atBRX1-1 and atBRX1-2 are involved in an alternative rRNA processing pathway in *Arabidopsis thaliana*

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ABSTRACT

Ribosome biogenesis is an essential process in all organisms. In eukaryotes, multiple ribosome biogenesis factors (RBFs) act in the processing of ribosomal (r)RNAs, assembly of ribosomal subunits and their export to the cytoplasm. We characterized two genes in *Arabidopsis thaliana* coding for orthologs of yeast BRX1, a protein involved in maturation of the large ribosomal subunit. Both atBRX1 proteins, encoded by AT3G15460 and AT1G52930, respectively, are mainly localized in the nucleolus and are ubiquitously expressed throughout plant development and in various tissues. Mutant plant lines for both factors show a delay in development and pointed leaves can be observed in the *brx1-2* mutant, implying a link between ribosome biogenesis and plant development. In addition, the pre-rRNA processing is affected in both mutants. Analysis of the pre-rRNA intermediates revealed that early processing steps can occur either in the 5' external transcribed spacer (ETS) or internal transcribed spacer 1 (ITS1). Interestingly, we also find that in *xrn2* mutants, early processing events can be bypassed and removal of the 5' ETS is initiated by cleavage at the P' processing site. While the pathways of pre-rRNA processing are comparable to those of yeast and mammalian cells, the balance between the two processing pathways is different in plants. Furthermore, plant-specific steps such as an additional processing site in the 5' ETS, likely post-transcriptional processing of the early cleavage sites and accumulation of a 5' extended 5.8S rRNA not observed in other eukaryotes can be detected.

Keywords: rRNA processing; ribosome biogenesis; Brx1; Xrn2; *Arabidopsis thaliana*; 60S subunit maturation

INTRODUCTION

In eukaryotes, the mature 18S, 5.8S, and 25/28S rRNAs are transcribed as a single precursor rRNA by RNA polymerase I (Woolford and Baserga 2013; Henras et al. 2014). The mature rRNAs in this transcript are flanked by external transcribed spacers (ETS) and are separated by internal transcribed spacers (ITS). The 5S rRNA is transcribed separately by RNA polymerase III and is incorporated into 60S pre-ribosomes during early ribosome assembly (Ciganda and Williams 2011). During and after transcription, the pre-rRNA is modified, processed, folded, and assembled with the ribosomal proteins (RP) (Watkins and Bohnsack 2012; Woolford and Baserga 2013; Turowski and Tollervey 2014). Pre-rRNA processing starts with the release of the primary transcript through an endonucleolytic cleavage in the 3' ETS, performed by Rnt1 in yeast and atRTL2 in *Arabidopsis thaliana* (Kufel et al. 1999; Comella et al. 2008). In yeast,

the 5' ETS is first removed by cleavages at sites A₀ and A₁ performed by the SSU processome in the 90S particle generating 33S and 32S pre-rRNAs, respectively (Dragon et al. 2002; Osheim et al. 2004). Separation of the precursors of the small and large ribosome subunits (SSU and LSU, respectively) is primarily achieved by A₂ cleavage in ITS1 that separates 90S into pre-40S and pre-60S particles containing the 20S and 27SA₂ pre-rRNAs (Bleichert et al. 2006; Henras et al. 2014). However, a minor pathway involving processing at a different cleavage site in ITS1, A₃, is discussed (Henras et al. 2014). In contrast, in human cells, most pre-rRNA transcripts are processed at site 2 in ITS1 (corresponding to site A₃ in yeast) and this cleavage often occurs before removal of the 5' ETS in human cells (Hadjilova et al. 1993; Henras et al. 2014). Less is known about pre-rRNA processing in plants, but a processing scheme was recently described for

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A. thaliana. It was suggested, that cleavage at site A₃ is the first processing event following P-site cleavage in the 5' ETS, which contains a specific insertion in *Arabidopsis* upstream of the P-site (Sáez-Vasquez et al. 2004; Zakrzewska-Placzek et al. 2010; Missbach et al. 2013; Weis et al. 2014), implying a processing pathway analogous to the major pathway in mammalian cells (Henras et al. 2014). However, an alternative processing route has also been suggested (Hang et al. 2014; Weis et al. 2014).

Pre-rRNA processing requires a multitude of proteinaceous and RNA RBFs (Woolford and Baserga 2013) and by genetic and proteomic studies >200 proteins functioning in ribosome assembly in yeast have been identified (for review, see Henras et al. 2008; Woolford and Baserga 2013). More recently inventories of RBFs have also been proposed for human cells (Wild et al. 2010; Tafforeau et al. 2013) and orthologs of >200 RBFs have been identified in plants (Xu et al. 2013; Ebersberger et al. 2014). However, the role of these putative RBFs in plant ribosome biogenesis still requires experimental confirmation, because the molecular functions of proteins may vary in different organisms. Indeed, we have recently shown that the orthologs of the yeast large ribosomal subunit GTPase 1 (Lsg1) (Kallstrom et al. 2003; Hedges et al. 2005) have diverged in its sequence and atLSG1-1 has lost its primary function in ribosome biogenesis, while atLSG1-2 still has its ancestral function in this process (Weis et al. 2014).

