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RESEARCH PAPER

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 detail 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 *AtNE3* 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 helixes 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/+*), CS24066 (*nse1-2/+*), CS334183 (*nse3-2/+*, 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 *pWOX5::GFP* line (Heidstra *et al.*, 2004; Blilou *et al.*, 2005) from Ben Scheres (Albert-Ludwigs University, Germany), and the *gWOX8-YFP* line (Ueda *et al.*, 2011) from Thomas Laux (Albert-Ludwigs University). The different marker lines were crossed with *nse1-1/+* and *nse3-1/+* and the progenies were selected through PCR and observed under a fluorescence microscope (Olympus FV1000). The *nse1-1/+* and *nse3-1/+* 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-SPYNE* and *pCAMBIA-SPYCE* vectors. Primers used in this test are listed in Supplementary Table S1 at *JXB* online.

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 download and displayed using PyMOL1.3.

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 (w/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 TH4-200) 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/+*, *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, 515–545 nm; PI: excitation, 559 nm; emission, 505–530 nm.

To detect dead cells in the root tip, seeds were germinated and cultured on non-selective half-strength Murashige and Skoog medium for 5 d, the roots of the seedlings were mounted onto glass slides with 40 μg ml^{-1} PI, and then observed and imaged using a confocal microscope (Olympus FluoView FV1000).

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.* (2015). 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 helixes 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 (Pebernard *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-$YFP^{N/C}$ and the full $AtNSE3^{C/N}$ were observed in the nucleus of the transformed cells, and similar results were obtained for AtNSE1 187-312-$YFP^{N/C}$ (Figs 1A, 2). Consistent with these results, there was no signal in the assays of AtNSE1 1-186-$YFP^{N/C}$ and the full $AtNSE3^{C/N}$. Consequently, the WH1 and WH2 motifs located in the N-terminal of AtNSE1 (1–186 aa) were not required for binding with AtNSE3. Similarly, we found that both AtNSE1 187-$240^{N/C}$ and AtNSE1 241-$312^{N/C}$ could interact with $AtNSE3^{C/N}$, 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-$111^{N/C}$ and AtNSE3 1-$24^{N/C}$ could interact with $AtNSE1^{C/N}$ (Figs 1B, 3). In contrast, neither AtNSE3 25-$111^{N/C}$, AtNSE3 25-$237^{N/C}$, nor AtNSE3 112-$237^{N/C}$ could bind with $AtNSE1^{C/N}$ (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 helixes. The BiFC results indicated that AtNSE2 41-$249^{N/C}$ and $AtNSE1^{C/N}$ could interact with each other in the nucleus, and AtNSE2 1-$40^{N/C}$ could also bind to the full AtNSE1 (Figs 1C, 4). Second, the YFP signals in the nucleus could also be observed in AtNSE2 83-$249^{N/C}$ and AtNSE2 41-$82^{N/C}$ with AtNSE,1 but AtNSE2 171-$249^{N/C}$ 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, the 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.

