RESEARCH PAPER

A partial loss-of-function mutation in an Arabidopsis RNA polymerase III subunit leads to pleiotropic defects

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Abstract

Plants employ five DNA-dependent RNA polymerases (Pols) in transcription. One of these polymerases, Pol III, has previously been reported to transcribe 5S rRNA, tRNAs, and a number of small RNAs. However, in-depth functional analysis is complicated by the fact that knockout mutations in Pol subunits are typically lethal. Here, we report the characterization of the first known viable Pol III subunit mutant, nrpc7-1. This mutant was originally isolated from a forward genetic screen designed to identify enhancers of the autoimmune mutant snc1, which contains a gain-of-function mutation in a nucleotide-binding leucine-rich repeat (NLR) immune receptor-encoding gene. The nrpc7-1 mutation occurs in an intron–exon splice site and results in intron retention in some NRPC7 transcripts. There is a global disruption in RNA equilibrium in nrpc7-1, exemplified by the altered expression of a number of RNA molecules, some of which are not reported to be transcribed by Pol III. There are developmental defects associated with the mutation, as homozygous mutant plants are dwarf, have stunted roots and siliques, and possess serrated leaves. These defects are possibly due to altered small RNA stability or activity. Additionally, the nrpc7-1 mutation confers an NLR-specific alternative splicing defect that correlates with enhanced disease resistance, highlighting the importance of alternative splicing in regulating NLR activity. Altogether, these results reveal novel roles for Pol III in maintaining RNA homeostasis, adjusting the expression of a diverse suite of genes, and indirectly modulating gene splicing. Future analyses using the nrpc7-1 mutant will be instrumental in examining other unknown Pol III functions.

Key words: Alternative splicing, Arabidopsis, NRPC7, plant immunity, Pol III, RNA polymerase III, Rpc25.

Introduction

Transcription under both static and dynamic conditions requires the action of evolutionarily conserved multi-subunit enzymes known as DNA-dependent RNA polymerases (Pols). All eukaryotes possess three distinct RNA polymerases (Pols I, II, and III), each of which transcribes specific suites of genes (Cramer et al., 2008).

Pol I transcribes 45S rRNA, which is the precursor to 5.8S, 18S and 25S rRNAs. Pol II transcribes mRNAs as well as most small nuclear (sn)RNAs and micro (mi)RNAs. Pol III was previously thought to be primarily required for the transcription of ‘housekeeping’ genes such as those encoding 5S rRNA and tRNAs. However, recent reports indicate that the
Pol III transcriptome is more diverse than formerly assumed (Dieci et al., 2007). There are two additional plant-specific RNA polymerases, Pol IV and Pol V, which are required for the biogenesis and functional activity of small interfering (si) RNAs (Haag and Pikaard, 2011). As knockout mutations in the genes encoding the subunits of Pol I, II, and III are lethal, there is a dearth of functional analysis of plant Pols.

While there are a number of published studies examining global transcriptomic changes in plants under various conditions (e.g. Nagano et al., 2012, Woo et al., 2012, Zhu et al., 2013), the literature to date has largely focused on the roles played by Pol II-transcribed RNAs in regulating plants’ responses to stimuli. Stimulus-induced alteration of expression of protein-coding genes has been extensively documented. Numerous recent reports have highlighted the importance of miRNAs in regulating a broad spectrum of biological processes including development (Wu, 2013), flowering time (Spanudakis and Jackson, 2014), drought stress (Ding et al., 2013), metal toxicity (Gupta et al., 2014), immunity (Staiger et al., 2013), and phytohormone crosstalk (Curaba et al., 2014), among others. Furthermore, the biosynthesis, functional mechanisms, and degradation pathways of miRNAs have been well studied (Rogers and Chen, 2013).

Comparatively little is known about Pol III-transcribed RNAs and how they aid plants in responding to intrinsic and extrinsic signals. An RNA molecule with significant sequence and structural similarity to SS rRNA was found to regulate alternative splicing of certain pre-mRNAs in Arabidopsis (Hammond et al., 2009). Intriguingly, studies in a variety of eukaryotes indicate that Pol III-transcribed non-coding RNAs may play regulatory roles in addition to their housekeeping functions (Hu et al., 2012).

Among the various stimuli to which plants are subjected, biotic stress in the form of pathogenic infection requires that plants be able to respond rapidly and initiate signalling cascades specific to the type of pathogen being encountered. While plants possess physical barriers and broad spectrum resistance that is activated by conserved features of pathogenic microbes, many pathogens are able to deliver infection-promoting effector molecules into the plant cell, thereby bypassing this layer of plant immunity (Bigeard et al., 2015). However, the plant genome contains a large number of genes encoding nucleotide-binding leucine-rich repeat proteins (NLRs; also referred to as Nod-like receptors due to their N-termini: some possess a Toll-interleukin 1 receptor (TIR) domain and are thus termed TNLs, while others contain a coiled-coil (CC) domain and are referred to as CNLs.

NLR-mediated signalling must be tightly controlled under both resting and induced conditions, as improper signalling through this pathway may lead to either enhanced disease susceptibility or autoimmunity. However, the regulatory mechanisms underlying NLR-mediated signalling are only partially understood. A successful forward genetic suppressor screen previously conducted in our lab used the gain-of-function autoimmune TNL mutant suppressor of npr1, constitutive 1 (snc1; Li et al., 2001; Zhang et al., 2003) to search for positive regulators of immunity (Johnson et al., 2012). More recently, we have undertaken a forward genetic screen to identify negative regulators of NLR-mediated immunity.

