The *MIDASIN* and *NOTCHLESS* genes are essential for female gametophyte development in *Arabidopsis thaliana*

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ABSTRACT

Female gametophyte development in *Arabidopsis thaliana* follows a well-defined program that involves many fundamental cellular processes. In this study, we report the involvement of the *Arabidopsis thaliana* *MIDASIN1* (*AtMDN1*) gene during female gametogenesis through the phenotypic characterization of plants heterozygous for an insertional *mdn1* mutant allele. The *MDN1* yeast ortholog has previously been shown to encode a non-ribosomal protein involved in the maturation and assembly of the 60S ribosomal subunit. Heterozygous *MDN1/mdn1* plants were semisterile and *mdn1* allele transmission through the female gametophyte was severely affected. Development of *mdn1* female gametophyte was considerably delayed compared to their wild-type siblings. However, delayed *mdn1* female gametophytes were able to reach maturity and a delayed pollination experiment showed that a small proportion of the female gametophytes were functional. We also report that the *Arabidopsis* *NOTCHLESS* (*AtNLE*) gene is also required for female gametogenesis. The NLE protein has been previously shown to interact with MDN1 and to be also involved in 60S subunit biogenesis. The introduction of an *AtNLE*-RNA interference construct in *Arabidopsis* led to semisterility defects. Defective female gametophytes were mostly arrested at the one-nucleate (FG1) developmental stage. These data suggest that the activity of both *AtMDN1* and *AtNLE* is essential for female gametogenesis progression. [Physiol. Mol. Biol. Plants 2010; 16(1) : 3-18] E-mail : dp.matton@umontreal.ca

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INTRODUCTION

The reproductive phase of the angiosperm life cycle is characterized by a short-lived and reduced gametophytic generation (haploid), which contrasts with the dominant sporophytic generation (diploid), represented by the flowering plant. Moreover, development of the male and female gametophytes mostly depends on the parent sporophyte, taking place enclosed within the anther and the ovule, respectively. The sporophytic structures first produce sexually differentiated spores through meiosis of diploid spore mother cells. Whereas numerous microspores are produced in the anther, only one functional megaspore survives in the ovule. Gametogenesis then initiates from the resulting haploid spores to give rise to the mature gametophytes by only

a few mitotic divisions (Bruckhin *et al.*, 2005; Drews and Yadegari, 2002). The mature male gametophyte, represented by the pollen grain, consists of three cells: a vegetative cell harboring two sperm cells (McCormick, 1993). The mature female gametophyte, represented by the embryo sac, is of the *Polygonum* type in about 70 % of flowering plant species, including *Arabidopsis* (Drews and Yadegari, 2002; Huang and Russell, 1992; Willemse and van Went, 1984). This type of embryo sac arrangement consists of seven cells: the egg cell flanked by two synergids at the micropylar pole, three antipodals at the opposite chalazal pole, and a diploid central cell. The sperm cells of the pollen grain are transported via a pollen tube into the embryo sac to fertilize the egg cell and the central cell, which initiates the formation of a diploid embryo and a triploid endosperm respectively.

The female gametophyte develops according to a well-defined program that involves many fundamental

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cellular processes (Christensen *et al.*, 1997; Schneitz *et al.*, 1995). In *Arabidopsis*, the functional megaspore undergoes three rounds of mitosis without cytokinesis, during which the nuclei migrate to specific positions to produce an eight-nucleate coenocytic embryo sac. Subsequent cellularization as well as fusion of the two polar nuclei of the future central cell give rise to a mature seven-celled embryo sac, as described earlier. The three antipodals finally degenerate around flower anthesis resulting in a four-celled embryo sac. Mutations disrupting one of these cellular processes are likely to affect female gametogenesis and show reduced transmission through the female gametophyte to the next sporophytic generation (Drews *et al.*, 1998; Feldmann *et al.*, 1997; Grossniklaus and Schneitz, 1998). Mutants showing defects during female gametogenesis progression have been mostly isolated through genetic screens in *Arabidopsis* and the identified genes disrupted in these mutants are involved in diverse cellular functions (Brukhin *et al.*, 2005; Christensen *et al.*, 2002; Christensen *et al.*, 1998; Drews and Yadegari 2002; Feldmann *et al.*, 1997; Howden *et al.*, 1998; Pagnussat *et al.*, 2005). For example, the underexpression of the *ARABINOGLACTAN PROTEIN 18* (*AGP18*) gene by RNA interference leads to a defect in the transition of the megaspore to a female gametophytic developmental program (Acosta-Garcia and Vielle-Calzada, 2004). Insertional mutations in *NOMEGA* (Kwee and Sundaresan, 2003) and *ANAPHASE PROMOTING-COMPLEX 2* (*APC2*) (Capron *et al.*, 2003), encoding two different components of the E3 ligase anaphase promoting complex/cyclosome (APC/C) involved in cell cycle progression, result in the arrest of female gametogenesis after the first nuclear division. Disruption of *PROLIFERA* (*PRL*), encoding a Mcm7-like licensing factor essential for DNA replication (Springer *et al.*, 1995), and targeted degradation of the *CHROMATIN-REMODELING PROTEIN 11* (*CHR11*) transcripts through RNA interference (Huanca-Mamani *et al.* 2005) also cause a premature arrest in nuclear division. In the *slow walker 1* (*swa1*) mutant, which is disrupted in a gene encoding a component of the large nucleolar U3 complex required for 18S ribosomal RNA biogenesis, nuclear proliferation in the embryo sac is delayed and asynchronized (Shi *et al.*, 2005). Mutant alleles of the plant retinoblastoma homologue *RBR1*, a negative regulator of cell division, lead to excessive nuclear proliferation in the female gametophyte (Ebel *et al.*, 2004).

In the present study, we report that disrupting the expression of the *Arabidopsis MIDASIN1* (*AtMDN1*)

gene, encoding a AAA ATPase and MIDAS domains containing protein, causes semisterility by delaying the female gametophyte development. In *Saccharomyces cerevisiae*, MDN1/REA1 was identified with NLE/RS44 in the same protein complexes as non-ribosomal factors participating in 60S ribosomal subunit maturation (Bassler *et al.*, 2001; Gavin *et al.*, 2002; Nissan *et al.*, 2002). The wild potato *Solanum chacoense* ScMDN1 ortholog was recently shown to interact with ScNLE in yeast two-hybrid assays (Chantha and Matton, 2007). Consistent with an involvement in a common cellular process, we also show that the introduction of an *AtNLE*-RNA interference construct in *Arabidopsis* also leads to a semisterility phenotype caused by defects in female gametophyte development. The female gametogenesis defects caused by the *mdn1* mutation and the *AtNLE-RNAi* construct are consistent with a function in a basic cellular process such as ribosome biogenesis.

