DSP1 and DSP4 Act Synergistically in Small Nuclear RNA 3’ End Maturation and Pollen Growth

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Small nuclear RNAs (snRNAs) play essential roles in spliceosome assembly and splicing. Most snRNAs are transcribed by the DNA-dependent RNA polymerase II (Pol II) and require 3’-end endonucleolytic cleavage. We have previously shown that the Arabidopsis (Arabidopsis thaliana) Defective in snRNA Processing 1 (DSP1) complex, composed of at least five subunits, is responsible for snRNA 3’ maturation and is essential for plant development. Yet it remains unclear how DSP1 complex subunits act together to process snRNAs. Here, we show that DSP4, a member of the metallo-β-lactamase family, physically interacts with DSP1 through its β-Casp domain. Null dsp4-1 mutants have pleiotropic developmental defects, including impaired pollen development and reduced pre-snRNA transcription and 3’ maturation, resembling the phenotype of the dsp1-1 mutant. Interestingly, dsp1-1 dsp4-1 double mutants exhibit complete male sterility and reduced pre-snRNA transcription and 3’-end maturation, unlike dsp1-1 or dsp4-1. In addition, Pol II occupancy at snRNA loci is lower in dsp1-1 dsp4-1 than in either single mutant. We also detected miscleaved pre-snRNAs in dsp1-1 dsp4-1, but not in dsp1-1 or dsp4-1. Taken together, these data reveal that DSP1 and DSP4 function is essential for pollen development, and that the two cooperatively promote pre-snRNA transcription and 3’-end processing efficiency and accuracy.

Small nuclear RNAs (snRNAs; Hadjiovol et al., 1966), a class of noncoding RNAs, are the basal components of the spliceosome and play essential roles in pre-mRNA splicing (Black et al., 1985; Ray et al., 1997; Guo et al., 2009). Their biogenesis involves transcription and subsequent processing steps. In Arabidopsis (Arabidopsis thaliana), the DNA-dependent RNA polymerase II (Pol II) synthesizes the primary snRNA transcripts (pre-snRNAs) U1, U2, U4, U5, and U12, but not U6, which is transcribed by Pol III (Carbon et al., 1987; Vankan and Filipowicz, 1988). Following transcription, pre-snRNAs are subjected to endonucleolytic cleavage at specific sites to remove the RNA fragment transcribed beyond the 3’-end of mature snRNAs.

In metazoans, snRNA 3’-end maturation requires the Integrator Complex (INT; Baillat et al., 2005). INT contains at least 14 subunits (Baillat et al., 2005; Chen and Wagner, 2010), associates with the C-terminal domain of the largest subunit of Pol II, and depends on transcription for complex formation. It cotranscriptionally cleaves pre-snRNAs upstream of the required 3’-box RNA motif (Uggen and Murphy, 2003, 2004; Baillat et al., 2005; Chen and Wagner, 2010). Integrator Subunit 11 (INT11) is a putative member of the metallo-β-lactamase (MBL)/β-CASP family of RNA endonucleases, is homologous to the cleavage and polyadenylation of snRNAs.
specificity factor 73 kD (CPSF73) that catalyzes pre-mRNA 3′-end cleavage, and is considered to be the enzyme that cleaves pre-snRNAs at the 3′ end. INT9 is also a CPSF73 homolog, but lacks key amino residues critical for endonuclease activity (Baillat et al., 2005; Li et al., 2016). INT9 and INT11 form a heterodimer through their C-terminal domains and this interaction is critical for the 3′-end processing efficiency of pre-snRNAs (Albrecht and Wagner, 2012; Wu et al., 2017). Besides snRNA 3′ maturation, INT also functions in other biological processes, including transcription termination of mRNAs, maturation of some viral-derived microRNAs, prevention of viral infection, biogenesis of enhancer RNAs, and dynein localization at the nuclear envelope (Jodoin et al., 2013; Gardini et al., 2014; Stadelmayer et al., 2014; Lai et al., 2015; Skaar et al., 2015; Xie et al., 2015; Li et al., 2016). Consistent with the importance of these functions, mutations in INT subunits often result in embryo lethality (Hata and Nakayama, 2007; Rutkowski and Warren, 2009; Ezzeddine et al., 2011; Kapp et al., 2013).

Plant snRNA 3′ maturation does not depend on transcription, although it requires a 3′ box motif whose sequence differs from its metazoan counterpart (Connelly and Filipowicz, 1993). In plants, the Defective in snRNA Processing 1 (DSP1) complex and the CPSF complex, both of which contain CPSF73-I, are responsible for the 3′-end cleavage of pre-snRNAs and pre-mRNAs, respectively. The DSP1 complex is composed of at least four additional subunits, DSP1 to DSP4 (Liu et al., 2016). Disruption of DSP1, DSP3, DSP4, or CPSF73-I, but not DSP2, impairs pre-snRNA processing, resulting in increased accumulation of pre-snRNAs. However, as in int mutants, the accumulation of mature snRNAs is not altered in the dsp mutants. Interestingly, Pol II occupancy and transcription of pre-snRNAs are reduced in dsp1, suggesting that DSP1 may also promote snRNA transcription. Supporting this, DSP1 was shown to bind snRNA gene promoters. Furthermore, all available null dsp1-2, dsp2-1, dsp3-2, and cpsf3-1 mutants are embryonically lethal, while dsp1 and cpsf3-1 have defective pollen development (Xu et al., 2006; Liu et al., 2016). These observations suggest that the DSP1 complex plays multiple important roles in development.