Here, we report the characterization of nrpc7-1, a partial loss-of-function allele of the gene encoding the Arabidopsis orthologue of yeast RpoC5, a Pol III subunit. This mutant was isolated from our MUSE (MUTANT, sncl-ENHANCING) forward genetic screen conducted in the modifier of sncl 4 (mos4) sncl double mutant background. A null mutation in NRPC7 is lethal, while a mutation in an intron–exon splice site junction gives rise to intronic retention in some NRPC7 transcripts, resulting in viable mutant plants. While the nrpc7-1 mos4 sncl triple mutant displays enhanced resistance against the virulent oomycete pathogen Hyaloperonospora arabidopsidis (H.a.) Noco2, the nrpc7-1 single mutant exhibits wild-type-level resistance. This correlates with the altered splicing of SNC1 observed in the triple mutant but not in the single mutant. Morphologically, the nrpc7-1 mutant is dwarf and has serrated leaves, short roots, and stunted siliques, although flowering time does not appear to be affected. The expression and potentially activity of a number of RNAs are distorted in nrpc7-1, contributing to its developmental defects. In keeping with its known function, we observed that the NRPC7 protein localizes to the nucleus. This is the first reported viable Pol III subunit mutant in Arabidopsis.

Materials and methods

Plant growth conditions and mutant isolation

Plants were grown either on soil or on half-strength Murashige and Skoog (MS) medium supplemented with 1% sucrose and 0.3% phytagel. All plants were grown under long day conditions (16 h light–8 h dark) at 22 ºC in climate-controlled chambers. The muse4 mutant was isolated from the MUSE screen, described previously (Huang et al., 2013).

Total RNA extraction and analysis

Approximately 0.1 g tissue was collected from 2-week-old seedlings grown on ½ MS, and the Ambion ToTALLY RNA Total RNA Isolation Kit (Thermo Fisher Scientific) was used to extract total RNA. For the comparison of rRNA levels, total RNA was run on ½ MS, and the Ambion ToTALL Y RNA Total RNA Isolation Kit (Thermo Fisher Scientific) was used to extract total RNA grown on ½ MS, and the Ambion ToTALL Y RNA Total RNA Isolation Kit (Thermo Fisher Scientific) was used to extract total RNA.

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Regarding RNA purity and quality, we used the Bioanalyzer 2100 and RNA 6000 Nano LabChip kit (Agilent Technologies) to evaluate RNA. Total RNA was run in parallel with RNA standards. RNA samples were assessed for integrity by electrophoresis on a 1% agarose gel. The integrity of the RNA samples was determined by comparing the density of the 18S and 28S rRNA bands on the gel. The presence of 18S and 28S rRNA bands indicates that the RNA is of high quality.
Preparation of transgenic plants and confocal microscopy

Full length At1g06790 genomic DNA, including 766 bp upstream of the start codon, was amplified via PCR, cloned into the pCAMBIA1305 vector, and transformed into muse4 mos4 snc1 using the floral dip method (Clough and Bent, 1998). The full length genomic fragment was also cloned into a pCambia1305 vector and transformed into muse4 mos4 snc1 using the floral dip method. Transgenic plants were selected for on ½ MS plates containing 50 mg ml−1 hygromycin. Confocal images of wild type (negative control), 35S::X-GFP (positive control), and NRCPC7-GFP transgenic seedlings were obtained using a PerkinElmer Ultraview VoX spinning disc confocal system (PerkinElmer) mounted on a Leica DM1000 B inverted microscope equipped with a Hamamatsu 9100-02 electron multiplier CCD camera (Hamamatsu). An argon 488 nm laser line with a complementary (522/36) emission band-pass filter to detect GFP or a 561 nm laser with a complementary (595/50) emission band-pass filter to detect propidium iodide was used. Images were acquired with a ×63 (water) objective lens. The nuclei and the cell wall were stained using 10 μM propidium iodide (Calbiochem) for 1 min, rinsed with water, and mounted on a slide and coverslip prior to imaging.

Yeast complementation

Full length MUSE4 cDNA was cloned into the yeast expression vector p425-GPD with primers 5′-CGGGATCCATGTTTATCTTTAGCAGGC-3′ and 5′-ACGCGTACGTCACGTCTCTCTGTGATT TGTTAGCTGGAATCGAA-3′ for tRNA-Gly; and LeuF 5′-TGTCAAGAGTGGGTTTGAACC-3′ and LeuR 5′-TCAGGATGCAGGGTGGTCTAAA-3′ for tRNA-Leu. Primers used for amplification of SNC1, RPS4, SR30, PAD4, and ACTIN7 were previously described (Zhang et al., 2003; Cheng et al., 2009; Xu et al., 2012).

Infection assays

H. a. Noco2 infection was performed by spraying 2-week-old soil-grown seedlings with a spore suspension with a concentration of 10⁵ spores per millilitre of water. Inoculated seedlings were grown for 7 d at 18 °C in a growth chamber with ~80% humidity and a 12h light–12h dark cycle. Sporulation was then quantified using a haemocytometer to count the number of spores from five plants shaken in 1 ml of water. Five replicates were performed for each of three independent trials. P.s.m. ES4326 infection was performed by infiltrating the abaxial leaf surface of 4-week-old soil-grown seedlings with bacteria suspended in 10 mM MgCl₂ (OD₆₀₀=0.0005). Leaf punches were collected at day 0 and day 3, and serial dilutions were performed and plated on LB medium. Plates were incubated at 28 °C for 24 h before colony forming units were measured.

Positional cloning and Illumina whole-genome sequencing

Positional cloning of muse4 was performed by crossing the muse4 mos4 snc1 triple mutant (generated in the Col-0 ecotype) with wild type Landsberg erecta. Twenty-four F2 plants homozygous for all three mutations were used for crude mapping, and approximately 500 F3 plants homozygous for muse4 and snc1 and heterozygous for mos4 were used for fine mapping. The markers used in mapping were derived from insertion/deletion polymorphisms between the Col-0 and Ler Arabidopsis ecotypes (Jander et al., 2002; http://www.arabidopsis.org). After determining that the mutation must be located on the top of chromosome 1 between 1.4 MB and 2.75 MB, extracted genomic DNA from muse4 mos4 snc1 was sequenced using the Illumina sequencing platform.