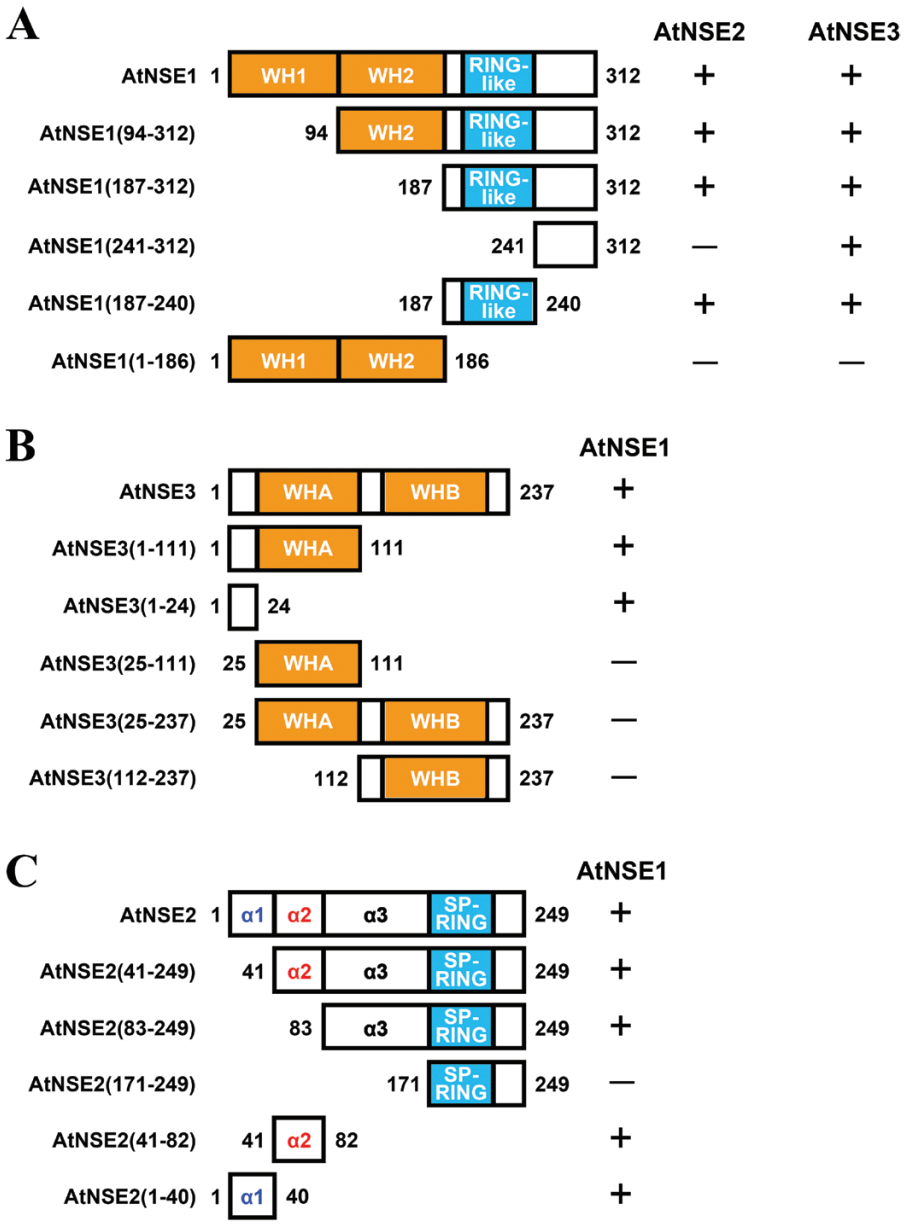

Fig. 1. Summary of the truncations of the Arabidopsis AtNSE1/2/3 proteins and the effects on their interactions. Schematic diagrams are shown of (A) the regions of AtNSE1 required interactions with AtNSE2 and AtNSE3, (B) the regions of AtNSE3 required for interaction with AtNSE1, and (C) the regions of AtNSE2 required for interaction with AtNSE1. + indicates that proteins can interact each other; – indicates that the proteins cannot interact.

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,

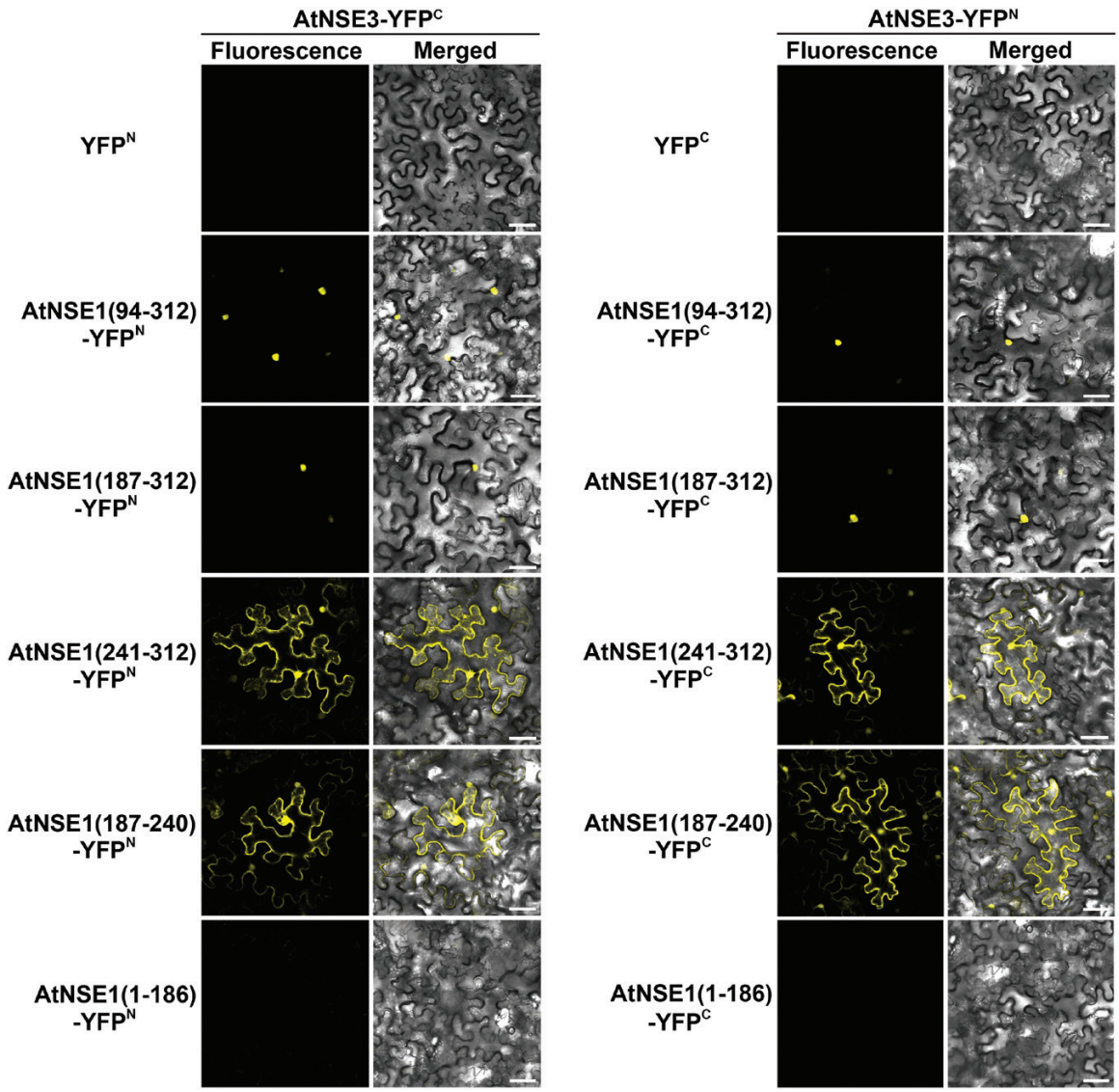

Fig. 2. Bimolecular fluorescence complementation assays between different truncated AtNSE1 regions and full-length AtNSE3. The assays were conducted in tobacco leaf cells. AtNSE1(94–312), AtNSE1(187–312), AtNSE1(241–312), and AtNSE1 (187–240) were able to interact with full-length AtNSE3, whilst AtNSE1(1–186) had no association with AtNSE3. YFP^C, YFP C-terminal fragment (aa 156–239); YFP^N, YFP N-terminal fragment (aa 1–155). Scale bars are 50 μm.

with the proportion of embryos with abnormal embryo proper and suspensor again increasing at the later stages of development (Table 1). We therefore concluded that the functions of *AtNSE1* and *AtNSE3* are non-redundant during early embryo development.

