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 25S/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 humans cells, most pre-rRNA transcripts are processed at site 2 in ITS1 (corresponding to site A2 in yeast) and this cleavage often occurs before removal of the 5′ ETS in human cells (Hadjiolova et al. 1993; Henras et al. 2014). Less is known about pre-rRNA processing in plants, but a processing scheme was recently described for...
A. thaliana. It was suggested, that cleavage at site A₂ 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 Rrp1 (Horsey 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 Rp2 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 aminoterminus (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 Rp2 and Has1 also be found in the yeast complexes isolated by TAP-tagged Rrp1 (Horsey et al. 2004).
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* | Yeast protein | Complex |
|------|-----------|---------------|---------|
| AT4G04940 | 0.929 | UTP21 | 90S |
| AT5G24940 | 0.929 | FIBRILLARIN 2 | 90S |
| AT4G05410 | 0.928 | RRP9 | 90S |
| AT3G15530 | 0.928 | UTP7 | 90S |
| AT3G23620 | 0.925 | RPF2 | 60S |
| AT3G18660 | 0.922 | HAS1 | 90S |
| Coexpression analysis—AT3G52930 (atBRX1-2) | | | |
| AT5G24940 | 0.980 | FIBRILLARIN 2 | 90S |
| AT1G31660 | 0.977 | ENP2 | 90S |
| AT5G08180 | 0.976 | NAP1 | 90S |
| AT3G32620 | 0.976 | RPF2 | 60S |
| AT4G12600 | 0.975 | SNUP13 | 90S |
| AT2G40360 | 0.975 | RPB1 | 60S |

*aString score.*
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. 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) or not with p22 (annealing between P 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 185′-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 A2 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
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 RNA 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 P1 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–

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**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. 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. (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 35S (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.
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′ occurs 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 (Wooldford 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; Taftoreau 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 (5.8SS and 5.8S1, 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′
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′–A2 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 (Woollford 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 xrr2 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-atFB2-mCherry was used as a nuclear 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, 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.

Sucrose density gradient

Sucrose density gradients were performed as described (Hsu et al. 2014) because we observed inefficient release of nuclear 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 material was ground and directly resuspended in cracking buffer (% (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 InParanoid (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 subjected to Western blotting using the atBRX1 antibody. Western blots of biological replicates were quantified using ImageJ.

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 μL RNase-free H2O. Five microliters 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 Stratalinker 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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