Results

The isolation, characterization, and identification of the muse4/nrpc7-1 mutant

The MUSE screen was designed to identify enhancers of the dwarf autoimmune mutant snc1 and has been described previously (Huang et al., 2013). To avoid potential lethality resulting from dramatically enhanced autoimmunity, the snc1 suppressor mos4 was included in the genetic background of all mutant lines. For analysis of differentially expressed miRNAs, all known Arabidopsis miRNAs were downloaded from miRBase (Release 20 from www.mirbase.org; Griffiths-Jones et al., 2008). PERL scripts were used to determine the expression level of known miRNAs in the small RNA libraries and then normalize these counts per million (RPMs). miRNAs with <10 RPMs in both nrpc7-1 and wild type libraries were removed. The differentially expressed miRNAs were identified by comparing expression in the nrpc7-1 library with wild type. The Audic–Claverie method was used to calculate P-values (Audic and Claverie, 1997), which were subsequently adjusted as described by Benjamini and Hochberg (1995) to determine the false discovery rate (FDR). To qualify as a differentially expressed miRNA, both a fold change >2 between wild type and nrpc7-1 and an FDR <0.05 were necessary.

Small RNA library construction and sequencing

Small RNAs within the size range of 15-40 nt were fractionated from total RNAs by 15% polyacrylamide gel electrophoresis. These small RNAs were then ligated with the 3′ and 5′ adapters sequentially using the Small RNA Sample Preparation Kit (Illumina) according to the manufacturer’s instructions. A reverse transcription reaction followed by a low cycle PCR was performed to obtain final products for deep sequencing. The wild type and muse4 libraries were barcoded and sequenced in one channel on an Illumina HiSeq2000.

Analysis of small RNA high throughput sequencing Data

PERL scripts were used to process small RNA raw reads as per Lertpanyasampatha et al. (2012). To summarize, reads were passed through Illumina’s quality control filter before being sorted into bins based on their barcodes and having their adaptor sequences removed. SOAP2 was used to map reads within the size range of 20–24 nt to the Tair10 Arabidopsis genome (Li et al., 2009). Differential small RNA regions were identified as previously described (Dinh et al., 2014). For analysis of differentially expressed miRNAs, all known Arabidopsis miRNAs were downloaded from miRBase (Release 20 from www.mirbase.org; Griffiths-Jones et al., 2008). PERL scripts were used to determine the expression level of known miRNAs in the small RNA libraries and then normalize these counts per million (RPMs). miRNAs with <10 RPMs in both nrpc7-1 and wild type libraries were removed. The differentially expressed miRNAs were identified by comparing expression in the nrpc7-1 library with wild type. The Audic–Claverie method was used to calculate P-values (Audic and Claverie, 1997), which were subsequently adjusted as described by Benjamini and Hochberg (1995) to determine the false discovery rate (FDR). To qualify as a differentially expressed miRNA, both a fold change >2 between wild type and nrpc7-1 and an FDR <0.05 were necessary.
construct was used to visualize PR2 gene expression in seedlings, and GUS staining was much stronger in the triple mutant than in mos4 snc1 (Fig. 1B). Consistent with this observation, qPCR demonstrated that expression of PR1 and PR2 is elevated in the triple mutant (Fig. 1C).

To examine whether the muse4 mutation alters resistance to the virulent oomycete strain Hyaloperonospora arabidopsidis Noco2, 2-week-old triple mutant seedlings were spray-inoculated with this pathogen. The enhanced resistance observed in snc1 but lost in mos4 snc1 was found to be reconstituted in the triple mutant (Fig. 1D). Together, these data indicate that muse4 restores all examined snc1-like phenotypes in the mos4 snc1 background.

A positional cloning strategy was employed to determine the molecular lesion responsible for the observed phenotypes. The muse4 mos4 snc1 mutant, which was generated in the Col-0 ecotype, was crossed to Landsberg erecta (Ler). From the F2 population, 24 plants displaying the triple mutant morphology were selected for crude mapping, which identified a linkage to the top of chromosome 1. Several F2 plants heterozygous at the top of chromosome 1 (but homozygous for snc1 and mos4 to prevent interference by these loci) were used to generate a fine mapping population of approximately 500 plants. The mutation was narrowed down to between the markers T7A14 (1.4 MB) and F22O13 (2.75 MB). Genomic DNA was extracted from muse4 mos4 snc1 triple mutant plants and sequenced using the Illumina whole-genome sequencing platform. The sequencing results were compared with the Arabidopsis reference genome, and five genes in this region were found to contain mutations (Fig. 2A).

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![Characterization of the muse4 mos4 snc1 triple mutant.](https://academic.oup.com/jxb/article-abstract/67/8/2219/2885027)

Fig. 1. Characterization of the muse4 mos4 snc1 triple mutant. (A) Morphology of soil-grown plants of the indicated genotypes, photographed 4 weeks post-germination. Scale bar represents 1 cm. (B) pPR2-GUS expression in seedlings of the indicated genotypes grown on 1/2 MS medium for 10 d. (C) PR1 and PR2 gene expression in the noted genotypes, as determined by qPCR. ACTIN7 expression serves as a loading control. (D) Growth of H.a. Noco2 on indicated genotypes 7 d post-inoculation with 1 x 10^5 spores innoculum ml^-1. Values represent the average of 4 replicates of 5 plants each ±SD. **P<0.01; ***P<0.001.
mutations in three of these genes are located in introns and the mutation in one gene was found to be silent, therefore the mutation in the remaining gene (At1g06790) was selected as the most likely candidate for muse4. This gene encodes the Arabidopsis orthologue of the yeast Pol III subunit Rpc25, NUCLEAR RNA POLYMERASE C, SUBUNIT 7 (NRPC7; Ream et al., 2015), and the muse4 mutation is at the intron–exon junction just before the sixth exon (Fig. 2B).