While DSP4 has sequence similarities with INT9, it does not interact with CPSF73-I, a homolog of INT11 (Liu et al., 2016). Until now, no DSP4 mutant allele has been analyzed, and the function of DSP4 in pre-snRNA 3′ maturation and development is still not understood. Here, we show that an amorphic dsp4-1 mutation impairs growth and male fertility and reduces pre-snRNA transcription and 3′-end processing in Arabidopsis. These phenotypes resembled those of dsp1. Interestingly, dsp1-1 dsp4-1 double mutants are completely male sterile. Pre-snRNA 3′-end processing and transcription is further reduced in dsp1-1 dsp4-1 relative to dsp1-1 or dsp4-1. Moreover, the cleavage accuracy of the pre-snRNA 3′ end is reduced in dsp1 dsp4 when compared with the wild type or single mutants. These results, together with the fact that DSP4 interacts with the ARM domain of DSP1 through its β-Casp domain, demonstrate that DSP4 and DSP1 cooperatively promote snRNA transcription and 3′ maturation, and regulate pollen and plant development.

RESULTS

The dsp4-1 Mutation Impairs Development and Male Gametophyte Transmission

We previously showed that knockdown of DSP4 with artificial microRNAs causes developmental defects. To further evaluate the function of DSP4, we obtained a dsp4-1 allele (SALK_005904) that contains a transfer DNA insertion in the 10th intron of DSP4 (Supplemental Fig. S1A). Reverse transcription quantitative PCR (RT-qPCR) analysis using specific primers that span the 10th intron revealed that the DSP4 transcript levels in dsp4-1 were greatly reduced relative to that in ecotype Columbia of Arabidopsis (Col; the wild type). Moreover, the size of the DSP4 transcript was longer in dsp4-1 than in Col (Supplemental Fig. S1B). Sequencing analysis showed that the increased size of the DSP1 transcript was caused by retention of the 11th intron that led to a premature stop codon (Supplemental Fig. S1C). Like DSP4 knockdown lines, dsp4-1 had delayed growth and fertility; several aborted seeds were detected in dsp4-1 siliques (Fig. 1A, A and B).

To demonstrate that dsp4-1 is responsible for the observed phenotypes, a wild-type copy of the DSP4 genomic DNA fused with a GFP reporter gene driven by its native promoter (pDSP4::DSP4-GFP) was transformed into dsp4-1. Expression of the wild-type DSP4 rescued the developmental defects of dsp4-1.

The ratio of heterozygous versus wild-type plants in the progeny of DSP4/dsp4-1 crosses was 1.24:1, which is less than the 2:1 ratio expected by Mendelian inheritance (Supplemental Table S1). This result suggested that, like DSP1 (Liu et al., 2016), DSP4 may also reduce gametophyte transmission. To determine whether dsp4-1 affects female or male gametophyte transmission, we performed reciprocal crosses between DSP4/dsp4-1 and the wild type. When DSP4/dsp4-1 was used as a pollen donor, the gametophyte penetration of dsp4-1 was distorted (Supplemental Table S2). In contrast, when DSP4/dsp4-1 was used as the female parent, dsp4-1 transmitted normally, suggesting that male, but not female, gametophyte transmission was impaired. To verify the influence of DSP4 on pollen development, we examined pollen viability using Alexander staining (Chen et al., 2016). Only a small number of purple-stained (viable) pollen were found in dsp4-1 anthers, suggesting that most grains were sterile (Fig. 1C). Furthermore, scanning electron microscopy (SEM) analysis showed that more than half of the pollen grains of DSP4/dsp4-1 were shrunk and irregular in shape (Fig. 1D) compared with those of the wild type. Consistently, most dsp4-1 pollen grains failed to germinate in vitro (Fig. 1E). The pDSP4::DSP4-GFP transgene was able to
rescue pollen structure, viability, and germination in
\( \text{dsp4-1} \) (Fig. 1, C–E), demonstrating that \( \text{DSP4} \) is re-
quired for pollen development.

Next, we examined whether the expression pattern of
\( \text{DSP4} \) is consistent with its function in pollen develop-
ment. We generated a transgenic plant expressing a
GUS reporter gene under the control of the \( \text{DSP4} \) pro-
moter. Histochemical staining showed that GUS was
weakly expressed in leaves and roots, but not in stems,
emerging flowers, and unfertilized ovules and eggs
(Fig. 1G; Supplemental Fig. S1D). High GUS expression
was detected in pollen (Fig. 1F), in agreement with a
role for \( \text{DSP4} \) in pollen development. GUS was also
detected in fertilized eggs and developing embryos
(Fig. 1F), suggesting that \( \text{DSP4} \) may have an additional
role in embryo development. Indeed, we found that
aborted \( \text{dsp4-1} \) seeds contained embryos arrested at the
globular stage (Supplemental Fig. S1E).

**DSP4 Interacts with the ARM Domain of DSP1 via Its \( \beta \)-Casp Domain**

As DSP4 interacts with DSP1, and is required for
snRNA 3′-end maturation and development, it is pos-
sible that it participates in snRNA processing and
development as a component of the DSP1 complex. To
evaluate this possibility, we first examined whether
\( \text{DSP4} \) localized in the nucleus, where snRNA 3′-end
processing occurs. We analyzed GFP localization in
\( \text{dsp4-1} \) plants harboring the \( \text{pDSP4::DSP4-GFP} \) trans-
gene and found that, indeed, the signals were enriched
in the nucleus of pollen and embryo cells (Supplemental
Fig. S2, A–C).

