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AtNSE1 and AtNSE3 are required for embryo pattern formation and maintenance of cell viability during Arabidopsis embryogenesis

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Abstract

Embryogenesis is an essential process during seed development in higher plants. It has previously been shown that mutation of the Arabidopsis non-SMC element genes AtNSE1 or AtNSE3 leads to early embryo abortion, and their proteins can interact with each other directly. However, the crucial regions of these proteins in this interaction and how the proteins are cytologically involved in Arabidopsis embryo development are unknown. In this study, we found that the C-terminal including the Ring-like motif of AtNSE1 can interact with the N-terminal of AtNSE3, and only the Ring-like motif is essential for binding with three α motifs of AtNSE2 (homologous to AtMMS21). Using genetic assays and by analysing molecular markers of cell fate decisions (STM, WOX5, and WOX8) in mutant nse1 and nse3 embryos, we found that AtNSE1 and AtNSE3 work non-redundantly in early embryo development, and that differentiation of the apical meristem and the hypophysis fails in the mutants, which have disrupted auxin transportation and responses. However, the upper cells of the suspensor in the mutants seem to have proper embryo cell identity. Cytological examination showed that cell death occurred from the early embryo stage, and that vacuolar programmed cell death and necrosis in the nse1 and nse3 mutant embryos led to ovule abortion. Thus, AtNSE1 and AtNSE3 are essential for maintaining cell viability and growth during early embryogenesis. Our results improve our understanding of the functions of SMC5/6 complex in early embryogenesis in Arabidopsis.

Keywords: Arabidopsis, AtNSE1, AtNSE3, embryo, pattern formation, programmed cell death.

Introduction

Seed development is a crucial stage of the life cycle in higher plants and correct embryo development is a critical part of this process. In Arabidopsis, embryo development starts from the fertilization of the egg cell by the sperm cell (Dumas and Rogowsky, 2008). The zygote then undergoes a series of highly regulated cell divisions until the mature embryo is formed. Embryo patterning occurs at an early stage, including the establishment of the apical–basal and radial axes together with initiation of the shoot meristem and specification of the hypophysis (Zhang and Laux, 2011; Lau et al., 2012). Thus, the early development of the embryo is a critical process.

Apical–basal patterning occurs earlier than radial patterning, and factors that play an important role in the process include many transcript factors, auxin transport and responses, and peptide signaling (Lau et al., 2012). Members of the WUSCHEL-related homeobox (WOX) transcription factors are crucial
regulators during this process. WOX2 and WOX8 are both expressed in the egg cell and zygote, and then WOX8 is transferred into the basal cell while WOX2 remains in the apical cell after the first asymmetric division of the zygote. In the 8-cell embryo, WOX8 is restricted to the suspensor cells (Haecker et al., 2004), and it has been reported that WRKY2-dependent WOX8 transcription links zygote polarization with the early patterning of the embryo (Ueda et al., 2011).

In addition to WOX genes, auxin-dependent pathways also function in establishing the apical–basal axis. One well-known mechanism is a system of two interconnected feedback loops formed by MONOPTEROS (MP) and BODENLOS (BDL). MP encodes an auxin responsive factor (ARF), while BDL encodes an auxin (AUX)/IAA inhibitor, both of which are expressed in the apical cell lineage (Hardtke and Berleth, 1998; Hamann et al., 2002; Lau et al., 2011), and auxin-dependent degradation of BDL is important for this regulation process (Lau et al., 2011). Thus, the regulation of auxin transport is important during early embryo patterning. PIN proteins are efflux carriers that are responsible for polar auxin transport, and their intracellular location is therefore considered to be a good predictor of auxin flux (Jenik et al., 2007). Thus, the precise location of PIN proteins in the embryo has an important impact on early embryo patterning.

Specification of the shoot meristem and the hypophysis are also essential processes during early embryogenesis, during the course of establishment of the first stem cells (ten Hove et al., 2015). Generation of new organs depends on the stem cells that originated from the shoot and root apical meristems (SAM and RAM, respectively). SHOOT MERISTEMLESS (STM) is a class I KNOTTED-like homeodomain transcription factor. STM acts as a repressor of differentiation across the whole SAM and, together with WUSCHEL, it is required to maintain the SAM (Long et al., 1996; Lenhard et al., 2002). WUSCHEL RELATED HOMEOBOX 5 (WOX5) is recognized as a marker gene for the root organization center and is specifically expressed in the quiescent center (QC) (Sarkar et al., 2007). It has been reported that the signaling peptide CLAVATA3/-EMBRYO- SURROUNDING REGION 40 (CLE40) and the receptor-like kinases ARABIDOPSIS CRINKLY4 (ACR4)/CLAVATA1 (CLV1) are involved in negative regulation of WOX5 in the maintenance of the root apical meristem (De Smet et al., 2008; Stahl et al., 2009, 2013). However, more detailed experimental evidence is still needed to improve our understanding of the molecular mechanisms and regulatory networks in early embryogenesis.