The mutation at an intron–exon junction of NRPC7 results in intron retention and is responsible for the muse4 phenotypes

To verify that the mutation in NRPC7 is responsible for the muse4 phenotypes, a full-length wild type copy of the gene driven by its native promoter and fused to the GFP-encoding gene at its 3′ end was transformed into the single mutant, which was generated by backcrossing the triple mutant to Col-0 and selecting plants homozygous for wild type SNC1 and MOS4 that retained the serrated leaf phenotype and dwarf size. Eight independent T2 lines displayed wild type morphology, and one representative line can be seen in Fig. 3A. These data suggest that NRPC7 can fully complement the muse4 phenotypes, and therefore that MUSE4 is indeed NRPC7.

We hypothesized that the muse4 mutation in the intron–exon junction of NRPC7 results in retention of the preceding intron. To test this, we designed primers flanking the intron of interest and amplified cDNA from wild type and muse4. A strong band of the expected size (465 bp) was observed in wild type while in muse4 two bands were observed, one of the expected size and one slightly larger (Fig. 3B). The larger band was excised from the gel and the PCR product was purified and sequenced. As predicted, sequencing revealed that the larger band corresponded to a transcript in which the intron preceding the muse4 mutation had been retained (Fig. 3C).

Despite strong sequence similarity between NRPC7 and knownRpc25 proteins in other species (Supplementary Fig. S1 at JXB online), the NRPC7 gene failed to complement a temperature-sensitive rpc25 yeast knockout line (Supplementary Fig. S2), suggesting divergence between the plant and yeast NRPC7.

It is expected that a knockout mutation in NRPC7 would be embryo lethal, as a previous study showed that loss-of-function mutations in RNA polymerase subunits are not transmitted maternally (Onodera et al. 2008). Indeed, when we let the heterozygous nrpc7 T-DNA insertion line CS1001213 self-fertilize and then planted the progeny, we identified 23 wild type plants lacking the insertion, 46 heterozygotes, and 0 plants that were homozygous for the insertion, matching the expected 1:2:0 (wild type:heterozygote:homozygote) ratio for a lethal mutation. We also performed reciprocal crosses between this heterozygous T-DNA insertion line and muse4 and found that none of the F1 progeny contained the T-DNA insertion, indicating that the T-DNA/muse4 heterozygotes are not viable. These results, combined with the data in Fig. 3B showing that muse4 still produces some properly spliced transcripts without intron retention, as well as the fact that muse4 is a recessive mutation, suggest that muse4 is a partial loss-of-function allele of NRPC7. Therefore, we renamed muse4 as nrpc7-1 and the T-DNA allele as nrpc7-2.

Splicing of SNC1 is altered in the nrpc7-1 mos4 snc1 background

In yeast, Rpc25 is required for Pol III transcription initiation (Zaros and Thuriaux, 2005). To assess whether Pol III function is affected by the nrpc7-1 mutation, we used real-time qPCR to determine whether expression of U6, a snRNA component of the spliceosome that is known to be transcribed by Pol III (Waibel and Filipowicz, 1990), is different in nrpc7-1 than in wild type. Relative to the expression levels of the Pol II-transcribed ‘housekeeping’ gene UBQ5, U6 expression is significantly lower in nrpc7-1 (Fig. 4A). To examine whether the nrpc7-1 mutation has a general effect on spliceosomal snRNA biosynthesis, the accumulations of Pol II-transcribed U1 and U2 snRNAs were also examined. While U1 accumulation is wild type-like, U2 expression is significantly reduced in nrpc7-1. This is likely due to an indirect effect of altered Pol III function on Pol II-transcribed genes.

The reduced expression of the spliceosome components U6 and U2 leads us to hypothesize that pre-mRNA splicing might be affected by the nrpc7-1 mutation. Specifically, as nrpc7-1 was isolated in our screen for snc1 enhancers, we hypothesized that
the mutation may affect the excision of introns from the SNC1 pre-mRNA transcript. The alternative splicing of a number of plant NLR-encoding genes, including SNC1, is known to affect their function in plant immunity (Yi and Richards, 2007; Xu et al., 2011). For SNC1, the second and third introns may be either retained or removed; therefore we used primers spanning these two introns to amplify the SNC1 transcript variants. A dramatic accumulation of the largest transcript variant (with both introns retained) was observed in nrpc7-1 mos4 snc1, although the SNC1 splicing pattern in the nrpc7-1 single mutant was indistinguishable from that observed in wild type (Fig. 4B and Supplementary Fig S3C). Alternative splicing defects were also observed in nrpc7-1 mos4 snc1, although the SNC1 splicing pattern in the nrpc7-1 single mutant was indistinguishable from that observed in wild type (Fig. 4B and Supplementary Fig S3C). Alternative splicing defects were also observed in nrpc7-1 mos4 snc1 for RESISTANT TO PSEUDOMONAS SYRINGAE 4 (RPS4), another NLR-encoding gene, and to a lesser degree for SR30, which encodes a serine/arginine-rich RNA-binding protein and is known to be alternatively spliced (Fig. 4B). The relative proportions of the transcript variants in the genotypes examined are shown in Fig. 4C. These data reveal significant alternative splicing defects caused by the Pol III subunit mutation.

To determine whether this splicing defect occurs at the level of basal splicing, transcripts of a gene that is not alternatively spliced (PHYTOALEXIN-DEFICIENT 4; PAD4) were also examined. No difference from the wild type splicing pattern was detected (Fig. 4B). Both snc1 and nrpc7-1 mos4 snc1 accumulated higher levels of PAD4 compared with wild type, which is consistent with previous reports that PAD4 is a defence-induced gene (Glazebrook 2001), whose expression is expected to be upregulated in autoimmune mutants.