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*

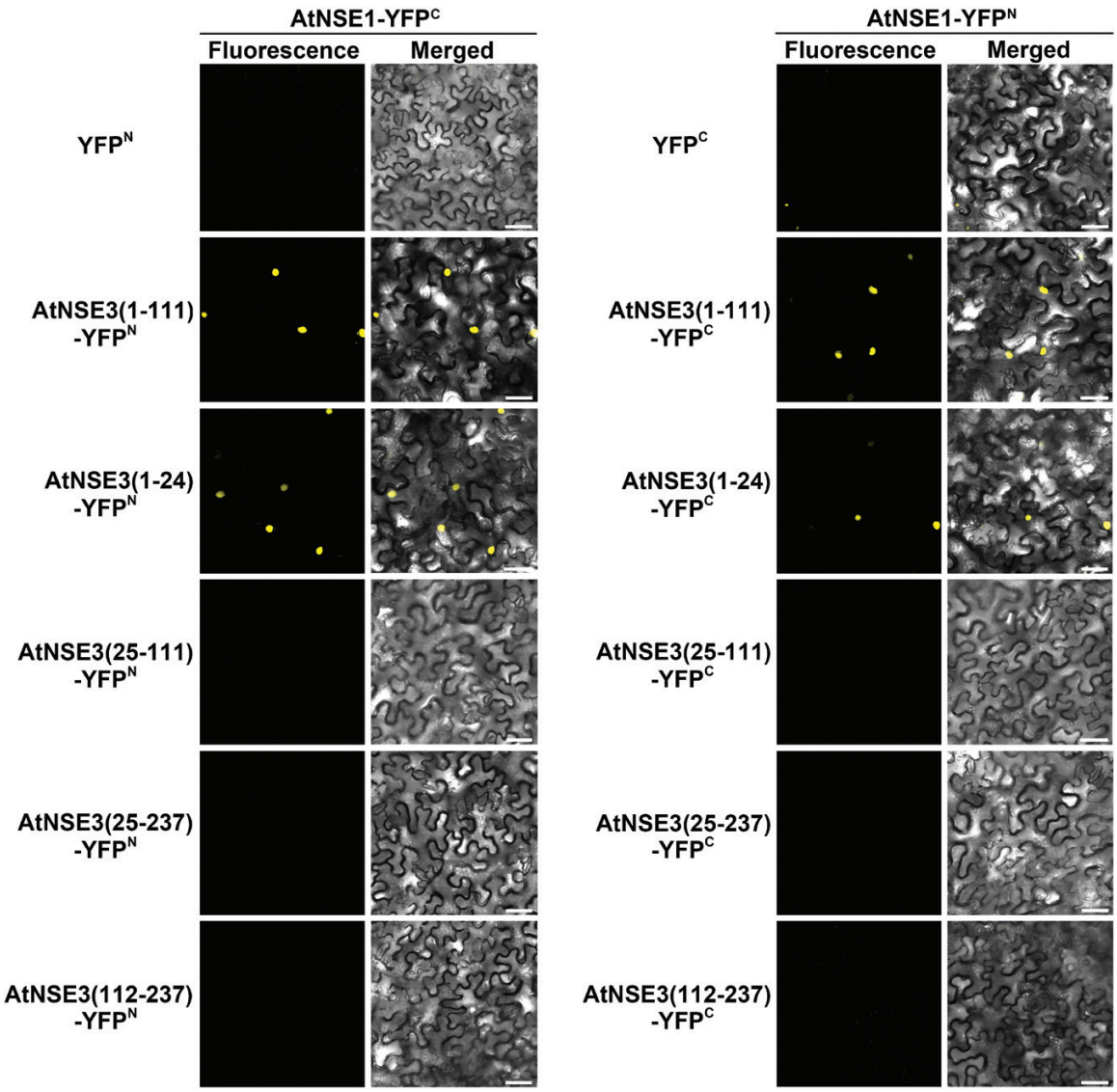

Fig. 3. Bimolecular fluorescence complementation assays between different truncated AtNSE3 regions and full-length AtNSE1. The assays were conducted in tobacco leaf cells. AtNSE3(1–111), and AtNSE3(1–24) were able to interact with full-length AtNSE1, whilst AtNSE3(25–237), AtNSE3(112–237), and AtNSE3(25–111) had no association with AtNSE1. YFP^C, YFP C-terminal fragment (aa 156–239); YFP^N, YFP N-terminal fragment (aa 1–155). Scale bars are 50 μm.

(*WOX5*) and *WOX8* 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.

