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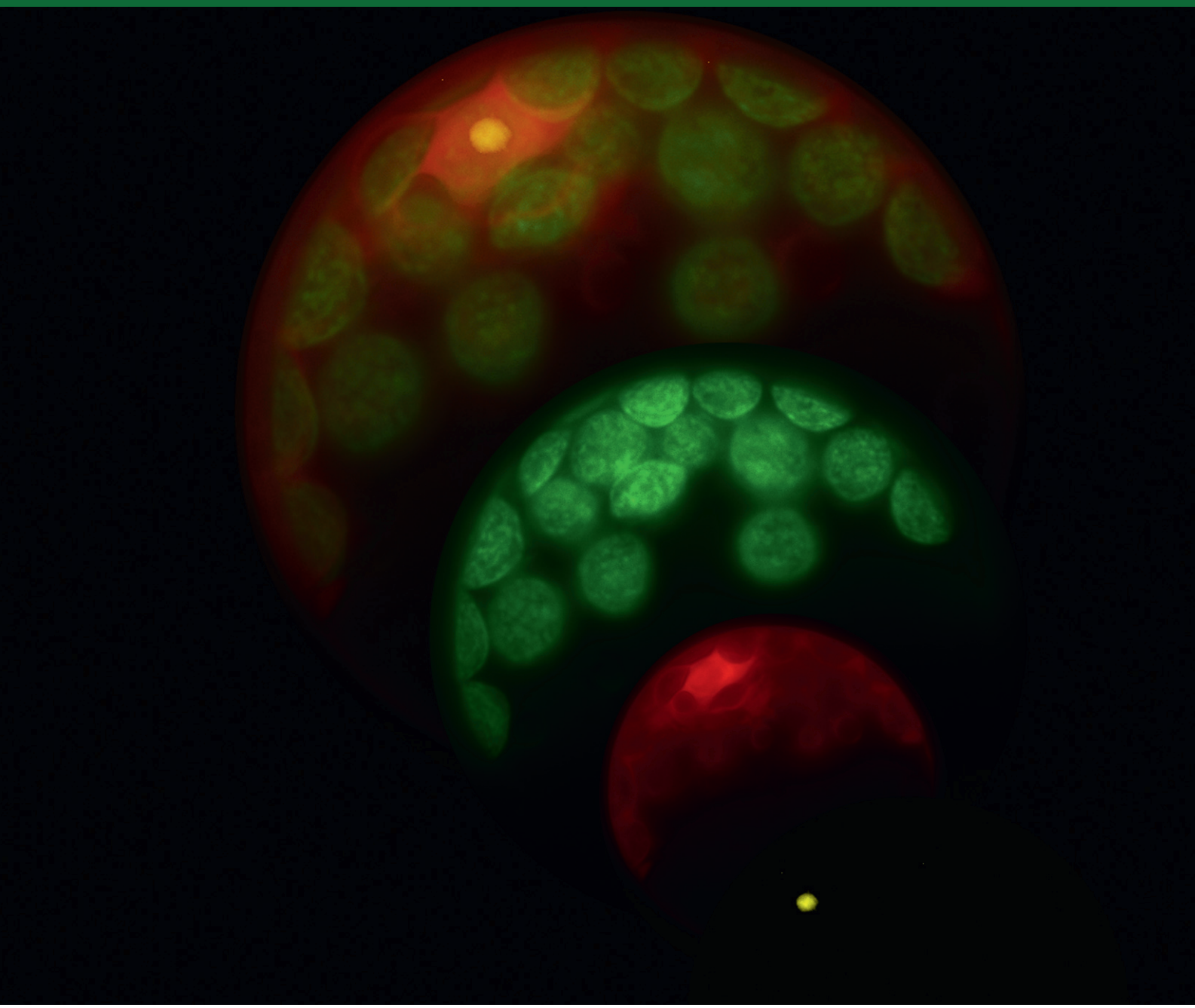

VOLUME 98 | NUMBER 2 | APRIL 2019
http://www.theplantjournal.com | ISSN 1365-313X
WILEY Blackwell

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ISSN 1365-313X (online)

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The Plant Journal is published semi-monthly (four volumes per annum), with two issues published each month. Subscription prices for 2019 are: Online only: US$7274 (The Americas), US$8488 (Rest of World), €4996 (Europe); £3936 (UK). Prices are exclusive of tax. Asia-Pacific GST, Canadian GST and European VAT will be applied at the appropriate rates. For more information on current tax rates, please go to www.wileyonlinelibrary.com/tax-vat. The price includes online access to the current and all online back files to January 1st 2015, where available. For other pricing options, including access information and terms and conditions, please visit www.wileyonlinelibrary.com/access.

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THE PLANT JOURNAL, (ISSN 0960-7412), is published semi-monthly (four volumes per annum). US mailing agent: Mercury Media Processing, LLC 1850 Elizabeth Avenue, Suite #C, Rahway, NJ 07065, USA. Periodical postage paid at Rahway, NJ.
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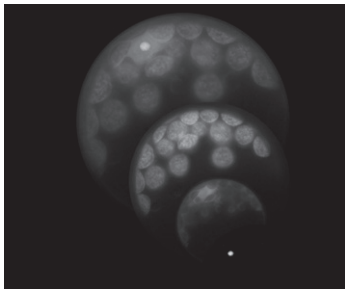

Front cover: The cover image shows an overlay and individual layers of *Arabidopsis thaliana* protoplast used for Bimolecular Fluorescence Complementation assay. In this case, interaction between AtCBF5 and AtTRB3 proteins was analysed. The biggest protoplast represents the final overlay of three layers, where the green one shows only chloroplasts, the red coloured protoplast symbolizes nuclear signal delivered by mRFP-VirD2NLS and finally, the yellow dot shows a specific interaction between selected proteins AtCBF5 and AtTRB3 localized in the nucleolus. For details, see article by Schořová *et al.* (pp.195–212).

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The Plant Journal (2019) **98,** 193–194 doi: 10.1111/tpj.14328

RESEARCH HIGHLIGHT

The journey to the end of the chromosome: delivering active telomerase to telomeres in plants

Lee Sweetlove and Crisanto Gutierrez

Linked article: This is a Research Highlight about Šárka Schořová *et al.* To view this article visit https://doi.org/10.1111/tpj.14306.

Linear chromosomes offer many advantages over circular DNA for transcription and replication of large genomes, hence their prevalence in eukaryotes. But the linear arrangement of the DNA has a massive Achilles heel: the terminal ends, or telomeres, are unstable and prone to mutation. Moreover, DNA replication cannot proceed to the end of a linear DNA molecule because the synthesis of Okazaki fragments needs RNA primers to bind ahead of the lagging strand. Eukaryotes deal with both of these problems by adding repetitive DNA sequences to the telomeres that act as a disposable buffer, protecting terminal genes from being truncated during replication and from mutation. Because the telomere is shortened during each DNA replication, it is necessary to resynthesise telomere DNA using an enzyme, telomerase reverse transcriptase.

Our understanding of telomere biology is dominated by research into human telomeres. This is understandable due to the links between telomere biology and cellular mortality, ageing and a range of diseases including cancer. However, telomere biology in plants shows some specific differences to humans which may be crucial in our understanding of telomere biology in general. For example, telomerase activity in plant cells is well balanced with the cellular proliferation rate. The reversible regulation of telomerase activity is thought to be important in this context: its activity is turned off in differentiated tissues and turned on during cell periods of active cell replication, for example, during regeneration of plant tissues. Understanding the mechanism for this reversible regulation of telomerase activity could be beneficial in biomedical applications of telomere biology in humans.

But where to start? From protozoans and humans, it was known that telomerase was a ribonucleoprotein, carrying its own RNA molecules that are complementary to the telomere repeats and are used as a template for telomere elongation, catalysed by the reverse transcriptase activity of the enzyme. But, in addition, a number of accessory proteins are required to deliver functional telomerase to the telomeres, to regulate its activity and to protect the elongated telomere from DNA repair enzymes. These components assemble into two distinct complexes known as shelterin and CST. Functional homologues of the CST complex have been identified in plants, but the same is not true for the shelterin complex. In plants, not all of the homologues of the six core shelterin components exist, and only some of them seem to be associated with telomeres *in vivo*. The goal, therefore was to identify undiscovered telomerase accessory proteins in plants and to establish how active telomerase is formed and regulated.

Jiří Fajkus and his research group at Masaryk University, have been working on plant telomeres for over 20 years. A key member of his team in the hunt for plant telomerase-associated proteins has been Petra Procházková Schrumpfová, first as a Ph.D. student and then through several postdoc periods. Working in Arabidopsis, the group had already established that Telomere Repeat Binding proteins (TRB) were involved in recruitment of telomerase to the telomeres. These proteins are specific to plants and contain an N–terminal Myb-like domain which is responsible for specific recognition of telomeric DNA. Attention turned to the plant homologues of two human telomere associated proteins called Pontin and Reptin after they turned up in a pull-down of TERT, the catalytic subunit of Arabidopsis telomerase, in an experiment done in collaboration with Eva Sýkorová's group at the Institute of Biophysics in Brno.

The plant Pontin and Reptin homologues are encoded by *RuvBL1* and *RuvBL2a*, respectively. But despite the fact that RuvBL proteins were isolated from plant cells as TERT-associated, Jiří and his team were not able to prove a direct interaction between TERT and RuvBL as had already been described in mammals. Serendipity then intervened. During their characterisation of RuvBL interactions with TERT, they used several proteins as negative controls. Surprisingly, one of the supposed negative controls showed reproducibly positive interaction with RuvBL proteins. It was in this way that they discovered that TRB proteins interact with RuvBL. Knowing that TRB proteins directly interact with TERT they started to closely characterise the trimeric complex TERT-TRB-RuvBL and that is

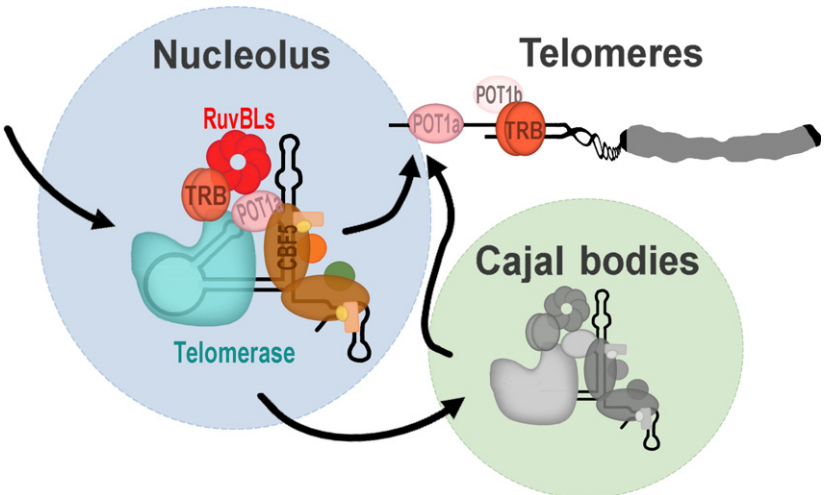

Figure 1. RuvBL1 and RuvBL2a, are orthologues of human Pontin and Reptin, respectively, in Arabidopsis.
Besides their mutual interactions, RuvBL1 associates with the catalytic subunit of telomerase (TERT) in the nucleolus *in vivo*. In contrast to mammals, interactions between TERT and RuvBL proteins in Arabidopsis are not direct but are mediated by one of the Telomere Repeat Binding (TRB) proteins. The plant orthologue of human dyskerin, named CBF5, is indirectly associated with TRB proteins but not with the RuvBL proteins in the plant nucleus/nucleolus, and interacts with the Protection of telomere 1 (POT1a) in the nucleolus or cytoplasmic foci.

the focus of the highlighted paper which is drawn from the MSc and PhD research of Šárka Schořová with Petra Procházková Schrumpfová and Jiří Fajkus as joint corresponding authors. The work also involved Lenka Záveská Drábková, a postdoc from David Honys's group at the Institute of Experimental Botany in Prague who did phylogenetic analysis of the RuvBL family in plants. That collaboration started late one afternoon during a short-term visit of Petra Procházková Schrumpfová to David Honys's lab that was focused on a completely different scientific topic. Such is the nature of science and scientists!

In this highlighted paper (Schořová *et al.*, 2019), a combination of BiFC, yeast-two hybrid and pull-down assays confirmed that there is no direct interaction between RuvBLs and TERT, but that the interaction is mediated by TRBs as an intermediary. It was also shown that RuvBL proteins form hetero- and homo-oligomers *in vivo*. Proof of the importance of RuvBL1/2 for telomerase biogenesis was provided by analysis of Arabidopsis knockout lines which had substantially reduced telomerase activity in flower buds (a rapidly proliferating tissue with high telomerase requirement). This crucial experiment turned out to be the hardest part of the research, with identification of knockout alleles a real struggle. Jiří and Petra say that they had to genotype hundreds of individual plants from several lines and were only able to identify a few heterozygous individuals of each gene with homozygous mutants being lethal.

Further protein interaction experiments identified another protein in the complex: CBF5, a homologue of mammalian dyskerin, a known telomerase-associated protein. Cell biological analyses were able to place all of these proteins in the nucleolus and some of them in Cajal bodies and, combined with previous studies, the authors were able to put together the most complete picture of the plant telomerase complex to date, as shown in Figure 1.

One of the most interesting facets of this picture is the similarities and divergence between plants and humans. On the one hand, identification of Reptin and Pontin in Arabidopsis and their conservation in humans, shows that the factors involved in telomerase biogenesis and function are evolutionary ancient. On the other, the interactions and mechanism of action of plant Reptin and Pontin is different than in human cells. The TERT subunit of Arabidopsis telomerase does not interact directly with Reptin and Pontin but through TRBs which in human cells are telomere-associated proteins but not TERT-accessory factors. This reveals that different mechanisms have evolved although using basically the same set of factors, a finding that would justify a similar study in other eukaryotic lineages to define the evolutionary history of complex formation between telomeric repeats, TERT, accessory factors and shelterin proteins.