In Arabidopsis, At5g21140 and At1G34770 are respectively the homologous genes of NSE1 (non-SMC element 1) and NSE3 in yeast, which are reported as the conserved subunits of the structural maintenance of chromosome (SMC) 5/6 complex. NSE1 and NSE3 play crucial roles in cell viability in yeast (McDonald et al., 2003; Pebernard et al., 2004; Losada and Hirano, 2005; Watanabe et al., 2009). It has been reported that mutation of AtNSE2 (homologous to MMS21), which encodes a subunit of the SMC5/6 complex, leads to cell death in homologous seedlings, and it is very important for maintenance of the root stem cell niche and for embryo patterning (Xu et al., 2013). In addition, loss function of SMC5 causes seed abortion (Watanabe et al., 2009). In a previous study, we found that mutation of AtNSE1 and AtNSE3 resulted in abnormal formation of the embryo at early stages of development and ultimately to aborted seed (Li et al., 2017). However, the biological functions of AtNSE1 and AtNSE3 in early embryo development were not clear. In addition, although it has been found that NSE1 dimerizes with NSE3 independently of the NSE1 Ring-like motif in yeast (Pebernard et al., 2008), the details of their interaction and the relationship between NSE1 and other subunits such as NSE2/MMS21 are still not known in higher plants.

In this study, we determined that the AtNSE1 C-terminal and AtNSE3 N-terminal are sufficient for their interaction, and the Ring-like domain is required for binding with the three α helices of the AtNSE2/MMS21 N-terminal. We also found that AtNSE1 and AtNSE3 play essential roles in early embryogenesis non-redundantly. We found that in the nse1-1 and nse3-1 mutants, not only was maintenance of the SAM and QC disturbed, but also that the destiny of the suspensor cells might have been altered to become cells of the embryo proper. Loss function of AtNSE1 and AtNSE3 induced vacuolar programmed cell death and necrosis in mutant embryos, which led to ovule abortion. These results suggest that AtNSE1 and AtNSE3 play a significant role in maintaining embryo patterning and cell viability during early embryo development.

**Materials and methods**

**Plant material and growth conditions**

*Arabidopsis thaliana* ecotype Columbia (Col-0) was used as the background material. The T-DNA insertion mutants CS16151 (nse1-1/1), CS24066 (nse1-2/1), CS334183 (nse3-2/1), an individual line obtained from a set of lines of CS451171) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/), and N734712 (nse3-1/+) was obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/).

The marker lines pDR5rev::3XVENUS-N7, pPIN1::PIN1-GFP and pSTM::STM-VENUS (Heisler et al., 2005) were obtained from Elliot Meyerowitz (California Institute of Technology, USA), the pWOX3::GFP line (Heidstra et al., 2004; Billo et al., 2005) from Ben Scheres (Albert-Ludwigs University, Germany), and the pWOX8-YFP line (Ueda et al., 2011) from Thomas Laux (Albert-Ludwigs University). The different marker lines were crossed with nse1-1/1 and nse3-1/1 and the progenies were selected through PCR and observed under a fluorescence microscope (Olympus FV1000). The nse1-1/1 and nse3-1/1 mutants carrying the homozygous fluorescence markers were used for the subsequent experiments.

All plants were grown in a greenhouse at Wuhan University at 22 ± 2 °C under a 16/8 h light/dark photoperiod.

**Yeast two-hybrid and bimolecular fluorescence complementation assays**

Cloning of the full-length ORFs (without stop codons) of AtNSE1, AtNSE2, and AtNSE3 for yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays was performed according to the method described in our previous study (Li et al., 2017). For the different truncated regions, each fragment was separately subcloned into the pCAMBIA-SPYCE and pCAMBIA-SPYCE header vectors. Primers used in this test are listed in Supplementary Table S1 at JXB online.

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Homology modeling

The sequences of the AtNSE1 (TAIR accession no. At5g21140), AtNSE2 (At3g15150), and AtNSE3 (At1g34770) proteins were downloaded from the Arabidopsis Information Resource (http://www.arabidopsis.org/). Their 3D structures were modeled as described in our previous study (Li et al., 2017). The PDB files of the modeled proteins were downloaded and displayed using PyMOL 1.3.

Ovule clearing for assessment of abnormalities

Siliques were sampled from 3–7 d after pollination (DAP). The fresh ovules were dissected from the siliques using forceps and mounted in Hoyer’s solution [chloral hydrate:glycerol:water, 8:1:2 (v/v/v)] for between 30 min to 6–8 h depending on the embryo developmental stage (Berleth and Jürgens, 1993). The cleared ovules were then imaged using a differential interference contrast microscope (Olympus TH2-200) equipped with CCD sensors of a SPOT Digital Microscope Camera ( Diagnostic Instruments) and assessed for abnormalities. At each sampling time, the total number of embryos and the number of abnormal embryos were counted, and the proportions of the different types of abnormal embryos were calculated.