Next, we sought to further confirm and characterize
the physical interaction between \( \text{DSP4} \) and DSP1 by
determining the putative protein domains that mediate
the interaction. \( \text{DSP4} \) is a homolog of CPSF73, but it is
catalytically inactive. It contains a MBL-fold metallo-
hydrolase domain (amino acids 101–280), a \( \beta \)-Casp
domain (MBL-associated CPSF-73 Artemis SNM1/
PSO2; amino acids 374–478), and a C-terminal region.
Targeting these three domains, we generated three
truncated \( \text{DSP4} \) proteins, DSP4-tr1 (amino acids 1–348),
DSP4-tr2 (amino acids 333–485), and DSP4-tr3 (amino
acids 483–699; Fig. 2A). DSP1 contains three clusters of
Armadillo/\( \beta \)-catenin-like repeats (ARM; ~40 amino
acids for each repeat), which provide solvent-accessible
surfaces for binding of other substrates. We construc-
ted three truncated versions of DSP1, DSP1-tr1 (amino
acids 1–230), DSP1-tr2 (amino acids 224–504), and
DSP1-tr3 (amino acids 498–1133), to cover these three

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**Figure 1.** \( \text{dsp4-1} \) causes pleiotropic developmental
defects. A, Twenty-five-day-old plants with nine-rosette
leaves of Col, \( \text{dsp4-1} \), and \( \text{dsp4-1} \) harboring the
\( \text{pDSP4::DSP4-GFP} \) transgene. B, Developing seeds in
siliques of various genotypes. C, Alexander staining of
pollen grains in anthers of various genotypes. D, Pollen
structures of various genotypes detected by SEM. E, In
vitro germination of pollen of various genotypes. Images
were obtained at 8 h after incubation in BK medium.
F, Histochemical GUS staining of pollen (left), embryo sacs
(middle), and embryos (right) of plants containing the
\( \text{pDSP4::GUS} \) transgene. G, Histochemical staining of
GUS in the siliques of plants containing the \( \text{pDSP4::GUS} \)
transgene. DAE, days after emasculation; DAP, days after
pollination. Images in E and G are representative of one
out of five plants analyzed. Scale bars = 5 mm (A), 1 mm
(B), 30 \( \mu \)m (C), 10 \( \mu \)m (D), 20 \( \mu \)m (E), 0.1 mm (F), and
0.5 mm (G).
ARM clusters (Fig. 2B). We first examined the interaction of truncated DSP1 proteins with truncated DSP4 proteins using bimolecular fluorescence complementation (BiFC). The coexpression of DSP1-tr2-cYFP and DSP4-tr2-nYFP, where no other pairs of truncated proteins were coexpressed (Fig. 2C), resulted in YFP fluorescence signals, suggesting that the second ARM domain of DSP1 and the β-Casp domain of DSP4 are responsible for the DSP1-DSP4 interaction. We next used coimmunoprecipitation (co-IP) to confirm this possibility. Coexpressed, in Nicotiana benthamiana, three truncated DSP4 proteins fused with a MYC tag at their C termini, with DSP1-tr2-GFP, or three GFP-fused truncated DSP1 proteins with DSP4-tr2-MYC. After IP, we could detect DSP1-tr2-GFP in DSP4-tr2-MYC and DSP4-tr2-MYC in the DSP1-tr2-GFP precipitates (Fig. 2, D and E), demonstrating that the β-Casp domain of DSP4 and the ARM cluster 2 of DSP1 are essential and sufficient to mediate the DSP1-DSP4 interaction.

**Figure 2.** The β-Casp domain of DSP4 and the second ARM cluster of DSP1 mediate the DSP1-DSP4 interaction. A and B, Schemes of full-length and truncated DSP4 (A) and DSP1 (B) used for testing the DSP1-DSP4 interaction. C, The interactions of various forms of DSP4 with truncated DSP1 was detected by BiFC in N. benthamiana epidermal cells. Paired DSP1-trs-cYFP and DSP4-trs-nYFP fusion proteins were infiltrated into N. benthamiana leaves. The green fluorescence indicates the BiFC signal (originally YFP fluorescence), and the red indicates autofluorescence of chlorophyll. Scale bar = 10 μm. D, Co-IP of DSP4-tr2 with truncated DSP1 proteins. E, Co-IP of DSP1-tr2 with truncated DSP4 truncation proteins. IP was performed using antibodies recognizing MYC (D) or GFP (E). After IP, truncated DSP4-MYC and DSP1-GFP were detected by western blot using antibodies against MYC and GFP, respectively. Input, total protein before IP.

dsp1-1 dsp4-1 Double Mutants Have Severe Developmental Defects

The interaction of DSP4 with DSP1 raised the possibility that they function as part of a complex in pre-snRNA 3’ maturation and development. To test this, we examined the genetic interaction between dsp1-1 and dsp4-1. We constructed a dsp1-1 dsp4-1 double mutant by crossing the two single mutants. We were able to identify dsp1-1 dsp4-1 plants in the F2 population, but with an extremely low ratio of penetration (2:300), which indicates an impaired male and/or female gametophyte transmission. To test this hypothesis, we made reciprocal crosses of dsp1-1/dsp1-1 DSP4/dsp4-1 or DSP1/dsp1-1 dsp4-1/dsp4-1 with the wild type. The transmission rate of the double mutations was dramatically reduced relative to that of single mutations when the double mutants were used as pollen donors (Supplemental Table S3). In contrast, this double mutation had a nearly normal penetration rate when the wild type was used as the pollen donor. These results suggest that dsp1-1 dsp4-1 further reduced male gametophyte transmission rate relative to the single mutations.