One possible reason to explain the variety of mechanisms suggested by this study is the specific organization, and possibly the 3D structure, of TERT RNA (TER) molecules which may limit the ability of TERT to interact directly with them or require other bridging proteins, as it occurs in Arabidopsis. Differences in the subnuclear localization of telomeric sequences may be also important. For Jiří and his team, work will continue to unpick the regulation of synthesis of both basic subunits of telomerase, TER and TERT, their intracellular trafficking and assembly into the holoenzyme complex, together with a number of associated factors.

REFERENCE

Schořová, Š., Fajkus, J., Záveská Drábková, L., Honys, D. and Procházková Schrumpfová, P. (2019) The plant Pontin and Reptin homologues, RuvBL1 and RuvBL2a, colocalize with TERT and TRB proteins *in vivo*, and participate in telomerase biogenesis. *Plant J.* **98**, 195–212.

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The Plant Journal (2019) **98,** 195–212 doi: 10.1111/tpj.14306

The plant Pontin and Reptin homologues, RuvBL1 and RuvBL2a, colocalize with TERT and TRB proteins *in vivo*, and participate in telomerase biogenesis

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Received 26 November 2018; revised 8 February 2019; accepted 26 February 2019; published online 4 March 2019.
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SUMMARY

Telomerase maturation and recruitment to telomeres is regulated by several telomerase- and telomere-associated proteins. Among a number of proteins, human Pontin and Reptin play critical roles in telomerase biogenesis. Here we characterized plant orthologues of Pontin and Reptin, RuvBL1 and RuvBL2a, respectively, and show association of *Arabidopsis thaliana* RuvBL1 (AtRuvBL1) with the catalytic subunit of telomerase (AtTERT) in the nucleolus *in vivo*. In contrast to mammals, interactions between AtTERT and AtRuvBL proteins in *A. thaliana* are not direct and they are rather mediated by one of the *Arabidopsis thaliana* Telomere Repeat Binding (AtTRB) proteins. We further show that plant orthologue of dyskerin, named AtCBF5, is indirectly associated with AtTRB proteins but not with the AtRuvBL proteins in the plant nucleus/nucleolus, and interacts with the Protection of telomere 1 (AtPOT1a) in the nucleolus or cytoplasmic foci. Our genome-wide phylogenetic analyses identify orthologues in RuvBL protein family within the plant kingdom. Dysfunction of *AtRuvBL* genes in heterozygous T-DNA insertion *A. thaliana* mutants results in reduced telomerase activity and indicate the involvement of AtRuvBL in plant telomerase biogenesis.

Keywords: telomerase assembly, Pontin, Reptin, AtTERT, AtTRB, AtRuvBL, AtPOT1a, nucleolus, *Arabidopsis*.

Linked article: This paper is the subject of a Research Highlight article. To view this Research Highlight article visit https://doi.org/10.1111/tpj.14328.

INTRODUCTION

Telomeres are nucleoprotein structures at the ends of eukaryotic chromosomes that protect linear chromosomes. Telomeric structures are formed by telomeric DNA, RNA, histones, and a number of other proteins that bind telomeric DNA, either directly or indirectly, together forming the protein telomere cap (Fajkus and Trifonov, 2001; de Lange, 2005; Schrumpfová *et al.*, 2016a). The core component of the telomere cap in mammals is a six-protein complex called shelterin. The specific telomeric double-stranded DNA binding of the shelterin is mediated by its TRF1 and TRF2 (Telomere Repeat Binding Factors 1 and 2) components through their Myb-like domain of a telobox type (Bilaud *et al.*, 1996; Peška *et al.*, 2011). In *Arabidopsis thaliana* Telomere Repeat Binding (AtTRB) proteins, that contain Myb-like domain of a telobox type and bind plant telomeric repeats *in vitro* (Schrumpfová *et al.*, 2004; Mozgová *et al.*, 2008), were found to colocalize with telomeres *in situ* and *in vivo* (Dvořáčková *et al.*, 2010; Schrumpfová *et al.*, 2014; Dreissig *et al.*, 2017), directly interacted with the telomerase reverse transcriptase (AtTERT) (Schrumpfová *et al.*, 2014) and physically interacted with AtPOT1b (Protection Of Telomeres 1) (Schrumpfová *et al.*, 2008). Moreover, shortening of telomeres was observed in *attrb* knockout mutants (Schrumpfová *et al.*, 2014, 2019; Zhou *et al.*, 2018).

Telomere- or telomerase-associated proteins can regulate lengths of telomere tracts by affecting the assembly of active telomerase complex or by modulation of the accessibility of telomeres to telomerase. The process of maturation and recruitment of human telomerase is partially understood (Schmidt and Cech, 2015; MacNeil *et al.*, 2016; Schmidt *et al.*, 2016). However, a similar description of telomerase assembly and recruitment to the telomeres in plants is still missing which would allow to distinguish between general and specific features of these processes.

Among a number of proteins, which were identified as associated with human telomerase, proteins named RuvBL (RuvB-like), that share limited sequence similarity to the bacterial RuvB helicase, were also identified. RuvBL proteins belong to the evolutionarily highly conserved AAA+ family (ATPase Associated with various cellular Activities) that are involved in ATP binding and hydrolysis (Matias *et al.*, 2006). Eukaryotic RuvBL1 (Pontin, TIP49a, Rvb1, TAP54α) and RuvBL2 (Reptin, TIP48, TIP49b, Rvb2, TAP54β) participate in many diverse cellular activities like chromatin remodeling (Jha *et al.*, 2008), transcriptional regulation (Ohdate *et al.*, 2003; Gallant, 2007), oncogenic transformation (Osaki *et al.*, 2013), epigenetic regulations (Gallant, 2007) or DNA-damage signaling (Rosenbaum *et al.*, 2013). RuvBL1 and RuvBL2 can also play a role in the assembly of box C/D or H/ACA of small nucleolar RNAs (snoRNAs) with specific proteins to form functional ribonucleoprotein particles (RNPs) (Watkins *et al.*, 2004; McKeegan *et al.*, 2007; Boulon *et al.*, 2008; Zhao *et al.*, 2008). Participation of RuvBL1 and RuvBL2 proteins in diverse cellular processes, as well as their association with specific interactors, can vary among cytoplasm, nucleus and nucleolus (Izumi *et al.*, 2012). RuvBL1 and also RuvBL2 monomers can assemble into different oligomeric forms, including hexameric structure with a central channel, or dodecamer composed of two hetero-hexameric rings with alternating RuvBL1 and RuvBL2 monomers (Torreira *et al.*, 2008; Niewiarowski *et al.*, 2010). RuvBL structure suggests that these proteins can act as scaffolding proteins, which explains their appearance in various cellular protein complexes (Matias *et al.*, 2006; Mao and Houry, 2017).

Mammalian RuvBL1 and RuvBL2, also termed as Pontin and Reptin, respectively, were found to play a critical role in telomerase biogenesis. Telomerase is a ribonucleoprotein enzyme complex composed of two core subunits: the catalytic telomerase reverse transcriptase (TERT) protein subunit and the telomerase RNA (TR) subunit (containing a box H/ACA motif). It performs the addition of telomeric DNA repeats onto the telomeres (Greider, 1996; Zhang *et al.*, 2011). Proper assembly of TERT with TR into a functional complex is a stepwise regulated process governed also by multiple associated proteins (Schmidt and Cech, 2015; MacNeil *et al.*, 2016). Human TR (hTR), as well as other box H/ACA snoRNPs, is associated with conserved scaffold proteins: dyskerin, NHP2, NOP10, NAF1 in the nucleoplasm, where NAF1 is replaced by GAR1 before the hTR RNP complex reaches the nucleolus. Association of hTR RNP with hTERT is proceeded in the nucleolus and the subsequent formation of catalytically active telomerase holoenzyme is localized into the Cajal bodies (CBs) (MacNeil *et al.*, 2016) that are evolutionary conserved mobile nuclear substructures involved in the RNA modification and the RNP assembly processes (Cioce and Lamond, 2005). Venteicher *et al.* (2008) demonstrated that hRuvBL1 (Pontin) and hRuvBL2 (Reptin) are interdependent proteins and are recruited to hTERT complexes through the association between hTERT and hRuvBL1. Additionally, they showed that both hRuvBL1 and hRuvBL2 directly interact with dyskerin and may help to assemble or remodel a nascent hTERT/hTR/dyskerin complex. The scaffold proteins, including dyskerin, together with hRuvBL1 and hRuvBL2, are required for a proper assembly of hTR RNP and are involved in the biogenesis of telomerase.

A homologue of human RuvBL1 from *A. thaliana* has been already described by Holt *et al.* (2002). They observed that plants with reduced *AtRuvBL1* (AT5G22330) mRNA levels had morphological defects and suggested that AtRuvBL1 was required in meristem development. Moreover, they observed that T-DNA insertion mutation in *AtRuvBL1* gene was lethal. In our laboratory, AtRuvBL1 protein and also one of two AtRuvBL2 homologues, named AtRuvBL2a (AT5G67630), were purified together with AtTERT using Tandem Affinity Purification (TAP) from *A. thaliana* suspension cultures (Majerská *et al.*, 2017).

In this study, we examined a mutual interaction of AtRuvBL1-AtRuvBL2a proteins and demonstrated that AtRuvBL proteins are associated with AtTERT in the nucleolus *in vivo*. However, in contrast to mammalian counterparts, interactions between AtTERT and AtRuvBL proteins are not direct and are likely to be mediated by one of the AtTRB proteins. We prove that AtTRB3 protein physically interacts with AtRuvBL1 and simultaneously with AtTERT. We further show that in plants, similarly to mammals, telomerase assembly is a dynamic process, as is supported by our observation that AtCBF5, a plant orthologue of dyskerin, is in the plant nucleus/nucleolus indirectly associated with three of AtTRB proteins, but not with the AtRuvBL proteins, and interacts with the AtPOT1a in the cytoplasmic or nucleolus foci. Heterozygous T-DNA insertion mutants in *AtRuvBL1* or *AtRuvBL2a* genes show reduced telomerase activity indicating the potential involvement of AtRuvBL proteins in telomerase assembly in *A. thaliana*. To identify new homologues of RuvBL protein family and elucidate their evolutionary relationships, we performed a survey of 83 plant species (80 angiosperms, one gymnosperm and two bryophytes).

RESULTS

AtRuvBL proteins form homomers or mutually interact

RuvBL1 and RuvBL2 proteins from mammals and yeast can co-exist in different monomeric or oligomeric complexes comprising dimers, trimers, hexamers or double-hexamers that can be composed as mixed multimers (Torreira *et al.*, 2008; Niewiarowski *et al.*, 2010; Queval *et al.*, 2014). Each RuvBL monomer contains three basic domains (DI, DII, DIII) (Figure 1a). Domain I (DI) together with domain III (DIII) represent the AAA+ core and are sufficient to form hexameric rings. In the AAA+ domain, the Walker A/B motifs are responsible for ATP binding and hydrolysis, while sensor I/II motifs sense whether the protein is bound to di- or triphosphates. Domain II (DII) corresponds to an insertion that is unique to RuvBL in comparison with other AAA+ family members (Silva-Martin *et al.*, 2016).