NLR-mediated signalling is often regulated by modulating transcription and/or translation of NLRs. As such, we examined whether SNC1 expression and protein accumulation are affected by the nrpc7-1 mutation. SNC1 expression was found to be slightly reduced in nrpc7-1 as compared with wild type, while SNC1 protein levels were wild type-like (Supplementary Fig S3). Similarly, the accumulation of SNC1 in nrpc7-1 mos4 snc1 was not dramatically higher than that observed in mos4 snc1. Taken together, these data indicate that SNC1 alternative splicing, but not overall gene expression or translation, is affected by the nrpc7-1 mutation.

The nrpc7-1 single mutant does not have altered immune responses

Since alterations in the splicing of SNC1 were observed in the nrpc7-1 mos4 snc1 triple mutant but not the nrpc7-1
Fig. 4. Splicing defects in *nrpc7-1*. (A) Quantitative real-time qPCR was used to determine the expression of Pol II-transcribed U1 and U2 snRNAs, as well as Pol III-transcribed U6, relative to UBQ5. Bars represent the averages of three technical replicates of two biological replicates ±SD. **P<0.01. (B) An analysis of SNC1, RPS4, SR30, and PAD4 splicing patterns in the indicated genotypes was performed using RT-PCR. Transcripts were amplified using 40 cycles. Numbers indicate transcript variants from largest to smallest. Schematic diagrams of the expected splicing events are shown to the right, with horizontal lines representing introns, black boxes representing exons, and white boxes representing alternatively retained exons that result in a premature stop codon. (C) Quantification of the alternative transcript variants in (B) across genotypes. Band intensities were quantified using ImageJ.

nrpc7-1 has global defects in RNA levels

Pol III transcribes tRNA, 5S rRNA, and assorted other non-coding RNAs. To further explore how Pol III function is affected by the *nrpc7-1* mutation, we examined the expression of a variety of RNAs by qPCR (Fig. 5A). Relative to UBQ5, 5S rRNA and three representative tRNAs (coding for Gln, Gly, and Leu, respectively) showed significantly reduced accumulation in *nrpc7-1* compared with wild type. In addition, when total RNA was run on a 2% agarose gel, altered relative proportions of the various rRNAs were consistently observable in association with the *nrpc7-1* allele (Fig. 5B). Relative to Pol I-transcribed 25S rRNA, there appears to be a lower abundance of chloroplast 16S and 23S rRNA associated with the *nrpc7-1* allele. These data suggest that in addition to Pol III transcribed genes, the *nrpc7-1* mutation also affects abundance of other RNAs, likely through indirect mechanisms.

Small RNA libraries were then prepared from *BG2* plants (Col-0 with the *pPR2-GUS* reporter gene construct that is present in the *nrpc7-1* background) and two independently isolated *nrpc7-1* single mutant lines. Analysis of these small RNA libraries indicated that a number of miRNAs are differentially expressed in the mutant. Those miRNAs that exhibited a two-fold or greater change in expression are shown in Supplementary Fig. S5A. To validate these results, three representative miRNAs were selected for northern blot analysis. Although the data from the small RNA libraries indicated that expression of both *miR159* and *miR166* is reduced in *nrpc7-1* while *miR398* expression is increased, no significant alterations in the levels of these miRNAs were consistently observed via northern blotting (Supplementary Fig. S5B), suggesting that any differences that exist between the mutant and wild type are too subtle to be detected by this method.

The general disruption in RNA equilibrium combined with the striking serrated leaf phenotype and dwarf morphology of the *nrpc7-1* mutant led us to hypothesize that the expression of (i) the *CUP-SHAPED COTYLEDONS (CUC)* genes,
which are targeted by miR164 (Mallory et al., 2004), and (ii) the HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP) genes, which are targeted by miR165/166 (Rhoades et al., 2002), might be altered in the mutant. Expression of a CUC2 transcript resistant to miR164 cleavage was previously shown to result in enhanced leaf serration; the same morphological phenotype was observed in plants containing loss-of-function mutations in the Pol II-transcribed MIR164 (Nikovics et al., 2006).

Overexpression of either miR165 or miR166 results in reduced expression of the HD-ZIP genes, which corresponds with dwarf morphology and altered rosette leaf morphology that is similar to that observed in nrpc7-1 (Jung and Park, 2007).

Real-time qPCR was used to determine that CUC1 and CUC2 accumulation is elevated in nrpc7-1 (Fig. 5C), although no alteration in miR164 levels were observed in nrpc7-1 based on our small RNA library data. Expression of the HD-ZIP genes PHABULOSA (PHB) and REVULOTA (REV) was found to be decreased in nrpc7-1 (Fig. 5C). No changes in miR165 levels were observed in nrpc7-1, and although miR166 expression was elevated in the mutant according to the small RNA library sequencing data (Supplementary Fig. S5A), no detectable change in miR166 levels was consistently measurable by northern blotting (Supplementary Fig. S5B). The altered expressions of CUC1, CUC2, PHB, and REV in the absence of detectable changes in miR164, miR165, and miR166 abundances suggests that in nrpc7-1 the activity of a number of small RNAs may be affected; alternatively, the nrpc7-1 mutation may indirectly affect Pol II-mediated transcription of certain genes, including CUC1, CUC2, PHB, and REV, although the mechanism behind this specificity is unclear. Many of the RNAs that seem to be differentially expressed in nrpc7-1 are not transcribed by Pol III (Fig. 5), suggesting that this mutation results in a disruption of the global RNA equilibrium and homeostasis.