Another RBF involved in 60S subunit biogenesis in yeast is “biogenesis of ribosomes in *Xenopus* 1” (Brx1). Brx1 belongs to the Imp4 superfamily and was first characterized in *Xenopus laevis* and yeast, where it was found to interact with rRNA and mutants affected pre-rRNA processing (Kaser et al. 2001). 35S, 27SA₂, and the aberrant 23S pre-rRNA accumulated after depletion of Brx1 in yeast (Kaser et al. 2001). The physical interaction of yeast Brx1 with pre-ribosomes was subsequently confirmed by pull down of the 66S preribosomal particles by TAP-tagged Rpl1 (Horseley et al. 2004). In addition, it was demonstrated that the anchoring of Brx1 to the preribosomal particle in yeast depends on Ebp2 (Shimoji et al. 2012), which in turn is essential for 25S rRNA maturation (Huber et al. 2000). Moreover, it was proposed that Tif6, Brx1, and Ebp2 are involved in the formation of the Rpf2 subcomplex, which plays a role in assembly of the central protuberance close to the top of the 60S subunit (Talkish et al. 2012).

In *A. thaliana*, we discovered two orthologs to yeast Brx1. Both are ubiquitously expressed and are mainly localized in the nucleolus. Mutant plant lines for atBRX1-1 and atBRX1-2, *brx1-1*, and *brx1-2*, respectively, show developmental abnormalities and defects in pre-rRNA processing. Interestingly, we observed accumulation of a specific subset of pre-rRNAs in the *brx1* mutant lines. We also reinvestigated the pre-rRNA processing pathway in *xrn2* mutant plants on our high-resolution agarose gels. These mutants show significant alterations in the pre-rRNA accumulation, while

plant development remains unaffected (Zakrzewska-Placzek et al. 2010). Based on these data, we present a revised overview of pre-rRNA processing in *A. thaliana* and discuss it in comparison to the yeast and mammalian pathways. Our findings suggest that two alternative pre-rRNA processing pathways coexist in *A. thaliana* and that both the A₂ and A₃ cleavage sites in ITS1 are used to separate pre-40S and pre-60S particles. Interestingly, mutation of the atBRX1 orthologs only affects one of these pathways.

RESULTS

Two orthologs of Brx1 in *A. thaliana* are constitutively expressed

Two orthologs of yeast Brx1 have been identified in *A. thaliana* (atBRX1-1, AT3G15460; atBRX1-2, AT1G52930). They share 41% and 40% sequence similarity with their yeast ortholog, respectively, and are themselves 86% similar in sequence. Both show the same architecture to the yeast protein but additionally possess a charged extension at the aminoterminal (Fig. 1A). This prompted us to investigate whether they are expressed in a tissue-specific manner. We isolated RNA from wild-type *A. thaliana* plants at different developmental stages and from different organs and analyzed the abundance of mRNAs coding for both orthologs by qRT-PCR (Fig. 1B; Missbach et al. 2013; Weis et al. 2014). The abundance of the *BRX1-1* mRNA was comparable in most tissues and only in roots and flowers of old plants, tissues with a higher demand for newly synthesized ribosomes, expression of the *BRX1-2* mRNA appears to be enhanced (Fig. 1B). However, by statistical analysis this increase was not found to be significant.

Furthermore, we analyzed the presence of both atBRX1 proteins at different developmental stages and in different organs by Western blotting using an antibody generated against a mixture of full-length atBRX1-1 and atBRX1-2 (Fig. 1C). As a control, we analyzed the abundance of atRPL5 (Fig. 1C) using a specific antibody generated against full-length atRPL5. We found that both atBRX1 proteins are expressed in all analyzed tissues, but comparison to atRPL5 revealed a decline of atBRX1 abundance after day 18 in nonreproductive tissues like rosette, stem, and hypophyll (Fig. 1C). Thus, the protein abundance in different tissues likely reflects the demand for ribosome biogenesis, as it is high in young developing tissues and reduced in leaves, stem, hypophyll, and seeds, which are tissues with reduced rate of cell division. Interestingly, inspection of the coexpression analysis using String 9.1 (Franceschini et al. 2013) shows that both *BRX1* genes are coexpressed with genes that are orthologs of yeast RBFs (Table 1). Like Brx1, all these orthologs are involved in the maturation of 90S and 60S preribosomal complexes and at least Rpf2 and Has1 have also been found in the yeast complexes isolated by TAP-tagged Rpl1 (Horseley et al. 2004).

FIGURE 1. *A. thaliana* has two orthologs of the yeast ribosome biogenesis factor Brx1. (A) A schematic alignment of atBRX1-1, atBRX1-2, and scBRX1 is shown. Negatively and positively charged amino acids are colored in red and blue, respectively. Note that both atBRX1 proteins have a charged amino-terminal insertion. (B) The expression of *BRX1-1* and *BRX1-2* mRNAs at the indicated developmental stages was analyzed by qRT-PCR. The results are the mean of four independent biological replicates and standard deviation is shown. By *t*-test no change with $P < 0.05$ was observed. (Seeds imb) Imbibed seeds. (C) Total proteins were extracted from the tissues and at the developmental stages indicated, separated on SDS-PAGE and subjected to Western blot using atBRX1 (top) and atRPL5 (bottom) antibodies. Arrowheads on the right indicate the migration of atBRX1-1 (filled) and atBRX1-2 (open). The intensity of detected bands was analyzed and the ratio of atBRX1-2 to atBRX1-1 and total atBRX1 to atRPL5, normalized to day 5, is indicated. (cc) Cell culture.