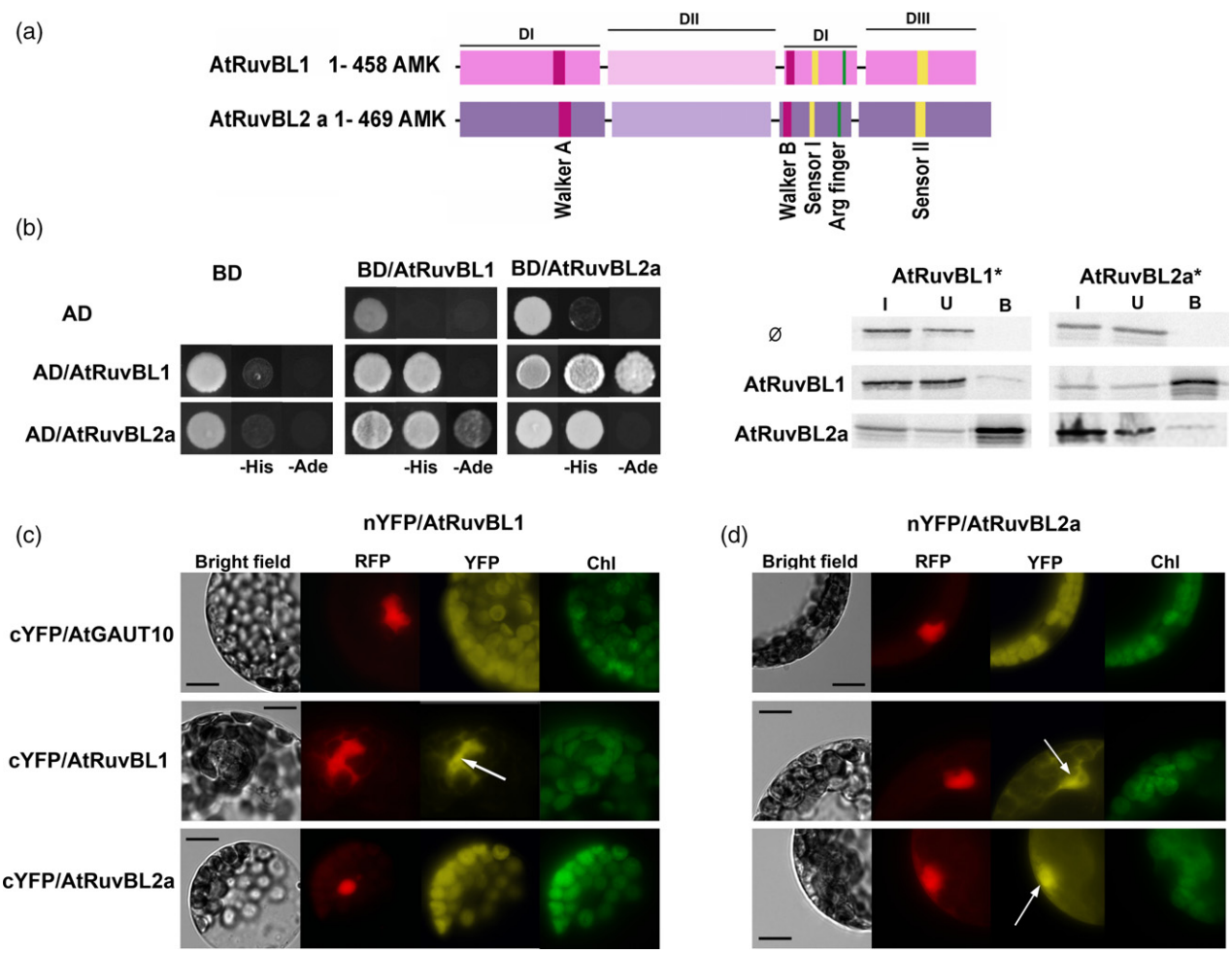

Figure 1. AtRuvBL proteins can form homo- or hetero-oligomers.
(a) Schematic representation of the conserved motifs of the RuvBL proteins from *Arabidopsis thaliana*. DI, DII, DIII, Domain I, II, III; Walker A/B, Walker motifs; Sensor I/II, sensors; Arg finger, arginine finger. AtRuvBL2a and AtRuvBL2b form closely related sequence pairs.
(b) Y2H system is used to assess homo- or heteromerization of AtRuvBL proteins. Two sets of plasmids carrying the indicated protein fused to either the GAL4 DNA-binding domain (BD) or the GAL4 activation domain (AD) are constructed and introduced into yeast strain PJ69-4a carrying reporter genes His3 and Ade2. Clear AtRuvBL1 and also AtRuvBL2a homomerization is detected on histidine-deficient plates. Mutual interaction between AtRuvBL1 and AtRuvBL2a is detected not only on histidine-deficient plates but also under stringent adenine selection. Co-transformation with an empty vector (AD, BD) serves as a negative control.
(c) Co-IP is performed with the TNT-RRL expressed AtRuvBL1* and AtRuvBL2a* (^{35}S-labelled*, prey) mixed with their protein counterparts AtRuvBL1 and AtRuvBL2a, fused with Myc-tag (anchor) and incubated with anti-Myc antibody. In the control experiment, the AtRuvBL* proteins are incubated with Myc-antibody and protein G-coupled magnetic beads in the absence of partner protein. Input (I), Unbound (U) and Bound (B) fractions are collected and run in 12% SDS-PAGE gels. Mutual AtRuvBL1 and AtRuvBL2a interactions appear to be stronger than entirely homo-interactions between AtRuvBL proteins.
(d) BiFC confirms homo- and also mutual heteromerization of AtRuvBL1 and AtRuvBL2a proteins. *A. thaliana* leaf protoplasts are co-transfected with 10 μg of each of the plasmids encoding nYFP-tagged or cYFP–tagged AtRuvBL1, AtRuvBL2a or AtGAUT10 clones (as negative control) and simultaneously with mRFP–VirD2NLS clone. Bright Field (left); RFP, mRFP–VirD2NLS (red fluorescent protein fused with nuclear localization signal) labels cell nuclei and determines transfection efficiency; YFP, yellow fluorescent protein signals indicate specific protein–protein interactions (PPI) also marked by white arrows; Chl, chloroplast autofluorescence is marked by green pseudocolor, chloroplast autofluorescence is also visible in the YFP channel. Scale bars = 10 μm.

To examine whether plant homologues of RuvBL proteins form homomers or mutual heteromers as their mammalian counterparts, or exist only as monomers, we performed several assays for protein–protein interactions (PPIs): yeast two-hybrid system (Y2H), co-immunoprecipitation (Co-IP) and bimolecular fluorescence complementation (BiFC).

First, we tested AtRuvBL1 and AtRuvBL2a homo-interactions. BiFC assay performed in *A. thaliana* leaf protoplasts, which enables direct visualization of protein interactions in living cells, demonstrated that AtRuvBL1 and AtRuvBL2a form homodimers or homomultimers *in vivo*. These results were confirmed using a GAL4 based Y2H assay, in which interactions took place inside the nucleus. We observed a clear homomeric interaction of AtRuvBL1 proteins as well as of AtRuvBL2a proteins in Y2H mating assay. The homomerization was further verified by Co-IP experiments in which proteins were expressed in the Coupled Transcription/Translation Rabbit Reticulocyte Lysate (TNT-RRL) System using the same vectors as in Y2H (Figure 1b, c).

Additionally, we expanded our BiFC study (Majerská *et al.*, 2017) and tested heteromerization of AtRuvBL1 and AtRuvBL2a not only in *Nicotiana tabacum* BY-2 protoplasts, but also in *A. thaliana* leaf protoplasts (Figure 1d). Analysis of subcellular localization of the AtRuvBL1-AtRuvBL2a interactions further showed that one reciprocal interaction of nYFP/AtRuvBL1 and cYFP/AtRuvBL2a was negative, and cYFP/AtRuvBL1 and nYFP/AtRuvBL2a showed nuclear, but not nucleolar localization, maybe due to the presence of a tag that may induce conformational changes of the AtRuvBL proteins (Cheung *et al.*, 2010). Using Y2H assay, we confirmed clear interaction not only on histidine-deficient (−His) plates but also under stringent adenine (−Ade) selection. Both Y2H and Co-IP experiments revealed that mutual AtRuvBL1−AtRuvBL2a interaction seemed stronger than pure homomerization of

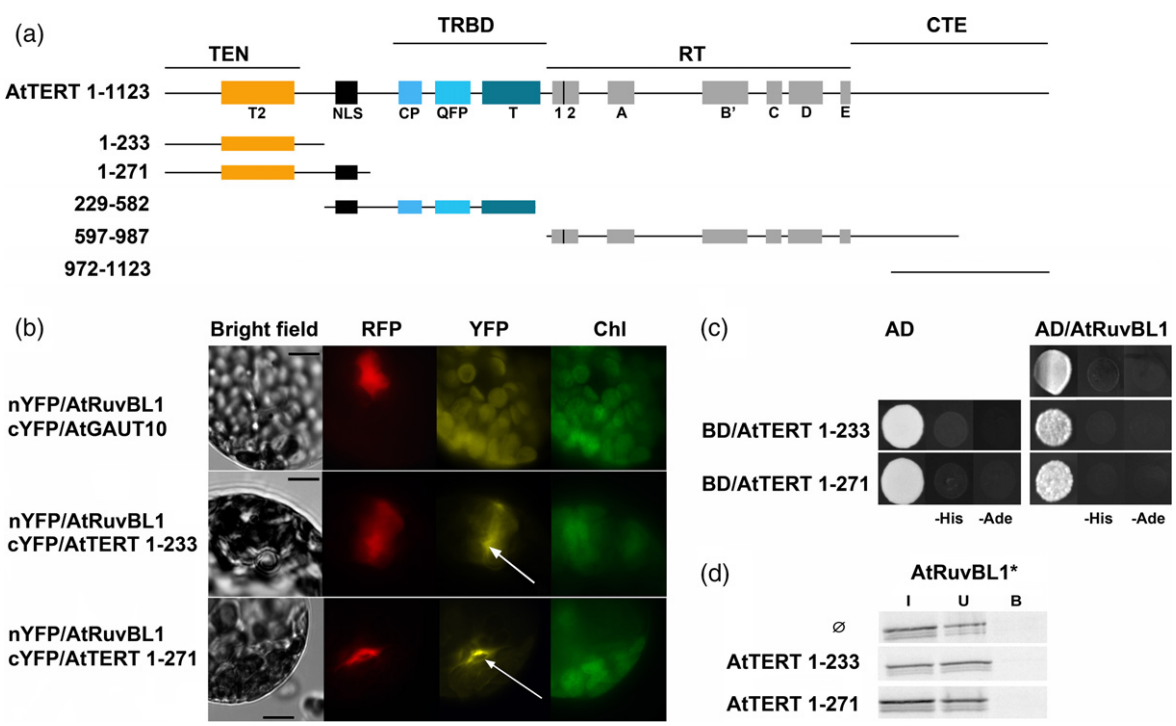

Figure 2. AtRuvBL1 interacts indirectly with N-terminal part of *Arabidopsis thaliana* catalytic subunit AtTERT. The analyses were performed as described in Figure 1.

(a) Schematic depiction of the plant catalytic subunit of telomerase (AtTERT) showing functional motifs. The regions of structural domains TEN (telomerase essential N-terminal domain), TRBD (RNA-binding domain), RT (reverse transcriptase domain) and CTE (C-terminal extension) are depicted above the conserved RT motifs (1, 2, A, B′, C, D and E), telomerase-specific motifs (T2, CP, QFP and T) and a NLS (nucleus localisation-like signal). All the depicted AtTERT fragments were used in protein–protein interaction analysis (amino acid numbering is shown). All AtTERT fragments were fused with activation domains (AD/BD or nYFP/cYFP) and used for further BiFC, Y2H and Co-IP analysis.

(b) BiFC in *A. thaliana* leaf protoplasts were used to detect the interaction between AtRuvBL1 and all AtTERT fragments from schematic depiction. Here we show PPI interaction (white arrows) of two N-terminal fragments of AtTERT (AtTERT 1−233 and AtTERT 1−271) and AtRuvBL1 located in the nucleolus. AtGAUT10, negative control; RFP, nucleus marker; YFP, detects PPI; Chl, Chloroplast autofluorescence. Scale bars = 10 μm.

(c) Y2H system fails to detect the interactions between AtRuvBL1 protein and N-terminal fragments of AtTERT (AtTERT 1−233 and AtTERT 1−271). BD, GAL4 DNA-binding domain; AD, GAL4 activation domain.

(d) Co-IP analysis does not detect interactions between AtTERT fragments and AtRuvBL1 protein which were demonstrated by BiFC. I, Input; U, Unbound; B, Bound fractions; asterisks*, ^{35}S-labelling.

AtRuvBL proteins. These results showed that RuvBL1 and RuvBL2a proteins from *A. thaliana* are able to form both homo- and heteromers, as well as their homologues in diverse organisms, although they preferably form heteromers.

AtRuvBL1 and AtTERT colocalize in the nucleus but contrary to mammalian homologues do not interact directly

Human RuvBL proteins are involved in the biogenesis and maturation of human telomerase complex. Human hRuvBL1 directly interacts with hTERT catalytic subunit. hRuvBL2 does not exhibit direct interaction with hTERT and seems to be recruited to an hTERT complex through bridging hRuvBL1 molecules (Venteicher *et al.*, 2008). To gain a deeper insight whether direct RuvBL-TERT interaction is conserved throughout the higher eukaryotes, we applied the above described Y2H, Co-IP and BiFC techniques. As TERT is a high-molecular-weight protein (approximately 130 kDa), we used the Gateway-compatible donor vectors carrying the AtTERT fragments that were described in Lee *et al.* (2012) and Zachová *et al.* (2013) (Figure 2a). We observed a clear nuclear interaction between AtRuvBL1 protein and AtTERT N-terminal fragments covering AtTERT domains localized in positions 1-233 and 1-271 in the *A. thaliana* leaf protoplasts using BiFC (Figure 2b). These results supported the observation from tobacco BY2 culture protoplasts where N-terminal fragments of AtTERT interact with AtRuvBL1 (Majerská *et al.*, 2017). As the central reverse transcriptase (RT) domain of hTERT is implicated in hRuvBL1 binding (Venteicher *et al.*, 2008), we expanded our interest to the other AtTERT fragments. However, no interactions were detected between AtRuvBL1 protein and AtTERT fragments localized in positions 229–582, 597–987 and 972–1123, therefore covering RT or C-terminal domains of AtTERT. Likewise, no interaction was observed between any of AtTERT fragments and AtRuvBL2a protein (Figure S1).

Notably, interactions of the N-terminal fragments between AtTERT domains and AtRuvBL1 were not confirmed in Y2H or Co-IP (Figure 2c, d). This discrepancy can be caused by the fact that the BiFC analysis detects the presence of proteins within the same macromolecular complex even in the absence of a direct contact between the proteins fused to the cYFP and nYFP fragments. The presence of proteins within the visualized macromolecular complex generally indicates that they participate in the same biological process (Kerppola, 2009). Our data show the interaction between AtRuvBL1 and AtTERT is localized in the nucleus and supports the suggestion of Majerská *et al.*, that AtRuvBL1-AtTERT interaction is mediated by an unknown partner and occurs in plant cells but not in RRL lysate or yeast system.

AtRuvBL proteins physically interact with AtTRB proteins

Previously, we have described that members of plant-specific group of AtTRB proteins physically interact with the N-terminal part of AtTERT and colocalized with telomeres *in situ* (Schrumpfová *et al.*, 2004, 2014; Mozgová *et al.*, 2008; Dreissig *et al.*, 2017; Zhou *et al.*, 2018). AtTRB1 interaction with double-stranded telomeric DNA is mediated by the Myb-like domain, while the H1/5 domain is involved in DNA sequence-non-specific DNA-protein interactions, interaction with AtPOT1b (Schrumpfová *et al.*, 2008) and in the multimerization of AtTRB1 (Mozgová *et al.*, 2008) (Figure 3a).