NRPC7 localizes to the nucleus

As part of the Pol III complex, NRPC7 is predicted to localize to the nucleus. To examine its localization, we used the complementing nrpc7-1 lines containing the transgene with NRPC7 fused to GFP under the control of the native promoter, described above. We analyzed cotyledon and root tissue using confocal microscopy, and GFP fluorescence was visible throughout the nucleus as it co-localized with nuclei stained with propidium iodide (Fig. 6). Additionally, there appeared to be intense fluorescent foci within the nucleus and along the plasma membrane.
nrpc7-1 has pleiotropic developmental defects

The roles various small RNAs play in the regulation of plant development have been well studied. As nrpc7-1 has large impacts on small RNA levels and, potentially, RNA activities, we examined the developmental phenotypes of the mutant. As described earlier, nrpc7-1 has serrated leaves (Figs 3A and 7A), and its growth is stunted (Fig. 7B). When grown on half-strength MS medium, nrpc7-1 plants also have significantly shorter roots than wild type plants (Fig. 7C). The siliques of nrpc7-1 were consistently found to be smaller (Fig. 7D). Flowering time was measured using several different assays, but a significant difference between nrpc7-1 and wild type was only observed when measuring the number of days until the primary stalk reached 6 cm (Fig. 7E), which is likely a reflection of the restricted growth of the mutant rather than an actual delay in flowering time. While the number and arrangement of the floral organs are wild type-like, the texture of the sepals is bumpy and irregular (Fig. 7F). These results show that the nrpc7-1 mutation is associated with a number of pleiotropic developmental defects.

Discussion

We demonstrated that Pol III function is altered by the partial loss-of-function mutation nrpc7-1 by showing that the expressions of Pol III-transcribed U6 snRNA, 5S rRNA, and a number of tRNAs are reduced in the mutant (Figs 4A and 5A). Pol II-transcribed U2 snRNA, but not U1 snRNA, also had reduced expression in nrpc7-1 (Fig. 4A), indicating that the transcriptional defects in the mutant extend to genes not directly transcribed by Pol III. The decreased accumulation of U6 and U2 snRNA led us to hypothesize that spliceosome functionality is impaired in nrpc7-1 and that alternative splicing of SNC1 is consequently affected, thereby explaining why this mutation was isolated from a screen for enhancers of the autoimmune mutant snc1. Indeed, SNC1 splicing is defective in the nrpc7-1 mos4 snc1 triple mutant background, in that there is a dramatic accretion of a transcript variant that retains both the second and third introns (Fig. 4B, C). A similar pattern was observed for the NLR-encoding gene RPS4. The second intron of SNC1 contains an in-frame premature stop codon, and thus retention of this intron should yield a
truncated version of the protein. Previous reports have shown that an accumulation of the N-termini of TNL proteins is sufficient to activate cell death and immunity (Weaver et al., 2006; Swiderski et al., 2009). This finding, combined with our data showing that transcription and translation of SNC1 are not enhanced by the nrpc7-1 mutation (Supplementary Fig. S3A, B), suggests that the modification in SNC1 splicing could be the primary cause of the sncl enhancing effects of the nrpc7-1 mutation in the mos4 sncl background (Fig. 1).

It is notable that the nrpc7-1 single mutant does not differ from wild type in either alternative splicing (Fig. 4B, C) or disease resistance (Supplementary Fig. S4). This suggests that the mos4 mutation is required in the genetic background for the nrpc7-1-associated splicing defects to become obvious. MOS4 is an integral component of the evolutionarily conserved MOS4-associated complex (MAC) that functions together with the splicesome to regulate pre-mRNA splicing (Johnson et al., 2011). Mutations in mos4 and other MAC components have previously been shown to affect the alternative splicing of both SNC1 and RPS4 (Xu et al., 2012), and are associated with a suppression of SNC1-dependent immune signalling (Palma et al., 2007). In this study we demonstrated that there is an increased accumulation of the intron-retaining SNC1 transcripts in mos4 sncl compared with sncl (Fig. 4C). However, this accumulation is radically enhanced in the nrpc7-1 triple mutant. One possible explanation for these results is that the mos4 mutation and, to a lesser extent, the nrpc7-1 mutation individually disrupt splicing efficiency, reducing the pool of the functional full-length SNC1 transcript variant with both introns excised but not increasing the production of alternative variants beyond the threshold required for immune activation. However, when these two mutations are combined in the sncl background, splicesome activity is markedly disturbed and the accumulation of intron-retaining SNC1 transcript variants is sufficiently high to yield enough truncated SNC1 to activate defence responses.

In addition to the splicing defects observed in nrpc7-1, the accumulations of rRNAs and tRNAs appear to be considerably distorted (Fig. 5A, B). Ribosomal protein gene dosage was recently found to have an effect on embryonic stem cell differentiation in mice (Fortier et al., 2015), indicating that alterations in the abundance of ribosome components can dramatically alter developmental progression. Homozygous nrpc7-1 plants exhibit certain phenotypes that may be associated with impaired stem cell differentiation including short roots (Fig. 7C) and delayed emergence of the first true leaves. This suggests that the sensitivity of ribosome function to changes in its subunit levels, as well as its role in regulating stem cell differentiation, may be conserved in plants, although the data in support of this are preliminary and additional experiments are required to fully explore this hypothesis.

We also detected a reduction in the accumulation of the chloroplast 16S and 23S rRNAs relative to Pol I-transcribed 25S rRNA (Fig. 5B). A similar rRNA abundance pattern was recently reported for atybe Y-1, a mutant allele of an endoribonuclease required for chloroplast rRNA processing and development that also exhibits pale green leaves and delayed development (Liu et al., 2015). Although the mechanism by which alterations in a Pol III subunit result in changes to the transcriptional regulation of Pol I-transcribed genes and the chloroplast genome is unclear, the light green colour of the nrpc7-1 single mutant (Fig. 3A) further suggests that this mutation may be associated with impaired chloroplast function.

