Virology 489 (2016) 233–242

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Screening a yeast library of temperature-sensitive mutants reveals a role for actin in tombusvirus RNA recombination

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ARTICLE INFO

Article history:
Received 20 July 2015
Returned to author for revisions
15 August 2015
Accepted 14 December 2015
Available online 14 January 2016

ABSTRACT

Genetic recombination in RNA viruses drives the evolutionary arms race with host's antiviral strategies and recombination also facilitates adaptation of viruses to new hosts. In this paper, the authors used tombusvirus and a temperature-sensitive (ts) mutant library of yeast to identify 40 host proteins affecting viral recombination in yeast model host. Subsequent detailed analysis with two identified actin-related proteins, Act1p and Arp3p, has revealed that the wt actin network helps TBSV to maintain low level viral recombination. Pharmacological inhibition of actin in plant protoplasts confirmed the role of the actin network in tombusvirus recombination. An in vitro approach revealed the altered activity of the tombusvirus replicase in the presence of mutated Act1p. The authors show more efficient recruitment of a cellular DEAD-box helicase, which enhances tombusvirus recombination, into the membrane-bound replicase in Act1p mutant yeast. Overall, this work shows that the actin network affects tombusvirus recombination in yeast and plant cells.

Introduction

Emergence of new viruses and strains adapted to a new host can be facilitated by high frequency mutations and genetic RNA recombination (Aaziz and Tepfer, 1999; Sztuba-Solinska et al., 2011; Worobey and Holmes, 1999). RNA recombination helps viruses to repair truncated or mutated viral RNA genomes, thus increasing the infectivity of RNA viruses (Guan and Simon, 2000; Hema et al., 2005; Nagy et al., 1997; Rao and Hall, 1993). Viral RNA recombination joins two or more noncontiguous segments of the same RNA or two separate RNAs together (Bujarski, 2013; Nagy and Simon, 1997). Viral recombination could lead to mutations, sequence insertions, duplications, deletions, rearrangements, gaining new cis-acting or coding sequences or formation of new sequences.

The importance of viral RNA recombination has been well-documented for many viruses. The viral replicase-driven template-switching type recombination is the major mechanism, albeit RNA joining/ligation has also been documented (Cheng and Nagy, 2003; Cheng et al., 2005; Figlerowicz et al., 1997; Gmyl et al., 2003; Kim and Kao, 2001; Nagy et al., 1995; Nagy and Simon, 1997; Panaviene and Nagy, 2003). Interestingly, research on Tombusviruses firmly established that various cellular pathways and factors are also major drivers of viral recombination (Chuang et al., 2015; Nagy, 2011).

The role of the host cell in RNA virus recombination is intensively studied with the help of *Tomato bushy stunt virus* (TBSV), a tombusvirus, based on the development of various unique approaches including the use of yeast (*Saccharomyces cerevisiae*) model host (Nagy et al., 2012; Nagy and Pogany, 2006, 2010, 2012; Panavas et al., 2005b; Rajendran and Nagy, 2006; Serviene et al., 2006, 2005). Systematic genome-wide screens in yeast have identified more than 30 host genes that affected TBSV RNA recombination (Jaag et al., 2010, 2007; Li et al., 2008; Serviene et al., 2006, 2005). Currently, three different cellular pathways have been characterized for their roles in TBSV recombination. The first pathway involves cellular endo- and exoribonucleases that cleave the viral RNA, leading to partially-degraded viral RNA molecules, called degRNAs (Fig. 1A). DegRNAs are then used by the viral replicase as templates for template-switching recombination (Cheng et al., 2006; Serviene et al., 2005). This pathway includes cellular endoribonucleases, and the cytosolic Xrn1p 5′-to-3′ exoribonuclease (Xrn4 in plants), which suppresses TBSV recombination via rapidly degrading TBSV degRNAs (Fig. 1A) (Cheng et al., 2007, 2006; Jaag and Nagy, 2009; Jiang et al., 2010).

The second pathway is based on Pmr1 Ca^{++}/Mn^{++} pump that controls Ca^{++}/Mn^{++} levels in the cytosol. Interestingly, the increased cytosolic Mn^{++} level in *pmr1Δ* cells induces high frequency RNA recombination via increasing the template-switching