According to these findings, AtTRB proteins might be components of a putative shelterin-like complex in plants that modulates access of the telomerase to telomeres (Schrumpfová *et al.*, 2016a, 2019). Our BiFC analysis revealed the AtTRB3 protein interaction with both AtRuvBL1 and AtRuvBL2a proteins in the nucleus (Figure 3b). These interactions were confirmed by Y2H (Figure 3c) and also by Co-IP (Figure 3d), in which AtTRB3

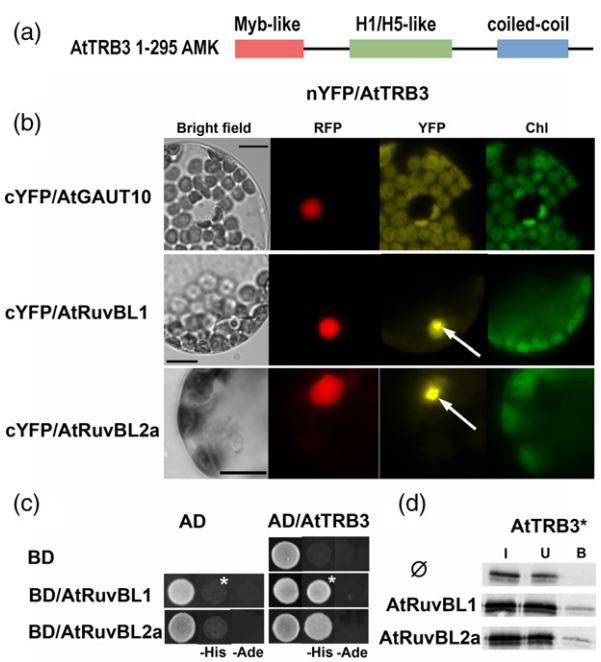

Figure 3. AtTRB3 proteins directly interact with AtRuvBL1 and AtRuvBL2a proteins. The methods are performed as is described in Figure 1.
(a) Schematic representation of the conserved motifs of the AtTRB3 protein from *Arabidopsis thaliana*. Myb-like, Telobox-containing Myb domain; H1/H5, histone-like domain; coiled-coil, C-terminal domain.
(b) BiFC shows interaction between AtTRB3 and both AtRuvBL proteins. PPIs marked by white arrows are localized in the nucleus. AtGAUT10, negative control; RFP, nucleus marker; YFP, detects PPI; Chl, Chloroplast autofluorescence. Scale bars = 10 μm.
(c) Y2H results show interactions between AtTRB3 and both AtRuvBL proteins on His- deficient plates. BD, GAL4 DNA-binding domain; AD, GAL4 activation domain; asterisks*, 5 mM 3-aminotriazol.
(d) Co-IP results confirm direct interactions between radioactively labelled AtTRB3 and Myc-tagged AtRuvBL proteins. I, Input; U, Unbound; B, Bound fractions; asterisks*, 35S-labelling.

protein was radioactively labelled by ^{35}S-methionine and mixed with its putative protein partners AtRuvBL1 or AtRuvBL2a fused with Myc-tag, and incubated with anti-Myc antibody. Clear nuclear interaction of AtTRB2 and AtRuvBL2a, but not of AtTRB2 with AtRuvBL1, was detected by BiFC and verified by Y2H and Co-IP. However, the nuclear interaction of AtTRB1 with AtRuvBL1 observed in BiFC seems to be indirect, as it was not proven by Y2H or Co-IP assays, but indicates that both proteins are present in the same macromolecular complex (Figure S2). Collectively, direct interactions of AtTRBs with AtTERT, as well as with AtRuvBL proteins, imply the role of AtTRB proteins as mediators of the interactions between AtRuvBL proteins and AtTERT telomerase subunit *in vivo*.

AtTRB3 protein mediates interaction between AtRuvBL1 and AtTERT

Our data, showing the indirect interaction between the N-terminal part of AtTERT and AtRuvBL1, suggested that this interaction could be mediated by AtTRB3 protein. We performed Co-IP assay with all three proteins of interest (Figure 4). Two prey proteins AtRuvBL1 and AtTRB3, were labelled with ^{35}S-methionine during the expression in TNT–RRL system. N-terminal fragment of AtTERT (AtTERT 1-271), fused with Myc-tag as an anchor, was expressed in TNT–RRL system in non-radioactive form ensuring a better resolution of the prey proteins in the 12% SDS-PAGE separation. Radioactively labelled AtTERT fragment was expressed in parallel tube to affirm the proper AtTERT 1-271 expression. The complex was captured with anti-Myc-antibody and protein G-coupled magnetic beads. Several negative controls were performed, where some of the monitored proteins were not present, to ensure specificity of the AtRuvBL1–AtTRB3–AtTERT complex. From these negative controls, it is evident that AtRuvBL1 protein neither directly interacts with the AtTERT 1–271 fragment nor is non-specifically bound to the magnetic beads. Conversely, the presence of AtTRB3 in immunoprecipitation mixture resulted in reproducible and significant increase of the AtRuvBL1 in the immunoprecipitated complex. So, it is evident that AtRuvBL1 is recruited to the AtTERT complex through an interaction with AtTRB3 protein, which mediates interaction of both proteins, AtTERT and AtRuvBL1.

Plant homologue of mammalian dyskerin AtCBF5 associates with AtTRB proteins in the plant nucleus

Mammalian protein dyskerin is a core component of mature and functional telomerase complex (He *et al.*, 2002; Schmidt and Cech, 2015; MacNeil *et al.*, 2016). Dyskerin binds the H/ACA box of small nuclear and nucleolar RNAs (sn- and sno-RNAs) and belongs to conserved scaffold proteins of human hTR (MacNeil *et al.*, 2016). Plant homologue AtCBF5 (also named AtNAP57) is localized within nucleoli and CBs (Lermontova *et al.*, 2007) and associates with enzymatically active telomerase RNP particles in an RNA-dependent manner (Kannan *et al.*, 2008).

Here we observed a clear indirect interaction of AtCBF5, fused with cYFP, with all three examined nYFP/AtTRB proteins using BiFC technique (Figure 5). As has already been discussed above, BiFC analysis can detect the presence of proteins within the same macromolecular complex even without a direct contact between the proteins fused with cYFP/nYFP (Kerppola, 2009). We assume that the interactions between AtCBF5 and AtTRBs are indirect because we

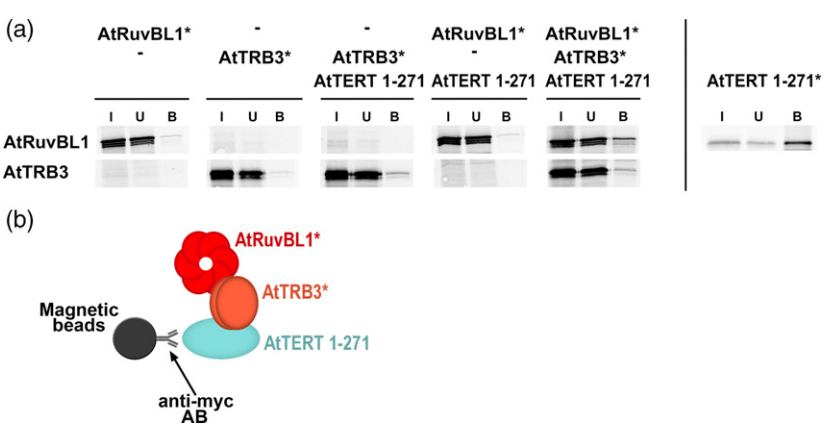

Figure 4. AtTRB3 protein is mediator of AtRuvBL1 and AtTERT interaction.
(a) Co-Immunoprecipitation of the three proteins of interest. Two proteins AtRuvBL1 and AtTRB3 are radioactively labelled by ^{35}S-methionine (marked with asterisks) during the expression in TNT-RRL lysate and subsequently incubated with non-radioactive Myc-tagged AtTERT 1–271 fragment and anti-Myc antibody. In the control experiments, the proteins are incubated with Myc-antibody and protein G-coupled magnetic beads in the absence of one or both partner proteins. Radioactively labeled AtTERT fragment is expressed in parallel tube as a control of the expression. From penult column it is evident that the presence of AtTRB3 results in significant increase of the AtRuvBL1 in the immunoprecipitated complex. I, Input; U, Unbound; B, Bound fractions were collected and run in 12% SDS-PAGE gels.
(b) Schematic depiction of the putative protein complex formed by proteins AtRuvBL1, AtTRB3 and AtTERT. AtRuvBL1 is depicted in its presumed hexameric form and AtTRB3 in its dimeric form.

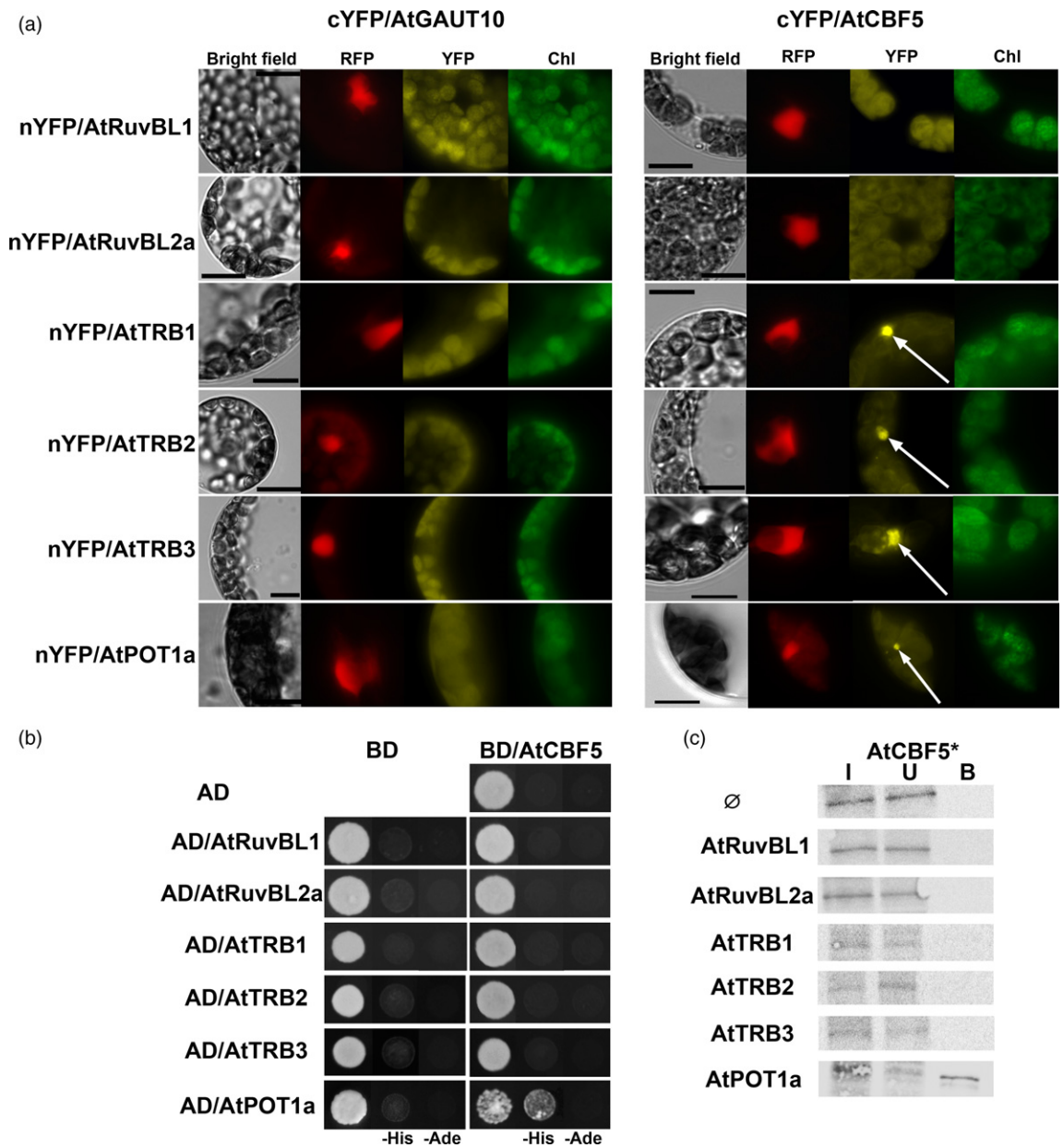

Figure 5. AtCBF5 associates with AtTRB proteins indirectly. The methods are done in the same manner as in Figure 1.
(a) BiFC assay shows indirect interaction between AtCBF5 and three proteins from AtTRB family (AtTRB1-3). AtCBF5 interacts also with AtPOT1a protein. PPIs are marked by white arrows. AtGAUT10, negative control; RFP, nucleus marker; YFP, detects PPI; Chl, Chloroplast autofluorescence. Scale bars represent 10 μm.
(b) Y2H assay analysis does not detect the interaction between AtCBF5 and AtTRB proteins which was found by BiFC. AtCBF5 protein interacts only with AtPOT1a on histidine deficient plate (-His). BD, GAL4 DNA-binding domain; AD, GAL4 activation domain.
(c) Co-IP analysis shows interaction only between AtCBF5 and AtPOT1a protein, fused with Myc-tag and incubated with Myc-antibody and protein G-coupled magnetic beads. I, Input; U, Unbound; B, Bound fractions; asterisks*, ^{35}S-labelling.

were not able to confirm the AtCBF5–AtTRBs interactions observed by BiFC in Y2H mating assay. Also Co-IP did not reveal any iteraction between proteins expressed in TNT–RRL system, fused with Myc-tag (AtRuvBL1, AtRuvBL2a, AtTRB1, AtTRB2 and AtTRB3) and with radioactively labelled AtCBF5 as a prey. Additionally, no interaction was detected between AtCBF5 and any of AtRuvBL proteins neither in BiFC nor in Y2H or Co-IP. As a positive control we used the interaction between AtCBF5 and AtPOT1a. Here we show that the AtCBF5 interacts with AtPOT1a not only in Y2H and Co-IP, as was shown in Kannan *et al.* (2008), but also in the plant nucleus using BiFC assay. In addition to the nucleolar localization of AtPOT1a–AtCBF5 interactions, we also observed this interaction in several

nuclear and cytoplasmic foci (Figure S3). Further, we observed a weak interaction between AtPOT1a–AtRuvBL1 proteins in Y2H and Co-IP assays but not in BiFC system (Figure S4). As a negative control in BiFC assay, we co-transfected protoplasts with cYFP/AtGAUT10. AtGAUT10 protein did not interact with any of the proteins of interest fused with nYFP: AtRuvBL1, AtRuvBL2a, AtTRB1, AtTRB2, AtTRB3 or AtPOT1a. Co-transformation with an empty vector (AD, BD) served as a negative control in Y2H experiments. In Co-IP experiment, the AtCBF5 proteins were incubated with Myc-antibody and protein G-coupled magnetic beads in the absence of partner protein as negative control. Together, we conclude that AtTRB proteins are associated in very close proximity with AtCBF5, the plant homologue of mammalian dyskerin, in the plant nucleus. However, at the same time, AtCBF5 is not localized in the nearby complex with the AtRuvBL proteins *in vivo*.