MATERIALS AND METHODS

Plant material and growth conditions

Plants were grown in a growth chamber at 20–22 °C under a 16 h light/8 h dark cycle. Columbia (Col) and Wassilewskija (Ws) ecotypes of *Arabidopsis thaliana* were used for *AtMDN1* and *AtNLE* characterization, respectively. Seeds collected from agro-infiltrated plants were surface sterilized in 70 % ethanol and 0.05 % Tween-20 for 5 min, followed by 3 washes in 95 % ethanol, and allowed to dry completely under a sterile flow hood. Seeds were plated on MS medium (½X MS medium, 0.1 % sucrose, 0.6 % agar, pH 5.7) without antibiotic or supplemented with 25 µg/ml kanamycin and 100 µg/ml carbenicillin, and stratified in darkness at 4 °C for 2 days. Seedlings were transferred to soil and grown in growth chambers in the same conditions as stated above.

Cloning of *AtNLE*

Total RNA (1 µg) extracted from flowers at anthesis from *Arabidopsis* WS ecotype was reverse-transcribed by using the First strand cDNA synthesis kit for RT-PCR (AMV) (Roche Diagnostics, Laval, QC, Canada) with oligo-p(dT)₁₅ primer, according to the manufacturer's instructions. The *AtNLE* cDNA sequence was PCR-amplified with primers NleWS-1 (5'-ATTACACAGGTCTCTTTGCGAAGCTC-3') and NleWS-2 (5'-TCCACCGCAAAAACCTATCCACAAC AATA-3'), designed based on the *AtNLE* Columbia sequence available in GenBank. PCR products corresponding to the *AtNLE* expected size were extracted

from the agarose gel and cloned into the pCR®4-TOPO vector using the TOPO TA cloning kit (Invitrogen, Burlington, ON, Canada).

Generation of *AtNLE-RNAi* lines

A 768 bp fragment of *AtNLE* from *Arabidopsis* WS ecotype (corresponding to positions 322–1089 in the At5g52820 cDNA sequence) was PCR amplified from cloned cDNA with the NLEWS3 primer (5'-GAGACTCGAGGGATCCCAGGCGGAAGCTGTTCTTTG-3'), containing the XhoI and BamHI restriction sites, and the NLEWS4 primer (5'-GAGAGGCGCGCCGGTACCTACAAGCTGTTGATGACCGG-3'), containing the AscI and KpnI restriction sites. The PCR fragment was first cloned in the sense orientation using the BamHI and KpnI sites of the silencing pDarth vector under the control of a double enhancer CaMV 35S promoter (O'Brien *et al.*, 2002). To ensure specific RNAi targeting of the *NLE* gene, the abovementioned sequence used for RNAi was compared against the *Arabidopsis* transcript database (TAIR9). The longest nucleotide sequence identity obtained with other genes was 18 nt, thus too short to trigger an RNAi response (Elbashir *et al.*, 2001). The resulting plasmid was then used for a second cloning of the same PCR fragment in the antisense orientation using the AscI and XhoI sites. The plasmid was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. *Arabidopsis* plants (WS ecotype) were transformed using the vacuum infiltration method with slight modifications (Clough and Bent, 1998). One third of a 3 ml overnight pre-culture of *Agrobacterium* carrying the silencing plasmid was used to inoculate 200 ml YEP (1 % yeast extract, 1 % peptone, 0.5 % NaCl, pH 7.0) supplemented with 50 µg/ml kanamycin at 28 °C until OD₆₀₀ > 1.2. Cells were harvested by centrifugation at 5500 rpm for 10 min and resuspended in 10 ml infiltration medium (½X MS medium, 5 % sucrose, 44 nM benzylaminopurine, 0.02 % Silwet L-77, pH 5.7) and the volume was made to 600 ml. A beaker containing the inoculum was placed in a vacuum chamber. Plants were inverted and immersed into the bacteria suspension such that the inflorescences were completely submerged. Vacuum was applied for about 5 min until bubbles were drawn from plant tissues. Infiltrated plants were grown in a growth chamber at 22 °C under 16 h light and 8 h dark. Seeds were collected and selected for kanamycin resistance, as described above. Transformation with the *AtNLE-RNAi* construct was confirmed by PCR amplification using the primers described above.

Isolation and gel blot analysis of RNA

Total RNA extraction and RNA gel blot analysis procedures were done as described previously (Lagace *et al.*, 2003). RNA for RT-PCR analysis of *AtNLE* RNAi lines was isolated using the RNeasy® plant mini kit (Qiagen, Mississauga, ON, Canada). For RNA gel blot analysis, 10 µg of total RNA for each tissue samples were separated on gel. Probes were derived from partial *ScMDN1* cDNA or full length *AtNLE* cDNA labeled with α-[³²P]-dATP (ICN Biochemicals, Irvine, CA, USA) using the High Prime DNA Labeling kit (Roche Diagnostics). A ³²P-labelled 18S probe was used as a control. Membranes were exposed at -85 °C with intensifying screens on Kodak Biomax MR film (Interscience, Markham, ON, Canada).

Segregation Analysis

Seeds collected from heterozygous *mdn1* self-cross and reciprocal crosses between heterozygous *mdn1* mutant and wild type plants were germinated on MS medium as described above. Genomic DNA from seedlings was extracted and PCR amplified for genotyping using the REDExtract-N-Amp™ Plant PCR Kit (Sigma-Aldrich, Oakville, ON, Canada). Presence of the WT *AtMDN1* allele was determined by amplification with the primers Midas1 (5'-AACTTACAACCTGCCTGTTC-3') and Midas2 (5'-AGAATTCCATCAGACCAAGC-3'). Presence of the *mdn1* mutant allele containing the T-DNA insertion was determined by amplification with the primers Lb1 (5'-GCGTGGACCGCTTGCTGC AACT-3') and Midas2. PCR reactions were performed under the following conditions: 1 cycle of 94 °C/4 min; 35 cycles of 94 °C/30 sec, 54 °C/30 sec, 72 °C/1 min; 1 cycle of 72 °C/4 min.