Compared with dsp1-1 or dsp4-1, dsp1-1 dsp4-1 had increased sterility, a strong reduction in size, and an increase in crimped leaves (Fig. 3, A and B). Double mutants also produced almost no viable pollen, as seen by the lack of purple Alexander staining in anthers (Fig. 3C). The grains produced were shrunken, irregular, and adhered together (Fig. 2, D and E), demonstrating that the β-Casp domain of DSP4 and the ARM cluster 2 of DSP1 are essential and sufficient to mediate the DSP1-DSP4 interaction.
DSP1 and DSP4 Synergistically Act in 3′ End Cleavage of Pre-snRNAs

The increased accumulation of pre-snRNAs and the reduced abundance of snRNAs in dsp1-1 dsp4-1 relative to single mutants suggest that DSP1 and DSP4 additively affect 3′-end cleavage of pre-snRNAs. To test this possibility, we examined the effect of dsp1-1 dsp4-1 on in vitro 3′-end cleavage of pre-U2.3 snRNA. The [32P]-labeled pre-U2.3 snRNA, with a poly-G tail that prevents 3′-end trimming, was incubated with nuclear protein extracts from int metazoan mutants (Tao et al., 2009; Liu et al., 2016). Interestingly, both RT-qPCR and northern-blot analyses showed that the levels of mature snRNAs were reduced in dsp1-1 dsp4-1 compared with the wild type and single mutants (Fig. 4, B and E). The introduction of DSP4 without the β-Casp domain (pDSP4::DSP4ΔCasp) into the dsp4-1 mutant failed to rescue the phenotypes or pre-snRNA level, thus providing evidence for the importance of the DSP4-DSP1 interaction in snRNA maturation (Supplemental Fig. S4).

DSP1 and DSP4 Synergistically Affect the Accumulation of Pre-snRNAs and snRNAs

Next we examined if DSP1 and DSP4 cooperatively function in snRNA 3′-end maturation. We first tested the accumulation of various pre-snRNAs, including U1a, U2.3, U4.2, and U5-6 in single and double mutants. As expected, the levels of all pre-snRNAs generated from various snRNA loci were uniformly increased in dsp1-1 and dsp4-1, relative to the wild type. Furthermore, all pre-snRNAs were dramatically increased in dsp1-1 dsp4-1 compared with single mutants (Fig. 4A). A ribonuclease (RNase) protection assay further confirmed this result (Fig. 4, C and D), indicating that DSP1 and DSP4 may synergistically affect pre-snRNA processing. We also examined the effect of DSP1 and DSP4 on the various mature snRNAs. Neither dsp1-1 nor dsp4-1 altered the abundance of mature snRNAs (Fig. 4, B and E), consistent with observations in other dsp mutants and in int metazoan mutants (Tao et al., 2009; Liu et al., 2016). Interestingly, both RT-qPCR and northern-blot analyses showed that the levels of mature snRNAs were reduced in dsp1-1 dsp4-1 compared with the wild type and single mutants (Fig. 4, B and E). The decreased abundance of snRNAs in dsp1-1 dsp4-1 was further reduced in dsp1-1 dsp4-1 compared with the wild type. Relative to DSP1-1 or DSP4-1, the level of U2 was further reduced in dsp1-1 dsp4-1 (Fig. 5A), suggesting a cooperative effect of DSP1 and DSP4 on pre-snRNA 3′-end processing.

Next, we used an in vivo GUS reporter system to validate the synergistic effect of DSP4 and DSP1 on pre-snRNA processing. In this system, a GUS reporter gene was inserted downstream of the 3′ box within the pre-U2 gene containing the promoter, the coding region, and the 3′ box (pU2::pre-U2-GUS). This system has been used to monitor the effect of dsp1-1 on pre-snRNA 3′ cleavage efficiency, because GUS protein levels are inversely proportional to the cleavage upstream of the 3′ box within pre-U2-GUS (Liu et al., 2016). We crossed three independent stable transgenic wild-type lines harboring pU2::pre-U2-GUS with dsp1-1, dsp4-1, and dsp1-1 dsp4-1, in parallel, and monitored GUS expression...
in these four genotypes. As expected, GUS levels were increased in dsp1-1 and dsp4-1 relative to the wild type due to impaired 3'-end maturation of pre-snRNAs (Fig. 5B), with three biological replicates giving similar results. We also observed that GUS expression levels and GUS activity were further increased in dsp1-1 dsp4-1 compared with single mutants (Fig. 5, C and D). These results demonstrate that DSP4 is essential, and acts cooperatively with DSP1, in pre-snRNA 3'-end cleavage.

We further examined the cleavage sites of mature snRNAs. Total RNA was attached with RNA adaptors, then reverse transcribed to single DNA strands. The randomly selected snRNAs from the U1, U2, U4, and U5 gene families were cloned using nested primers, then sequenced to examine the cleavage sites. In the wild type, dsp1-1, and dsp4-1, cleavage occurred upstream of the 3' box (Table 1). However, in dsp1-1 dsp4-1, a portion of snRNAs (6%-10%) was miscleaved at the 3' box (Fig. 5E; Table 1). This result reveals that the accurate 3' cleavage of pre-snRNA requires the cooperative action of DSP1 and DSP4.

DSP4 Impairs the Occupancy of Pol II and DSP1 on snRNA Loci

We have shown that dsp1-1 affects the occupancy of Pol II in the promoter and coding regions of snRNAs but not in the 3' box. Since DSP4 acts synergistically with DSP1, we investigated whether DSP4 and DSP1 also cooperatively influence Pol II occupancy at various regions of pre-snRNAs, using the chromatin immunoprecipitation (ChIP) assay with antibodies recognizing RPB2, the second largest subunit of Pol II. We also included the downstream region of the 3' box in the experiment because it has been shown that impaired snRNA 3' maturation can lead to pre-snRNA 3'-end extension (Fukudome et al., 2017). Like dsp1-1, dsp4-1 also reduced Pol II occupancy at the promoters and coding regions of U1a and U2.3, but not at the 3' box and downstream regions (Fig. 6A). In contrast, Pol II had comparable occupancy levels at the Actin2 loci in plants of all genotypes, or was not associated with the Pol II C1 loci, which is an intergenic DNA region (Supplemental Fig. S3). These results suggest that, like DSP1, DSP4 is required for proper occupancy of Pol II at snRNA loci. Compared with single mutants, dsp1-1 dsp4-1 further reduced the association of Pol II with the promoters and coding regions of U1a and U2.3. A higher occupancy of Pol II at the 3' box and the downstream region was also observed in dsp1-1 dsp4-1 relative to other genotypes. These data suggest that DSP1 and DSP4 may synergistically impact Pol II accumulation at snRNA genes.

