2003), under the control of the cauliflower mosaic virus 35S promoter, as described previously (Mitsuda et al., 2006). Each DNA construct was introduced into the pBCKH plant expression vector (Mitsuda et al., 2006) by LR reaction of the Gateway system (Invitrogen). The vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the transformed bacteria were then infiltrated into *Arabidopsis* plants by the floral dip method (Clough and Bent, 1998) to obtain the CRES-T lines of *Arabidopsis*. The CRES-T library established 10 transgenic lines for each transcription factor. For a forward genetic approach, we used the CRES-T library to isolate transcription factors that regulate seed morphology.

Generation of *ProNARS1:NARS1-SRDX* and *ProNARS2:NARS2-SRDX* Transgenic Plants

For the generation of *ProNARS1:NARS1-SRDX* and *ProNARS2:NARS2-SRDX*, we amplified the 5′ upstream regions of *NARS1* and *NARS2*, which consisted of 3039 and 2972 bp from the site of translational initiation, using the primers CR245pF2A, CR245pRB, CR248pFA, and CR248pRB (see Supplemental Table 1 online). The amplified fragments of 5′ upstream and the protein-coding regions (described above) of *NARS1* and *NARS2* genes were introduced into the *Asc*I/*Bam*HI sites and the *Sma*I site, respectively, of a promoterless vector, pSRDX-NOS_Ent, which was derived from p35SSRDXG. Each DNA construct was introduced into pBCKH. *Arabidopsis* plants were then transformed with the vectors, as described above.

Light Microscopy, Confocal Laser Scanning Microscopy, and Electron Microscopy

Seed shapes were examined using a stereoscopic microscope (model SZX12; Olympus) equipped with a CCD camera (model DP20; Olympus).

To see the morphology of embryos and seed coats, developing seeds were made transparent by incubating them in solution (chloral hydrate: water:glycerol [10:2.5:1, w/v/w]), after which they were inspected using a microscope (Axioskop 2 plus system; Carl Zeiss) with a CCD camera (model VB-7010; Keyence).

For analysis of subcellular localization of enhanced yellow fluorescent protein (EYFP)-tagged NAC genes, the tissues were incubated in the dark at 22°C for 22 to 24 h. The fluorescent images of EYFP were observed using a confocal laser scanning microscope (model LSM 510 META; Carl Zeiss). The exported data were processed using Adobe Photoshop CS2 (Adobe Systems).

The developing seeds were vacuum-infiltrated for 30 min with a fixative consisting of 4% paraformaldehyde and 1% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4. The fixed samples were then postfixed with 0.5% osmium tetroxide in the same cacodylate buffer for 2 h. After washing with the same buffer, the specimens were dehydrated in a graded ethanol series at room temperature. Subsequent procedures for embedding and sectioning were the same as those described previously (Hayashi et al., 1998). For histological studies, thin sections that were stained with toluidine blue were examined with a light microscope (Axioskop 2 plus system). For ultrastructural studies, the ultrathin sections were stained with 0.4% uranyl acetate and lead citrate and were then examined using a transmission electron microscope (model H-7600; Hitachi High-Technologies) operated at 80 kV.

The surface of dry seeds was observed with a scanning electron microscope (model Miniscope TM-1000; Hitachi High-Technologies) without fixation.

Transient Expression of EYFP-NARS1 and EYFP-NARS2

To determine the subcellular localization of NARS1 and NARS2, each full-length coding region was cloned into the entry vector pENTR/D-TOPO (Invitrogen). For amplification of the coding regions of *NARS1* and *NARS2*, the following primers were used: At3g15510TOPO_F1 and At3g15510-TOPO_R1 for *NARS1* and At1g52880TOPO_F1 and At1g52880TOPO_R1 for *NARS2* (see Supplemental Table 1 online). The resulting constructs were transferred, by LR reaction as described above, into the pUGW42 vector to express EYFP-tagged NARS1 or NARS2 under the control of cauliflower mosaic virus 35S (Nakagawa et al., 2007). The EYFP-tagged NAC genes were transiently expressed in epidermal cells of onion (*Allium*

cepa) using a particle gun, as described previously (Matsushima et al., 2004).