Both atBRX1 proteins are nucleolar and associate with preribosomes

Next, the subcellular localization of both atBRX1 proteins was analyzed. carboxyterminally GFP tagged atBRX1-1 and atBRX1-2 proteins were transiently overexpressed in protoplasts under the control of the 35S promoter (Fig. 2A). Full-length fusion protein expression was confirmed by Western blotting using an anti-GFP antibody (Fig. 2A). atBRX1-1-GFP shows one band of the expected size, while atBRX1-2-GFP shows a double band suggesting that the protein expressed from this construct is partially unstable (Fig. 2A). Both proteins are mainly localized to the nucleolus as judged from the overlay of the GFP signal of atBRX1-GFP

proteins and the fluorescence of Fibrillarin-mCherry as a nucleolar marker (Fig. 2A). These results are consistent with the nucleolar localization of yeast and mammalian BRX1 (Kaser et al. 2001).

The interaction of both atBRX1 proteins with ribosomes was studied using sucrose density gradient centrifugation (Fig. 2B). With the conditions used here, polysomes and 80S ribosomes were unstable, but distinct peaks corresponding to fractions containing mature and pre-rRNAs, ribosomal proteins, and the RBF eIF6-2 clearly demonstrated the separation of the (pre-)40S and (pre-)60S particles (Fig. 2B). Interestingly, both atBRX1 proteins comigrate in the fractions corresponding to pre-60S complexes (Fig. 2B) suggesting that they are both involved in ribosome biogenesis.

Individual BRX1 proteins are important for plant development

We selected one SALK and one GABI-KAT transfer DNA (T-DNA) insertion line for *BRX1-1* and *BRX1-2*, respectively (Fig. 3A), to further investigate the functions of both proteins in vivo. Screening PCRs on genomic DNA and sequencing analysis (Missbach et al. 2013) revealed a single back-to-back T-DNA insertion in both lines, *brx1-1* and *brx1-2* (Fig. 3A,B). In addition, *brx1-1* and *brx1-2* are homozygous lines as the wild-type allele was no longer detectable (Fig. 3B, lanes 6 and 12, respectively). As expected, we detected a transcript upstream of the T-DNA insertion for both lines (Fig. 3C, first row of panels), but a full-length transcript was not detected in either plant line (Fig. 3C, third row of panels). However, in *brx1-2* a transcript downstream from the insertion exists as well (Fig. 3C, lane 4, panel 2), likely caused by the 35S promoter in the T-DNA close to the left border. Most important, the homozygosis of both lines was confirmed by Western blot using the atBRX1 antibody on protein extracts from wild-type and mutant flowers (Fig.

TABLE 1. Coexpression analysis with String 9.1

Gene	STRING S ^a	Yeast protein	Complex
Coexpression analysis—AT3G15460 (<i>atBRX1-1</i>)			
AT4G04940	0.929	UTP21	90S
AT5G52490	0.929	FIBRILLARIN 2	90S
AT4G05410	0.928	RRP9	90S
AT3G10530	0.928	UTP7	90S
AT3G23620	0.925	RPF2	60S
AT3G18600	0.922	HAS1	90S
Coexpression analysis—AT1G52930 (<i>atBRX1-2</i>)			
AT5G52490	0.980	FIBRILLARIN 2	90S
AT1G31660	0.977	ENP2	90S
AT5G08180	0.976	NAN1	90S
AT3G23620	0.976	RPF2	60S
AT4G12600	0.975	SNU13	90S
AT2G40360	0.975	ERB1	60S

^aString score.

FIGURE 2. Both atBRX1 proteins are nucleolar and associated with pre-60S complexes. (A) *A. thaliana* protoplasts were cotransformed with plasmids coding for Fibrillarin-mCherry and either atBRX1-1-GFP (top) or atBRX1-2-GFP (bottom). mCherry fluorescence (left) and GFP fluorescence (second panel) were merged with the autofluorescence of chlorophyll (third panel). A differential interference contrast (DIC) image is also shown (fourth panel). A representative image is given. Note that the nuclear signals may partially be due to overexpression of fusion proteins from the 35S promoter. (Right) Transformed protoplasts were lysed and subjected to Western blot using a GFP antibody. (B) Sucrose density gradient centrifugation using total cell lysate from *A. thaliana* cell culture was performed. The UV absorption profile is shown on top. Mature rRNAs in the fractions were visualized by ethidium bromide (EtBr) staining. Pre-rRNAs were detected by Northern blot (NB). Western blot (WB) analysis was carried out using the antibodies indicated on the left. Arrowheads indicate the migration of atBRX1-1 (filled) and atBRX1-2 (open).

3D). The corresponding full-length protein was not detected in *brx1-1* and *brx1-2*, confirming that these lines are suitable to analyze the consequence of an absence of atBRX1-1 and atBRX1-2 in vivo. Nevertheless, atBRX1-1 accumulates slightly in the *brx1-2* mutant (Fig. 3D) possibly to compensate for the loss of atBRX1-2. This suggests that both atBRX1 orthologs are redundant.