Association of AtRuvBLs, AtTRBs and AtTERT indicates the formation of their complex in the nucleolus

During the assembly of a fully functional complex of the human telomerase, the mature hTR gets recruited to the nucleolus where it binds the hTERT complex. Both of the core telomerase components, hTR and also hTERT, are previously processed by several proteins, including hRuvBL1 and hRuvBL2. It has already been published that in the interphase, the AtTRB proteins showed preferential localization to the nucleus and specially to the nucleolus (Dvořáčková *et al.*, 2010). In comparison with the mammalian nucleoli, plant nucleoli are larger, more frequently undergo fusions, and sometimes have a central clear region, often called the nucleolar vacuole, the size of which depends on nucleoli transcriptional activity (Shaw and Brown, 2012; Stepinski, 2014).

We analyzed the subcellular localizations of the interactions of our proteins of interest: AtTERT 1-271, AtRuvBL1, AtRuvBL2a, AtTRB3 and AtCBF5 fused with nYFP- or cYFP-tag in routinely performed BiFC experiments. The nucleoli were marked by control plasmid mRFP–AtFibrillarin 1 (Pih *et al.*, 2000). Figure 6 shows interactions between AtRuvBL1–AtTERT, AtTRB3–AtTERT, AtRuvBL1–AtTRB3 and AtRuvBL2a–AtTRB3, which occupy distinct areas within the plant nucleus that match to the plant nucleolus. The number of the PPIs foci localized exclusively in the nucleolus is listed in the Table S1. Similar patterns of nuclear or nucleolar PPI localization is visible also in Figure S5 where the whole nucleus was marked by mRFP–VirD2NLS. However, the AtCBF5–AtTRB3 interaction showed different localization pattern than the other examined PPIs. AtCBF5–AtTRB3 interaction seems to be localized in nucleoli and sometimes in additional nuclear bodies at the periphery or outside the nucleoli, which is consistent with localization of free AtCBF5 (Lermontova *et al.*, 2007). Together, our data indicate formation of AtRuvBLs–AtTRBs–AtTERT complex in the nucleolus.

Dysfunction of *AtRuvBL* genes reduces telomerase activity

In human cells, the hRuvBL1 and hRuvBL2 proteins associate with a significant population of hTERT molecules that do not yield high-level telomerase activity, measured by Telomere Repeat Amplification Protocol (TRAP). The depletion of hRuvBL1 and hRuvBL2 markedly impaired telomerase RNP accumulation and diminished human telomerase activity (Venteicher *et al.*, 2008). To assess whether mutations in *AtRuvBL* genes have any impact on telomerase activity in *A. thaliana*, we set to perform TRAP assay on telomerase extracts isolated from T-DNA insertion mutant lines. Extensive search of several T-DNA

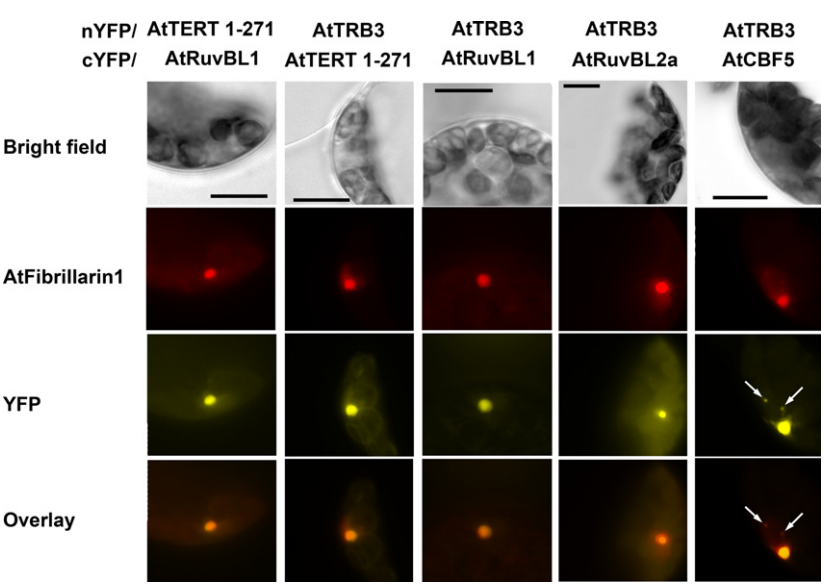

Figure 6. Association of AtRuvBLs, AtTRBs and AtTERT in the nucleolus in *A. thaliana* leaf protoplasts. Protoplasts are co-transfected with mRFP–AtFibrillarin 1 encoding RFP that labels nucleolus and simultaneously with each of the plasmids encoding nYFP-tagged or cYFP-tagged AtRuvBL1, AtRuvBL2a, AtTERT 1–271, AtTRB3 or AtCBF5 to determine PPI localization. AtRuvBL1–AtTERT, AtTRB3–AtTERT, AtRuvBL1–AtTRB3 or AtRuvBL2a–AtTRB3 interactions show nucleolar localization. Plant homologue of mammalian dyskerin, AtCBF5, is associated with AtTRB3 in the nucleolus and in additional nuclear bodies at the periphery of the nucleolus. RFP, marked nucleus; YFP, detects PPI; Scale bars = 10 μm.

insertion lines, which are available from several plant databases, revealed only two suitable plant lines with a limited number of heterozygous mutant plants but with no homozygous mutant plants: SAIL_397_C11 (*AtRuvBL1*) and GK-543F01 (*AtRuvBL2a*). In an additional seven tested T-DNA insertion plant lines we did not detect any viable mutant or heterozygous plants for *AtRuvBL1* or *AtRuvBL2a* genes (Table S2). Furthermore, genotypic ratios of offspring of individual heterozygous plants did not follow the expected Mendelian genotypic ratio. The observed ratio for *AtRuvBL1*$^{+/-}$ and for *AtRuvBL2a*$^{+/-}$ plants was (51:21:0) and (91:10:0), respectively, instead of (1:2:1) (Figure 7a) and the cause of this phenomenon will be further investigated.

Quantitative TRAP assay performed with telomerase extract isolated from flower buds of individual *AtRuvBL2a* heterozygous plants demonstrated that relative telomerase activity showed apparent reduction in comparison with telomerase extract from wild-type *A. thaliana* (Col-0$^{+/+}$) buds (Figure 7b). T-DNA insertion mutation in *AtRuvBL1* gene was lethal (Holt *et al.*, 2002) but we detected viable heterozygous *AtRuvBL1* plants. These plants showed a milder reduction of telomerase activity than *AtRuvBL2a*$^{+/-}$ plants, which supports the assumption that AtRuvBL1 protein is essential for meristem development (Holt *et al.*, 2002).

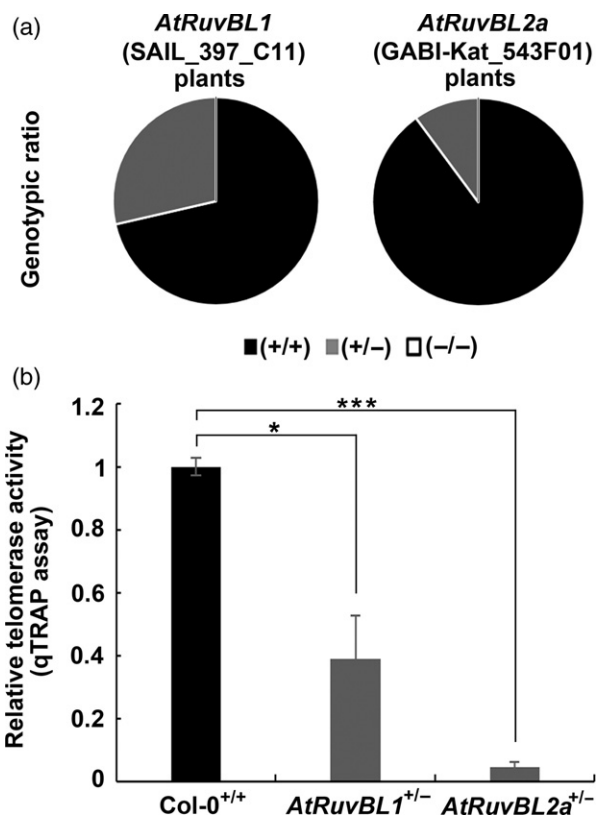

Figure 7. Reduction of relative telomerase activity in heterozygous *AtRuvBL* mutant plants.

(a) Genotypic ratio of the offspring of heterozygous *AtRuvBL1* and *AtRuvBL2a* T-DNA insertion mutant plants. Homozygous mutant plants in *AtRuvBL* genes are fully absent and even the number of heterozygous plants does not follow the Mendelian genotypic ratio.

(b) Samples isolated from *AtRuvBL1*$^{+/-}$ and *AtRuvBL2a*$^{+/-}$ buds are analyzed in three technical replicates by quantitative TRAP. Data are related to wild-type Col-0 sample (telomerase activity in Col-0 buds are arbitrarily chosen as 1). Relative telomerase activity is reduced in both *AtRuvBL1*$^{+/-}$ and especially in *AtRuvBL2a*$^{+/-}$ samples. $P < 0.05$ are considered as significant. Single asterisk denotes $0.01 < P < 0.05$. Three asterisks denote $0.01 < P < 0.001$.

Human RuvBL proteins are direct interactors of transcription factor MYC that is required for expressing many genes involved in cell-cycle transition events and proliferation (Wood *et al.*, 2000). hRuvBL2 regulates MYC-dependent transcription of *TERT* via targeting the hTERT promoter (Wood *et al.*, 2000; Li *et al.*, 2010; Flavin *et al.*, 2011; Zhao *et al.*, 2014). We analyzed the levels of *AtTERT* transcripts in *AtRuvBL1* and *AtRuvBL2a* heterozygous plants to detect whether the decrease of telomerase activity was caused by the negative regulation of AtTERT promoter i.e. the decrease of the abundance of *AtTERT* transcripts. We did not observe significant changes in transcripts of *AtTERT* gene in *AtRuvBL1* heterozygous mutant plants compared with the wild-type *A. thaliana*. Instead, we observed very slight, though significant, increase in *AtTERT* transcripts in *AtRuvBL2a* heterozygous mutant plant lines (Figure S6).

Due to the difficulties in maintaining the heterozygous *AtRuvBL* plant lines for several subsequent generations, we were not able to analyze the transgenerational effects of reduced telomerase activity on telomere lengths in plants heterozygous in *AtRuvBL1* and *AtRuvBL2a* genes. However, in the analyzed generation of *AtRuvBL1*$^{+/-}$ and *AtRuvBL2a*$^{+/-}$ plants that were descendants of heterozygous predecessors, we did not detect any significant changes in telomere lengths compared with the wild-type plants using Terminal Restriction Fragment analysis (TRF) (Figure S7).

Together, we conclude that the depletion of AtRuvBL1 and especially of AtRuvBL2a proteins reduces telomerase activity which suggests a conserved role of AtRuvBL proteins in maturation of functional telomerase complex across the mammals and also plants.

Identification and phylogenetic analysis of the RuvBL family in plants

RuvBL proteins, showing association with TERT in human cells, represent a group of proteins well conserved across all eukaryotic kingdoms, including Fungi, Animalia or Plantae.

Here, we present a genome-wide analysis of RuvBL proteins in 80 vascular plant species, one gymnosperm and two bryophytes, totally 83 taxa, that were analyzed for the presence of all three basic domains (DI, DII, DIII). The evolutionary relationships among the RuvBL proteins were

determined using maximum likelihood analyses based on multiple alignments producing a phylogenetic tree depicting the relationships among all currently accessible RuvBL sequences. The evolutionary hypotheses from these analyses were highly congruent. RuvBL protein family was divided in two distinct groups based on the similarity of sequences and branch length. Sequence similarity between RuvBL1 and RuvBL2 is generally low, about 35–40% while the sequence similarity within RuvBL subfamilies is about 80%. For instance, in *A. thaliana* AtRuvBL2a and AtRuvBL2b share 82% similarity. On the other hand, AtRuvBL1 with AtRuvBL2a or AtRuvBL1 with AtRuvBL2b share 37.5 and 38.8% similarity, respectively. However, only a subset of RuvBL1 was clearly separated (100% BS; blue branch in Figure 8). Surprisingly, based on BLAST search, RuvBL1 was found only in dicots and basal angiosperms (*Amborella trichopoda*) up to now, RuvBL2 was represented in both, dicots and monocots from angiosperms, but also in gymnosperms (*Picea sitchensis*) and bryophytes (*Physcomitrella patens* and *Marchantia polymorpha*). The number of the homologues varied from 1 to 8 (Data S1 and S2).