Cell viability assays

Fertilized ovules at different developmental stages (3, 4, and 5 DAP) of the nse1-1/+ and nse3-1/+ mutants were collected, and embryos were isolated according to the method described previously by Yu and Zhao (2012). Using a hand-made micropipette, homozygous embryos were selected under an inverted microscope and stained with 5 μg ml−1 FDA (fluorescein diacetate, Sigma) and 10 μg ml−1 PI (propidium iodide, Sigma) mixed dyes to observe the activity of cells, which were imaged under a confocal microscope (Olympus FluoView FV1000); FDA: excitation, 488 nm; emission, 505–530 nm; PI: excitation, 559 nm; emission, 515–545 nm. To detect dead cells in the root tip, seeds were germinated and cultured under an inverted microscope (Olympus TH2-200) equipped with CCD sensors of a SPOT Digital Microscope Camera (Diagnostic Instruments) and assessed for abnormalities. At each sampling time, the total number of embryos and the number of abnormal embryos were counted, and the proportions of the different types of abnormal embryos were calculated.

Transmission electron microscopy

Ovules at 4 DAP in wild-type siliques and aborted white ovules in nse1-1/+ and nse3-1/+ siliques were isolated, fixed, and embedded as described by Chen et al. (2013). Ultrathin sections (60–70 nm) were cut using a MTX ultramicrotome (RMC), and examined and images under a transmission electron microscope (JEM-1400plus; JEOL).

Results

The Ring-like domain of AtNSE1 is essential for interactions with the N-terminal domain of AtNSE3 and three conserved α helices of AtNSE2

In our previous work, we demonstrated that AtNSE1 and AtNSE3 can interact directly with each other (Li et al., 2017). In yeast, it is known that the Ring-like domain is not essential for the NSE1–NSE3 interaction, and that a minimal region of NSE3, namely a disordered N-terminal region, is required for the interaction (Pebbernard et al., 2008). However, details of the interaction in Arabidopsis remain unknown.

3D structure modeling of Arabidopsis AtNSE1 or AtNSE3 indicated that both of these proteins are very similar to the human homolog. There were two conserved WH domains and a Ring-like domain in AtNSE1, while AtNSE3 had a conserved MAGE domain including WHA and WHB domains (Supplementary Fig. S1A, B, E, F). To identify the regions that were responsible for the AtNSE1–AtNSE3 interaction, we used BiFC assays in a Nicotiana benthamiana leaf epidermal cell transient transformation system. The yellow fluorescent protein (YFP) signals of AtNSE1 94–312-YFPN/C and the full AtNSE3C/N were observed in the nucleus of the transformed cells, and similar results were obtained for AtNSE1 187–312-YFPN/C and AtNSE1 241–312N/C could interact with AtNSE3C/N, although the YFP signals expanded to the whole cells (Figs 1A, 2). Therefore, the C-terminal of AtNSE1, including the Ring-like domain, was essential for the interaction with AtNSE3. On the other hand, both AtNSE3 1–111N/C and AtNSE3 1–249N/C could interact with AtNSE1C/N (Figs 1B, 3). In contrast, neither AtNSE3 25–111N/C, AtNSE3 25–237N/C, nor AtNSE3 112–237N/C could bind with AtNSE1C/N (Figs 1B, 3). These results indicated that the 24 amino acids of the N-terminal of AtNSE3 were efficient for the AtNSE1–AtNSE3 interaction.

It is known that AtNSE2 (also known as a SUMO ligase) is an important subunit of the SMC5/6 complex and that it can bind to AtSMC5 in Arabidopsis (Xu et al., 2013). Like AtNSE1, mutation of AtNSE2 can also lead to defects in embryo development; however, the relationship between them is not known. To shed light on this, we first carried out Y2H assays to test for an interaction between AtNSE1 and AtNSE2. The results indicated that the full AtNSE1 and AtNSE2 could interact directly with each other in yeast (Fig. 1A, C, Supplementary Fig. S2A), and this was also confirmed by BiFC assays in N. benthamiana leaf epidermal cells (Fig. 1A, C, Supplementary Fig. S2B). A series of BiFC assays were then performed to determine the critical regions required for the AtNSE1–AtNSE2 interaction. First, the 3D structure modeling results had shown that AtNSE2 had a conserved helix bundle region of an N-terminal and a SP-RING motif of the C-terminal similar to the homolog in Saccharomyces cerevisiae (Supplementary Fig. S1C, D) (Duan et al., 2009), and this helix bundle consisted of three α helices. The BiFC results indicated that AtNSE2 41–249N/C and AtNSE1C/N could interact with each other in the nucleus, and AtNSE2 1–40N/C could also bind to the full AtNSE1 (Figs 1C, 4). Second, the YFP signals in the nucleus could also be observed in AtNSE2 83–249N/C and AtNSE2 41–82N/C with AtNSE1, but AtNSE2 171–249N/C could not interact with AtNSE1 directly (Figs 1C, 4). Taken together, these results suggested that the N-terminal helix bundle was essential for binding with AtNSE1. In addition, we also used BiFC to investigate the crucial region of AtNSE1 during the AtNSE1–AtNSE2 interaction. The results indicated that only the Ring-like domain of AtNSE1 was necessary for binding with AtNSE2 (Figs 1A, 5).