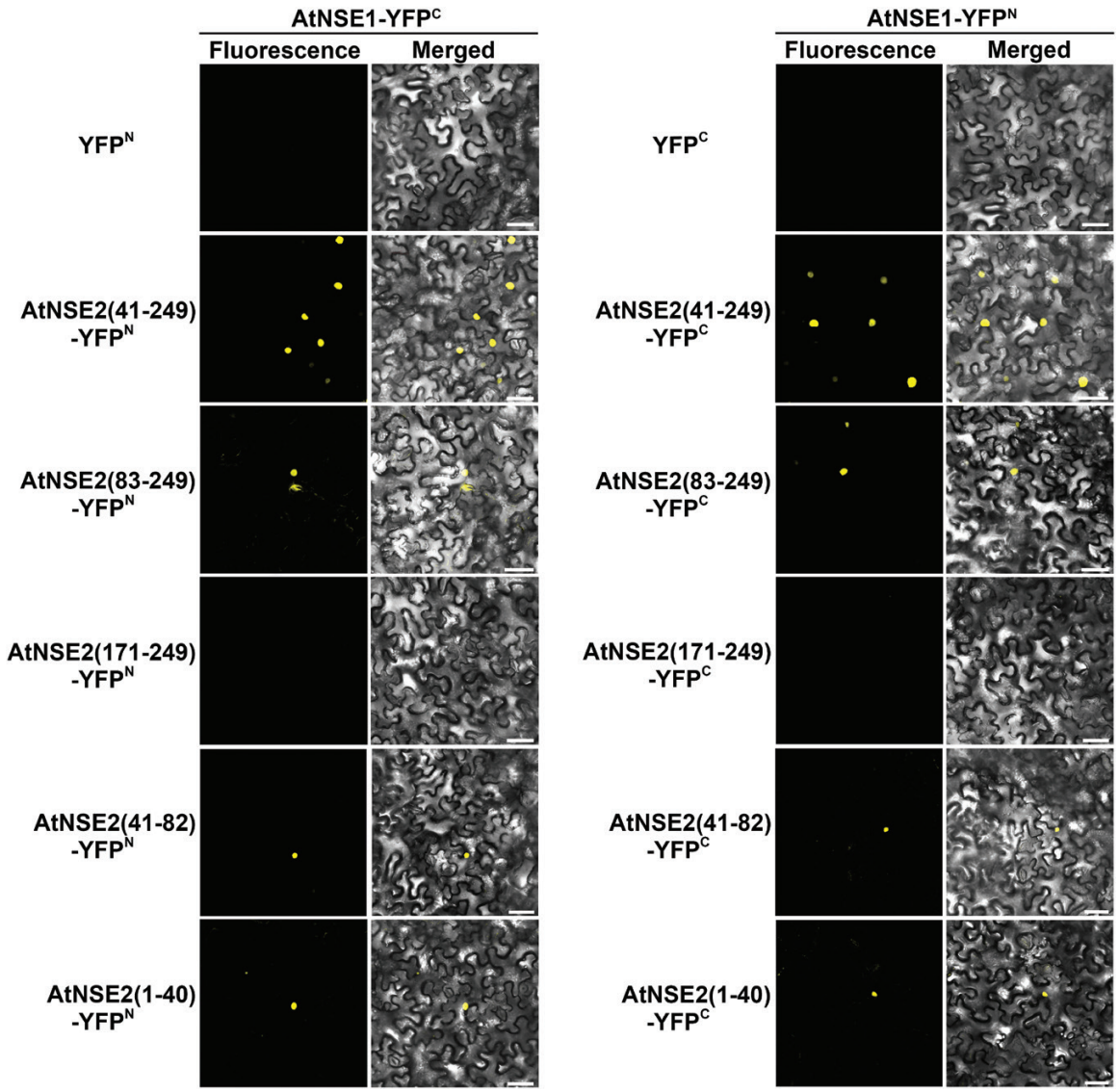

Fig. 4. Bimolecular fluorescence complementation assays between different truncated AtNSE2 regions and full-length AtNSE1. The assays were conducted in tobacco leaf cells. AtNSE2(41–249), AtNSE2 83–249) AtNSE2(41–82), and AtNSE2(1–40) can were able to interact with full AtNSE1, whilst AtNSE2(171–249) had no association with AtNSE1. YFP^C, YFP C-terminal fragment (aa 156–239); YFP^N, YFP N-terminal fragment (aa 1–155). Scale bars are 50 μm.

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 transportation and responses of auxin were disrupted in the *nse1-1*

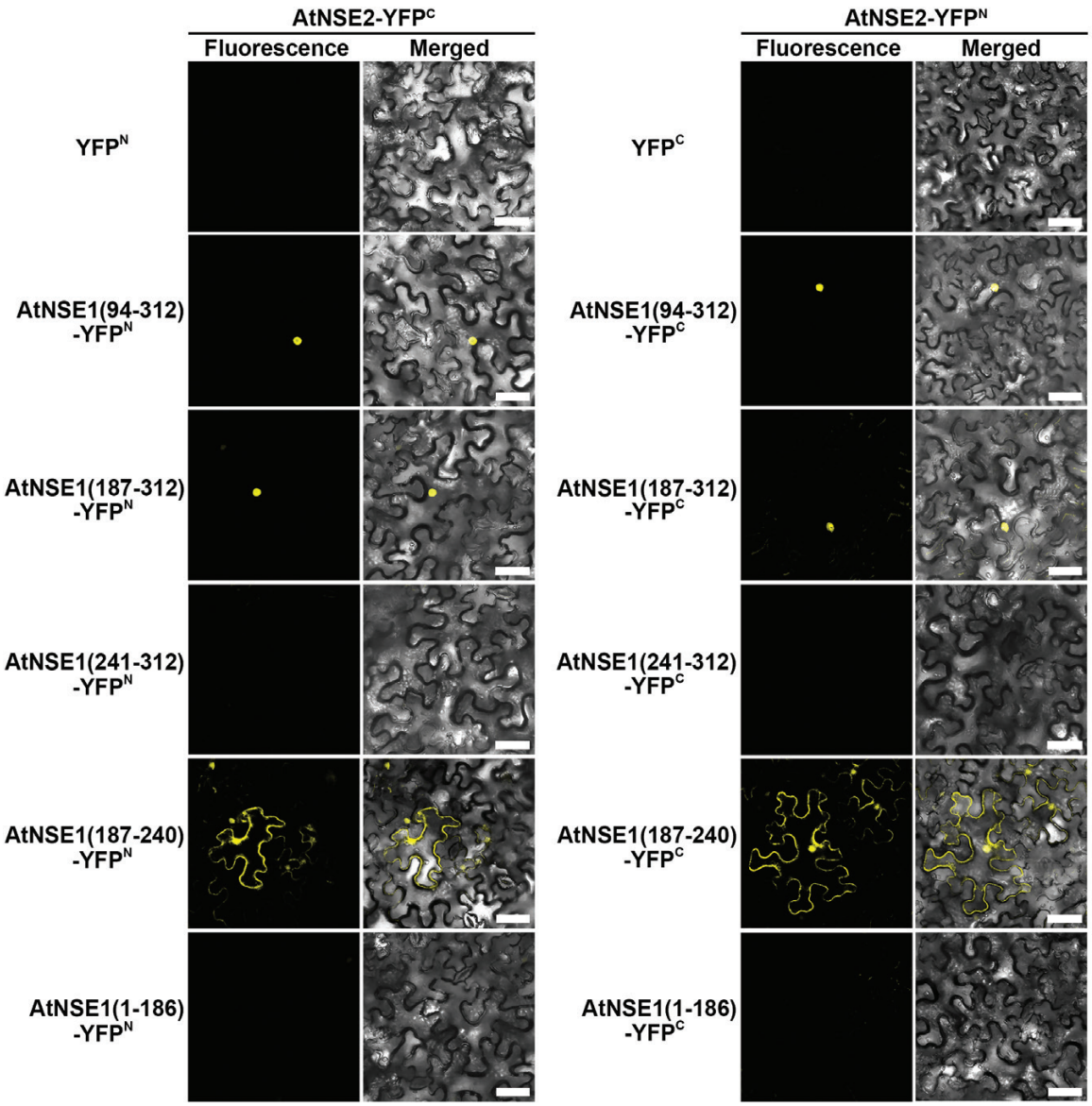

Fig. 5. Bimolecular fluorescence complementation assays between different truncated AtNSE1 regions and full-length AtNSE2. The assays were conducted in tobacco leaf cells. AtNSE1(94–312), AtNSE1(187–312), and AtNSE1(187–240) were able to interact with full AtNSE2, whilst AtNSE1(241–312) and AtNSE1(1–186)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

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 *IAA*s, *ARF*s, and *YUC*s (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.