Next, we analyzed the phenotype of the two mutant lines. We observed a growth delay in both lines (Fig. 3E, inset) and an overall slower growth rate of *brx1-2* (Fig. 3E). Further-

more, development of the first rosette leaves was impaired in both lines (Fig. 3F), but was also more pronounced for *brx1-2* (Fig. 3F). Interestingly, *brx1-2* showed a slight pointed leaf phenotype (Fig. 3G) that has previously been observed in ribosomal proteins mutants (Byrne 2009; Horiguchi et al. 2011). Development of all following rosette leaves as well as flowering was also more delayed in *brx1-2* than in *brx1-1*. However, crossing of both mutants did not lead to a double homozygous mutant (not shown), supporting that the atBRX1 orthologs are redundant.

brx1 mutants affect pre-rRNA processing

To gain insight into whether the atBRX1 proteins are required for rRNA processing, we isolated total RNA from wild-type, *brx1-1* and *brx1-2* seedlings, resolved it on high-resolution agarose-glyoxal (Fig. 4B) or polyacrylamide gels (Fig. 4E) and detected pre-rRNAs by Northern blotting using a variety of probes hybridizing to different regions of the pre-rRNA transcripts (Zakrzewska-Placzek et al. 2010; Missbach et al. 2013; Weis et al. 2014). Importantly, probes annealing between each of the known/proposed cleavage sites in the pre-rRNA were used enabling each of the intermediate detected to be defined (Fig. 4A).

With all probes shown in Figure 4A, the 35S pre-rRNA, extending from site P in the 5' ETS and extending into the 3' ETS, can be detected (Fig. 4B,D). A pre-rRNA (33S[P']) can be detected with probes p2 (annealing between P' and the mature 5' end of 18S rRNA), p3, p42, p43, p4, and p5 (ITS2) but not with p22 (annealing between P₁ and P') or p6 (3' ETS) (Fig. 4B,D). The 5' end of 33S[P'] is therefore generated by cleavage at site P' and this precursor lacks the 3' ETS. Although this intermediate has been observed previously (Zakrzewska-Placzek et al. 2010; Missbach et al. 2013; Weis et al. 2014), the processing steps/cleavage sites by which it is generated have remained unclear until now. Furthermore, p3, p42, p43, and p5 recognize the recently described 32S pre-rRNA (Hang et al. 2014) that comigrates with 33S[P'] under these conditions (Fig. 4B,D). We also observed the 27SA pre-rRNA with probes p42, p43 (between A₂ and A₃), and p5 (Fig. 4B,D). Interestingly, the 5' end of this intermediate was previously thought to be generated by A₃ cleavage; however, our data show that 27SA pre-rRNAs extending to A₂ (27SA₂) can also be detected (see p42 and p43). In addition, the 27SB pre-rRNA was observed to accumulate significantly. This is also the case in yeast where it is proposed that downstream preribosomal remodeling steps are rate-limiting for pre-rRNA processing (Woolford and Baserga 2013). Furthermore, multiple 5' extended precursors of the 18S rRNA, named P-A₃, P'-A₃ and 18S-A₃, can be detected with probes p23, p22, p2, p3, p42, and p43 (Fig. 4B,D). The 3' ends of all of these pre-rRNAs are extended to site A₃ because they are recognized by p43, which anneals immediately upstream of site A₃. Interestingly, the 20S precursor of the 18S rRNA that extends from the 5' end of 18S to the A₂ cleavage site and is

FIGURE 3. Characterization of *brx1* mutant plants. (A) The gene model of *BRX1-1* and *BRX1-2* with the T-DNA position of the mutants is shown. The positions of the oligonucleotides used in B (filled triangle) and C (open triangle) are indicated below. (B) The segregation of the T-DNA insertion in the indicated mutants (lanes 4–6 and 10–12) was analyzed by PCR on genomic DNA (gDNA) with the oligonucleotides shown in A. LB: T-DNA left border primer. Wild-type (WT) gDNA was analyzed as control (lanes 1–3 and 7–9). (C) The presence of *BRX1* mRNA was analyzed by RT-PCR using RNA isolated from wild type (lanes 1,3), *brx1-1* (lane 2), and *brx1-2* (lane 4) and the oligonucleotides shown in A. Actin (*ACT2*) mRNA was amplified as loading control. (D) The presence of the atBRX1 protein was determined by Western blotting using the atBRX1 antibody on protein extracts from wild type (WT, lane 1), *brx1-1* (lane 2) and *brx1-2* (lane 3). atRPL5 served as loading control (α RPL5). (E) The rosette diameter was determined for at least 10 independent, randomly selected, plants of each genotype as indicated. Inset shows the growth curve between days 4 and 16. The standard deviation is shown. (F) Developmental stages according to Boyes et al. (2001). (G) Pictures of the plant lines indicated on top were taken on days 9, 15, and 19. Scale bar: 10 mm.

highly abundant in yeast, is not observed in *A. thaliana* wild-type seedlings. On the polyacrylamide gel, the conserved 3' extended 5.8S precursors 7S and 6S can be seen with probe p5 (Fig. 4E). In addition, a 5' extended 5.8S pre-rRNA containing the mature 3' end of 5.8S can be detected with p4.