Taken together, these results indicated that the C-terminal region including the Ring-like motif of AtNSE1 played an important role in the interaction with the N-terminal 24 amino acids of AtNSE3, and only the Ring-like motif of AtNSE1 was critical for binding with the helix bundle region of AtNSE2.
AtNSE1 and AtNSE3 work non-redundantly in early embryo development

In our previous work, we found that the homozygous mutants of AtNSE1 and AtNSE3 were embryo lethal, and that these two proteins could interact with each other (Li et al., 2017), which suggested that their mutants might have similar phenotypes. Using ovule clearing, we found that the defective phenotypes of the nse1 and nse3 mutant embryos during seed formation were different from the wild-type but were very similar to each other (Fig. 6A–C). It was noticeable that the abnormal mutant embryos could be divided into two types: defective only in the embryo proper, and defective in both the embryo proper and the suspensor (Table1). We found that each single-mutant had ~25% abnormal embryos between 3–7 DAP but the proportions of the two types changed over time, with an increased proportion of the second type being observed as the embryos developed. This indicated that abnormal cell divisions in the mutant embryos became increasingly serious as development progressed. Eventually, even the boundary between the embryo proper and the suspensor became indistinguishable (Fig. 6B7–8, 6C7–8). To obtain a better understanding of the functional relationship between AtNSE1 and AtNSE3, we crossed nse1-1/+ with nse3-1/+ and examined the phenotype of the double-mutant. We found 43.65% (n=1024) abortive white seeds in the nse1-1/+ nse3-1/+ line in the F2 progeny (Supplementary Fig. S3), which conformed to the expected 43.75% ratio according to Mendel’s second law. Similar to the single-mutants, aborted embryos of the double-mutant appeared after the 8-cell embryo stage (Fig. 6D) and the abnormal embryos could again be divided into the two types,
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AtNSE1 and AtNSE3 are essential for maintenance of meristem activity during embryogenesis

AtNSE2 functions in stem-cell niche maintenance in the Arabidopsis root, even at the embryogenesis stage (Xu et al., 2013). Our results showed that AtNSE1 interacted with AtNSE2. In the embryos of the nse1 and nse3 mutants, the cotyledons could not be differentiated and an irregular shape of pro-embryos occurred from the beginning of the 8-cell stage. These results suggested that AtNSE1 and AtNSE3 may also have important roles in the maintenance of meristem activity.

To further investigate the embryo defects in nse1-1 and nse3-1, we crossed them with some embryo-specific marker lines and observed their expression patterns in embryos. In Arabidopsis, SHOOT MERISTEMLESS (STM) is expressed in the shoot meristem and is required for maintenance of the meristematic cell function (Long and Barton, 1998). We used translational fusion STM to the YFP variant VENUS (pSTM::STM-VENUS) (Heisler et al., 2005) and examined the expression of the fusion proteins in the mutant embryos. In the wild-type, expression of pSTM::STM-VENUS was not detected in the globular embryos, but it appeared at the heart-shaped stage (Fig. 7A, B). The nse1-1 and nse3-1 mutants had no obvious shoot meristems, and the STM signal could not be detected in some of the embryos (Fig. 7D, F) but it did appear in some others (Fig. 7C, E). Therefore, we could conclude that mutation of AtNSE1 or AtNSE3 interfered with the expression of the STM gene, which resulted in a loss of the meristem activity.