Transactivation Analysis

The full-length coding regions of *NARS1* and *NARS2* were amplified using primer sets At3g15510inf_F1 and At3g15510inf_R1 for *NARS1* and At1g52880inf_F1 and At1g52880inf_R1 for *NARS2*. The partial coding regions of the NAC domains were amplified using primer sets At3g15510inf_F1 and At3g15510Ninf_R1 for *NARS1* and At1g52880inf_F1 and At1g52880Ninf_R1 for *NARS2*. The partial coding regions except for the NAC domains were amplified using primer sets At3g15510Cinf_F1 and At3g15510inf_R1 for *NARS1* and At1g52880Cinf_F1 and At1g52880inf_R1 for *NARS2*. These primers are shown in Supplemental Table 1 online. Each of these amplified fragments was inserted between *Eco*RI and *Pst*I sites of pGBKT7 (Clontech). The constructs, which encoded a fusion protein with the GAL4 DNA binding domain of yeast, were transformed into *Saccharomyces cerevisiae* strain AH109, which has GAL4-responsive reporter genes *HIS3*, *ADE2*, and *MEL1*. The transformed cells were grown on SD plates that contained 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside and lacked either His or adenine. Transactivation ability was determined by evaluating either the growth of yeast cells or the color of colonies.

Isolation of RNA and RT-PCR

Total RNA was isolated from maturing *Arabidopsis* siliques, seeds, or dissected seeds with the RNeasy Plant Mini kit (Qiagen). cDNA was synthesized from total RNA with Ready-To-Go RT-PCR beads (GE Healthcare Bio-Science). PCR was performed with the cDNAs and ExTaq polymerase (Takara). Primer sets used are shown in Supplemental Table 1 online.

Immunoblot Analysis

Siliques of wild-type and *nars1 nars2* plants were harvested at sequentially different stages. One-tenth of the total protein extracted from each silique was subjected to immunoblot analysis with anti-δVPE antibody (diluted 1:5000) (Nakaune et al., 2005) and a peroxidase-conjugated goat antibody against rabbit IgG (Pierce; diluted 1:2000). The signals were detected using an ECL detection system (GE Healthcare Bio-Science). Alternatively, the proteins were stained with Coomassie Brilliant Blue R 250.

In Situ Hybridization

In situ hybridization was performed as described by Takada et al. (2001). *NARS1* and *NARS2* probes were prepared from 555- and 426-bp regions, respectively, of cDNA fragments excluding the NAC domain. *δVPE* and *PDF1* probes were designed to generate 616- and 602-bp fragments, respectively, from specific regions of cDNA. These fragments were amplified by PCR and subcloned into pCR-Blunt II-TOPO vector (Invitrogen). Primer sets used for amplification are shown in Supplemental Table 1 online. Hybridization was performed at 45°C. Western Blue (Promega) was used as a substrate for signal detection.

Phylogenetic Analysis

Protein sequences of NAC III subfamily genes were obtained from The Arabidopsis Information Resource database (http://www.Arabidopsis.org) and aligned by ClustalW implemented in MEGA (version 4; http://www.megasoftware.net). A phylogenetic tree of the NAC III subfamily was constructed with the aligned protein sequences by MEGA using the neighbor-joining method with the following parameters: Poisson correction, complete deletion, and bootstrap (1000 trials, random seed). The alignment used to generate the tree is shown in Supplemental Data Set 1 online.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: NARS1 (At3g15510, NM_112419), NARS2 (At1g52880, NM_104166), NAP (At1g69490, NM_105616), At1g61110 (NM_104792), At3g04070 (NM_111278), At5g08790 (NM_147856), At5g63790 (NM_125774), At1g77450 (NM_106394), At1g01720 (NM_100054), At1g52890 (NM_104167), At3g15500 (NM_112418), At4g27410 (NM_118875), δVPE (At3g20210, NM_112912), PDF1 (At2g42840, NM_129845), and ACT2 (At3g18780, NM_112764).