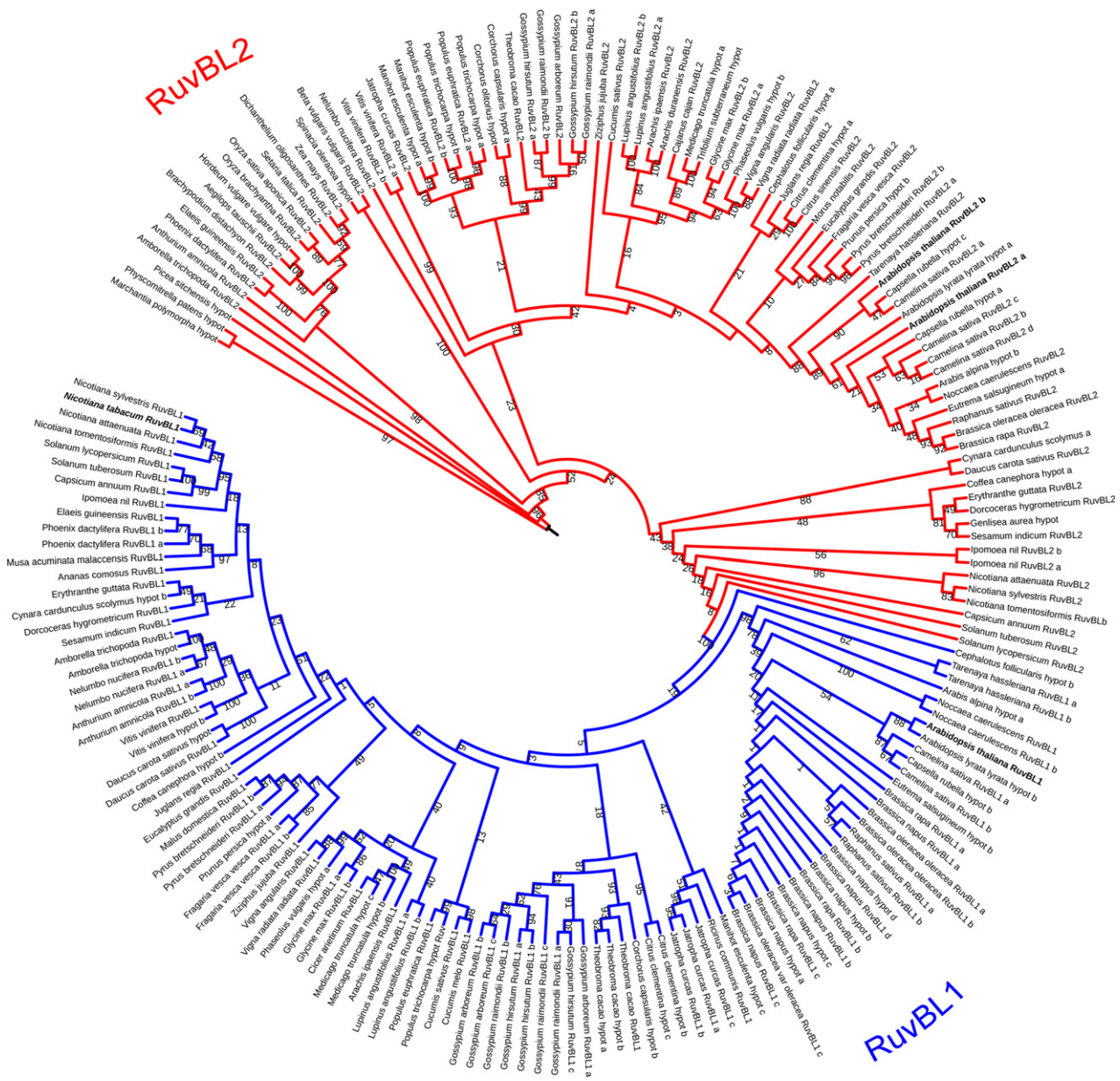

Figure 8. Phylogenetic analysis of the RuvBL family in plants.
Unrooted phylogenetic tree of 190 proteins sequences of RuvBL family with enumerated plant species. Numbers above branches means bootstrap support values. Orthologues from *Arabidopsis thaliana* and *Nicotiana tabacum* are in bold letters.

DISCUSSION

The formation of functional and enzymatically active telomerase, a multisubunit RNP complex, is a dynamic process governed by number of cofactors. In mammals, hRuvBL1 and hRuvBL2 proteins, Pontin and Reptin, respectively, are present in early steps of telomerase RNP biogenesis. We characterized plant homologues of RuvBL proteins: AtRuvBL1 and AtRuvBL2a, previously co-purified together with telomerase protein subunit AtTERT from *A. thaliana* suspension cultures (Majerská *et al.*, 2017). Here we show that AtRuvBL1 protein colocalizes with N-terminal part of AtTERT subunit of plant telomerase also *in vivo*. However, in contrast with the AtRuvBL mammalian counterparts, their interaction in plants seems to be indirect. Association of AtRuvBL proteins with AtTERT in the plant nucleolus appears to be bridged by telomeric AtTRB proteins. Requirement of AtRuvBL proteins for a proper telomerase assembly is endorsed by the fact that depletion of AtRuvBL1 and especially of AtRuvBL2a protein, reduces telomerase activity in plants heterozygous for *AtRuvBL1* or *AtRuvBL2a* genes. Moreover, AtTRB proteins are associated in the plant cell with a homologue of mammalian dyskerin, AtCBF5, that plays a role in telomerase RNP biogenesis and directly interacts with AtPOT1a protein. AtTRB proteins thus play a role of interaction hubs not only in telomere chromatin structure but also in telomerase biogenesis. AtRuvBL proteins are able to multimerize, which is analogous to the situation in mammalian cells, and our data show preference to form mutual heteromers. Detailed summary of protein–protein interactions between AtRuvBLs, AtTRBs, AtTERT fragments, AtPOT1s and AtCBF5 proteins, that have been detected using BiFC, Y2H or Co-IP assays in this and other relevant publications, are given in the Table 1.

Our detailed phylogeny proved that RuvBL proteins are evolutionarily conserved in land plants and implied plausible functional conservation of the RuvBL proteins. However, further biochemical validation of the possible conservation of mutual RuvBL–TRB interaction across the plant kingdom can be limited by the fact that the number of paralogues varies from 1 to 8 members in between RuvBL proteins. The multiplication of genes of the same family is not surprising as, in many plant families, the polyploidy (i.e. whole-genome duplication, WGD), resulting in retention of multiple gene paralogs may lead to their sub-functionalization, neo-functionalization or partial or full redundancy (Mandakova and Lysak, 2008; Freeling, 2009). These limitations might be deteriorated by the fact that the AtRuvBL proteins can be involved in a similar biochemical pathway but their interaction partners might slightly differ (this paper; Venteicher *et al.*, 2008).

RuvBL proteins are involved in various cellular processes

The exact function even of mammalian RuvBL proteins is still quite unknown as they interact with many molecular complexes with vastly different downstream effectors (Mao and Houry, 2017). Among others, hRuvBL2 was shown to regulate hTERT promoter likely through the regulation of MYC (c-myc), the transcription factor for *TERT* (Wood *et al.*, 2000; Li *et al.*, 2010; Flavin *et al.*, 2011; Zhao *et al.*, 2014). We observed no significant changes in transcripts of *AtTERT* gene in *AtRuvBL1* heterozygous mutant plants, however we detected a very slight increase in transcripts of *AtTERT* gene in *AtRuvBL2a* heterozygous plants. Although the transcript levels of *AtTERT* gene were slightly increased in *AtRuvBL2a* heterozygous plant lines, we observed a very significant reduction of telomerase activity

Table 1 A summary table of protein–protein interactions

	AtRuvBL1	AtRuvBL2	AtTRB1	AtTRB2	AtTRB3	AtTERT (1-233)	AtTERT (1-271)	AtTERT (1-582)	AtTERT (229-582)	AtTERT (597-987)	AtTERT (958-1123)	AtCBF5
AtRuvBL1	●●●$^{\alpha}$											
AtRuvBL2a	●●●$^{\alpha\eta}$	●●●$^{\alpha}$										
AtTRB1	●xx$^{\alpha}$	xxx$^{\alpha}$	-●●$^{\beta}$									
AtTRB2	xxx$^{\alpha}$	●●●$^{\alpha}$	-●●$^{\beta}$	–●–$^{\delta}$								
AtTRB3	●●●$^{\alpha}$	●●●$^{\alpha}$	-●●$^{\beta}$	–●–$^{\delta}$	–●–$^{\delta}$							
AtTERT (1-233)	●xx$^{\alpha\eta}$	xxx$^{\alpha}$	-x-$^{\omega}$	-x-$^{\omega}$	-x-$^{\omega}$	–x–$^{\eta}$						
AtTERT (1-271)	●xx$^{\alpha\eta}$	xxx$^{\alpha}$	●●●$^{\gamma}$	●●●$^{\gamma}$	●●●$^{\gamma}$	–x–$^{\eta}$	–●–$^{\eta}$					
AtTERT (1-582)	---	---	-●●$^{\gamma}$	-●●$^{\gamma}$	-●●$^{\gamma}$	–x–$^{\eta}$	–●–$^{\eta}$	–x–$^{\eta}$				
AtTERT (229-582)	xxx$^{\alpha}$	xxx$^{\alpha}$	●x●$^{\gamma\omega}$	●x●$^{\gamma\omega}$	●x●$^{\gamma\omega}$	–x–$^{\eta}$	–●–$^{\eta}$	–●–$^{\eta}$	–●–$^{\eta}$			
AtTERT (597-987)	xxx$^{\alpha}$	xxx$^{\alpha}$	-x-$^{\omega}$	-x-$^{\omega}$	-x-$^{\omega}$	–x–$^{\eta}$	–x–$^{\eta}$	–x–$^{\eta}$	–x–$^{\eta}$	–x–$^{\eta}$		
AtTERT (958-1123)	xxx$^{\alpha}$	xxx$^{\alpha}$	---	---	---	–x–$^{\omega}$	–x–$^{\omega}$	–x–$^{\omega}$	–x–$^{\omega}$	–x–$^{\omega}$	---	
AtCBF5	xxx$^{\alpha}$	xxx$^{\alpha}$	●xx$^{\alpha}$	●xx$^{\alpha}$	●xx$^{\alpha}$	---	---	---	---	---	---	---
AtPot1a	x●●$^{\omega}$	xxx$^{\omega}$	xx-$^{\omega}$	xx-$^{\omega}$	xx-$^{\omega}$	-●●$^{\zeta}$	●●●$^{\eta\zeta}$	–x–$^{\zeta}$	–x–$^{\zeta}$	–x–$^{\zeta}$	–x–$^{\zeta}$	●●●$^{\alpha\varepsilon}$
AtPot1b	xxx$^{\omega}$	xxx$^{\omega}$	-●●$^{\beta}$	-●-$^{\beta}$	-●-$^{\beta}$	–x–$^{\zeta}$	–x–$^{\zeta}$	---	---	---	---	xxx$^{\omega}$

●●●	BiFC, Y2H, Co-IP
●	INTERACTION
x	NO interaction
–	n.a. = not analysed
●	this publication
●	other relevant publications
α	this publication
β	Schrumpfova et al., 2008
γ	Schrumpfova et al., 2014
δ	Schrumpfova et al., 2004
ε	Kannan et al., 2008
ζ	Rossignol et al., 2007 (TERT fragments differ)
η	Majerská et al., 2017
ω	data not shown

Summary table shows all interactions between AtRuvBLs, AtTRBs, AtTERT fragments, AtPOT1s and AtCBF5 proteins that are detected using BiFC, Y2H or Co-IP assays.