Given that the basal regions of embryos were abnormal in the nse1 and nse3 mutants, we carried out a comparative analysis of the expression patterns of WUSCHEL-LIKE HOMEOBOX5...
Li et al. using the transcription fusions pWOX5::GFP and gWOX8::YFP. The well-characterized WOX5 gene is initially expressed specifically in the hypophysis of globular embryos, and then subsequently in the quiescent center (QC) during the heart-shaped stage, and it is involved in the maintenance of root stem cells (Haecker et al., 2004; Sarkar et al., 2007). In contrast to the wild-type (Fig. 7G, H), the expression of WOX5 was not restricted to a specific location in the mutant embryos. Instead, it was randomly expressed in the embryo proper and in the suspensors (Fig. 7I–L), showing that the location of its expression was extended and that the QC was out of control in a manner that led to the disruption of stem cell proliferation. WOX8 is known to be expressed specifically in suspensor cells and is essential for establishing the apical–basal axis in Arabidopsis (Haecker et al., 2004; Wu et al., 2007). We found that WOX8 was expressed throughout the suspensor in the wild-type (Fig. 7M, N). In contrast, whilst the WOX8 signal was detected throughout the suspensor in some embryos of the nse1-1 and nse3-1 mutants (Fig. 7O, Q), it was only observed in the basal cells of the suspensors in others (Fig. 7P, R). These results indicated that suspensor cell development might have been defective in the mutants, and this was particularly pronounced in the upper cells of the suspensor adjacent to the QC. Hence, mutation of AtNSE1 or AtNSE3 led to disrupted expression of WOX5 and WOX8, and to cells in the QC having disordered and excessive division. In addition, expression of the embryo proper-specific WOX5 extended to the upper region of the suspensor, while the expression of the suspensor-specific WOX8 was confined to the only the basal cells rather than throughout the whole suspensor.

Overall, the expression patterns of the disrupted marker genes suggested that AtNSE1 and AtNSE3 are required in shoot and root meristem maintenance, and in suspensor development during Arabidopsis embryogenesis.

**Fig. 3.** Bimolecular fluorescence complementation assays between different truncated AtNSE3 regions and full-length AtNSE1. The assays were conducted in tobacco leaf cells. AtNSE3(1–111), and AtNSE3(1–24) were able to interact with full-length AtNSE1, whilst AtNSE3(25–237), AtNSE3(112–237), and AtNSE3(25–111) had no association with AtNSE1. YFP<sup>C</sup>, YFP C-terminal fragment (aa 156–239); YFP<sup>N</sup>, YFP N-terminal fragment (aa 1–155). Scale bars are 50 μm.
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Auxin transport and responses in the nse1-1 and nse3-1 mutants are disrupted during embryo development

Auxin is an important phytohormone in plant development, and the regulation of auxin maxima is critical for the establishment of embryo patterning (Möller and Weijers, 2009; Balzan et al., 2014). To determine whether the defects observed in the nse1-1 and nse3-1 mutants were associated with changes in auxin transport and/or responses, we examined the localization of pPIN1::PIN1-GFP and the expression of pDR5rev::3XVENUS-N7 in the mutant embryos. PIN1 is an essential auxin efflux transporter that mediates the establishment of the maxima through polar localization (Blilou et al., 2005). In the wild-type globular embryos, pPIN1::PIN1-GFP was expressed only in apical cells, and its expression was polarized in the plasma membrane facing the basal embryo pole (Fig. 8A). At the heart-shaped stage, PIN1-GFP was expressed in the developing vasculature and cotyledon primordia (Fig. 8B). In the nse1-1 and nse3-1 mutants, however, the PIN1-GFP signal occurred irregularly in the central region of the embryo proper and even across the whole embryo proper (Fig. 8C, E). In addition, the signal was also apparent in the suspensor cells (Fig. 8D, F).

DR5, a synthetic auxin-responsive promoter, is often fused with a GFP tag and can be used as a marker to visualize the spatial pattern of auxin responses during embryogenesis (Friml et al., 2003). Consistent with the results for PIN1-GFP, expression of pDR5rev::3XVENUS-N7 in the nse1-1 and nse3-1 mutants was not concentrated in the hypophysis of the globular or heart-shaped embryos as seen in the wild-type (Fig. 8G, H), but instead extended to almost all of the embryo proper and the suspensor cells (Fig. 8I–L).

Overall, these results indicated that both the transport and responses of auxin were disrupted in the nse1-1 and
and nse3-1 embryos. Furthermore, in our previous study (Li et al., 2017), RNA-seq data for ovules at 7 DAP indicated that many genes related to auxin signaling were clearly down-regulated, including IAs, ARFs, and YUCs (Supplementary Table S2). This may suggest AtNSE1 and AtNSE3 are required for normal auxin biogenesis, translocation, and transduction.

**Loss of cell viability in embryos of the nse1-1 and nse3-1 mutants**

Our observations showed that mutations of AtNSE1 or AtNSE3 led to ovule death. The embryos in the abnormal white ovules displayed delayed development and the cells showed disorganized proliferation. These phenotypes suggested that cell division may have been disrupted in the aborted embryos, and that cell viability may have been affected. To test this, we isolated a series of homozygous embryos and different development times and double-stained them with fluorescein diacetate (FDA) and propidium iodide (PI). The wild-type embryonic cells had no PI signal except for some suspensor cells at later stages (Fig. 9A–C). Most of the cells in the mutant embryos at 3 DAP remained viable, but there were some that showed a PI signal, indicating that they were dead (Fig. 9D, G, J, M). An increasing number of dead cells appeared in the mutants at 4 DAP and 5 DAP (Fig. 9E, F, H, I, K, L, N, O). In addition, we observed that the pattern of cell division was completely disordered. These results indicated that the viability of the embryo cells was affected from an early developmental stage, and it increased as development continued. Thus, both AtNSE1 and AtNSE3 are essential for early embryo cell growth and division.