Supplemental Data

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

Supplemental Figure 1. Dry Seed Aberrant Shapes of Transgenic Plants That Expressed Chimeric Repressors of NAC III Transcription Factors.

Supplemental Figure 2. NARS1 and NARS2 Have the Ability to Act as Transcription Factors.

Supplemental Figure 3. Coexpression Network within NAC Transcription Factors.

Supplemental Table 1. Primer Sets Used in This Study.

Supplemental Data Set 1. Text File of the Alignment Used to Generate the Phylogenetic Tree in Figure 1.

ACKNOWLEDGMENTS

We are grateful to Tsuyoshi Nakagawa (Shimane University) for his kind donation of a Gateway vector (pUGW42) and to the ABRC for providing seeds of *Arabidopsis* T-DNA insertion mutants (SM_3_28017 and WiscDsLox364F11). We thank Keiichiro Hiratsu (National Institute of Advanced Industrial Science and Technology; current affiliation is the National Defense Academy of Japan) for his technical support in pSRDX-NOS_Ent vector construction. We thank Yukie Kimura, Naomi Ujiie, and Yoshimi Sugimoto (National Institute of Advanced Industrial Science and Technology) for their skillful assistance in plant cultivation. This work was supported by the Core Research for Evolutional Science and Technology of the Japan Science and Technology Corporation, by Grants-in-Aid for Scientific Research (Grants 16085203 and 17107002) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan, by the Global Center of Excellence Program "Formation of a Strategic Base for Biodiversity and Evolutionary Research: from Genome to Ecosystem" of MEXT, and by a research fellowship to T.K. from the Japan Society for the Promotion of Science (Fellowship 19004891).

Received April 20, 2008; revised September 10, 2008; accepted September 21, 2008; published October 10, 2008.

REFERENCES

Abe, M., Takahashi, T., and Komeda, Y. (1999). Cloning and characterization of an L1 layer-specific gene in *Arabidopsis thaliana*. Plant Cell Physiol. **40:** 571–580.

Arabidopsis Genome Initiative (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature **408:** 796–815.

Beeckman, T., Rycke, R.D., Viane, R., and Inze, D. (2000). Histological study of seed coat development in *Arabidopsis thaliana*. J. Plant Res. **113:** 139–148.

Chaudhury, A.M., Ming, L., Miller, C., Craig, S., Dennis, E.S., and Peacock, W.J. (1997). Fertilization-independent seed development in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA **94:** 4223–4228.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

Colombo, L., Franken, J., Van der Krol, A.R., Wittich, P.E., Dons, H. J., and Angenent, G.C. (1997). Downregulation of ovule-specific MADS box genes from petunia results in maternally controlled defects in seed development. Plant Cell **9:** 703–715.

Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., and Lepiniec, L. (2003). Proanthocyanidin-accumulating cells in Arabidopsis testa: Regulation of differentiation and role in seed development. Plant Cell **15:** 2514–2531.

Duval, M., Hsieh, T.F., Kim, S.Y., and Thomas, T.L. (2002). Molecular characterization of AtNAM: A member of the Arabidopsis NAC domain superfamily. Plant Mol. Biol. **50:** 237–248.

Garcia, D., Fitz Gerald, J.N., and Berger, F. (2005). Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in Arabidopsis. Plant Cell **17:** 52–60.

Garcia, D., Saingery, V., Chambrier, P., Mayer, U., Jurgens, G., and Berger, F. (2003). Arabidopsis haiku mutants reveal new controls of seed size by endosperm. Plant Physiol. **131:** 1661–1670.

Guo, Y., and Gan, S. (2006). AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J. **46:** 601–612.

Hayashi, M., Toriyama, K., Kondo, M., and Nishimura, M. (1998). 2,4-Dichlorophenoxybutyric acid-resistant mutants of Arabidopsis have defects in glyoxysomal fatty acid beta-oxidation. Plant Cell **10:** 183–195.

Hiratsu, K., Matsui, K., Koyama, T., and Ohme-Takagi, M. (2003). Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in Arabidopsis. Plant J. **34:** 733–739.