The reduced Pol II occupancy at the promoter and coding regions of snRNAs in single or double mutants indicates that DSP1 and DSP4 may also positively regulate snRNA transcription. To test this possibility, we monitored pre-U2 transcription using a pU2:pre-U2m-GUS transgene. Since the pre-U2m-GUS RNA contains a mutated 3' box and cannot be cleaved there, its transcript levels or GUS levels in transgenic plants will not be affected by 3' cleavage, and thus, it can be used to monitor pre-snRNA expression. We transformed this transgene into the wild type, crossed three independent stable transgenic lines into dsp1-1, dsp4-1, and dsp1-1 dsp4-1, in parallel, and examined the expression levels of GUS and pre-U2m-GUS in lines with the pre-U2m-GUS transgene, in all genotypes. GUS staining, RT-qPCR, and GUS activity analyses revealed elevated levels of pre-U2m-GUS in dsp1-1, dsp4-1, and dsp1-1 dsp4-1 compared with the wild type (Fig. 6, C–E), with three biological replicates giving similar results. In addition, the levels of pre-U2m-GUS were lower in dsp1-1 dsp4-1 than in dsp1-1 or dsp4-1. These data are consistent with the ChIP results and show that DSP1 and DSP4 additively promote snRNA transcription.
DISCUSSION

We have previously shown that the DSP1 complex is responsible for snRNA 3′ maturation (Liu et al., 2016). However, the precise roles of DSP components in this process, and how these proteins coordinately catalyze snRNA 3′ maturation, remained unknown. In this study, we showed that DSP4 is an essential component of the DSP complex, evidenced by the reduced pre-snRNA processing efficiency in dsp4-1. Moreover, DSP4 promotes snRNA transcription, given its positive impact on Pol II occupancy at the promoters of snRNAs and accumulation of pre-snRNAs. Our results also demonstrated that DSP1 and DSP4 synergistically influence CPSF73-I activity in snRNA 3′-end cleavage, given the reduced pre-snRNA cleavage efficiency and accuracy in dsp1 dsp4 double mutants relative to dsp1 or dsp4.

What is the function of DSP4 in pre-snRNA 3′-end cleavage? It has been known that CPSF73 and its homolog CPSF100 form a heterodimer, which is required for the endonuclease activity that cleaves pre-mRNA during the polyadenylation process (Kolev et al., 2008; Sullivan et al., 2009). Like CPSF73, DSP4 is a catalytically inactive endonuclease of the MBL/β-CASP family. By analogy, DSP4 could act with CPSF73-I to form a functional endonuclease for pre-snRNA 3′-end cleavage. However, DSP4 does not interact with CPSF73-I. Instead, DSP4 interacts with DSP1, which also interacts with CPSF73-I. Moreover, these three proteins coexist in a complex (Liu et al., 2016). These results raise the possibility that DSP1 mediates the association between DSP4 and CPSF73-I, which in turn facilitates CPSF73-I activity. This model predicts that DSP1 acts as a scaffold for the assembly of an active endonuclease for pre-snRNA cleavage. Supporting this model, the DSP1 homolog in metazoans, INT4, interacts with and stabilizes the INT9-INT11 heterodimer, which is required for efficient pre-snRNA cleavage (Albrecht et al., 2018). Based on this model, we expected that dsp1 dsp4 would have an impact on snRNA processing similar to that observed for dsp1 or dsp4. However, we observed a synergistic effect of DSP1 and DSP4 on pre-snRNA processing activity. In addition, we found that the cleavage accuracy of pre-snRNAs by CPSF73-I is impaired in dsp1 dsp4. These results suggest that DSP1 and/or DSP4 have additional roles in regulating CPSF73-I cleavage efficiency and in defining cleavage sites for CPSF73-I. In metazoans, INT does not promote snRNA transcription, but in plants, DSP1 and DSP4 appear to play cooperative roles in recruiting Pol II to the promoters of snRNA genes. These results suggest that the mechanisms promoting snRNA transcription differ between plants and metazoans, despite the common need for the snRNA activating protein complex (Guirou and Murphy, 2017).

DSP4 also plays important roles in development. Interestingly, we find that DSP4 is highly expressed in pollen but not in ovules. In agreement, DSP4 is required for male germline development. However, the levels of mature snRNAs in dsp4 are comparable with those in the wild type, suggesting that DSP4 has functions other than in snRNA biogenesis. DSP1 and DSP4 appear to have a synergistic effect on general and male germline development, given the increased severity of phenotypes in dsp1 dsp4 relative to single mutants. Although it is possible that the enhanced developmental defects of dsp1 dsp4 are due to reduced snRNA levels, other possibilities exist. Both DSP1 and DSP4 mutations affect male gametophyte development, while srd2, a mutant with defective snRNA transcription, was affected exclusively in the female gametophyte (Ohtani et al., 2008). This opposite phenotype also supports the position that DSP1 and DSP4 act on snRNA maturation and germline development through parallel pathways.
It is possible that DSP1 and DSP4 have cooperative effects on the metabolism of RNAs other than snRNAs. Indeed, INT could affect transcription termination of some mRNAs and the biogenesis of enhancer RNAs in metazoans (Gardini et al., 2014; Stadelmayer et al., 2014; Lai et al., 2015; Skaar et al., 2015). Moreover, 3’ extended transcription of snRNAs can produce protein-coding transcripts from downstream snRNA loci (Fukudome et al., 2017). It is tempting to speculate that impaired 3’-end cleavage in dsp1 dsp4 results in extended transcription of snRNAs and biogenesis of protein-coding transcripts, which may disrupt normal development.