![Fig. 5. Bimolecular fluorescence complementation assays between different truncated AtNSE1 regions and full-length AtNSE2. The assays were conducted in tobacco leaf cells. AtNSE1(94–312), AtNSE1(187–312), and AtNSE1(187–240) were able to interact with full AtNSE2, whilst AtNSE1(241–312) and AtNSE1(1–186) had no association with AtNSE2. YFP, YFP C-terminal fragment (aa 156–239); YFP, YFP N-terminal fragment (aa 1–155). Scale bars are 50 μm.](image-url)
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Loss function of AtNSE1 and AtNSE3 induces programmed cell death

It has been reported that there are two types of programmed cell death (PCD) in plants according to the ultrastructural characteristics of the dead cells, namely vacuolar cell death and necrosis, and shrunken protoplasts and autophagosomes, respectively, are their characteristic markers (van Doorn et al., 2011; Minina et al., 2013). We observed that there were many dead cells in the homozygous embryos of both the nse1-1 and
nse3-1 mutants (Fig. 10). Interestingly, both shrunken protoplasts (Fig. 10E, H) and autophagosomes (Fig. 10F, I) appeared in nse1-1 and nse3-1, suggesting that both vacuolar cell death and necrosis occurred in the mutant embryos. Thus, AtNSE1 and AtNSE3 play an essential role in maintaining cell viability in early embryo development in Arabidopsis.

**Discussion**

*Novel interactions exist between AtNSE1, AtNSE2, and AtNSE3*

The SMC5/6 complex was initially characterized in yeast (Fousteri and Lehmann, 2000; Sergeant et al., 2005), and it is well known for its role in maintaining genome stability (De Piccoli et al., 2009). In addition to the core subunits SMC5 and SMC6, there are six other non-SMC elements (NSEs) that have been identified in yeast, humans, and Arabidopsis, and the whole complex is made up of three sub-complexes, namely NSE2-SMC5-SMC6, NSE1-NSE3-NSE4, and NSE5-NSE6, which act as specialized functional modules in yeast (Diaz and Pecinka, 2018). Although the interactions of the SMC5/6 complex are clear in yeast and humans, the situation is less well understood in Arabidopsis. It is known that the Ring-like domain of NSE1 is not essential for binding with the N-terminal of NSE3 in yeast, while the N-terminal of NSE1 is necessary for the interaction with NSE3 (Pebernard et al., 2008).

In contrast to yeast and humans, in Arabidopsis we found that the Ring-like domain of AtNSE1, but not the N-terminal region, was sufficient for binding with AtNSE3 (Figs 1, 2). In common with yeast and humans, we found that the AtNSE3 N-terminal, not the MAGE domain, played an essential role in the interaction with AtNSE1. NSE2, on the other hand, is known to bind to SMC5 in fungi, animals, and plant (Fousteri and Lehmann, 2000; Potts and Yu, 2005; Duan et al., 2009; Xu et al., 2013). Structural analysis has shown that the N-terminal domain of NSE2 contributes to SMC5 binding, whereas its C-terminal domain contains a variant RING structure and has no contact with SMC5 (Duan et al., 2009). All of the three α helix regions contribute to the NSE2–SMC5 interaction (Duan et al., 2009). Although AtNSE2 has been found to bind with AtSMC5 in Arabidopsis (Xu et al., 2013), the association of AtNSE1 and AtNSE2 has not been characterized before. In this study, we found that AtNSE1 could directly bind to AtNSE2 (Supplementary Fig. S2), and that the Ring-like domain of AtNSE1 was required for the interaction with the three α helix regions of the AtNSE2 N-terminal (Figs 1, 4, 5). This interaction model indicated that the detailed structure of the SMC5/6 complex might have some differences in Arabidopsis compared with yeast and humans. It is known that the SP-RING domain of NSE2 gives it small ubiquitin-like modifier (SUMO) ligase activity (Zhao and Blobel, 2005). In vitro studies have shown that NSE2 adds SUMO modifications to numerous proteins, including SMC5, SMC6, NSE3.
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NSE4, and even to itself (Andrews et al., 2005; Potts and Yu, 2005, 2007; Zhao and Blobel, 2005; Pebernard et al., 2008; McAleenan et al., 2012; Diaz and Pecinka, 2018). In addition to the novel interaction between AtNSE1 and AtNSE2, it seems that AtNSE2 also could act on AtNSE1 through its SUMO ligase activity. However, more experimental data are required to explore this interesting possibility.