All pre-rRNA intermediates described above were also detected in both *brx1* mutants (Fig. 4B,E). The level of the 35S pre-rRNA was unaltered in the mutants and was therefore used to determine the relative accumulations of other pre-rRNA species in the different lines (Fig. 4C). In both mutants, the 33S(P'), 32S, and 27SA₂ pre-rRNAs accumulated ~2.5-fold compared with wild type, while the levels of P-A₃ were reduced by half (Fig. 4B,C). Consistent with

this, the ratio between P-A₃ and 32S was five times lower than the ratio determined in wild type (Fig. 4C). The ratio between 27SA₂ and 32S was unaltered in the mutants (Fig. 4C). Interestingly, the 20S pre-rRNA becomes visible in both *brx1* mutants, although at low levels (Fig. 4B). These results show that only a subset of the pre-rRNA intermediates are affected by mutation of *BRX1* and support a function of both atBRX1 proteins in pre-60S subunit maturation in *A. thaliana*.

Processing at site P can be bypassed

The new insights into the cleavages in both the 5' ETS and ITS1 that are used to generate pre-rRNA intermediates found

in wild-type plants imply that rRNA maturation could be more complex than previously thought. To gain further information about these pathways we also examined plant lines lacking the exonuclease atXRN2 that have previously been shown to exhibit severe pre-rRNA processing defects (Zakrzewska-Placzek et al. 2010). atXRN2 is one of the three homologs for yeast nuclear 5'→3' exonuclease Rat1/Xrn2. In yeast, this protein is involved in the maturation of 5.8S and 25S rRNAs (Henry et al. 1994; Geerlings et al. 2000), in RNA decay (Bousquet-Antonelli et al. 2000), as well as in transcription termination (Kim et al. 2004; El Hage et al.

2008). Mammalian XRN2 has been shown to be required for pre-rRNA processing at site 01/A' (Wang and Pestov 2011; Sloan et al. 2014). *xrn2* mutant plants do not show any visible phenotype, but accumulate 5' extended rRNA precursors that have not been efficiently processed at site P (Fig. 5; Zakrzewska-Placzek et al. 2010). Furthermore, in *xrn2* mutant plants, P₁ processing also appears to be impaired, because 5' ETS degradation intermediates extending between site P and P' accumulate in the mutant (Lange et al. 2011).

By Northern blot analysis, we observed a 5' ETS extended 35S and P-A₃ pre-rRNAs in the *xrn2* mutant (35S*, 5' ETS–

FIGURE 4. Accumulation of pre-rRNAs is affected in *brx1* mutants. (A) Shown is the primary pre-rRNA transcript of *A. thaliana* with the processing sites indicated by arrows on top. Probes (p) used for Northern blots are indicated below. Insertion: a 1083-nt sequence in the 5' ETS specific for *A. thaliana*. (ETS) External transcribed spacer, (ITS) internal transcribed spacer. The A₁₂₃B cluster is specific to *Brassicaceae* and was shown to be bound by the NF-B/D complex important for P-site cleavage (Sáez-Vasquez et al. 2004). (B) Total RNA from 18-day-old seedlings from wild-type (WT) and the *brx1* mutants was separated on a 1.2% agarose gel, transferred onto nylon membrane and hybridized with the probes indicated in A. Mature chloroplast rRNAs visible with ethidium bromide (EtBr) staining (23S-1 and 16S) are shown in gray. The names of the detected pre-rRNAs are given on the right. (C) Individual Northern blots as shown in B were quantified and the band intensity was normalized to the 35S pre-rRNA that is unaltered in the mutants. Wild-type values were set to one. (D) Scheme of the pre-rRNAs that are detected in *B*. Light gray boxes mark the newly defined 33S(P') and 27SA₂ pre-rRNAs. Italic: 5' extended pre-rRNAs seen in *xrn2* mutants (Fig. 5). (E) Total RNA from 18-day-old seedlings from wild-type (WT) and the *brx1* mutants was separated on an 8% acrylamide/8 M urea gel, transferred onto nylon membrane and hybridized with the probes indicated. Mature chloroplast rRNAs seen by ethidium bromide (EtBr) staining (23S-3, 5S, and 4.5S) are shown in gray. The names of the detected pre-rRNAs are given on the right.

FIGURE 5. Processing at site P can be bypassed. Total RNA from wild-type (WT) and *xrn2* mutant plants was analyzed as described in Figure 4B. The names of the pre-rRNAs detected are given on the right. Note that the 35S and P-A₃ pre-rRNAs are 5' ETS extended in the *xrn2* mutant (35S* and 5' ETS-A₃, respectively).

A₃) (Fig. 5), supporting the conclusion that atXRN2 is required for P-site cleavage and removal of an *Arabidopsis*-specific insertion in the 5' ETS (Zakrzewska-Placzek et al. 2010). Interestingly, the 33S(P') and P'-A₃ pre-rRNAs accumulated to normal levels in the *xrn2* mutant (Fig. 5), suggesting that P-site cleavage can be bypassed and downstream processing steps can occur normally leading to normal levels of the mature rRNAs. It is not clear whether cleavage of site P₁ is a prerequisite for P' processing as pre-rRNA intermediates specifically generated by this cleavage are not detectable, however, comparison to mammalian 5' ETS processing might suggest that cleavage at P₁ and P' occur almost simultaneously as has been proposed for A₀ and A₁ (Henras et al. 2014). This suggests that the mature 5' end of the 18S rRNA can be generated even if early 5' ETS processing is impaired.