Histological analysis

Arabidopsis floral organs were cleared with methyl salicylate as described previously (Estrada-Luna *et al.*, 2004). Siliques were dissected longitudinally with hypodermic needles (1 ml insulin syringes). Cleared ovules were observed on a Zeiss Axioimager M1 microscope (Carl Zeiss Canada, Toronto, ON, Canada) under differential interference contrast (DIC) optics and images were acquired with a Zeiss AxioCam MR digital camera. All images were processed for publication using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

DNA sequencing and sequence analysis

Approximately 200 ng (5 µl) of plasmid DNA and 15 µl of reaction mixture containing 8.5 µl of water, 3.5 µl

5X sequencing buffer, 2 µl primer at 0.8 µM, and 1 µl Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Streetsville, ON, Canada) was used for the sequencing reaction. Sequencing reactions were performed on a GeneAmp PCR System 9700 (Applied Biosystems), and the cycling conditions were: 96 °C, 10 sec; 50 °C, 5 sec, 60 °C, 4 min for 25 cycles. DNA sequencing was performed on an Applied Biosystem ABI 3100 automated sequencer. Sequence alignments were performed with the ClustalW module of the MacVector 8.1.2 software (Accelrys, San Diego, CA, USA). Database searches were conducted with the BLAST program on the National Center for Biotechnology Information web service (<http://www.ncbi.nlm.nih.gov>).

The GenBank accession number for the *AtMDN1* Columbia ecotype cDNA sequence is NM_105382. The *AtMDN1* gene corresponds to locus At1g67120 and the *AtNLE* gene corresponds to locus At5g52820. Accession numbers for ClustalW protein sequence analyses of *AtMDN1* orthologues are as follows: *Saccharomyces cerevisiae* (NP_013207); *Homo sapiens* (NP_05542); *Vitis vinifera* (XP_002274489); *Populus trichocarpa* (XP_002327931); *Ricinus communis* (XP_002521362); *Oryza sativa* cv. *japonica* (ABG66112).

RESULTS

AtMDN1 sequence analysis

Arabidopsis thaliana *MIDASIN1* (*AtMDN1*) is a single-copy gene and is located on chromosome 1 (locus At1g67120). Based on computer-predicted exon-intron boundaries, the *AtMDN1* coding region is comprised within 73 exons and consists of 16 011 bp (accession number NM_105382) (Figure 1A). Although, ESTs covering small portions of this sequence have been found, the predicted complete sequence has, however, not been confirmed experimentally. The *AtMDN1* gene is predicted to encode a large protein of 5337 amino acids with a calculated molecular weight of 584.7 kD. Its yeast ortholog is considered the largest protein in the yeast proteome (Garbarino and Gibbons, 2002).

A BLAST search of publicly available protein databases revealed that *AtMDN1* showed significant overall sequence similarities with sequences from *Saccharomyces cerevisiae* (also known as REA1) (21 % identity, 36 % similarity) and *Homo sapiens* (23 % identity, 40 % similarity). From other recently sequenced plants genomes, complete predicted MDN1 orthologous

sequences were retrieved from five species. Overall sequence identities (similarities in parenthesis) with *AtMDN1* were as follows: *Vitis vinifera* 51 % (65 %), *Populus trichocarpa* 49 % (65 %), *Ricinus communis* 47 % (64 %), and *Oryza sativa* 39 % (54 %). Comparison with the 816 aa C-terminal sequence of the *Solanum chacoense* ScMDN1 that was shown to interact with ScNLE (Chantha and Matton 2007), revealed a similar sequence identity range, from 57 to 40 % identity (71 to 55 % similarity) with the corresponding MDN1 C-terminal region from *V. vinifera*, *P. trichocarpa*, *R. communis*, *A. thaliana*, and *O. sativa*. The MDN1 protein comprises two highly conserved domains: an AAA ATPase domain in its N-terminal region and a MIDAS domain at its C-terminus (Figure 1B) (Garbarino and Gibbons, 2002). The most conserved domain was the C-terminally located MIDAS domain. Using the MIDAS domain only, a pairwise comparison showed that the *AtMDN1* MIDAS domain shared 72 % sequence identity (83 % similarity) with the corresponding sequence in *V. vinifera*, 70 % sequence identity (81 % similarity) with *P. trichocarpa* and *R. communis*, 68 % sequence identity (81 % similarity) with *S. chacoense*, 66 % sequence identity (82 % similarity) with *O. sativa*, and 41 % sequence identity (62 % similarity) with *H. sapiens* and yeast. Proteins containing AAA ATPase and MIDAS domains are involved in diverse cellular processes and frequently function in multiprotein complexes (Iyer *et al.*, 2004; Whittaker and Hynes, 2002). The AAA domain of MDN1 consists of six tandem ATPase domains, referred as AAA protomers, each characterized by several conserved motifs important for ATP sensing, binding, and hydrolysis (Fig. 1C). The AAA protomers are thought to fold altogether into a hexameric ring that changes in conformation upon ATP binding and hydrolysis (Garbarino and Gibbons, 2002; Iyer *et al.*, 2004). The MIDAS domain binds metal ions via a set of conserved sequence motifs that form a metal ion-dependent adhesion site (MIDAS) and is thought to mediate protein-protein interactions (Fig. 1D) (Whittaker and Hynes, 2002).

MDN1 was shown to be localized in the nucleus of yeast cells (Garbarino and Gibbons, 2002). Analysis of the *AtMDN1* amino acid sequence with the PredictNLS server [<http://cubic.bioc.columbia.edu>] revealed one potential nuclear localization signal, which is represented by a cluster of basic residues RKRRK (residues 791-795) located in the AAA domain. *AtMDN1*, like its yeast ortholog, is thus most probably localized in the nucleus.

Fig. 1. Genomic structure of *AtMDN1*, protein organization and sequence alignment of MDN1 orthologs. **A.** Genomic organization of the *AtMDN1* gene and position of the T-DNA insertion. Exons are shown as black boxes and introns are represented by gray lines. The T-DNA is inserted in exon 27, resulting in the interruption of the AAA-domain. **B.** Schematic diagrams of MDN1 protein orthologs. The AAA-domain and the MIDAS-domain are represented by dark gray and light gray boxes respectively. Sequence identities and similarities (brackets) between the AAA-domain and the MIDAS-domain of *Arabidopsis* MDN1 and orthologs (*Saccharomyces cerevisiae*, *Homo sapiens*, *Solanum chacoense*) are indicated above the corresponding boxes. **C.** Amino acid sequence alignment of selected AAA1 and AAA3 protomers of the AAA-domain of MDN1 orthologs. Conserved motifs that characterize members of AAA ATPase family are shown above the alignment. The Walker A and B motifs are part of the nucleotide binding pocket of AAA ATPases (Ramakrishnan *et al.* 2002). Short consensus sequences are indicated under the alignment. Critical residues involved in ATP sensing, binding, and hydrolysis are identified by arrowheads. Gray shades indicating level of consensus apply to sequence conservation for a single AAA promoter and not to comparison between different AAA promoters. **D.** Amino acid sequence alignment of the MIDAS-domain of MDN1 orthologs. Sequence motifs are underlined and consensus sequences are indicated under the alignment. In C and D sequence identity is highlighted in dark gray and similarity in light gray, dashes indicate gaps introduced to maximize homology, h indicates hydrophobic residues, x represents any residues.