Overall, we found some novel interactions between AtNSE1, AtNSE2, and AtNSE3 in Arabidopsis that differed from those found in fungal and mammal cells. This indicates that the pattern of organization of the SMC5/6 complex in Arabidopsis has some novel characteristics, which may provide more clues to its biological functions.

AtNSE1 and AtNSE3 are essential for embryo pattern formation in Arabidopsis.

NSE1 was first identified in budding yeast (Fujioka et al., 2002), while NSE3 was identified in yeast undergoing meiosis (Pebernard et al., 2004). They were then also characterized in

**Fig. 7.** Expression patterns of the embryo-specific marker genes STM, WOX5, and WOX8 in the Arabidopsis wild-type and the nse1-1 and nse3-1 mutants. STM-Venus was expressed only in the shoot apical meristem of the wild-type embryos (A, B), but in the mutants it could be expressed in other cells (C, E) or in no cells (D, F). WOX5-GFP was expressed in quiescent center of the wild-type (G, H), but in the mutants it occurred in the embryo proper (I, K) or in the suspensor cells (J, L). WOX8-YFP was expressed throughout the suspensor cells of the wild-type (M, N) and in some embryos of the mutants (O, Q), but in other mutant embryos it was only expressed in the basal cells of the suspensor (P, R). Scale bars are 20 μm.
Drosophila melanogaster (Li et al., 2013), Dictyostelium (Taniura et al., 2015), and in humans (Potts and Yu, 2005; Taylor et al., 2008). More recently, it has been reported that the SMC5/6 complex is required for early embryo development in Drosophila (Tran et al., 2016) and that expression of Smc5 during oocyte growth is crucial for the early stages of embryogenesis in mice (Hwang et al., 2017). As components of the SMC5/6 complex, NSE1 and NSE3 play important roles in cell proliferation and meiosis (Fujioka et al., 2002; Pebernard et al., 2004); however, their functions in plant embryo development have not been fully described. Similar to previous findings in yeast, we found that mutations of AtNSE1 and AtNSE3 resulted in disordered cell division in embryos and they were not able to complete organ differentiation. We found that both cell division and organ primordial differentiation in the mutants were affected (Fig. 6). These effects were concomitant with disruption of the expression of STM and WOX5 (Fig. 7), and we suggest that was the cause of the disordered proliferation of the stem cells. It has been reported that mutation of AtNSE2 also causes WOX5 expression to be diffused across adjacent cells (Xu et al., 2013), indicating that AtNSE2 is involved in root stem-cell niche maintenance. We therefore considered that the AtSMC5/6 complex might play a crucial role in stem cell maintenance; however, we found that AtNSE1 and AtNSE3 were also involved in maintenance of the shoot stem cells. It is known that auxin is very important for embryogenesis, and we found that the expression patterns of PIN1 and DR5 were completely disrupted in the nse1-1 and nse3-1 mutants. In addition, previous RNA-seq results (Li et al., 2017) showed that both auxin biogenesis and signaling were down-regulated significantly in the mutants (Supplementary Table S2). However, more data on the genetic relationship between AtNSE1/3 and the auxin-mediated pathways is still required. KEGG analysis of the RNA-seq results also showed plant hormone transduction pathways were down-regulated significantly in the mutants (Supplementary Fig. S4), which may suggest that mutations of AtNSE1 and AtNSE3 have a general effect on hormone regulation.

It is not clear why we observed changes in the expression patterns of the marker genes that we studied, although it is likely that damage to DNA played a role. It is noteworthy that AtNSE2 may bind to chromosomes through an interaction with the SMC5/6 complex and thus function in the regulation of gene expression (Xu et al., 2013). AtNSE2 and a NSE6 homologue, SN11, have been shown to interact with the E2F/DP pathway and to participate in regulation of the cell cycle in Arabidopsis (Liu et al., 2016; Wang et al., 2018), which might suggest that the SMC5/6 complex also functions in regulation.
AtNSE1/3 are required in maintaining cell viability during embryogenesis

of gene expression. It is therefore possible that both DNA damage and the SMC5/6 complex contribute to the disrupted expression patterns in the mutant embryos.

AtNSE1 and AtNSE3 are crucial factors for maintaining directional differentiation of suspensor cells

Suspensor cells originate from the basal cell formed after the first asymmetric cell division of the zygote (ten Hove et al., 2015). Although the suspensor degenerates at the late stage embryogenesis, it is essential for early embryo development (Yeung and Meinke, 1993; Kawashima and Goldberg, 2010).