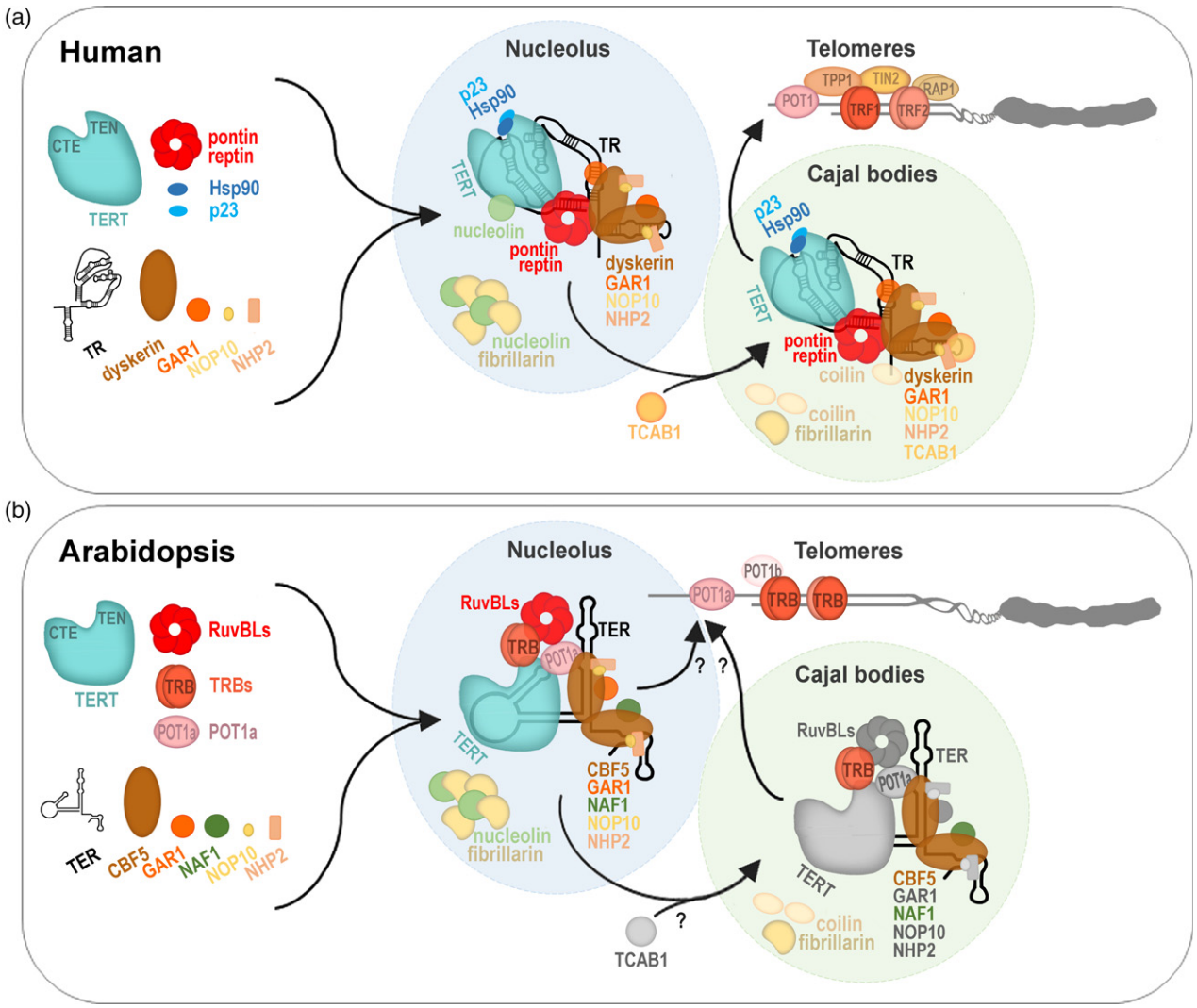

Figure 9. Comparative model of telomerase assembly in human and *Arabidopsis*.
(a) Human TR binds dyskerin, NHP2, NOP10, and GAR1 and human TERT associates with the chaperones Hsp90 and p23. The telomerase RNP is retained into the nucleoli through the interaction between TERT and nucleolin. Assembly of TR and TERT into catalytically active telomerase is aided by Pontin (hRuvBL1) and Reptin (hRuvBL2) AAA+ ATPases. Telomerase is recruited to Cajal bodies (CBs) by its interaction with TCAB1. The CBs will colocalize with telomeres, and telomerase is recruited to telomeres by the interaction with the shelterin component TPP1 (MacNeil *et al.*, 2016; Lim *et al.*, 2017).
(b) Arabidopsis CBF5, GAR1, NOP10, NHP2, but in contrast with human cells also NAF1, were localized into the plant nucleolus (Pendle *et al.*, 2005; Lermontova *et al.*, 2007). In the plant nucleolus, we observe colocalization of TERT with RuvBL AAA+ ATPases complex bridged by telomeric TRB proteins, as well as the interaction of telomeric protein POT1a with CBF5. *Arabidopsis* telomeres cluster at the periphery of the nucleolus which is mediated by the presence of nucleolin. Recruitment of the mature telomerase complex to telomeres with or without commitment of CBs in *Arabidopsis* needs further investigation. Proteins that were already proven as associated with CBs are highlighted in color in Cajal bodies. Proteins that have not yet been experimentally proven as CBs associated are marked with black and white.

in these plants. Telomerase activity was reduced also in *AtRuvBL1* heterozygous T-DNA insertion plant lines. To verify whether the regulation of telomerase activity was affected due to the compromised assembly of telomerase complex rather than due to regulation of transcript levels of *AtTERT* gene in AtRuvBL-dependent manner, however, needs further investigation.

The participation of RuvBL proteins in heterogeneous cellular process as well as their association with specific interactors can vary between cytoplasm, nucleus and nucleolus (Mao and Houry, 2017). It seems that, also in *A. thaliana*, the function of AtRuvBL proteins is not specific only to the telomerase assembly, as they were suggested as regulators of disease resistance (R) genes (Holt *et al.*, 2002). It has already been published that AtRuvBL1 is essential in meristem development (Holt *et al.*, 2002), the function consistent with its function in telomerase assembly observed in this work. Our extensive, but unsuccessful,

effort to receive homozygous mutant plants in several T-DNA insertion lines mutant in the *AtRuvBL1* and *AtRuvBL2a* genes also indicated the essentiality of AtRuvBL proteins in various cellular processes in plants. Furthermore, genotypic ratio of offspring of individual heterozygous plants does not follow the Mendelian genotypic ratio, indicating that both AtRuvBL proteins are essential regulators of plant development. Therefore, we suggest to investigate the function of AtRuvBLs in plant sporophyte or female gametophyte development in future studies.

Nucleolus localization of telomerase assembly complex

Telomere maintenance requires a proper assembly of the TERT and TR components of telomerase into RNP as well as a number of cofactors involved in maturation, stability and subcellular localization of telomerase. In mammals, the association of hTR RNP with hTERT proceeds in the nucleolus during the early S-phase (Lee *et al.*, 2014). Assembled and catalytically active telomerase RNP separates from the nucleoli and is transported to CBs during the S-phase for subsequent recruitment to telomeric chromatin and telomere extension (Figure 9a) (MacNeil *et al.*, 2016). Association of hTERT with human RuvBL proteins, Pontin and Reptin, peaks in S-phase, which may reflect cell-cycle regulation of total TERT and/or assembly of telomerase on telomeres (Venteicher *et al.*, 2008). RuvBL1 and RuvBL2a proteins, together with, for example, Fibrillarin 1 and many other proteins, were purified and identified in nucleoli isolated from *A. thaliana* cell culture protoplasts (Pendle *et al.*, 2005). Our data indicated that plant homologues of human Pontin and Reptin, the AtRuvBL proteins, are associated in the plant nucleolus with AtTERT, together with AtTRB proteins (Figure 9b). AtTRB proteins are highly dynamic and during the interphase, they are preferentially localized to the nucleolus or nuclear bodies of different size (Dvořáčková, 2010). AtTRBs behave as typical nucleolar resident proteins, being largely dispersed at prophase, coinciding with nucleolar disassembly. However, a small but detectable amount of the protein remains associated with the chromatin throughout mitosis (Azum-Gelade *et al.*, 1994; Dvořáčková *et al.*, 2010). Similarly, to the AtTRB proteins, also the N-terminal part of AtTERT was detected in the nucleoli in *A. thaliana* (Rossignol *et al.*, 2007; Zachová *et al.*, 2013).

In mammals, the telomerase RNP is retained in nucleoli through the interaction between hTERT and nucleolin in the dense fibrillar component (Khurts *et al.*, 2004; Lee *et al.*, 2014). In *A. thaliana*, null mutants for the nucleolar protein NUCLEOLIN 1 cause telomere shortening on all chromosome arms (Pontvianne *et al.*, 2016). Telomeres in *A. thaliana* do not form a Rabl conformation, as in some other species, but telomeres and their flanking regions strongly associated with the nucleolus in a rosette-like organization (Armstrong *et al.*, 2001; Fransz *et al.*, 2002; Roberts *et al.*, 2009; Pontvianne *et al.*, 2016; Schrumpfová *et al.*, 2016a). Our data indicated the presence of AtTERT–AtTRB–AtRuvBL complex in the nucleolus. Nucleolar localization of the AtTERT–AtTRB–AtRuvBL complex together with the close proximity of telomeres to the nucleolus, suggested the conservation of the recruitment of the maturating telomerase to the nucleolus during the telomerase assembly. Figure 9 shows a comparative model of the assembly and localization of telomerase in mammalian and plant cells.

Plausible conservation of the telomerase trafficking pathway

Cajal bodies are spherical suborganelles localized in the nucleoplasm either in the vicinity of the nucleolus and/or they are present free. The function of CBs is not completely understood, but they were implicated mainly in snRNAs synthesis and processing. CBs also contribute to the biogenesis of telomerase. In S-phase, CBs colocalize with telomeres and facilitate recruitment of the mature mammalian telomerase complex to the telomeres. Human dyskerin, hNHP2, hNOP10 and hGAR1, that displaces hNAF1 in the hTR RNP, belong to conserved scaffold proteins, which colocalize with CBs and are involved in hTR RNP assembly (MacNeil *et al.*, 2016). Expression of putative *AtGAR1*, *AtNOP10*, *AtNHP2* genes encoding protein components of the H/ACA box snoRNP complex correlate with that of *AtCBF5*, a plant homologue of dyskerin (Lermontova *et al.*, 2007). AtCBF5 directly interacts with AtNAF1 (Lermontova *et al.*, 2007) and has been identified as a component of the enzymatically active *A. thaliana* telomerase RNP (Kannan *et al.*, 2008). AtCBF5 localizes in nucleoli and sometimes in additional nuclear bodies at the periphery or outside the nucleoli, but AtCBF5 also colocalizes with TMG-capped snRNA, a marker for CBs (Lermontova *et al.*, 2007).

Here we show that plant dyskerin, AtCBF5, indirectly interacts with AtTRB proteins in the plant nucleolus or in other nuclear bodies. It has already been published that AtTRBs are located not only in the nucleolus but also in nuclear bodies of different size, some of which might be CBs (visualized by a marker protein Coilin) (Dvořáčková, 2010). Dvořáčková detected significant colocalization of AtTRB1 with Coilin present in the CBs adjacent to the nucleolus. However, no colocalization was detected between signals corresponding to the AtTRB1 and free CBs in the nucleoplasm. Presence of AtTRB1 protein entirely in the CBs adjacent to the nucleoli implies a potential conservation of the trafficking pathway during the telomerase maturation, which comprises movement of maturating telomerase complex through nucleolus to CBs and finally to the telomeres. Notably, not all the organisms (e.g. budding yeast and ciliates) rely on the CBs trafficking since telomerase RNAs from these species do not have H/ACA or CAB box motifs, and further studies

are needed to prove this hypothesis. We observe that interaction between AtCBF5 and AtPOT1a is localized mostly in the nucleolus but in few cases also in cytoplasmic foci. The cytoplasmic localization is not surprising as it has already been shown that plant AtPOT1a and AtPOT1b, as well as their human homologue hPot1, are localized in the nucleus, as well in the cytoplasm (Chen *et al.*, 2007; Rossignol *et al.*, 2007).

The assembly of hTR RNP to the telomerase holoenzyme is not fully elucidated and it is highly complex multistep process. Therefore, the absence of the interaction between AtCBF5 and AtRuvBLs in the plant nucleus in our experiments is also not surprising. For example, Machado-Pinilla *et al.* (2012) showed that dyskerin was sandwiched between two hSHQ1 domains in the first steps of the biogenesis of telomerase. C-terminal tail of hCBF5 was essential for hSHQ1 release mediated by hRuvBLs. However, a stable interaction with the tails is not a part of the process because hRuvBLs bind to hCBF5 in a pull-down assay, even in the absence of its tail. Assembly of functional AtTER RNP, as well as the assembly of mammalian hTR RNP, is certainly a multistep process that may include AtTER, AtCBF5, AtTRBs, AtRuvBLs, AtPOT1a and many other factors, whose presence/participation/mutual interactions will be the subjects of our future research. Dynamics and complexity of mutual interactions can be demonstrated by the fact that we detect the interacting complex of AtCBF5–AtPOT1a in the nucleolus or in the cytoplasmic and nuclear foci using BiFC assay, while AtCBF5–AtTRBs interactions are localized entirely to the nucleoli and additional nuclear bodies. Moreover, association of AtTRB3 with AtTERT and AtRuvBLs is entirely localized to the nucleolus.

Concluding remarks

Homologues of the mammalian Pontin and Reptin, named RuvBL proteins, as well as TRB proteins, might be involved in diverse processes in the plant cell. AtTRB proteins are not only components of terminal complex associated with telomeres and catalytic subunit of telomerase, AtTERT (Schrumpfová *et al.*, 2016a, 2019), but they also serve as epigenetic regulators that potentially impact the transcription status of thousands of genes as subunits of epigenetically active multiprotein complexes (Lee and Cho, 2016; Schrumpfová *et al.*, 2016b; Zhou *et al.*, 2016; Dokládal *et al.*, 2018; Tan *et al.*, 2018). AtRuvBL1 protein has been assumed as a regulator of R genes so far and is essential in meristem development (Holt *et al.*, 2002). Here we suggest involvement of AtRuvBL proteins in telomerase assembly pathway in *A. thaliana*. We detected new interactions of AtTRB proteins with AtRuvBL proteins, localized the AtTERT–AtTRB–AtRuvBL complex exclusively in the nucleolus and observed that heterozygous T-DNA insertion mutants in *AtRuvBL1* or *AtRuvBL2a* genes showed reduced telomerase activity. Further, our results showed interactions of AtCBF5, the plant orthologue of dyskerin, with AtTRB and AtPOT1, but not with the AtRuvBL proteins, which expanded our knowledge on the telomerase assembly process. Indispensability of the AtRuvBL proteins for the plant development was supported by our finding that homozygous *atruvbl1* and *atruvbl2a* mutant plants were not viable. Furthermore, we identified new homologues RuvBL proteins and analyzed their evolutionary relationships in plants. Altogether, our data show that the plant homologues of Pontin and Reptin, AtRuvBLs, and also AtTRB are involved in telomerase assembly and suggest conservation of telomerase trafficking pathway via the nucleolus to the telomeres in plants.

EXPERIMENTAL PROCEDURES

Searching transcriptomes and genomes for RuvBL homologues

RuvBL homologues were identified by BLASTP searches using *A. thaliana* proteins from the TAIR database (https://www.arabidopsis.org/) to query NCBI protein databases (http://www.ncbi.nlm.nih.gov/). The BLASTP searches used default parameters, adjusted to the lowest *E*-value. The duplicates from all searches were eliminated. We conducted an iterative search of the UniProt database (http://www.uniprot.org/) and the Phytozome version 11 database (https://phytozome.jgi.doe.gov) was next searched for proteins not found by BLASTP. We analyzed all sequences independently of their annotations, with no prior assumptions. Information summary of accession numbers for RuvBL are in Data S1 and S2.

Sequence alignment

Amino acid sequences were aligned using the Clustal Omega algorithm (Sievers *et al.*, 2011) in the Mobyle platform (Neron *et al.*, 2009), with homology detection by HMM-HMM comparisons (Soding, 2005). Protein isoforms with the same length were also used, because the differential expression patterns producing protein isoforms from various tissues suggested that isoforms could have different biological functions *in vivo* (Chen *et al.*, 2014).