The *mdn1* insertional mutant allele caused female semisterility

In order to define the function of *AtMDN1* during plant development, we analyzed the phenotypes associated with a T-DNA insertion mutant in the Columbia ecotype (Salk_057010) obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso *et al.*, 2003). The T-DNA insertion site was estimated to be located within exon 27 of *AtMDN1* in the SIGnal database (<http://signal.salk.edu/cgi-bin/tdnaexpress>). This location was confirmed by PCR amplification using gene specific primers in combination with a T-DNA left border primer (data not shown). The T-DNA is predicted to disrupt the AAA-domain of *AtMDN1* (Figure 1A), which is essential for the catalytic activity of the protein (Iyer *et al.*, 2004), therefore rendering the *AtMDN1* protein nonfunctional. The T-DNA insertional *AtMDN1* mutant allele is referred to as *mdn1* and was used here for further analysis. Plants were individually genotyped by PCR amplification since the kanamycin resistance marker comprised in the T-DNA was suppressed, an effect that can be obtained after several generations of growth (<http://signal.salk.edu>).

None of the parental plants was homozygous for *mdn1*, suggesting that the mutant allele could not be fully transmitted through the gametophytes and/or caused seed lethality. Heterozygous *MDN1/mdn1* plants showed no visible abnormalities in vegetative growth or development when compared to WT plants, which is expected for diploid lines carrying a recessive mutation. However, siliques produced by *MDN1/mdn1* plants were somehow shorter than the WT because of reduced seed set (Figure 2A, B). Quantitative determination of seed production showed that WT siliques were associated with nearly full seed set (5.1 % ovule abortion) while *MDN1/mdn1* siliques produced normal seeds as well as small and whitish ovules, representing signs of ovule abortion, in about equal proportions (Figure 2B, D). Crosses between *MDN1/mdn1* pistils and pollen from WT anthers produced siliques bearing about 50% aborted ovules while crosses between WT pistils and pollen from *MDN1/mdn1* anthers led to almost full seed set (Figure 2D). These results confirm that the semisterility phenotype observed in *MDN1/mdn1* plants is attributable to the female reproductive organs.

The *mdn1* mutation affects predominantly the female gametophyte

Semisterility originating from ovule abortion in a heterozygous mutant is often indicative of a

gametophytic mutation affecting the development or a reproductive function of the female gametophyte (Drews *et al.*, 1998; Feldmann *et al.*, 1997; Grossniklaus and Schneitz, 1998). In the case of a fully penetrant female-specific gametophytic mutation, the expected segregation ratio of presence to absence of the *mdn1* allele in the progeny is 1:1, as the pollen grains of a heterozygous plant carry either the *mdn1* or the *MDN1* allele and both types should fertilize in equal proportions WT female gametophytes exclusively, the *mdn1* female gametophytes being nonfunctional. Several female gametophytic mutations are however not specific and affect both female and male gametophytes, and as a consequence, show more aborted ovules than the expected 1:1 ratio (Drews and Yadegari, 2002). We determined that the segregation ratio of the *mdn1* allele in the F1 progeny of selfed *MDN1/mdn1* plants was 1:2.4 (*MDN1/mdn1*:*MDN1/MDN1*) ($n=136$) (Table 1). This distorted segregation ratio significantly lower than 1:1 suggests that *mdn1* is a general gametophytic mutation that affects the development and/or the reproductive function of both female and male gametophytes.

To determine to which extent the respective gametophytes were affected by *mdn1* in their ability to transmit their genes to the next generation, we analyzed the transmission efficiency of the *mdn1* allele through

Fig. 2. Semisterility in *MDN1/mdn1* mutant. **A,B.** Cleared pollinated pistils around 30 hours after flowering. **A.** WT pistil showing full seed set. **B.** *MDN1/mdn1* pistil. The asterisks indicate the aborted ovules. About half of the siblings develop into seeds. **C.** Mature *MDN1/mdn1* silique section. The asterisks show small and white aborted ovules. **D.** Frequency of ovule abortion in *MDN1/mdn1* mutant. Mature siliques obtained from crosses were opened and scored for the ovule abortion phenotype and mature seed production.

Table 1. Transmission efficiency (TE) of the *mdn1* allele

Parental genotypes female × male	Genotype of F ₁ plants			TE*
	<i>MDN1/mdn1</i>	<i>MDN1/MDN1</i>	<i>mdn1/mdn1</i>	
<i>MDN1/mdn1</i> × <i>MDN1/mdn1</i>	40	96	0	-
<i>MDN1/mdn1</i> × <i>MDN1/MDN1</i>	16	154	-	10.4 %
<i>MDN1/MDN1</i> × <i>MDN1/mdn1</i>	38	57	-	66.7 %

Seeds from each cross were collected and germinated. These F₁ plants were then tested for *mdn1* allele transmission by PCR using a combination of gene-specific and T-DNA left border specific primers as described in methods.

*TE = (Number of mutant plants/number of wild-type plants) × 100%, through female or male gametes.

either sex in the progeny of reciprocal crosses between *MDN1/mdn1* and WT plants. Transmission efficiency (TE) of the *mdn1* allele was significantly reduced through the male gametophyte (TE_{male}=66.7 %, *n*=94) but was more strongly reduced through the female gametophyte (TE_{female}=10.4 %, *n*=170) (Table 1). These data indicate that the *mdn1* allele affects more severely the female gametophyte than the male gametophyte and that the *mdn1* allele is mainly transmitted to the progeny by the male gametophyte. Despite a significant transmission of the *mdn1* allele through both gametophytes, homozygous plants were not recovered from the progeny of selfed *MDN1/mdn1* plants, suggesting that the *mdn1* mutation may also cause embryonic lethality.

Female gametophyte development is impaired by the *mdn1* mutation

To investigate which aspect of female gametophyte

development or reproductive function is compromised in *MDN1/mdn1* plants, we first analyzed by differential interference contrast (DIC) microscopy the phenotype of female gametophytes in cleared ovules at flower anthesis. Female gametophyte developmental stages (FG) are defined according to Christensen *et al.* (1997) and flower development stages are defined according to Smyth *et al.* (1990). Developmental studies in *Arabidopsis* have shown that female gametogenesis in ovules comprised within one individual pistil is fairly synchronous and this synchrony allows the use of the WT female gametophytes of a pistil to predict the developmental stage of the mutant gametophytes within the same pistil (Christensen *et al.*, 1997; Shi *et al.*, 2005).