It is known that conversion of cell fate can occur in the suspensor in the course of abnormal pro-embryo development in Arabidopsis. Diphtheria toxin A (DTA) is a highly toxic protein that is used to ablate specific cells and tissues. Local expression of DTA in the embryo proper leads to excessive proliferation of suspensor cells in Arabidopsis that can result in the formation of secondary embryos (Weijers et al., 2003), indicating that the suspensor can develop into an embryo if inhibition from the embryo proper is removed. The upper cells of the suspensor have the potential to develop into an embryo at the globular embryo stage in Arabidopsis, and this potential is often suppressed by the embryo proper (Gooh et al., 2015; Liu et al., 2015). In conifers, polar auxin transport is important for embryo development, which controls the suspensor fate and affects embryo pattern formation (Larsson et al., 2008). Interestingly, we found that WOX8 was expressed only in the basal cells in the nse1 and nse3 mutants rather than in the whole suspensor and that the expression of WOX5 was not restricted in the QC cells but extended.

Fig. 9. Cellular viability at different stages of embryo development in the Arabidopsis wild-type (WT) and nse1-1 and nse3-1 mutants. Viability was determined by double-staining with fluorescein diacetate/propidium iodide (FDA/PI), where green fluorescence indicates living cells and red fluorescence indicates dead cells. DAP, days after pollination. Scale bars are 20 μm.
to the upper suspensor cells as well as the embryo proper (Fig. 7). Consistent with these results, PIN1 and DR5 signals extended to the upper suspensor cells (Fig. 8). These results suggested that the cell fate of the upper suspensor cells was altered in the mutants, which might have been caused by the abnormal development of the embryo proper and by the disrupted transport of auxin. Thus, both AtNSE1 and AtNSE3 are required for the directional development of the suspensor cells during early embryogenesis in Arabidopsis.

**Fig. 10.** Mutations in Arabidopsis AtNSE1 and AtNSE3 lead to necrosis and vacuolar programmed cell death (PCD). TEM images of the ultrastructure of embryo cells of the wild-type (A–C) and the mutants nse1-1 (D–F) and nse3-1 (G–I). Both necrosis and vacuolar PCD occurred in nse1-1 (E, F) and nse3-1 (H, I). N, nucleus; V, vacuole; *, cell wall. Black arrows indicate autophagosomes; white double-headed arrows indicate detachment of the plasma membrane from the cell wall. Scale bars are 2 μm (B, E, F, H) and 5 μm (A, C, D, G, I).

AtNSE1 and AtNSE3 are required for maintaining cell viability during embryo development in Arabidopsis

Numerous studies have indicated that cell death in Arabidopsis root stem cells is a consequence of DNA double-strand breaks (DSBs) (Boltz et al., 2012; Xu et al., 2013; Yoshiyama et al., 2013; Horvath et al., 2017). Our previous work also showed that AtNSE1 and AtNSE3 mutations increased DSBs and caused cell death in the roots (Li et al., 2017); however, whether a similar phenomenon occurred in the embryos was not clear. The results from our current study showed that cell death occurred at a very early stage in the nse1 and nse3 mutant embryos (Fig. 9), suggesting that AtNSE1 and AtNSE3 are essential for maintaining cell viability during embryogenesis. Programmed cell death (PCD) occurs widely in plants, and it has been reported that there are two main types according to the ultrastructural characteristics of the dead cells, namely vacuolar PCD and necrosis (van Doorn et al., 2011; Minina et al., 2013). Here, we found that mutations in AtNSE1 and AtNSE3 resulted in both types of PCD (Fig. 10) and this appeared to be the direct cause of lethality leading in the mutant embryos. As it is known that the AtSMC5/6 complex is
conserved in DNA damage repair, we therefore suggest that PCD in the mutant embryos may be caused by unrepaired DNA damage.

Conclusions

Our study has indicated that the Ring-like domain of AtNSE1 is essential for its interaction with AtNSE2 and AtNSE3, and the interactions in Arabidopsis are different to those in yeast and humans. AtNSE1 and AtNSE3 are required for early embryo development, including determination of the morphology of the embryo proper and the suspensor. Cytological analysis of the nse1 and nse3 mutants showed that auxin transportation was disrupted and maintenance of stem cells failed. Programmed cell death occurred in the mutants from a very early stage and was apparent through almost the whole embryo development process. As components of the SMC5/6 complex, AtNSE1 and AtNSE3 play crucial roles in early embryogenesis by maintaining stem cell development. Our results provide a better understanding of the biological functions of the SMC5/6 complex during plant embryo development.

Supplementary data

Supplementary data can be found at JXB online.

Fig. S1. Homologous modelling of the protein structures of AtNSE1, AtNSE2, and AtNSE3.

Fig. S2. Yeast two-hybrid and BiFC assays for interactions between AtNSE1 and AtNSE2.

Fig. S3. Phenotypes of siliques of the nse1-1/+ nse3-1/+ double-mutant and the wild-type.

Fig.S4. KEGG pathway enrichment analysis of down-regulated genes in the ovules of the mutants (Li et al., 2017).

Table S1. List of primers used in the experiments.

Table S2. RNA-seq analysis results for genes with down-regulated expression in the nse1-1 and nse3-1 mutants compared to the wild-type (Li et al., 2017).

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