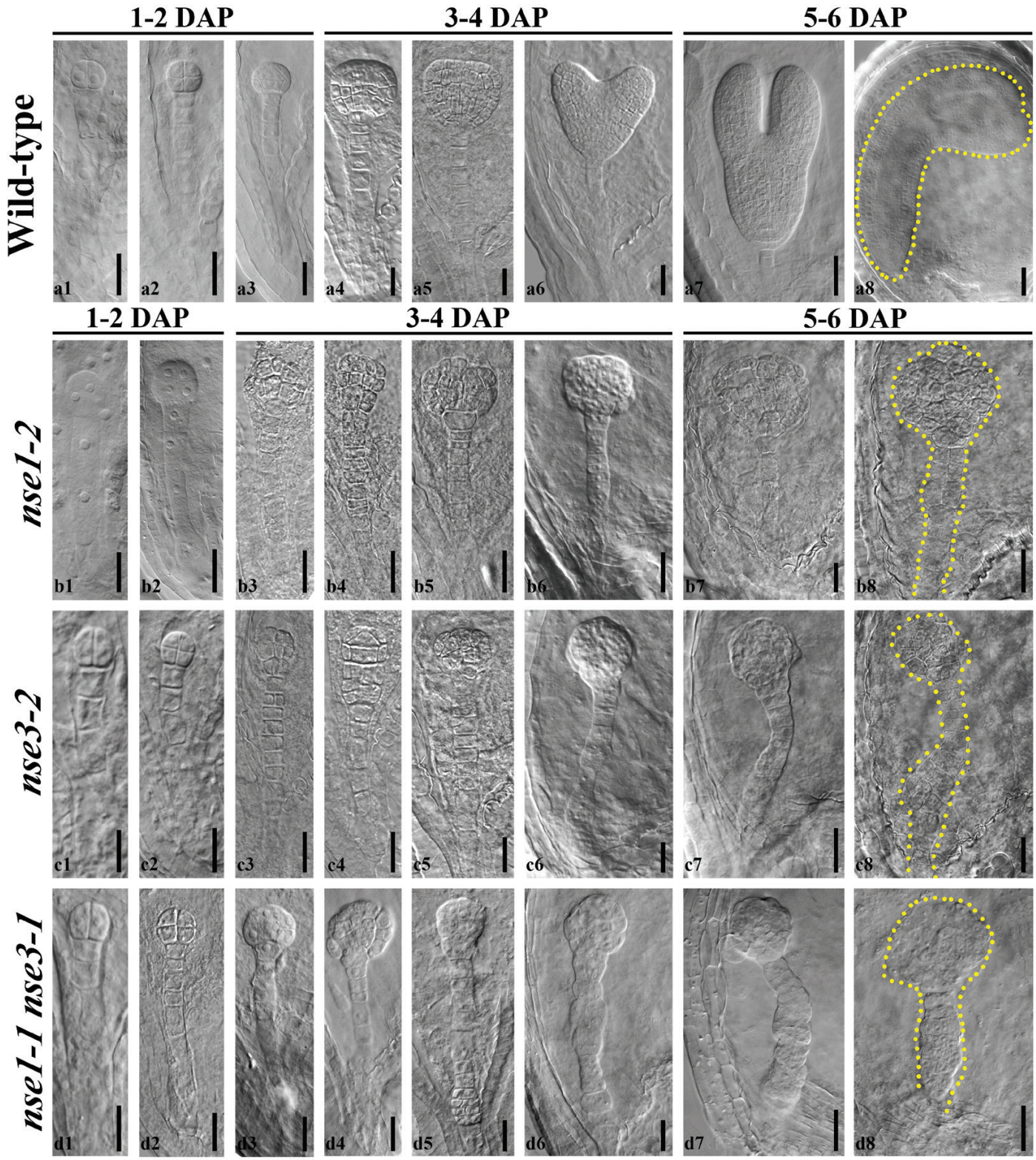

Fig. 6. Embryo development in Arabidopsis wild-type and *nse1-2*, nse*3-2*, and *nse1-1 nse3-1* mutants. Abnormal embryos occurred from 3 d after pollination (DAP) in *nse1-2* (B3), *nse3-2* (C3), and *nse1-1 nse3-1* (D3). Scale bars are 20 μm in A1–6, B1–7, C1–6, and D1–6, and 50 μm in the other images.

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

Table 1. *Occurrence of abnormal phenotypes in the Arabidopsis wild-type and* nse1-1/+ *and* nse3-1/+ *mutants during embryo development*

DAP	Genotype	*n*	Occurrence of abnormalities (%)		Aborted embryos (%)
			Embryo proper	Embryo proper and suspensor	
3	Wild-type	561	0	0	0.36
	nse1-1/+	457	23.41	1.09	24.50
	nse1-2/+	353	20.16	3.40	23.46
	nse3-1/+	675	22.67	1.04	23.71
	nse3-2/+	474	25.68	1.69	28.27
	nse1-1/+nse3-1/+	629	36.08	3.49	39.57
4	Wild-type	475	0	0	0.63
	nse1-1/+	654	17.18	8.71	25.89
	nse1-2/+	434	16.08	5.83	21.91
	nse3-1/+	514	16.93	7.00	23.93
	nse3-2/+	494	15.99	9.72	25.71
	nse1-1/+nse3-1/+	542	29.34	11.07	40.41
5	Wild-type	362	0	0	1.09
	nse1-1/+	410	4.39	20.98	25.37
	nse1-2/+	520	5.83	17.69	23.52
	nse3-1/+	471	4.25	19.75	24.00
	nse3-2/+	415	6.81	18.25	25.06
	nse1-1/+nse3-1/+	628	27.07	20.71	47.78
7	Wild-type	385	0	0	0.77
	nse1-1/+	657	0.53	25.23	25.76
	nse1-2/+	404	1.04	22.39	23.43
	nse3-1/+	572	0.18	25.28	25.46
	nse3-2/+	352	0.82	23.33	24.15
	nse1-1/+nse3-1/+	480	10.42	37.08	47.50

DAP: days after pollination. Embryos were sampled from at least nine siliques.