Johnson, C.S., Kolevski, B., and Smyth, D.R. (2002). TRANSPARENT TESTA GLABRA2, a trichome and seed coat development gene of Arabidopsis, encodes a WRKY transcription factor. Plant Cell **14:** 1359–1375.

King, S.P., Lunn, J.E., and Furbank, R.T. (1997). Carbohydrate content and enzyme metabolism in developing canola siliques. Plant Physiol. **114:** 153–160.

Luo, M., Dennis, E.S., Berger, F., Peacock, W.J., and Chaudhury, A. (2005). MINISEED3 (MINI3), a WRKY family gene, and HAIKU2 (IKU2), a leucine-rich repeat (LRR) kinase gene, are regulators of seed size in Arabidopsis. Proc. Natl. Acad. Sci. USA **102:** 17531–17536.

Matsushima, R., Fukao, Y., Nishimura, M., and Hara-Nishimura, I. (2004). NAI1 gene encodes a basic-helix-loop-helix-type putative transcription factor that regulates the formation of an endoplasmic reticulum-derived structure, the ER body. Plant Cell **16:** 1536–1549.

Mitsuda, N., Hiratsu, K., Todaka, D., Nakashima, K., Yamaguchi-Shinozaki, K., and Ohme-Takagi, M. (2006). Efficient production of male and female sterile plants by expression of a chimeric repressor in Arabidopsis and rice. Plant Biotechnol. J. **4:** 325–332.

Mitsuda, N., Seki, M., Shinozaki, K., and Ohme-Takagi, M. (2005). The NAC transcription factors NST1 and NST2 of Arabidopsis regulate secondary wall thickenings and are required for anther dehiscence. Plant Cell **17:** 2993–3006.

Nakagawa, T., Kurose, T., Hino, T., Tanaka, K., Kawamukai, M., Niwa, Y., Toyooka, K., Matsuoka, K., Jinbo, T., and Kimura, T. (2007). Development of series of Gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J. Biosci. Bioeng. **104:** 34–41.

Nakaune, S., Yamada, K., Kondo, M., Kato, T., Tabata, S., Nishimura, M., and Hara-Nishimura, I. (2005). A vacuolar processing enzyme, deltaVPE, is involved in seed coat formation at the early stage of seed development. Plant Cell **17:** 876–887.

Obayashi, T., Kinoshita, K., Nakai, K., Shibaoka, M., Hayashi, S., Saeki, M., Shibata, D., Saito, K., and Ohta, H. (2007). ATTED-II: A database of co-expressed genes and cis elements for identifying co-regulated gene groups in Arabidopsis. Nucleic Acids Res. **35:** D863–D869.

Sheen, J., Zhou, L., and Jang, J.C. (1999). Sugars as signaling molecules. Curr. Opin. Plant Biol. **2:** 410–418.

Takada, S., Hibara, K., Ishida, T., and Tasaka, M. (2001). The CUP-SHAPED COTYLEDON1 gene of Arabidopsis regulates shoot apical meristem formation. Development **128:** 1127–1135.

Tamura, K., Takahashi, H., Kunieda, T., Fuji, K., Shimada, T., and Hara-Nishimura, I. (2007). Arabidopsis KAM2/GRV2 is required for proper endosome formation and functions in vacuolar sorting and determination of the embryo growth axis. Plant Cell **19:** 320–332.

Weber, H., Borisjuk, L., Heim, U., Buchner, P., and Wobus, U. (1995). Seed coat-associated invertases of fava bean control both unloading and storage functions: Cloning of cDNAs and cell type-specific expression. Plant Cell **7:** 1835–1846.

Western, T.L., Burn, J., Tan, W.L., Skinner, D.J., Martin-McCaffrey, L., Moffatt, B.A., and Haughn, G.W. (2001). Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in Arabidopsis. Plant Physiol. **127:** 998–1011.

Windsor, J.B., Symonds, V.V., Mendenhall, J., and Lloyd, A.M. (2000). Arabidopsis seed coat development: Morphological differentiation of the outer integument. Plant J. **22:** 483–493.

Wobus, U., and Weber, H. (1999). Seed maturation: Genetic programmes and control signals. Curr. Opin. Plant Biol. **2:** 33–38.