Phylogenetic reconstruction

Maximum likelihood (ML) analyses of the matrices were performed in RAxML 8.2.4 (Stamatakis, 2014) to examine differences in optimality between alternative topologies. Using the Akaike information criterion as implemented in Modeltest 3.8 (Posada and Crandall, 1998), a GTR+I+Γ model was chosen as the best-fitting model, and 1000 replications were run for bootstrap values. The final data set for RuvBL contained 190 proteins of different species and length 576 bp. Phylogenetic trees were constructed and modified with iTOL v3.4 (Letunic and Bork, 2016).

Transgenic constructs

The Gateway-compatible donor and destination vectors carrying the AtTERT (AtTERT 1-233, 1-271, 229-582, 597-987, 958-1123) fragments were described in Zachová *et al.* (2013). The Gateway-compatible donor vectors carrying AtRuvBL1, AtRuvBL2a, AtPOT1a, AtPOT1b and AtGAUT10 were described in Majerská *et al.* (2017). The AtTRB1, 2 and 3 constructs have described previously (Schrumpfová *et al.*, 2014).

The cloned cDNA sequence of AtCBF5 (GC105080 from Arabidopsis Information Resource (http://www.arabidopsis.org/)) in pENTR223 was used as entry vector. For preparation of yeast two-hybrid (Y2H) and/or BiFC constructs, DNA fragments were introduced into the destination Gateway vectors pGBKT7-DEST, pGADT7-DEST (Horak *et al.*, 2008) and/or the pSAT5-DEST-c(175-end)EYFP−C1(B), pSAT4-DEST-n(174)EYFP−Cl (Lee *et al.*, 2012) using the LR recombinase reaction (Invitrogen, Carlsbad, CA, USA).

PCR-based genotyping of plant lines

Plants with annotated T-DNA insertion within *AtRuvBL1* gene (SAIL_397_C11, WiscDsLoxHs027_03G, WiscDsLoxHs117_06F, WiscDsLoxHs168_06D) and *AtRuvBL2a* gene (GK-543F01, SALK_071103, SALK_144539, SALK_144540, SAIL_500_C04) in the Col-0 background were used (Figure S8). To distinguish between wild-type plants and those that were heterozygous for the T-DNA insertion in the *AtRuvBL1* or *AtRuvBL2a* genes, we isolated genomic DNA from leaves by the standard protocol of Dellaporta *et al.* (1983). The genomic DNA was used for PCR analysis using MyTaq DNA polymerase (Bioline, http://www.bioline.com). The conditions used were in accordance with the manufacturer's instructions. The primers used were specific for T-DNA and *AtRuvBL1* or *AtRuvBL2a* genes (Table S3, Figure S9). Thermal conditions were 95°C for 1 min (initial denaturation), followed by 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min 20 sec, with a final extension at 72°C for 10 min.

RT-PCR

Total RNA was extracted from approximately 100 mg of frozen young leaves using an RNeasy plant mini kit (Qiagen, Venlo, Netherlands) and RNA samples were treated with TURBO DNA-free (Applied Biosystems/Ambion, http://www.lifetechnologies.com TURBO DNA-free). The quality and quantity of RNA were determined by electrophoresis on 1% w/v agarose gels and by measurement of absorbance using NanoDrop™ 2000/2000c spectrophotometer (https://www.thermofisher.com/). Reverse transcription was performed using random nonamers (Sigma-Aldrich, http://www.sigmaaldrich.com) with 1 μg RNA and Mu-MLV RT (New England Biolabs, https://www.neb.com/). Quantification of transcript levels of the *AtRuvBL1*, *AtRuvBL2a* (Figure S10) and *AtTERT* genes (Fojtová *et al.*, 2011) was carried out by FastStart I SYBR Green Master (Roche, Basel, Switzerland), a Rotorgene 6000 cycler (Qiagen) and using the Ubiquitin-10 gene as suitable references for quantitative analyses in *A. thaliana.* A 2 μl aliquot of cDNA, from two biological replicates, were added to the 20 μl reaction mix; the final concentration of each forward and reverse primer (sequences are given in Table S3) was 0.25 μM. Three technical replicates were done for each reaction that was measured in triplicates; the PCR cycle consisted of 15 min of initial denaturation followed by 40 cycles of 15 sec at 95°C, 20 sec at 56°C and 30 sec at 72°C. SYBR Green I fluorescence was monitored consecutively after the extension step (Fojtová *et al.*, 2011) sequences of primers are given in Table S3. Statistical analysis was performed using unpaired Student's *t*-test.

Quantitative TRAP assay

Protein extracts from buds were prepared as described by Fitzgerald *et al.* (1996). qTRAP analysis was performed as described in Herbert *et al.* (2006) using FastStart SYBR Green Master (Roche) and TS21 and TEL-PR primers. Samples were analyzed in triplicate. A 1 μl aliquot of extract diluted to 50 ng μl^{-1} protein concentration was added to the 20 μl reaction mix. Ct values were determined using the Rotorgene 3000 (Qiagen) machine software, and relative telomerase activity was calculated by the ΔCt method (Pfaffl, 2004).

TRF analysis

TRF analysis was performed as described previously (Ruckova *et al.*, 2008) using 500 ng genomic DNA isolated from 5 to 7 weeks old rosette leaves using NucleoSpin Plant II (Machery Nagel). Hybridized samples (Hybond XL, GE Healthcare, Chicago, IL, USA) by Southern hybridization method were radioactively marked by random priming, in which the telomeric probe was prepared according to a modified protocol from Ijdo *et al.* (1991). Telomeric signals were visualized using an FLA7000 imager (Fujifilm, Tokyo, Japan). Evaluation of fragment lengths was performed using a Gene Ruler 1 kb DNA ladder (Fermentas, http://www.thermoscientificbio.com/fermentas/) as the standard. Mean telomere lengths were calculated as described by Grant *et al.* (2001).

Yeast two-hybrid assay

Yeast two-hybrid experiments were performed using the Matchmaker TM GAL4-based two-hybrid system (Clontech, Kyoto, Japan) as described in Schrumpfová *et al.* (2014). *AtRuvBL1* and *AtRuvBL2a* constructs from pDONR/221 entry clones were subcloned into the Gateway-compatible destination vector pGBKT7-DEST (bait vector) and pGADT7-DEST (prey vector). cDNA sequences encoding *AtTERT* fragments from pDONR/221 entry clones and *AtCBF5* from PENTR223 entry clone were subcloned into the Gateway-compatible destination vector pGBKT7-DEST (bait vector). *AtPOT1a* constructs were subcloned from pDONR/221 entry clones into the Gateway-compatible destination vector pGADT7-DEST (prey vector). The pGADT7 prey vectors (Clontech) carrying *AtTRB1-3* and *AtPOT1a* have been described previously (Schrumpfová *et al.*, 2008). Successful co-transformation of each bait/prey combination into *Saccharomyces cerevisiae* PJ69-4a was confirmed on SD plates lacking Leu and Trp, and positive interactions were selected on SD medium lacking Leu, Trp and His (with or without 3-aminotriazol (3-AT)) or SD medium lacking Leu, Trp and Ade. Co-transformation with an empty vector and homodimerization of the AtTRB1 protein served as negative and positive control, respectively (Schrumpfová *et al.*, 2014). Protein expression was verified by immunoblotting in equal amounts of protein extracts separated by SDS-PAGE (12%), blotted onto nitrocellulose membrane, and probed with mouse anti-Myc (1:1000; Sigma-Aldrich) and mouse anti-HA (1:1000) primary antibodies binding to specific protein epitope tags of AD- and BD-fusion proteins, followed by an anti-mouse HRP-conjugated secondary antibodies (1:8000; Sigma-Aldrich) for chemiluminescence detection.

In vitro translation and co-immunoprecipitation

Additionally, the Y2H constructs were employed for verification in assay as described in Schrumpfová *et al.* (2008). Briefly, radioactively (^{35}S-Met) labelled proteins with hemagglutinin tag (HA) (pGADT7, pGADT7-DEST), as well as non-radioactively labelled protein partners with a Myc-tag (pGBKT7, pGBKT7-DEST) were separately expressed in the TNT Quick Coupled Transcription/Translation System (TNT-RRL) (Promega, Fitchburg, WI, USA) in 50 μl of each reaction according to the manufacturer's instructions. The co-immunoprecipitation procedure was performed as described by Schrumpfová *et al.* (2008) with 1 μg anti-Myc-tag polyclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) and incubated overnight at 4°C with 10 μl protein G magnetic particles (Dynabeads, Invitrogen-Dynal).

During the co-immunoprecipitation with three proteins of interest, two radioactively labeled proteins with HA-tag (AtRuvBL1, AtTRB3) and one non-radioactively labeled AtTERT 1-271 fragment with Myc-tag were expressed separately in TNT-RRL and incubated in the same manner as previous Co-IP together with protein G magnetic particles (Dynabeads, Invitrogen-Dynal) and 1 μg anti-Myc-tag polyclonal antibody (Sigma). Input, Unbound and Bound fractions were separated by 12% SDS-PAGE and analyzed by FLA7000 imager (Fujifilm).

Bimolecular fluorescence complementation

Arabidopsis thaliana leaf protoplasts were prepared and co-transfected with DNA (10 μg of each construct) as was described in Lee *et al.* (2012). The same entry vectors (pDONR/221, PENTR223), already used for *AtRuvBL1*, *AtRuvBL2a*, *AtTERT* fragments, *AtCBF5* and *AtPOT1a* Y2H constructs cloning (Majerská *et al.*, 2017) or entry vectors used for cloning *AtTRB1-3* (Schrumpfová *et al.*, 2008) were ligated into pSAT5-DEST-c(175-end)EYFP−C1(B), pSAT4-DEST-n(174)EYFP−CI vectors. As a negative control, we used the cYFP/AtGAUT10 construct. To control transformation efficiency and to label cell nuclei, we co-transfected a plasmid expressing mRFP fused to the nuclear localization signal of the VirD2 protein of *A. tumefaciens* (mRFP−VirD2NLS; Citovsky *et al.*, 2006). To label nucleolus we co-transfected a plasmid expressing mRFP fused to the to *AtFibrillarin 1* (Pih *et al.*, 2000). Transfected protoplasts were incubated in the light, at room temperature overnight, and then observed for fluorescence using a Zeiss AxioImager Z1 epifluorescence microscope equipped with filters for YFP (Alexa Fluor 488), RFP (Texas Red) and CY5 (chloroplast autofluorescence). The mRFP−VirD2NLS and AtGAUT10−cEYFP constructs for BiFC experiments were kindly provided by Prof. Stanton B. Gelvin (Purdue University, USA).

ACCESSION NUMBERS

AtRuvBL1 (AT5G22330); *AtRuvBL2a* (AT5G67630); *AtTERT* (AT5G16850); *AtTRB1* (AT1G49950); *AtTRB2,* formerly *TBP3* (AT5G67580); *AtTRB3*, formerly *TBP2* (AT3G49850); *AtCBF5* (AT3G57150); *AtPOT1a* (AT2G05210); *GAUT10* (AT2G20810); *AtFibrillarin 1* (AT5G52470).

ACKNOWLEDGEMENTS

This work was supported by the Czech Science Foundation (16-1137S), by the project SYMBIT, reg. number: CZ.02.1.01/0.0/0.0/15_003/0000477 financed by the ERDF, and by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601). We thank Inna Lermontova (IPK Gatersleben) for *AtCBF5* constructs. We further thank Eva Sýkorová and Vratislav Peška for providing us with the collection of *TERT* fragments and *Fibrillarin 1-RFP* vectors. We thank to Miloslava Fojtová for help with qTRAP assay. Jennifer McMahon is gratefully acknowledged for proofreading. The access to the computing and storage facilities owned by parties and projects contributing to the National Grid Infrastructure MetaCentrum provided under the program 'Projects of Large Infrastructure for Research, Development, and Innovations' (LM2010005) was highly appreciated, as was the access to the CERIT-SC computing and storage facilities provided under the program 'Center CERIT Scientific Cloud', part of the Operational Program Research and Development for Innovations, reg. no. CZ.1.05/3.2.00/08.0144. Core Facility Plant Sciences of CEITEC MU is acknowledged for the cultivation of experimental plants used in this paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

PPS designed the study, supervised the project and wrote the manuscript with support from SŠ, JF, LZD and DH.SŠ performed the experiments. LZD designed the phylogeny analysis. JF helped to supervise the project and wrote the manuscript. All authors discussed the results and contributed to the final manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. AtRuvBL1 does not interact with either the RT-domain or the C-terminus of AtTERT.

Figure S2. AtTRB2 proteins directly interact with AtRuvBL2a protein and AtTRB1 is associated with AtRuvBL1 in BiFC assay.

Figure S3. Nucleolar or cytoplasmic localization of AtPOT1a–AtCBF5 interactions.

Figure S4. Weak interaction between AtPOT1a and AtRuvBL1 proteins.

Figure S5. Association of AtRuvBLs, AtTRBs and AtTERT in the nucleolus in *A. thaliana* leaf protoplasts where the whole nucleus is marked.

Figure S6. Relative transcript levels of *AtTERT* gene in *AtRuvBL1* and *AtRuvBL2a* heterozygous mutant plants.

Figure S7. Terminal restriction fragment analysis.

Figure S8. Schematic illustration of specific primers and T-DNA insertion location within the *AtRuvBL1* and *AtRuvBL2a* genes.

Figure S9. Example of PCR analysis of genomic DNA isolated from wild-type (Wt) plants and heterozygous *AtRuvBL1* and *AtRuvBL2a* plants.

Figure S10. Relative *AtRuvBL1* and *AtRuvBL2a* transcript levels in heterozygous *AtRuvBL1* and *AtRuvBL2a* plants.

Table S1. The number of PPIs foci with exclusively nucleolar localization.

Table S2. List of T-DNA insertion lines.

Table S3. List of primers.

Data S1. List of the analyzed plant species sorted by phylogenetic system with number of homologues.

Data S2. List of the analyzed plant species for *AtRuvBL* homologues and their accession numbers.

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