DISCUSSION

atBRX1 proteins are important for development

Here, we identified and analyzed the two orthologs to the yeast RBF Brx1 that is involved in early pre-60S maturation (Kaser et al. 2001; Horsey et al. 2004; Shimoji et al. 2012). Both atBRX1 proteins are highly similar in sequence (Fig. 1A), ubiquitously expressed (Fig. 1B,C), localize to the nucleolus (Fig. 2A) and, interestingly, both are associated with the pre-60S ribosomal subunit (Fig. 2B). Furthermore, plant lines lacking either one of the atBRX1 proteins show the same pre-rRNA processing defects and a double homozygous mutant is not viable (Figs. 3, 4). These results suggest that the two atBRX1 proteins have redundant function. In contrast, many other RBFs with multiple orthologs have diverged in their sequence or subcellular localization and only one of the orthologs still has its ancestral function in ribosome biogenesis (Pontvianne et al. 2007; Comella et al.

2008; Lange et al. 2008; Zakrzewska-Placzek et al. 2010; Durut et al. 2014; Weis et al. 2014).

Interestingly, the pointed leaf phenotype in *brx1-2* (Fig. 3G) was previously observed for many RP mutants (Byrne 2009; Horiguchi et al. 2011) and a delay in early growth and development was shown for multiple RBF mutants (Pontvianne et al. 2007; Lange et al. 2011; Weis et al. 2014). These observations imply that ribosome biogenesis, in general, is closely coupled to early plant development, likely due to the high demand for newly synthesized ribosomes in this growth phase.

Pre-rRNA processing occurs by two alternative pathways in *A. thaliana*

The observed pre-rRNAs in wild-type plants including the newly identified 33S(P') and 27SA₂ pre-rRNAs (Fig. 4B,D), as well as the accumulation of a defined set of pre-rRNAs in the *brx1* mutants (Fig. 4B,C), suggest two alternative rRNA processing pathways for *A. thaliana* (Fig. 6) as it was also proposed for the other eukaryotic model organisms (Woolford and Baserga 2013; Henras et al. 2014).

In "Pathway 1" (Fig. 6), the 35S pre-rRNA is processed at the mature 3' end of 25S rRNA and at site P' in the 5' ETS, which yields the 33S(P') pre-rRNA (Fig. 4B). 33S(P') is then further processed to the 32S pre-rRNA. These two steps resemble the A₀ and A₁ cleavages in yeast (Dragon et al. 2002; Phipps et al. 2011). Interestingly, the large pre-rRNAs (35S, 33S, and 32S) are barely detectable in yeast, because processing at sites A₀, A₁, and A₂ takes place cotranscriptionally on >70% of all transcripts (Osheim et al. 2004; Koš and Tollervey 2010; Turowski and Tollervey 2014). In contrast, these pre-rRNAs can be well detected in *A. thaliana* (Figs. 4B, 5, Zakrzewska-Placzek et al. 2010; Missbach et al. 2013; Weis et al. 2014) suggesting that pre-rRNA processing mostly occurs post-transcriptionally under normal conditions as has been suggested for mammalian cells where 47S/45S, 43S, and 41S can also be readily detected in wild type (Preti et al. 2013; Tafforeau et al. 2013; Sloan et al. 2014). Next, pre-40S and pre-60S maturation is separated by cleavage at A₂ in ITS1 yielding the 20S and 27SA₂ pre-rRNAs. However, the 20S pre-rRNA is barely detectable in wild type (Fig. 4B) suggesting rapid processing of this intermediate. In contrast, the yeast 20S pre-rRNA is readily detectable as it is part of the late pre-40S subunit that undergoes tight quality control in the cytoplasm (Lebaron et al. 2012; Strunk et al. 2012; García-Gómez et al. 2014). The 27SA₂ pre-rRNA is further processed in a pathway similar to that used in yeast to generate the short and long forms of the 5.8S rRNA (5.8S_S and 5.8S_L, respectively) as well as the 25S rRNA (Fig. 6). Interestingly, a 5' extended 5.8S pre-rRNA can be detected in wild type (Fig. 4E; Zakrzewska-Placzek et al. 2010; Missbach et al. 2013), suggesting that 3' maturation of the 5.8S rRNA can naturally occur before maturation of the 5' end, which is different to yeast or mammalian cells. In yeast, a 5'

FIGURE 6. Two alternative rRNA processing pathways coexist in *A. thaliana*. A primary transcript is released by cleavage at site B₀ mediated by atRTL2. Further processing involving the exonuclease atXRN2 and processing of the 1083-nt insertion prepares site P for efficient cleavage by the BoU3 snoRNP complex. (*Left*) In Pathway 1, the 35S pre-rRNA is processed at site P' (33S[P']) and subsequently at the mature 5' end of 18S rRNA (32S), before A₂ cleavage releases the 20S and 27SA₂ pre-rRNAs. 20S pre-rRNA is rapidly matured to the 18S rRNA. (*Right*) Pathway 2 involves A₃ cleavage to separate pre-40S and pre-60S particles. The resulting P-A₃ fragment is subsequently processed at P₁, P', and the mature 5' end of 18S pre-rRNA before the final processing step at site D occurs in the cytoplasm. 27SA₂ (from Pathway 1) or 27SA₃ (Pathway 2) are processed at site B₁ before C₂ processing separates them into 7S and C₂-25S. The 7S pre-rRNA likely undergoes 3'→5' exonucleolytic trimming that yields 5.8S + 30, 6S, and finally the mature 5.8S rRNA. Furthermore, a 5' extended pre-5.8S rRNA is detectable in RNA from wild type, but its place in the processing scheme remains to be established. For further details and references see main text. Newly defined pre-rRNAs are marked by a gray box.