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http://dx.doi.org/10.1016/j.virol.2015.12.007
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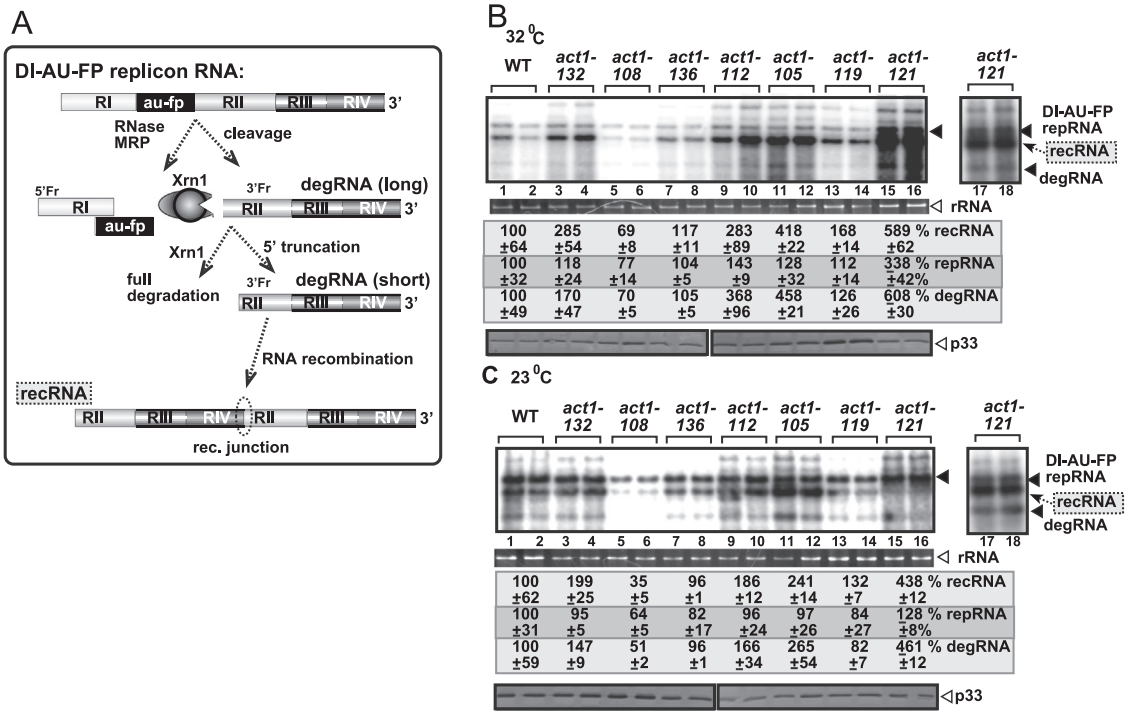

Fig. 1. The essential actin protein, Act1p, affects TBSV RNA recombination in yeast. (A) Scheme of the TBSV RNA recombination pathways in yeast. The first step is the cellular RNase MRP endoribonuclease-based cleavage of the replication-competent highly recombinogenic TBSV DI-AU-FP repRNA as shown. This results in the short 5′ fragment and the long 3′ fragment called "long degRNA". The long degRNA is further processed by the cellular Xrn1p 5′-3′ exoribonuclease via 5′ truncations to give rise to "short degRNAs". Altogether, these cellular nucleases generate a pool of replication-competent degRNAs that serve as templates in template-switching recombination events driven by the viral replicase, as shown. The sequence elements in a typical TBSV recRNA are also shown schematically. (B, C) Rapid accumulation of recRNAs in yeast expressing *act1*^{ts} mutants at 32 °C (semi-permissive temperature) (panel B) or at 23 °C (permissive temperature for yeast growth) (panel C). Top panel: Northern blot analysis of the accumulation of TBSV DI-AU-FP repRNA, recRNAs and degRNA at the 24 h time point. Note that the detection of recRNAs was less reliable in *act121*^{ts}, therefore, we show a second set of images on the right (lanes 17–18). Middle panel: The accumulation level of repRNA was normalized based on the ribosomal (r)RNA. Western blotting based measurement of the accumulation level of His_6-p33 (bottom panel). Each sample is obtained from independent yeast colonies. Note that the Western blots were developed the same time under the same conditions. The experiments were repeated two-to-three times.

activity of the viral replicase (Jaag et al., 2010). The third viral recombination pathway involves cellular DEAD-box helicases, which are co-opted into the viral replicase complex (VRC). These DEAD-box helicases affect viral RNA synthesis via locally unwinding viral RNA structures and controlling viral RNA-dependent RNA polymerase (RdRp):RNA interactions (Chuang et al., 2014; Kovalev et al., 2012a, 2012b; Kovalev and Nagy, 2014). The characterized helicases include the DDX3-like Ded1p (AtRH20 in plants), which suppresses the formation of recRNAs, and the eIF4AIII-like RH2 with replication and recombination promoting function (Chuang et al., 2014).