The serrated leaf phenotype observed in nrpc7-1 is likely linked to its elevated expression of CUC1 and CUC2 (Fig. 5C), which could be a result of reduced miR164 activity in the mutant background or an indirect effect of the nrpc7-1 mutation on Pol II function. There may also be a link between the nrpc7-1 mutant morphology and the decreased expression of the HD-ZIP genes (Fig. 5C). Other studies have demonstrated tentative links between the transcriptional activities of Pol II and Pol III. One study identified areas of the genome where protein-coding genes on one DNA strand overlapped with tRNA-coding genes on the opposite strand, and that their rates of transcription by Pol II and Pol III, respectively, were negatively correlated (Lukosz et al., 2013). Another study found that human RPPH1 is transcribed by both Pol II and Pol III, and identified a number of transcriptional activators that associate with both Pols (Faresse et al., 2012). Our data show that a disturbance in Pol III function affects the expression of non-Pol III-transcribed RNAs, indicating that the role of Arabidopsis Pol III in transcriptional regulation is more complex than previously assumed.

There are 12 core subunits of Arabidopsis Pol III, each of which has a homologue or is itself also a component in Pols I, II, IV, and V (Haag and Pikaard, 2011; Ream et al., 2015). There are also subunits specific to individual Pols. NRPC7 encodes a core Pol III subunit with homologues in each of the other Pols, and shares significant sequence similarity with Rpe25 proteins from other model organisms (Supplementary Fig. S1). However, Arabidopsis NRPC7 failed to complement a temperature-sensitive rpe25 yeast knockout line (Supplementary Fig. S2), suggesting that the functional conservation of this protein by itself between yeast and plants is limited. This is not entirely unprecedented. Rpe25 is known to form a dimer with Rpe17 within the Pol III complex (Siat et al., 2003). The protein–protein interaction surface of NRPC7 may be sufficiently evolutionarily divergent so as to prohibit it from dimerizing with yeast Rpe17. Although the function of the protein complex is conserved, an individual component of the complex may still be divergent enough that it fails to complement a knockout of its orthologue in a distant organism.

In summary, we have demonstrated that a perturbation in Pol III function results in modified gene splicing as well as alterations in the abundances and potentially activities of a number of RNA molecules. These effects extend to several RNAs reported to be transcribed by other polymerases, revealing a novel role for Pol III in modulating the expression of a larger complement of genes than previously described. Moving forward, the partial loss-of-function nrpc7-1 mutant provides a unique tool for performing other functional analyses of Pol III.

**Supplementary data**

Supplementary data are available at *JXB* online.

**Figure S1.** Sequence alignment of RPC25 from a broad range of species, based on BLAST analysis.

**Figure S2.** Yeast complementation with NRPC7.
Figure S3. SNCl gene and protein expression in nrpc7.
Figure S4. Immune characterization of nrpc7-I single mutant plants.
Figure S5. RNA defects in nrpc7-I.

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References

Audic S, Claverie JM. 1997. The significance of digital gene expression profiles. Genome Research 7, 986–995.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate – a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society. Series B, Statistical Methodology 57, 289–300.

Bigeard J, Colcombet J, Hirt H. 2015. Signaling Mechanisms in Pattern-Triggered Immunity (PTI). Molecular Plant 8, 521–539.

Cheng YT, Germain H, Wiemer M, Bi DL, Xu F, Garcia AV, Wirthmueller L, Despres C, Parker JE, Zhang YL, Li X. 2009. Nuclear pore complex component MOS7/Nup88 is required for innate immunity and nuclear accumulation of defense regulators in Arabidopsis. The Plant Cell 21, 2503–2516.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. The Plant Journal 16, 735–743.

Cramer P, Armache KJ, Baumli S, et al. 2008. Structure of eukaryotic RNA polymerases. Annual Review of Biophysics 37, 337–352.

Curaba J, Singh MB, Bhalla PL. 2014. miRNAs in the crosstalk between phytohormone signalling pathways. Journal of Experimental Botany 65, 1425–1438.

Dieci G, Fiorino G, Castelnuovo M, Teichmann M, Pagano A. 2007. The expanding RNA polymerase III transcriptome. Trends in Genetics 23, 614–622.

Ding YF, Tao YL, Zhu C. 2013. Emerging roles of microRNAs in the mediation of drought stress response in plants. Journal of Experimental Botany 64, 3077–3086.

Dinh TT, Gao L, Liu XG, Botany 23, 84, 64, 3077–3086.

E1004446. Hammond MC, Wachter A, Breaker RR. 2009. A plant SS ribosomal RNA mimic regulates alternative splicing of transcription factor IIIA pre-miRNAs. Nature Structural & Molecular Biology 16, 541–549.

Hammond MC, Wachter A, Breaker RR. 2009. A plant SS ribosomal RNA mimic regulates alternative splicing of transcription factor IIIA pre-miRNAs. Nature Structural & Molecular Biology 16, 541–549.

He SS, Wu J, Chen L, Shan G. 2012. Signals from noncoding RNAs: Unconventional roles for conventional pol III transcripts. International Journal of Biochemistry & Cell Biology 44, 1847–1851.

Huang Y, Chen XJ, Liu YA, Roth C, Copeland C, McFarlane HE, Huang S, Lipka V, Wiemer M, Li X. 2013. Mitochondrial ATPM16 is required for plant survival and the negative regulation of plant immunity. Nature Communications 4, 2558.

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL. 2002. Arabidopsis map-based cloning in the post-genome era. Plant Physiology 129, 440–450.

Johnson KCM, DongOX, Huang Y, Li X. 2012. A rolling stone gathers no moss, but resistant plants must gather their moises. Cold Spring Harbor Symposium on Quantitative Biology 77, 259–268.

Johnson KCM, DongOX, Li X. 2011. The evolutionarily conserved MOS4-associated complex. Central European Journal of Biology 6, 776–784.

Jung JH, Park CM. 2007. MIR166/165 genes exhibit dynamic expression patterns in regulating shoot apical meristem and floral development in Arabidopsis. Planta 225, 1327–1338.