extended 5.8S pre-rRNA is only detectable when factors such as Rat1 or Rrp17, that are directly involved in 5' trimming of 5.8S rRNA precursors are deleted (Henry et al. 1994; Oeffinger et al. 2009). However, the relevance of this step for plant pre-rRNA processing, as well as its place in the presented processing scheme, is not yet clear (Fig. 6). Interestingly, the large pre-rRNAs belonging to the proposed Pathway 1 (33S [P'], 32S, 27SA₂, and 20S) specifically accumulate in both *brx1* mutants (Fig. 4B,C) supporting the conclusion that they belong to one processing pathway.

In "Pathway 2," the 35S pre-rRNA is cleaved at site A₃ to separate pre-40S and pre-60S maturation resulting in the P-A₃ pre-rRNA (comparable to 30S in mammalian cells) and the 27SA₃ pre-rRNAs (Fig. 6). The P-A₃ fragment is then processed to P'-A₃ and 18S-A₃ (Fig. 6). These fragments are comparable to the aberrant 23S, 22S, and 21S pre-rRNAs in yeast and 26S and 21S pre-rRNAs in mammalian cells (Henras et al. 2014). The 18S-A₃ pre-rRNA is then cleaved at site D by the homolog of the endonuclease Nob1 (Fatica et al. 2003; Veith et al. 2012; Missbach et al. 2013). It remains unclear whether the 18S-A₃ pre-rRNA is first processed to the 20S pre-rRNA before D-site cleavage or if atNOB1 can directly cleave the 18S-A₃ pre-rRNA.

An alternative pre-rRNA processing route starting comparable to Pathway 1 was proposed in parallel by Hang and co-workers who described the 32S pre-rRNA (Hang et al. 2014).

However, this proposed route would ultimately lead back to only one pathway in contrast to the two independent pathways described here as we have detected a 27SA₂ pre-rRNA. In addition, we identified 33S(P') as the starting point for Pathway 1.

In conclusion, pre-rRNA processing in *A. thaliana* likely follows two alternative routes (Fig. 6). Pathway 1 is analogous to the main processing pathway used in yeast (Woolford and Baserga 2013; Henras et al. 2014) whereas Pathway 2 is comparable to the major processing pathway observed in mammalian cells (Mullineux and Lafontaine 2012; Henras et al. 2014). While the alternative ITS1 processing pathways have also been described for both yeast and mammalian cells, in these organisms the majority of transcripts are processed via a single pathway (A₂ in yeast, A₃-like in mammals Woolford and Baserga 2013; Henras et al. 2014). Interestingly, both the A₂ and the A₃ processing sites are used in *A. thaliana* to separate the pre-40S and pre-60S particles (Fig. 6). Finally, in both pathways processing in the 5' ETS can be bypassed by cleavage at site P' as the newly identified 33S(P') and the P'-A₃ fragments are detected in the *xrn2* mutants (Fig. 5). In future, it will be highly interesting to identify other factors required for ribosome maturation in *A. thaliana* and to investigate further the pathways of pre-rRNA processing and pre-rRNA intermediates that are specific for plants.

MATERIALS AND METHODS

In vivo localization

For protein localization analysis, the coding sequences of atBRX1-1 and atBRX1-2 were amplified from cDNA and cloned into the pRTdS-vector (Missbach et al. 2013) to generate carboxy-terminal GFP fusions for expression in protoplasts. pRTdS-atFIB2-mCherry was used as a nucleolar marker (Missbach et al. 2013). *A. thaliana* leaf mesophyll protoplasts were isolated, transformed, and visualized as previously described (Sommer et al. 2011).

Protein and antibody generation

The coding sequences of atBRX1-1, atBRX1-2, atRPL5, and atRPS10 were amplified from cDNA and cloned into a modified pQE80 vector for amino-terminal fusion with a His₂₁ tag. Proteins were expressed in BL21 star pRosetta cells for 4 h at 37°C with 0.25 mM IPTG. All proteins were purified using NiNTA (Qiagen) under denaturing conditions in a buffer containing 25 mM Tris pH 7.5, 300 mM NaCl, and 6 M guanidium hydrochloride. The denatured atBRX1 proteins were mixed in a 1:1 molar ratio and used for antibody generation in rabbits (Dr. Pineda, Berlin). atRPL5 and atRPS10 were used to immunize one rabbit each. For antibody purification, the buffer of the atBRX1 proteins was exchanged to 0.1 M NaHCO₃, 0.5 M NaCl, and 8 M urea for coupling to CNBr Sepharose. The atBRX1 matrix was then prepared and the antibody was purified according to the manufacturer's protocol (GE Healthcare). For Western blotting, the atBRX1 antibody was used in a 1:5000 dilution. For atRPL5 and atRPS10, the serum was used directly for Western blotting at a 1:20,000 dilution.