**MATERIALS AND METHODS**

**Plant Materials**

All transfer DNA insertion mutants (dsp4-1 SALK_005904 and dsp1-1 SALK_036641) were obtained from the Arabidopsis Biological Resources Center (https://abrc.osu.edu). All mutants are in the Columbia (Col) genetic background.

**Plasmid Construction**

The 1.97 kb promoter region of DSP4 was cloned into pENTR/D/D-TOPO and subsequently cloned into pGWB43 (Nakagawa et al., 2007) to generate the pDSP4::GUS vector. The genomic fragments of DSP4 containing the promoter and coding regions were PCR amplified and cloned into pGWB4 to generate the pDSP4::GUS vector. The truncated gene sequences of DSP1 and DSP4 were cloned into pGWB4 and pGWB17 to generate DSP1-trs-GFP and DSP4-trs-MYC vectors, respectively. The primers used for plasmid construction are listed in Supplemental Table S4.

**Histochemical GUS Staining**

For GUS staining, seedlings, inflorescence, or embryos dissected from immature seeds were directly incubated overnight in the GUS staining buffer, in the dark, and at 37°C. After removing the chlorophyll in 70% (v/v) ethanol, the samples were dephosphorylated, and then ligated to RNA adaptor. The ligation products were further purified with ethanol and ethanol precipitated, and then used as templates for PCR. For GUS staining in mature seeds, RNA samples were extracted, purified, and then ligated to RNA adaptor. The ligation products were further purified with ethanol and ethanol precipitated, and then used as templates for PCR. For RNA samples, the ligation products were further purified with ethanol and ethanol precipitated, and then used as templates for PCR.

**Pollen Viability and Pollen Growth Assays**

Alexander staining was used to examine pollen viability as previously described by Xu et al. (2013). In vitro pollen growth and in vivo pollen germination assays were performed as described before in Chen et al. (2016). Briefly, for the in vitro pollen growth assay, mature anthers were collected and vortexed in Brewbaker and Kwack (BK) lipid medium. Then, the deposited pollen was spread on BK medium for 8 h, and observed by microscopy.

**Observation of Pollen Grain Structures**

Ultrastructures of pollen grain were examined according to Wang et al. (2017). Briefly, anthers at various developmental stages were fixed, dehydrated, embedded in resin (Epon812), and cut to semithin sections (0.6-0.8 μm) with a Leica microtome for optical analyses. Serial sections were stained with toluidine blue. The slides were observed on an Olympus BX51 microscope. To observe the ultrastructure, the same blocks used for the optical microscopy observations were cut to ~80-nm sections with a diamond knife. The sections were collected on grids and sequentially stained with uranyl acetate and lead citrate. Following contrast and washing, the sections were observed by transmission electron microscopy (BEOL).

**U2.3 Pre-snRNA In Vitro Processing Assay**

In vitro processing assays of pre-U2.3-polyG were performed as described before in Ugurel and Murphy (2004) and Liu et al. (2016). Briefly, pre-snRNA-polyG was generated by in vitro transcription using the T7 RNA polymerase. RNA substrates were purified using an 8% (w/v) polyacrylamide gel with 8 M urea, then 5˚α labeled with [P32] using T4 Polynucleotide Kinase (T4 PNK). Then, the pre-snRNA-polyG RNA was incubated for 60 min with 2 μg nuclear proteins extracted from various plants, in a reaction buffer containing 10 mM HEPES, pH 7.9, 30 mM KCl, 10% (v/v) glycerol, 20 mM creatine phosphate, 3 mM MgCl2, 3% (w/v) polyethylene glycol, and 1 mM diethiothreitol. Following the reaction, RNAs were extracted, purified, and resolved on 5% (w/v) PAGE gels with 8 M urea. Radioactive signals were detected with a PhosphorImager (GE Typhoon).

**RNase Protection Assay**

Synthesized antisense RNA was labeled with [P32] using T4 PNK. Five micrograms of total RNA extracted from inflorescences using the Trizol Reagent were incubated with radiolabeled RNA probes. RNase protection assays were performed using RNase T1 and RNase A as previously described by Carey et al. (2013). After the reactions, the final protected RNAs were separated in a 6% PAGE gel containing 8 M urea. Radioactive signals were detected with a PhosphorImager.

**Pre-snRNA Cleavage Site Analysis**

The 3’-end cleavage sites of pre-snRNAs were analyzed according to Liu et al. (2016). Briefly, RNA samples were dephosphorylated, and then ligated to a 3’ RNA adaptor. The ligation products were further purified with phenol/chloroform, ethanol precipitated, and then used as templates for RT (primer sequences are in Supplemental Table S4). The RT products were used as templates for next-PCR amplification. The resulting PCR products were cloned into pGEM-T Easy Vector for sequencing.

**ChIP Assay**

ChIP assays with anti-Fol II were performed as described before by Liu et al. (2016). Anti-RPB2 (Abcam) antibodies were used for IP. Enrichment of the target DNA loci relative to input were measured by qPCR with three biological replicates. The primers used in ChIP-PCR are listed in Supplemental Table S4.