Lertpanyasampatha M, Gao L, Kongswadworakul P, Vorboonjun U, Chrestin H, Liu YR, Chen XM, Narangajavana J. 2012. Genome-wide analysis of microRNAs in rubber tree (Hevea brasiliensisl L.) using high-throughput sequencing. Planta 236, 437–445.

Li RO, Yu C, Li YR, Lam TW, Yiu SM, Kristiansen K, Wang J. 2009. SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 25, 1966–1967.

Li X, Clarke JD, Zhang YL, Dong XN. 2001. Activation of an EDS1-mediated R-gene pathway in the snc1 mutant leads to constitutive, NPR1-independent pathogen resistance. Molecular Plant-Microbe Interactions 14, 1131–1139.

Li X, Kapos P, Zhang YL. 2015. NLs in plants. Current Opinion in Immunology 32, 114–121.

Liu J, Zhou W, Liu G, et al. 2015. The conserved endoribonuclease YbeY is required for chloroplast ribosomal RNA processing in Arabidopsis. Plant Physiology 168, 205–221.

Lukoszek R, Mueller-Roeppe B, Ignatova Z. 2013. Interplay between polymerase II- and polymerase III-assisted expression of overlapping genes. FEBS Letters 587, 3692–3695.

Malloy AC, Dugas DV, Bartel DP, Bartel B. 2004. MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Current Biology 14, 1035–1046.

Nomano AJ, Sato Y, Mihara M, Antonio BA, Motoyama R, Itoh H, Nagamura Y, Iwaza T. 2012. Deciphering and prediction of transcriptome dynamics under fluctuating field conditions. Cell 151, 1358–1369.

Nikovics K, Blein T, Peaucelle A, Ishida T, Morin H, Aida M, Laufs P. 2006. The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis. The Plant Cell 18, 2929–2945.

Onodera Y, Nakagawa K, Haag JR, Piikaad D, Miiki T, Ream T, Ito Y, Piikaad CS. 2008. Sex-biased lethality or transmission of defective transcription machinery in arabidopsis. Genetics 180, 207–218.

Palma K, Zhao QQ, Cheng Y, Bi DL, Monaghan J, Cheng W, Zhang YL, Li X. 2007. Regulation of plant innate immunity by three proteins in a complex conserved across the plant and animal kingdoms. Genes & Development 21, 1484–1493.

Ream TS, Haag JR, Pontvianne F, Nicora CD, Norbeck AD, Pasa-Tolic L, Piikaad CS. 2015. Subunit compositions of Arabidopsis RNA Polymerase I and III reveal Pol I- and Pol III-specific forms of the AC40 subunit and alternative forms of the 5S3 subunit. Nucleic Acids Research 43, 4163–4178.

Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. 2002. Prediction of plant microRNA targets. Cell 110, 513–520.

Rogers K, Chen XM. 2012. Signals from noncoding RNAs: Unconventional roles for conventional pol III transcripts. International Journal of Biochemistry & Cell Biology 44, 1847–1851.
Rpb7-like complex in yeast RNA polymerase III contains the orthologue of mammalian CGRP-RCP. Molecular and Cellular Biology 23, 195–205.

Spanudakis E, Jackson S. 2014. The role of microRNAs in the control of flowering time. Journal of Experimental Botany 65, 365–380.

Staiger D, Korneli C, Lummer M, Navarro L. 2013. Emerging role for RNA-based regulation in plant immunity. New Phytologist 197, 394–404.

Swiderski MR, Birker D, Jones JDG. 2009. The TIR domain of TIR-NB-LRR resistance proteins is a signaling domain involved in cell death induction. Molecular Plant-Microbe Interactions 22, 157–165.

Waibel F, Filipowicz W. 1990. U6 snRNA genes of Arabidopsis are transcribed by RNA polymerase-III but contain the same 2 upstream promoter elements as RNA polymerase-II-transcribed U-snRNA genes. Nucleic Acids Research 18, 3451–3458.

Weaver LM, Swiderski MR, Li Y, Jones JDG. 2006. The Arabidopsis thaliana TIR-NB-LRR R-protein, RPP1A: protein localization and constitutive activation of defence by truncated alleles in tobacco and Arabidopsis. The Plant Journal 47, 829–840.

Woo J, MacPherson CR, Liu J, Wang H, Kiba T, Hannah MA, Wang XJ, Bajic VB, Chua NH. 2012. The response and recovery of the Arabidopsis thaliana transcriptome to phosphate starvation. BMC Plant Biology 12, 62.

Wu G. 2013. Plant microRNAs and development. Journal of Genetics and Genomics 40, 217–230.

Xu F, Xu SH, Wiermer M, Zhang YL, Li X. 2012. The cyclin L homolog MOS12 and the MOS4-associated complex are required for the proper splicing of plant resistance genes. The Plant Journal 70, 916–926.

Xu SH, Zhang ZB, Jing BB, Gannon P, Ding JM, Xu F, Li X, Zhang YL. 2011. Transportin-SR is required for proper splicing of resistance genes and plant immunity. PLoS Genetics 7, e1002159.

Yi H, Richards EJ. 2007. A cluster of disease resistance genes in Arabidopsis is coordinately regulated by transcriptional activation and RNA silencing. The Plant Cell 19, 2929–2939.

Zaros C, Thuriaux P. 2005. Rpc25, a conserved RNA polymerase III subunit, is critical for transcription initiation. Molecular Microbiology 55, 104–114.

Zhang YL, Goritschnig S, Dong Xen X, Li X. 2003. A gain-of-function mutation in a plant disease resistance gene leads to constitutive activation of downstream signal transduction pathways in suppressor of npr1-1, constitutive 1. The Plant Cell 15, 2636–2646.

Zhu QH, Stephen S, Kazan K, Jin GL, Fan LJ, Taylor J, Dennis ES, Helliwell CA, Wang MB. 2013. Characterization of the defense transcriptome responsive to Fusarium oxysporum-infection in Arabidopsis using RNA-seq. Gene 512, 259–266.