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,

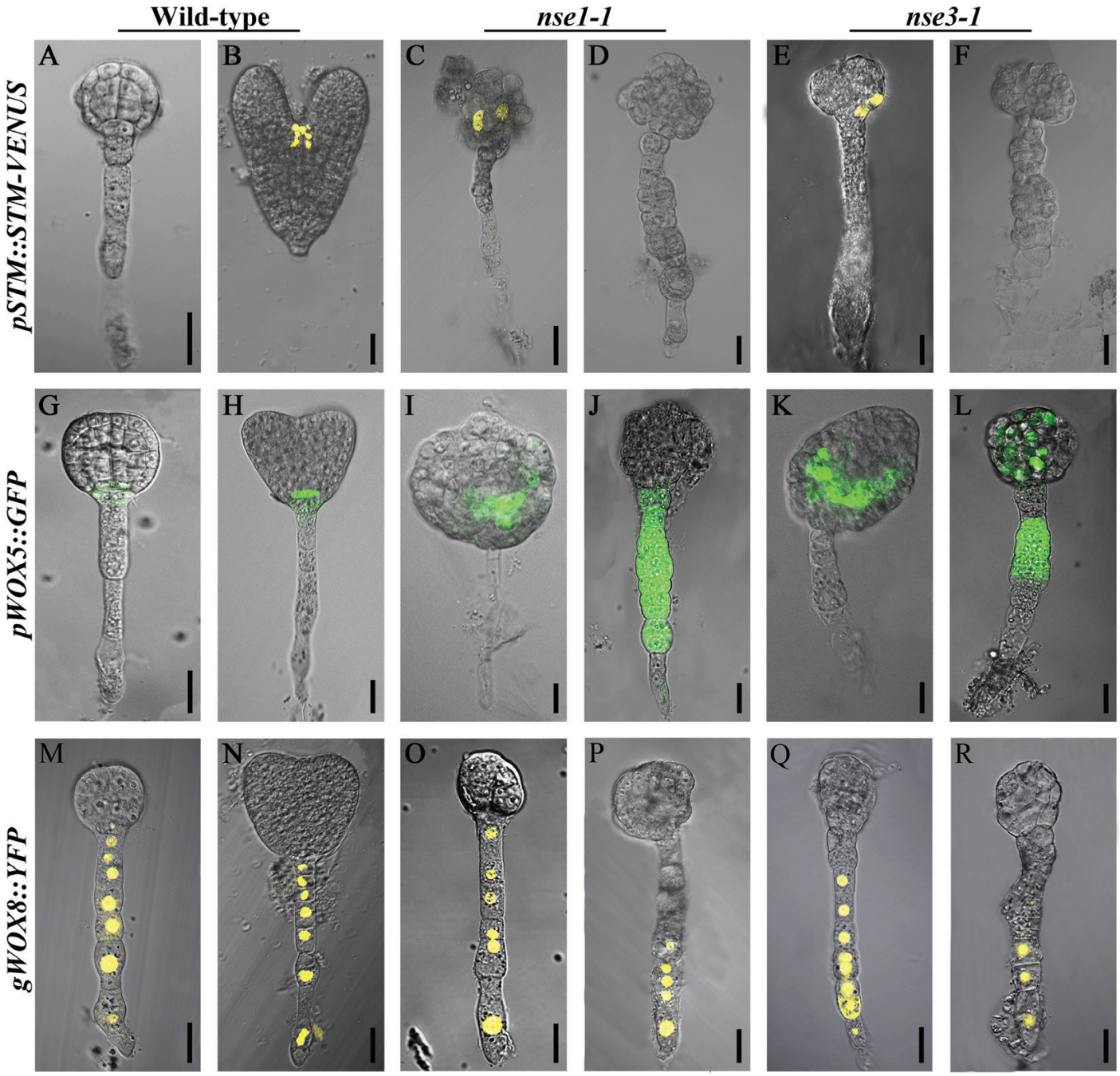

Fig. 7. Expression patterns of the embryo-specific marker genes *STM*, *WOX5*, and *WOX8* in the Arabidopsis wild-type and the *nse1-1* and *nse3-1* mutants. *STM-VENUS* was expressed only in the shoot apical meristem of the wild-type embryos (A, B), but in the mutants it could be expressed in other 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.