Among the female gametophytes examined from *MDN1/mdn1* pistils at flower anthesis (stage 13), about half were at the seven-celled (FG6) or four-celled (FG7) stages (Figure 3A,B; Table 2) while the other half covered a variety of earlier developmental stages, mostly

Table 2. Stages of female gametophyte or embryo development in *MDN1/mdn1* pistils

Floral stage	Pistil no.	Female gametophyte stages							Embryo stages		Total no.
		FG1	FG2	FG3	FG4	FG5	FG6	FG7	4-cell	8-cell	
Anthesis (stage 13)	A1				2	3	2	4			11
	A2			3	6	1	4	5			19
	A3			3	6	4	5	7			25
	A4		1	4	5	5	7	6			28
30 HAF	P1						3	2	1	5	11
	P2			1		1	3	4	1	11	20
	P3			1		1	3	5	3	8	21
	P4			1	1	1	4	7	4	9	27

Pistils from four different *MDN1/mdn1* plants were collected at the specified developmental stages and cleared. Ovules were dissected and analyzed by DIC for female gametophyte developmental stage determination.

Fig. 3. Female gametophyte and pollen grain development in *MDN1/mdn1* flowers at anthesis revealed by differential interference contrast (DIC) microscopy on cleared whole-mount preparations. **A.** A female gametophyte at seven-celled stage FG6. Two synergid nuclei (SYN), an egg cell nucleus (EN), a prominent secondary endosperm nucleus (SEN), and three antipodal nuclei (AN) represent the seven cells forming the embryo sac at this stage. The central vacuole (V) and the smaller vacuoles (v) of the synergids are visible. **B.** A female gametophyte at four-celled stage FG7. The antipodal cells have degenerated compared to stage FG6 (A). **C.** A female gametophyte at stage FG3. A chalazal nucleus (CN) and micropylar nucleus (MN) are separated by a large central vacuole (V). A smaller vacuole (v) is present at the chalazal pole. **D.** A female gametophyte at late stage FG4. Two chalazal nuclei (CN) and two micropylar nuclei (MN) are separated by a large central vacuole (V). A smaller vacuole (v) separates the two chalazal nuclei. **E.** A female gametophyte at stage FG5. Three antipodal nuclei (AN) are at the chalazal pole while two synergid nuclei (SYN) and an egg cell nucleus are at the micropylar pole. The chalazal polar nucleus (CPN) and the micropylar polar nucleus (MPN) are migrating toward each other. **F.** Pollen grains in a *MDN1/mdn1* anther. In all panels, female gametophytes are orientated with the micropylar pole left and the chalazal pole right. Bars = 10 µm.

including two-nucleate stage (FG3), four-nucleate stage (FG4), and eight-nucleate stages (FG5) (Figure 3C-E; Table 2). Because about half of the ovules were aborted in *MDN1/mdn1* siliques, it is likely that female gametophytes at the seven-celled (FG6) and four-celled (FG7) stages were WT whereas female gametophytes at earlier developmental stages bear the *mdn1* allele. Therefore, the *mdn1* mutation would affect female gametophyte development by retarding or arresting its development rather than affecting one of its reproductive functions.

Fig. 4. Female gametophyte development *MDN1/mdn1* pistils 30 hours after flowering revealed by differential interference contrast (DIC) microscopy of cleared whole-mount preparations. **A.** A female gametophyte at stage FG3. **B.** A female gametophyte at early stage FG5. Three antipodal nuclei (AN) are at the chalazal pole while two synergid nuclei (SYN) and an egg cell nucleus (EN) are at the micropylar pole. The chalazal polar nucleus (CPN) and the micropylar polar nucleus (MPN) are migrating toward each other. **C.** A female gametophyte at early seven-celled stage FG6. Recent fusion of the polar nuclei gives the secondary endosperm nucleus (SEN) an elongated shape. **D.** A female gametophyte at four-celled stage FG7. **E.** WT seed containing an embryo at octant stage. In all panels, female gametophytes are orientated with the micropylar pole on the left and the chalazal pole on the right. Bars = 10 µm.

Despite the reduced transmission of the *mdn1* allele through the male gametophyte, although not as severely as through the female gametophyte, no obvious defects in pollen grain morphology could be detected in *MDN1/mdn1* anthers by DIC microscopy when compared to the WT (Figure 3F).

***mdn1* female gametophyte development is delayed and can progress to maturity**

To determine whether the *mdn1* female gametophytes were arrested during the nuclear division stages or were delayed during their development and could further progress to the mature stage (FG7), we analyzed ovules from pistils at a later flower developmental stage. *MDN1/mdn1* pistils at about 30 hours after flowering (HAF) contained young developing seeds and small ovules in about equal proportions (Figure 2A). The developing seeds examined mostly contained octant stage embryos and some quadrant embryos (Figure 4E; Table 2), while undeveloped ovules from the same pistils contained female gametophytes at different developmental stages (Figure 4A–D; Table 2). These comprised two-nucleate stage (FG3), four-nucleate stage (FG4), eight-nucleate stage (FG5), seven-celled stage (FG6), and four-celled stage (FG7). About half of these delayed female gametophytes were at the four-celled stage (FG7) (Table 2) and showed a normal cellular constitution, with two synergids, one egg-cell, and a central cell (Figure 4D). Therefore, the *mdn1* mutation would primarily cause a delay rather than an arrest in female gametophyte development since a fraction of them can progress to maturity.

Delayed pollination of *MDN1/mdn1* pistils increased seed set

As a consequence of a slower development, the *mdn1* female gametophytes would not be mature when fertilization normally takes place in WT ovule siblings and hence remained unfertilized and eventually aborted. As shown above, several delayed *mdn1* female gametophytes can reach maturity and are likely to be functional. Also, as mentioned above, a small proportion of the female gametophytes can transmit the *mdn1* allele to their progeny (Table 1) and could possibly represent *mdn1* female gametophytes that reached maturity after a short delay and were then fertilized. If this is the case, we could expect that the *mdn1* allele would be transmitted to the progeny in higher proportions when pollination time is delayed. To test this hypothesis, a delayed pollination experiment was performed with a strict control on emasculation and pollination time.

MDN1/mdn1 flowers at stage 12b (Christensen *et al.*, 1997) were emasculated and pistils were pollinated with pollen from WT anthers 24 h (stage 13) and 48 h (stage 14–15) after emasculation (Smyth *et al.*, 1990). Seeds obtained from at least three independent plants for each pollination time were collected and scored for the presence of the *mdn1* allele. We determined that while 5% (10:190, $n=200$) of the progeny from the 24 h group segregated the *mdn1* allele, 15.5 % (31:169, $n=200$) of the progeny from the 48 h group segregated the *mdn1* allele. These results suggest that *mdn1* female gametophytes are not defective in their ability of being fertilized but are rather impaired in their developmental progression, which is delayed compared to their WT siblings.