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**Table 1. Ratio of miscleaved snRNAs in single and double mutants**

The RT-qPCR products of different snRNAs from dsp1-1 dsp4-1 were cloned into the pMD-18T vector, and 192 randomly selected clones for each snRNA were sequenced. The miscleaved ratio (MR) was calculated as the number of each cleaved type of snRNA clone divided by the total analysis number (192). The normal cleaved ratio of snRNAs (NR) includes three types of cleavage site (the −1 site, the middle site, and the +1 site).

| snRNA   | Col         | dsp1-1       | dsp4-1       | dsp1-1 dsp4-1 |
|---------|-------------|--------------|--------------|---------------|
|         | NR          | MR           | NR           | MR            | NR             | MR             | NR             | MR             |
| U1a     | 28.5:39.6:31.9 | 0           | 26.7:40.3:33.0 | 0            | 27.3:40.3:32.4 | 0              | 21.7:33.4:38.6 | 6.3            |
| U2.3    | 25.2:40.5:34.3 | 0           | 26.7:41.7:31.6 | 0            | 27.0:42.3:30.7 | 0              | 19.6:35.2:38.2 | 7.0            |
| U4.2    | 30.2:41.2:28.7 | 0           | 29.3:39.4:31.3 | 0            | 31.6:38.0:30.4 | 0              | 25.2:34.8:33.2 | 6.8            |
| U5-6    | 20.4:46.8:32.8 | 0           | 24.3:47.6:28.1 | 0            | 22.9:48.4:28.7 | 0              | 19.2:32.5:38.8 | 9.5            |

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**BiFC and the Co-IP assay**

DSP1 and DSP4 fragments were fused at their N termini with nYFP (pEarleyGate201-YN) and cYFP (pEarleyGate202-YC), respectively. GV3101 Agrobacterium cells transformed with different combinations of DSP1-trs-GFP and DSP4-trs-MYC were infiltrated into the leaves of *Nicotiana benthamiana*. Epidermal cells were examined with confocal microscopy (Leica TCS SP5). For the co-IP assay, a mixture of *Agrobacterium* containing different combinations of DSP1-trs-GFP and DSP4-trs-MYC were expressed in *N. benthamiana* leaves and then used for IP assays as described before by Zhang et al. (2013). The IP proteins were analyzed by western blot.

**Statistical Analyses**

The statistical analyses, including Student's t test, were performed by Excel 2010 software. The qPCR for each sample was replicated three times unless noted otherwise, the average values of 2-ΔΔCT were used to determine the differences, and the data were expressed as the mean ± SD. A significant difference was considered at *P* < 0.05 and extremely significant at *P* < 0.01.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Analyses of *dsp4-1* and the expression pattern of DSP4.

**Supplemental Figure S2.** Subcellular localization of DSP4.

**Supplemental Figure S3.** *dsp1-1* *dsp4-1* double mutants have complete male sterility.

**Supplemental Figure S4.** The importance of the DSP4-DSP1 interaction.

**Supplemental Figure S5.** Pol II occupancy at Act2 and Pol II C1 loci.

**Supplemental Table S1.** Segregation ratio in the offspring of DSP4/dsp4-1 plants.

**Supplemental Table S2.** Analysis of gametophyte transmission in heterozygous plants by reciprocal crosses.

**Supplemental Table S3.** Analysis of gametophyte transmission in double mutants by reciprocal crosses.

**Supplemental Table S4.** List of primers used in this study.

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**LITERATURE CITED**

Albrecht TR, Wagner EJ (2012) snRNA 3’ end formation requires heterogeneous association of integrator subunits. Mol Cell Biol 32: 1112–1123

Albrecht TR, Shevtsov SP, Wu Y, Mascibroda LG, Peart NJ, Huang KL, Sawyer IA, Tong L, Druz M, Wagner EJ (2018) Integrator subunit 4 is a “Symplekin-like” scaffold that associates with INTS9/11 to form the Integrator cleavage module. Nucleic Acids Res 46: 4241–4255

Baillat D, Hakimi MA, Nääär AM, Shilatifard A, Cooch N, Shiekhattar R (2005) Integrator, a multiprotein mediator of small nuclear RNA processing, associates with the C-terminal repeat of RNA polymerase II. Cell 123: 265–276

Black DL, Chabot B, Steitz JA (1985) U2 as well as U1 small nuclear ribonucleoproteins are involved in premessenger RNA splicing. Cell 42: 737–750

Carbon P, Murogo S, Ebel JP, Krol A, Tebb G, Mattaj IW (1987) A common octamer motif binding protein is involved in the transcription of U6 snRNA by RNA polymerase III and U2 snRNA by RNA polymerase II. Cell 51: 71–79

Carey MF, Peterson CL, Smale ST (2013) The RNase Protection Assay. Cold Spring Harbor Laboratory Press, New York

Chen J, Wagner EJ (2010) snRNA 3’ end formation: The dawn of the Integrator complex. Biochem Soc Trans 38: 1082–1087
Chen Y, Zou T, McCormick S (2016) 3'-adenosinylmethionine synthetase 3 is important for pollen tube growth. Plant Physiol 172: 244–253

Connelly S, Filipowicz W (1993) Activity of chimeric U small nuclear RNA (snRNA)/mRNA genes in transfected protoplasts of Nicotiana plumbaginifolia: U snRNA 3'-end formation and transcription initiation can occur independently in plants. Mol Cell Biol 13: 6403–6415

Ezzeddine N, Chen J, Walsenspiel B, Burch B, Albrecht T, Zhuo M, Warren WD, Marzluff WF, Wagner EJ (2013) A subset of Drosophila integrator proteins is essential for efficient U7 snRNA and spliceosomal snRNA 3'-end formation. Mol Cell Biol 31: 328–341

Fukudome A, Sun D, Zhang X, Koishi I (2017) Salt stress and CTD PHOSPHATASE-LIKE4 mediate the switch between production of small nuclear RNAs and mRNAs. Plant Cell 29: 3214–3233

Gardini A, Baille D, Cesaroni M, Hu D, Marinis JM, Wagner EJ, Lazar MA, Shilatifard A, Shiekhattar R (2014) Integrator regulates transcriptional initiation and pause release following activation. Mol Cell 56: 128–139

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell 5: 1217–1229

Guiro J, Murphy S (2017) Regulation of expression of human RNA polymerase II-transcribed snRNA genes. Open Biol 7: 170073