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

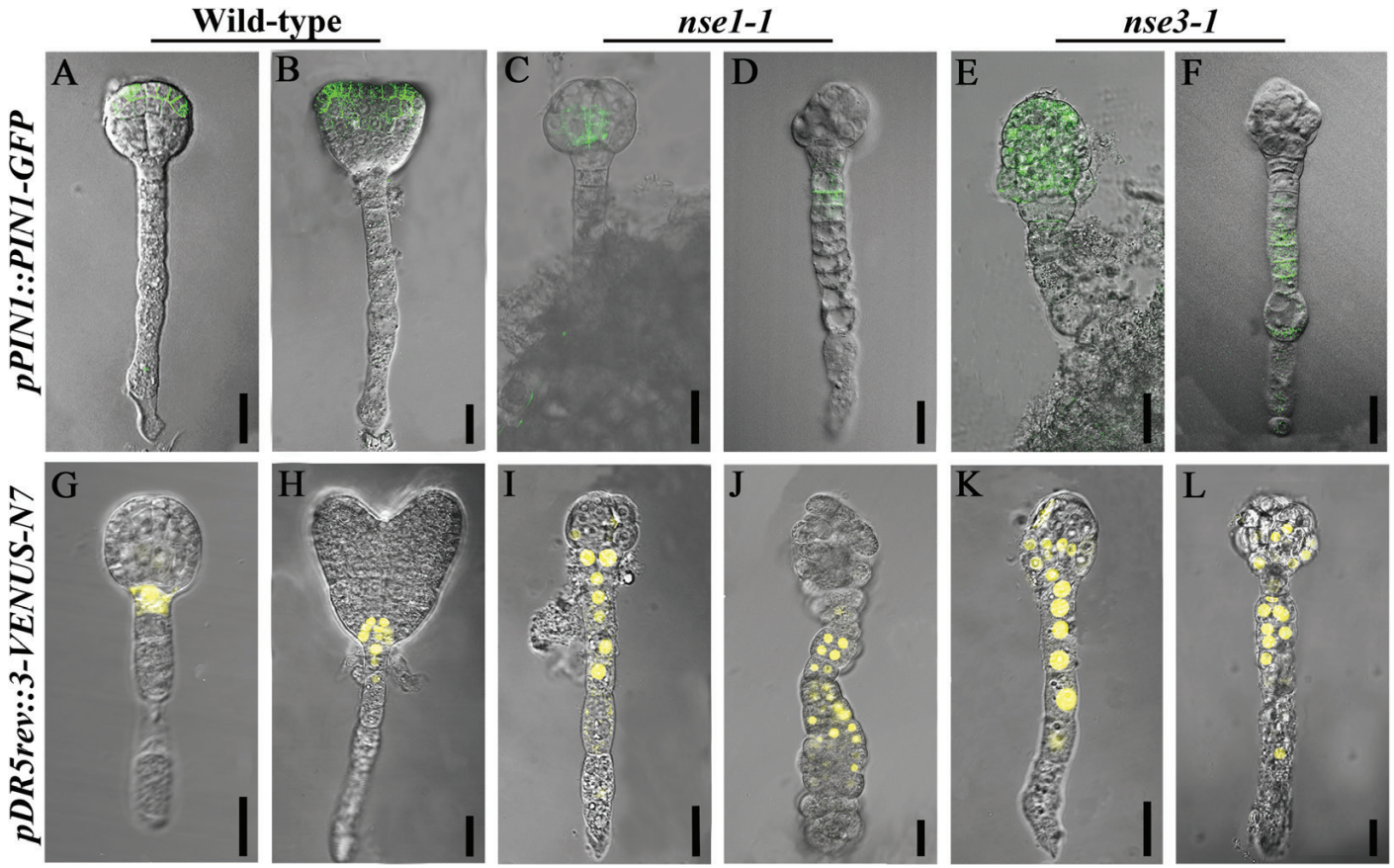

Fig. 8. Auxin transportation and responses are disrupted during embryo development in the Arabidopsis wild-type and *nse1-1* and *nse3-1*mutants. (A–F) The auxin efflux transporter PIN1 was detected using *pPIN1::PIN1-GFP*. Expression was detected only in the developing vasculature and cotyledon primordia of the embryo proper of wild-type embryos at the globular stage (A) and the early heart-shaped stage (B), but in the mutants it was detected in different cells of the embryo proper and also in suspensor cells (C–F). (G–L) DR5 is a synthetic auxin-responsive promoter and it was detected using *pDR5rev::3-VENUS-N7*. Expression was detected in the hypophysis of wild-type embryos at the globular (G) and the heart-shaped stages (H), but in the mutants expression extended to the embryo proper and the suspensor cells (I–L). 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, *SNI1*, have been shown to interact with the *E2F/DPa* 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