Expression of an *AtNLE* interference construct in transgenic plants caused female semisterility

Previous yeast two-hybrid screens have shown that the *S. chacoense* MDN1 (ScMDN1) ortholog interacts with the WD-repeat NOTCHLESS (ScNLE) protein (Chantha and Matton, 2007) and tandem affinity purification assays in yeast have isolated both proteins as components of the same pre-60S ribosomal protein complexes (Bassler *et al.*, 2001; Gavin *et al.*, 2002; Nissan *et al.*, 2002). We therefore wanted to determine if the *AtNLE* gene acts in the same developmental pathway as the *AtMDN1* gene in *Arabidopsis*. Because no insertional mutation in the *AtNLE* gene could be identified, an *AtNLE* RNA interference (RNAi) construct was generated and used to transform WT *Arabidopsis* Wassilewskija (WS) ecotype to generate silencing of endogenous gene activity. From the 21 primary transformants obtained, none showed any visible vegetative growth or developmental defects. However, several independent *AtNLE*-RNAi lines produced shorter siliques than the WT due to high levels of ovule abortion. Quantitative analysis revealed that 16 of the primary lines showed ovule abortion levels ranging on average from 15 % to almost 100 % (Figure 5B) while WT plants showed about 9 % ovule abortion. This semisterility phenotype was maintained in the next generation for all the lines analyzed. Crosses between *AtNLE*-RNAi pistils and pollen from WT anthers produced similar ovule abortion levels as in *AtNLE*-RNAi self crosses, while crosses between WT pistils and pollen from *AtNLE*-RNAi anthers led to almost full seed set (data not shown). These results therefore suggest that the semisterility phenotype observed in *AtNLE*-RNAi plants is attributable to the female reproductive organs.

Female gametophyte development is impaired in *AtNLE* RNAi lines

To investigate the nature of the developmental defect associated with the expression of the *AtNLE-RNAi* construct, we analyzed cleared ovules isolated from pistils of two transformed lines, *AtNLEi-3* and *AtNLEi-26*, that showed respectively an average of about 45 % and 85 % ovule abortion levels. We noticed in all the *AtNLE-RNAi* lines analyzed that the semisterility phenotype decreased in severity over time, as plants were getting older. For example, the firsts siliques produced by the *AtNLEi-26* line showed 100 % aborted ovules while siliques produced later during inflorescence development contained a few seeds. Since the semisterility phenotype was maintained over the following generation for all the lines tested (data not shown), the transgene could in all cases be sexually transmitted to the descendants. The partial recovery from the semisterility phenotype could be attributable to progressive transcriptional silencing of the *AtNLE-RNAi* transgene itself, hence reducing RNA interference effectiveness over time (Kerschen *et al.*, 2004). We first analyzed ovules from flowers at anthesis (stage 13) and defined the trend in female gametophyte developmental stages in data presented in Table 3. In WT siliques, the majority (~ 95 %) of ovules carried female gametophytes at the four-nucleate (FG4) stage or later, with the eight-nucleate (FG5) stage being predominant. In ovules of *AtNLEi-3* siliques, less than 30 % of the female gametophytes were at the four-nucleate (FG4) stage or later while the remaining ones were at earlier developmental stages. Among these delayed female gametophytes, the one-nucleate (FG1) was predominant (Figure 5C). As for *AtNLEi-26* siliques, the proportion of ovules bearing female gametophytes at the four-nucleate (FG4) stage or later was even lower (~16 %) than in the *AtNLEi-3* line. Delayed female gametophytes in the *AtNLEi-26* line were mostly at the one-nucleate (FG1) stage and, in addition, some were at the megaspore forming stage (FG0) (Figure 5D) or degenerated (Figure 5E). In both *AtNLEi-3* and *AtNLEi-26* lines, some female gametophytes could reach the mature stage (FG7) and had a normal embryo sac morphology. These results suggest that the *AtNLE-RNAi* construct caused a delay or an arrest in female gametophyte development.

To better understand the female gametophyte developmental defect in *AtNLE-RNAi* lines, we also analyzed cleared ovules from siliques at later

Fig. 5. Ovule abortion in *AtNLE* RNA interference lines and female gametophyte developmental defects at anthesis. **A.** Levels of ovule abortion observed in *AtNLE-RNAi* primary transformant lines. **B.** Female gametophyte at stage FG1 from a *AtNLEi-3* pistil. FM, functional megaspore; DM, degenerated megaspores. **C.** Female gametophyte at stage FG0 from a *AtNLEi-26* pistil. FM, functional megaspore; SM, sister megaspore during degeneration process. **D.** Degenerated embryo sac from a *AtNLEi-26* pistil.

developmental stages, after pollination occurred and presented the scores of female gametophyte developmental stages in Table 3. In WT ovules, the majority (more than 95 %) of the female gametophytes had reached the eight-nucleate (FG5) stage or later, with the seven-celled (FG7) stage being predominant. In ovules of *AtNLEi-3* siliques, about half of the female gametophytes were at the eight-nucleate (FG5) stage or later. The remaining half were at earlier developmental stages, being predominantly at around the one-nuclear (FG1) stage or degenerated. As for *AtNLEi-26* siliques, only about 25 % of the female gametophytes reached the eight-nucleate (FG5) or later stages. As in the *AtNLEi-3* line, delayed female gametophytes of the *AtNLEi-26* line were mostly at the one-nucleate stage (FG1) and a considerable proportion was degenerated. Both *AtNLEi-3* and *AtNLEi-26* siliques also comprised some ovules bearing fertilized embryo sacs. Also for these lines, the proportion of delayed female gametophyte observed is consistent with the frequency of ovule abortion reported above. Taken altogether, these results suggest that the *AtNLE-RNAi* construct caused a developmental arrest early during female gametophyte formation, mostly at the one-nuclear (FG1) stage, and a higher proportion of degenerated embryo sacs.

Table 3. Stages of female gametophyte development in *AtNLE-RNAi* pistils at flower anthesis (A) & after pollination (P)

Line	Pistil no.*	Deg.	Female gametophyte stages								Total	
			FG0	FG1	FG2	FG3	FG4	FG5	FG6	FG7		FG8
WT	A1						2	9	4		3	18
	A2				1	1	3	14	2	1		22
iNle 3	A1			13	9	3	5	3				33
	A2	1		16	2			14		1		34
iNle 26	A1			11	1	3	4	1		4	2	26
	A2	2	5	17	7	2	3					36
	A3	2		21	2	5	1					31
WT	P1						1	7	5	23	10	46
	P2					1				12	6	19
	P3						1	5	5	24	8	43
iNle 3	P1	2		6	1	2		1		6	5	23
	P2		3		3						11	17
	P3	8		11	1			1			18	39
iNle 26	P1	7		8			1	1		3	1	21
	P2	5		19	3	3	2	9				41
	P3	4	4	11	2	2	2			6	2	33

Pistils were collected at the specified developmental stages and cleared. Ovules were dissected and analyzed by DIC for female gametophyte developmental stage determination. *A, refers to pistils collected at flower anthesis and; P, refers to the second pollinated pistil produced before the flower at anthesis stage.