Guo Z, Karunatilaka KS, Rueda D, Hadjiolov AA, Venkov PV, Tsanev RG (2007) Development of series of gateway binary vectors, pGWBs, for realizing efficient transformation of Arabidopsis thaliana. Physiol Genomics 29: 321–328

Hadjiolov AA, Venkov PV, Tsanev RG (1966) Ribonucleic acids fractionation by density-gradient centrifugation and by agar gel electrophoresis: A comparison. Anal Biochem 17: 263–267

Hata T, Nakayama M (2007) Targeted disruption of the murine large nuclear KIAA1440/Ints1 protein causes growth arrest in early blastocyst stage embryos and eventual apoptotic cell death. Biochim Biophys Acta 1773: 1039–1051

Jodoin JN, Sbouli M, Albrecht TR, Lee E, Wagner EJ, Reversade B, Lee LA (2013) The snRNA-processing complex, Integrator, is required for ciliogenesis and dynein recruitment to the nuclear envelope via distinct mechanisms. Biol Open 2: 1390–1396

Kapp LD, Abrams EW, Marlow FL, Mullins MC (2013) The integrator complex subunit 6 (Ints6) confines the dorsal organizer in vertebrate embryogenesis. PLoS Genet 9: e1003822

Kolev NG, Yario TA, Benson E, Steitz JA (2008) Conserved motifs in both CPSF73 and CPSF100 are required to assemble the active endonuclease for histone mRNA 3'-end maturation. EMBO Rep 9: 1013–1018

Lai F, Gardini A, Zhang A, Shiekhattar R (2015) Integrator mediates the biogenesis of enhancer RNAs. Nature 525: 399–403

Li Y, Si L, Zhai Y, Hu Y, Hu Z, Bei JX, Xie B, Ren Q, Cao P, Yang F, et al (2016) Genome-wide association study identifies 8p21.3 associated with persistent hepatitis B virus infection among Chinese. Nat Commun 7: 11664

Liu Y, Li S, Chen Y, Kimberlin AN, Cahoon EB, Yu B (2016) snRNA 3'-end processing by a CPSF73-containing complex essential for development in Arabidopsis. PLoS Biol 14: e1002571

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, 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

Ohtani M, Demura T, Sugiyama M (2008) Differential requirement for the function of SRD2, an snRNA transcription activator, in various stages of plant development. Plant Mol Biol 66: 303–314

Ray R, Ray K, Panda CK (1997) Differential alterations in metabolic pattern of the six major UsnRNAs during development. Mol Cell Biochem 177: 79–88

Rutkowski RJ, Warren WD (2009) Phenotypic analysis of deflated/Ints7 function in Drosophila development. Dev Dyn 238: 1131–1139

Sanders PM, Bui AQ, Weterings K, McIntire KN, Hsu YC, Lee PY, Truong MT, Beals TP, Goldberg RB (1999) Anther developmental defects in Arabidopsis thaliana male-sterile mutants. Sex Plant Reprod 11: 297–322

Shaer JR, Ferris AL, Wu X, Saraf A, Khanna KK, Florens L, Washburn MP, Hughes SH, Pagano M (2015) The Integrator complex controls the termination of transcription at diverse classes of gene targets. Cell Res 25: 288–305

Stadelmayer B, Micas G, Gamot A, Martin P, Malirat N, Koval S, Raffel R, Sobbian B, Severac D, Riale S, et al (2014) Integrator complex regulates NELF-mediated RNA polymerase II pause/release and processing at coding genes. Nat Commun 5: 5531

Sullivan KD, Steiniger M, Marzluff WF (2009) A core complex of CPSF73, CPSF100, and Symplekin may form two different cleavage factors for processing of poly(A) and histone mRNAs. Mol Cell 34: 322–323

Tao S, Cai Y, Sampath K (2009) The Integrator subunits function in hematopoiesis by modulating Smad/BMP signaling. Development 136: 2757–2765

Ugenn P, Murphy S (2003) The 3’ ends of human pre-snRNAs are produced by RNA polymerase II CTD-dependent RNA processing. EMBO J 22: 4544–4554

Ugenn P, Murphy S (2004) 3’-box-dependent processing of human pre-U1 snRNA requires a combination of RNA and protein co-factors. Nucleic Acids Res 32: 2987–2994

Vankan P, Filipowicz W (1988) Structure of U2 snRNA genes of Arabidopsis thaliana and their expression in electroporated plant protoplasts. EMBO J 7: 791–799

Wang R, Xue JS, Yu YH, Liu SQ, Zhang JX, Yao XZ, Liu ZX, Xu XF, Yang ZN (2017) Fine regulation of ARF17 for anther development and pollen formation. BMC Plant Biol 17: 243

Wu Y, Albrecht TR, Baille D, Wagner EJ, Tong L (2017) Molecular basis for the interaction between Integrator subunits Ints9 and Ints11 and its functional importance. Proc Natl Acad Sci USA 114: 4394–4399

Xie M, Zhang W, Shu MD, Xu A, Lenis DA, DiMaio D, Steitz JA (2015) The host Integrator complex acts in transcription-independent maturation of herpesvirus microRNA 3’ ends. Genes Dev 29: 1552–1564

Xu R, Zhao H, Dinkins RD, Cheng X, Carberry G, Li QQ (2006) The 73 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF) complex affects reproductive development in Arabidopsis. Plant Mol Biol 61: 799–815

Xu XF, Wang B, Lou Y, Han WJ, Lu JY, Li DD, Li LG, Zhu J, Yang ZN (2015) Magnesium Transporter 5 plays an important role in Mg transport for male gametophyte development in Arabidopsis. Plant J 84: 925–936

Zhang S, Xie M, Ren G, Yu B (2013) CDC5, a DNA binding protein, positively regulates posttranscriptional processing and/or transcription of primary microRNA transcripts. Proc Natl Acad Sci USA 110: 17586–17593