Sucrose density gradient

Sucrose density gradients were performed as described (Hsu et al. 2014) because we observed inefficient release of nucleolar pre-ribosomes using our standard procedure (Missbach et al. 2013; Weis et al. 2014). Total cell lysate from *A. thaliana* cell culture was separated on a 10%–40% (w/v) sucrose gradient by centrifugation for 18 h at 100,000g in a TST41.14 rotor. Fractions were collected and analyzed as described previously (Weis et al. 2014).

Reverse transcription and qRT-PCR analysis

First strand cDNA from 1 µg of RNA was synthesized using M-MuLV reverse transcriptase (Thermo Scientific) according to the manufacturer's protocol. The relative expression of *BRX1-1* and *BRX1-2* mRNAs was quantified by qRT-PCR as previously described (Missbach et al. 2013).

Protein analysis in different tissues and developmental stages

For the analysis of the protein abundance of atBRX1-1 and atBRX1-2 in different developmental stages or different organs the tissue

TABLE 2. Northern probes used in this study

Probe	Position on pre-rRNA (relative to TIS)	Sequence (5' → 3')
p23	+1333 to +1356	GTTCCAATAATCTACCGAAGTAC
p22	+1709 to +1732	CTTTGGCGGGACTGAATCACTTCG
p2	+1802 to +1824	ACGGCAATCCCCGCCACATCC
p3	+3644 to +3668	GGTCGTTCTGTTTTGGACAGGTATC
p42	+3698 to +3722	GCAAGGAATCGGCTAAGAAACCGGC
p43	+3803 to +3826	CACTTTTCTGTCGGGGTTTTGTG
p4	+3894 to +3915	CGTTTTAGACTTCAGTTCGCAG
p5	+4069 to +4093	GGATGGTGAGGGACGACGATTGTG
p6	+7689 to +7718	CGTAAGGAGCTGTGCTTTGTTAGTGTAG
7SL	+2887 to +2908	CGGAGTCCTATAAGCAACATCC

material was ground and directly resuspended in cracking buffer (5% (w/v) SDS, 6 M urea, 40 mM Tris–HCl pH 6.8, 0.1 mM EDTA, 0.4 mg/mL bromophenol blue, 147 mM β-mercaptoethanol, 1 mM PMSF). After incubation at 60°C for 10 min and centrifugation at 20,000g for 5 min the supernatant was quantified using amido black protein quantification. Equal amounts of protein were loaded onto SDS-PAGE and subjected to Western blotting using the atBRX1 antibody. Western blots of biological replicates were quantified using ImageJ.

Plant growth and T-DNA analysis

The search for orthologs of scBrx1 was performed with In Paranoid (Ebersberger et al. 2014). T-DNA insertion lines for AT3G15460 (*BRX1-1*) and AT1G52930 (*BRX1-2*) were ordered from the Nottingham Arabidopsis Stock Centre (NASC; *brx1-1*: SALK_004020, *brx1-2*: GABI_771C02). Plants were grown in climate chambers under long day conditions (14 h photoperiod with 120 µmol/m²/sec; 21°C at day and 18°C at night). Genomic DNA for screening PCRs was isolated as previously described (Edwards et al. 1991). T-DNA mapping was performed as previously described (Missbach et al. 2013). The T-DNA insertion mutants were further verified by reverse transcription (RT) PCR and Western blotting. For the latter, one flower was ground in 50 µL cracking buffer using an EPPI-pestle before addition of another 100 µL cracking buffer and incubation at 60°C for 10 min. After centrifugation at 20,000g for 5 min, the supernatant (total protein) was analyzed by SDS-PAGE and Western blotting. Growth-stage based phenotypic analysis was performed as described (Boyes et al. 2001).

RNA isolation and Northern blotting

Total RNA from 18-d-old seedlings or flowers was isolated as previously described (Chomczynski and Sacchi 1987) with some modifications. Briefly, 100 mg of tissue was frozen in liquid nitrogen and ground to a fine powder. Three milliliters of Solution D (4 M guanidine thiocyanate, 25 mM sodium citrate pH 7.0, 147 mM β-mercaptoethanol, 0.5% (w/v) sodium lauroyl sarcosinate) and 300 µL of 2 M sodium acetate pH 4.0 were added and vigorously mixed. Three milliliters of acid saturated phenol (pH4.3, Sigma-Aldrich) and 600 µL chloroform were added, mixed, and incubated on ice for 15 min. After centrifugation, the aqueous phase was extracted once with

phenol:chloroform:isoamylalcohol (25:24:1) and once with chloroform, before precipitation with one volume isopropanol. RNA was pelleted, further purified using the NucleoSpin RNA kit (Macherey-Nagel) and eluted with 40 μ L RNase-free H₂O. Five micrograms of total RNA was resolved on a 1.2 (w/v) % agarose gel in BPTE buffer (30 mM Bis-Tris, 10 mM PIPES, 1 mM EDTA) or on an 8% polyacrylamide/8 M urea gel in TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) buffer before blotting on nylon N+ membrane (GE healthcare). RNAs were cross-linked to the membrane using a Stratilinker and probed with ³²P end-labelled oligonucleotide probes as previously described (Missbach et al. 2013; Weis et al. 2014). The probes used are listed in Table 2.

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