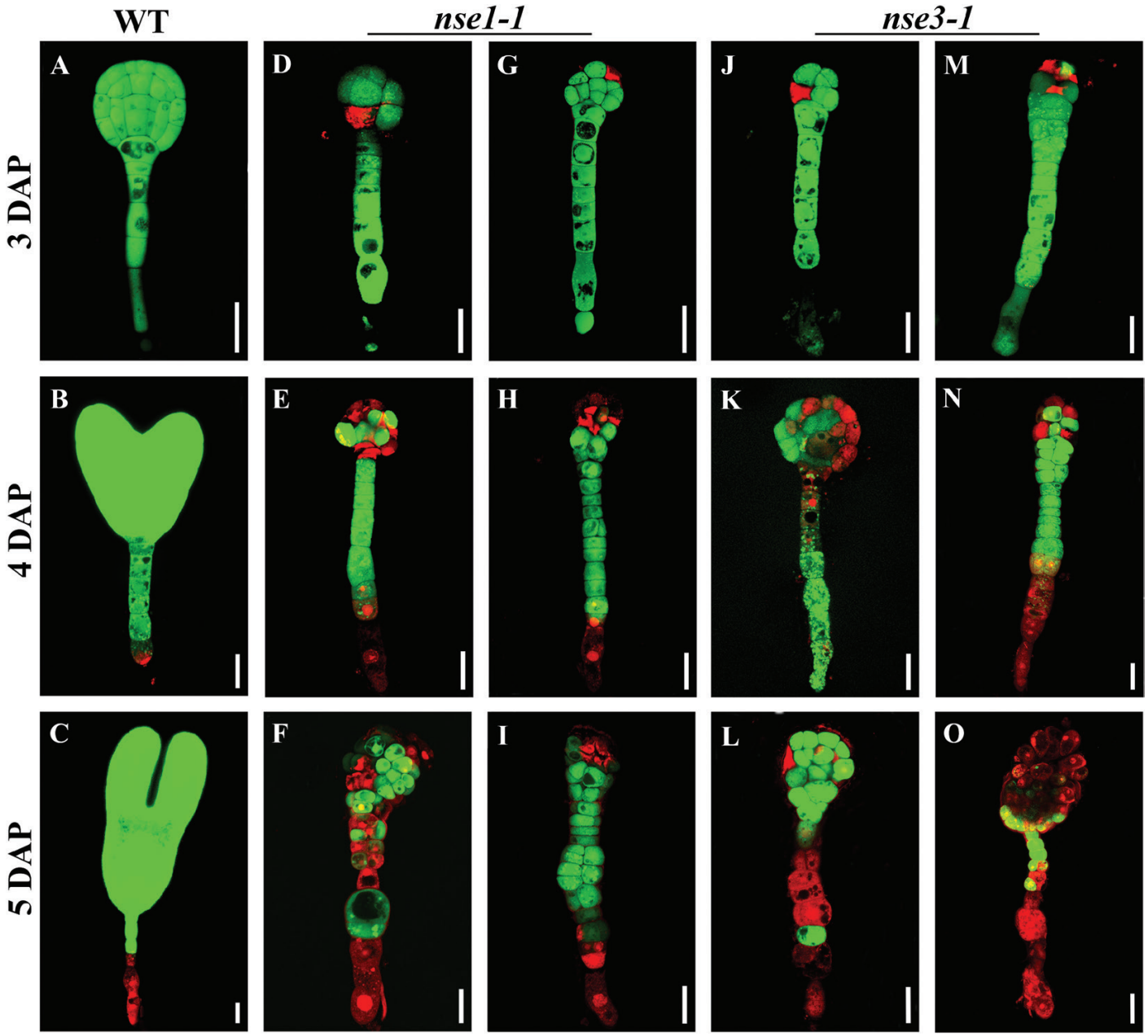

Fig. 9. Cellular viability at different stages of embryo development in the Arabidopsis wild-type (WT) and *nse1-1* and *nse3-1* mutants. Viability was determined by double-staining with fluorescein diacetate/propidium iodide (FDA/PI), where green fluorescence indicates living cells and red fluorescence indicates dead cells. DAP, days after pollination. Scale bars are 20 μm.

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

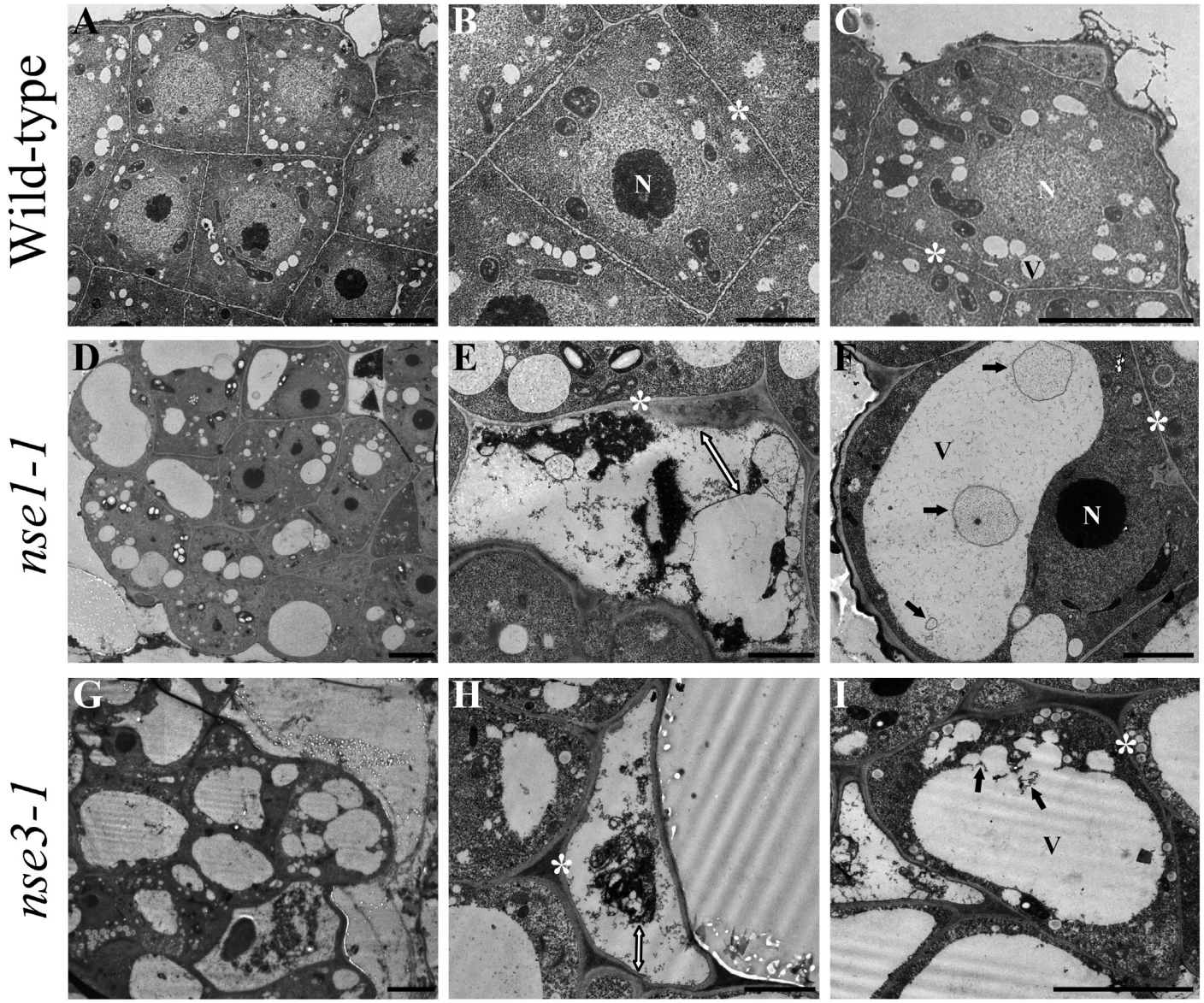

Fig. 10. Mutations in Arabidopsis *AtNSE1* and *AtNSE3* lead to necrosis and vacuolar programmed cell death (PCD). TEM images of the ultrastructure of embryo cells of the wild-type (A–C) and the mutants *nse1-1* (D–F) and *nse3-1* (G–-I). Both necrosis and vacuolar PCD occurred in *nse1-1* (E, F) and *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).

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.

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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