Fig. 6. *AtMDN1* and *AtNLE* expression profiles in different plant organs and tissues as generated by the GeneAtlas tool of the GENEVESTIGATOR *Arabidopsis* microarray database. Signal intensity values are indicated on the left. Graphical display from GeneAtlas tool has been modified in Adobe Illustrator to increase resolution.

***AtMDN1* and *AtNLE* are expressed throughout the plant**

If MDN1 and NLE interact with each other *in planta*, we would expect their expression domains in plant organs to overlap. Genes expression patterns obtained from publicly accessible databases (Zimmermann *et al.*, 2004) showed that both *AtMDN1* and *AtNLE* are ubiquitously expressed and show very similar expression profiles (Figure 6). *AtNLE* is however consistently expressed at higher levels than *AtMDN1*, being at least three times more abundant in all plant organs, except in the ovary where they are expressed at similar levels. Highest expression levels for both genes were found in tissues containing actively dividing cells, such as callus, shoot apex, radicle and hypocotyl. In flower organs more specifically, both *AtMDN1* and *AtNLE* were more highly expressed in the carpel. The constitutive and overlapping expression patterns of *AtMDN1* and *AtNLE* was confirmed by gel blot analysis performed on total RNAs isolated from various plant tissues (data not shown).

DISCUSSION

In *S. cerevisiae*, MDN1 (also known as REA1) and NLE (known as RSA4) were identified as non-ribosomal factors in the same pre-60S ribosomal complexes (Bassler *et al.*, 2001; Gavin *et al.*, 2002; Nissan *et al.*, 2002). Biogenesis of the 60S subunit is a highly coordinated process that progress from the nucleolus to the cytoplasm and that involves the participation of more than 70 trans-acting factors for the maturation of ribosomal RNAs and their assembly with ribosomal proteins (Fatica and Tollervey, 2002; Fromont-Racine *et al.*, 2003; Tschochner and Hurt, 2003). Ribosome biogenesis was mostly characterized in yeast but most of the trans-acting factors identified, including MDN1/REA1 and NLE/RSA4 (Chantha *et al.*, 2006), are well conserved in higher eukaryotes and ribosome biogenesis pathway is therefore expected to be similar in plants, animals and fungi (Fatica and Tollervey, 2002; Fromont-Racine *et al.*, 2003; Tschochner and Hurt, 2003). The *Solanum chacoense* MDN1 and NLE proteins were shown to interact together in two-hybrid assays, which brings support to the conservation of 60S subunit biogenesis process in plants (Chantha and Matton, 2007). In yeast, REA1 is associated with an intermediate pre-60S particle termed the Rix1-particle in the nucleoplasm. In a recent study, RSA4 was found as one of several factors that co-purify with the Rix-1 complex (Ulbrich *et al.*, 2009). The direct interaction in yeast between REA1 (MDN1) and RSA4 (NLE) was

confirmed through yeast two-hybrid assays and by immunoprecipitation of the tagged proteins, strongly supporting the interaction first observed in plants (Chantha and Matton, 2007). In this study, we report the functional characterization of the *AtMDN1* and *AtNLE* genes in *Arabidopsis* and show their involvement in female gametogenesis.

The *MDN1* gene encodes a large protein comprising an AAA ATPase domain in the N-terminal region and a MIDAS domain involved in protein interactions at its C-terminus (Garbarino and Gibbons, 2002; Whittaker and Hynes, 2002). Plant heterozygous for the *mdn1* insertional mutation showed a semisterility phenotype due to ovule abortion. The *mdn1* allele mainly impaired female gametogenesis. Analysis of cleared ovules at different floral developmental stages revealed that the development of *mdn1* female gametophytes was delayed compared to their WT siblings but could still progress to maturity. Moreover, our delayed pollination experiment showed that a small fraction of the *mdn1* female gametophytes could become functional and get fertilized. Interestingly, plants heterozygous for an insertional mutation in the *SLOW WALKER1* (*SWA1*) gene, which encodes a trans-acting factor involved in 40S ribosomal subunit biogenesis, caused a similar delay in female gametogenesis in *Arabidopsis*, with some delayed *swa1* female gametophytes becoming mature and functional (Shi *et al.*, 2005). These data altogether suggest that the *AtMDN1* gene is required for normal progression of female gametogenesis.

Additional observations revealed that the *AtMDN1* gene is also involved in other aspects of plant development. Reciprocal crosses showed that *AtMDN1* activity is required for male gametogenesis as well, although at a lesser extent than for female gametogenesis. This could be explained by the lower number of cell cycles involved in male gametophyte development, being two compared to three for female gametogenesis, if residual *AtMDN1* activity is meiotically inherited from the diploid spore mother cell. Evidences for the requirement of *AtMDN1* function during plant sporophytic development were also provided. No homozygous *mdn1* plant was recovered from self-crosses although the *mdn1* allele could be partially transmitted through both male and female gametophytes. Furthermore, *AtMDN1* was constitutively expressed throughout the plant, with higher expression levels in young tissues.

The identification of both NLE/RSA4 and MDN1/REA1 in 60S ribosomal subunit biogenesis in yeast

(Galani *et al.*, 2004; Nissan *et al.*, 2002) and their interaction in plants (Chantha and Matton, 2007) and in yeast (Ulbrich *et al.*, 2009), suggested their involvement in similar aspect of female gametophyte development. Since no insertional line could be identified for the *AtNLE* gene, we analyzed *Arabidopsis* plants transformed with an *AtNLE-RNAi* construct. Variable expression levels of endogenous *AtNLE* transcript were observed, although none of the lines showed an important change in *AtNLE* transcript accumulation. However, the majority (~ 75 %) of the primary transformed lines shared a semisterility phenotype with each line showing specific ovule abortion levels. This phenotype is also consistent with the semisterility phenotype reported previously in transgenic *S. chacoense* plants underexpressing the *ScNLE* gene (Chantha *et al.*, 2006). Moreover, the semisterility phenotype was maintained in the following generations. Previous studies reported that RNA interference is not always associated to detectable reduction in targeted gene transcript levels (Kerschen *et al.*, 2004) and that levels of residual targeted gene transcript are not necessarily correlated to degree of phenotype severity (Acosta-Garcia and Vielle-Calzada, 2004; Chantha *et al.*, 2006). In cleared ovules of the *AtNLE-RNAi* lines analyzed, female gametophytes in proportions corresponding to levels of aborted ovules were either degenerated or arrested at early developmental stages, including as early as during the megaspore forming stage (FG0) and with a predominance at the one-nucleate (FG1) stage. Therefore, *AtNLE* function is required from megaspore forming stages during the process of the female gametophyte formation.

Evidences suggest that the *AtNLE-RNAi* construct affected female gametophyte development from the sporophytic level. As shown for *AtMDN1*, *AtNLE* was constitutively expressed throughout the plant with highest expression levels in tissues containing actively dividing cells. Moreover, several of the generated *AtNLE-RNAi* lines produced siliques with 100 % ovule abortion levels, indicating that the semisterility defect could not solely be caused by *AtNLE-RNAi* expression in the female gametophyte. Since no morphological defects were observed in the sporophytic structures of the ovules, the sporophytic effect of the *AtNLE-RNAi* is likely here not physical. One possible explanation for the sporophytic contribution to the female gametogenesis defect induced by *AtNLE-RNAi* is that normal *AtNLE* activity in ovule sporophytic tissues, such as in the megaspore mother cell, could be required for female gametophyte development. Another possibility would be

that products generated by RNA interference from double-stranded *AtNLE* transcript accumulation in the sporophytic cells could cause post-transcriptional gene silencing in the megaspore and the developing female gametophyte, as was suggested previously (Acosta-Garcia and Vielle-Calzada, 2004). RNA interference products could either be transmitted during meiosis of the megaspore mother cell or be transported through plasmodesmata to the functional megaspore. The presence of plasmodesmata connecting the functional megaspore and the adjacent sporophytic nucellar cells creates an active communication bridge between these structures (Bajon *et al.*, 1999). Variability in ovule abortion levels associated to the different defective *AtNLE-RNAi* lines could be caused by variability in the penetrance of RNA interference in each line. Deciphering more precisely the functional contribution of gametophytic versus sporophytic *AtNLE* activity in female gametogenesis will required the use of specific promoters to drive the expression of the *AtNLE-RNAi* line in the female gametophyte.

As mentioned above, the defects in female gametogenesis produced in heterozygous *mdn1* and *AtNLE-RNAi* lines were different, being somehow more severe in *AtNLE-RNAi* lines than in the *mdn1* mutant. This phenotypic difference can be explained in several ways. Although NLE/RSA4 and MDN1/REA1 are involved in the same cellular process in yeast, they do not accomplish the same function during 60S subunit biogenesis and their respective function may not be equally essential. In yeast, while NLE/RSA4 represents one of the few trans-acting factors involved in all the maturation steps of the 60S ribosomal subunit, from early on in the nucleolus to the final maturation steps in the cytoplasm (Nissan *et al.*, 2002), MDN1/REA1 is only involved in late nucleoplasmic pre-60S complexes that are close to their export to the cytoplasm (Galani *et al.*, 2004). Another explanation would be that the function of NLE/RSA4 and/or MDN1/REA1 may not be restricted to 60S subunit biogenesis, as was determined for other 60S trans-acting factors (Tschochner and Hurt, 2003). In animals, the *NLE* gene was shown to be involved in the regulation of the Notch receptor activity (Cormier *et al.*, 2006; Royet *et al.*, 1998). The phenotypic difference could also be attributable to the different nature of the transformed lines studied. The developmental function of *AtMDN1* was analyzed through a heterozygous mutant line in which the WT allele could compensate for the non-functional mutant allele in sporophytic diploid cells. Residual *AtMDN1* activity provided by heterozygous

diploid megaspore mother cell could therefore contribute to female gametophyte development. The function of *AtNLE* was on the other hand analyzed through transformation with an *AtNLE* RNA interference construct and, as discussed above, activity of *AtNLE-RNAi* in sporophytic cells affected female gametophyte development.

The requirement of *MDN1* and *NLE* for normal female gametogenesis progression is consistent with data obtained from yeast and plant. In yeast, both *MDN1/REA1* and *NLE/RSA4* are essential since mutations in these genes lead to cell lethality while depletion of both proteins causes a significant slow growth phenotype (de la Cruz *et al.*, 2005; Galani *et al.*, 2004). Underexpression of *ScNLE* in *S. chacoense* by RNA interference was previously shown to cause a pleiotropic phenotype during plant sporophytic development, the major defect being the production of smaller organs due to reduced cell proliferation and cell enlargement (Chantha and Matton, 2007). However, unlike the sporophytic phenotype generated in *S. chacoense ScNLE*-RNA interference lines, no sporophytic defect was observed in *Arabidopsis AtNLE-RNAi* lines. This discrepancy could be explained by RNA interference showing different levels of effectiveness on the *NLE* gene in the sporophytic tissues of these two plant species. While clear reduction in *ScNLE* transcript levels was detected in *S. chacoense* transformants (Chantha *et al.*, 2006), this was not the case for *AtNLE* transcripts in *Arabidopsis* transformants. Another explanation could be that *Arabidopsis* sporophytic development is more sensitive to changes in *AtNLE* expression levels and could not survive to an important reduction in transcript levels. These sporophytic developmental defects altogether with female gametogenesis defects suggest an essential function of both *MDN1* and *NLE* for normal cellular progression.

To date, very few ribosomal trans-acting factors have been characterized for their function during plant growth and development. Available genetic studies on a few ribosomal trans-acting factors in plants however point to their importance in diverse aspects of female gametophyte development. For example, an insertional mutation in the *Arabidopsis* ortholog of yeast Nop10, a trans-acting factor involved in ribosomal RNA modifications during ribosomal biogenesis (Henras *et al.*, 1998), causes the production of female gametophyte with unfused polar nuclei (Pagnussat *et al.*, 2005). An insertional mutation in *SWA1*, encoding a trans-acting factor involved in 40S ribosomal subunit biogenesis, causes a delay in female gametogenesis (Shi *et al.*,

2005). Even though only one *AtMDN1* insertional T-DNA mutant line was available and examined for phenotype screening, the phenotypical similarities observed between the *mdn1* and *nle* mutant lines, as well as with other mutants affected in genes involved in ribosome biogenesis, strongly argue against an effect caused by other insertions in the *mdn1* mutant line examined, although this possibility cannot be entirely discarded at the moment. Our data indicate that *AtMDN1* and *AtNLE*, encoding orthologs of yeast 60S ribosomal subunit trans-acting factors, are also essential for the normal progression of female gametogenesis in *Arabidopsis*.

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