The p33 and p92^{pol} replication proteins of tombusviruses are directly translated from the genomic (g)RNA. The p92^{pol} is produced through translational readthrough of the p33 stop codon (Oster et al., 1998; Panaviene et al., 2003; Scholthof et al., 1995). The RdRp function of p92^{pol} depends on viral and host components, such as heat shock protein 70 and phospholipids (Panaviene et al., 2005; Panaviene et al., 2004; Pogany and Nagy, 2012, 2015). The abundant p33 RNA chaperone has a key role in recruitment of viral RNA template for replication and in the assembly of the membrane-bound VRCs (Monkewich et al., 2005; Panavas et al., 2005a; Pogany and Nagy, 2012; Pogany et al., 2008; Pogany et al., 2005; Stork et al., 2011). Both replication proteins are essential components of the tombusvirus VRCs (Panaviene et al., 2004; Serva and Nagy, 2006).

Various animal and plant viruses reorganize the actin network to optimize the intracellular environment to support viral replication and spread viral infections (Heinlein, 2015; Matthews et al., 2013; Taylor et al., 2011). For plant viruses, including TBSV, the actin network plays a role in cell-to-cell movement by facilitating the intracellular transport of viral movement protein–viral RNA complexes to the openings of plasmodesmata, narrow membranous connections between neighboring plant cells (Harries et al., 2009; Heinlein, 2015).

The actin network, including actin and actin-like molecules, is ubiquitous in cells. Actin molecules are present in monomeric (G-actin) and filamentous (F-actin) forms that allows for rapid reorganization of the actin network via an arsenal of actin binding proteins to initiate new actin strands or break apart existing filaments (Mishra et al., 2014; Smertenko et al., 2010). Yeast has one essential actin gene, called *ACT1*, which forms actin cables, highly motile actin patches and actin–myosin contractile ring. Altogether, the actin network is involved in numerous cellular processes, such as secretion, endocytosis, organellar movement, and it also affects cell shape and motility (Mishra et al., 2014; Smertenko et al., 2010).

In the current work, we have performed a systematic screen with TBSV based on a temperature-sensitive library of yeast mutants, which has led to the identification of 40 yeast genes affecting TBSV recombination. We have chosen the actin-related genes to provide evidence on the roles of the identified host proteins in TBSV recombination. We find that several mutations in Act1p lead to increased level of TBSV recombination. Similarly, pharmacological inhibition of the actin network in plant cells led to increased recombinant RNA accumulation, suggesting that the wt actin network helps TBSV to maintain low level of viral recombination. We observed that mutations in Act1p affected the in vitro activity of the viral replicase. Specifically, in the *ACT1* mutant yeast, we found more efficient recruitment of a cellular eIF4AIII-like RH2 DEAD-box helicase, which enhances tombusvirus

Table 1
The list and known cellular functions of the identified yeast genes affecting TBSV recombinatio.n based on yeast ts-library screen.

Gene	*Rec/repl*	*FUNCTION*
ABF1-101	755/281	DNA binding protein with possible chromatin-reorganizing activity involved in transcriptional activation, gene silencing, and DNA replication and repair
ACT1-121	973/278	Actin, structural protein involved in cell polarization, endocytosis, and other cytoskeletal functions
ARP3-G302Y	502/42	Essential component of the Arp2/3 complex, which is a highly conserved actin nucleation center
BET2-1	947/206	Beta subunit of Type II geranylgeranyltransferase required for vesicular transport between the ER and the Golgi; provides a membrane attachment moiety to Rab-like proteins Ypt1p and Sec4p
CDC4-1	478/215	F-box protein required for both the G1/S and G2/M phase transitions; modular substrate specificity ` factor which associates with core SCF (Cdc53p, Skp1p and Hrt1p/Rbx1p) to form the SCFCdc4 complex; SCFCdc4 acts as a ubiquitin-protein ligase directing ubiquitination of cyclin-dependent kinase (CDK) phosphorylated substrates.
CDC9-1	589/197	DNA ligase found in the nucleus and mitochondria, an essential enzyme that joins Okazaki fragments during DNA replication; also acts in nucleotide excision repair, and base excision repair.
CDC14-8	875/301	Protein phosphatase required for mitotic exit; required for rDNA segregation;
CDC20-3	65/93	Activator of anaphase-promoting complex/cyclosome (APC/C); APC/C is required for metaphase/anaphase transition; directs ubiquitination of mitotic cyclins,
CDC34-1	410/328	Ubiquitin-conjugating enzyme (E2); catalytic subunit of SCF ubiquitin-protein ligase complex
CDC37-1	843/146	Essential Hsp90p co-chaperone; necessary for passage through the START phase of the cell cycle; stabilizes protein kinase nascent chains and participates along with Hsp90p in their folding
DBF2-2	447/167	Ser/Thr kinase involved in transcription and stress response
DED1-199	1183/201	ATP-dependent DEAD-box RNA helicase; required for translation initiation of all yeast mRNAs;
ESA1-L254P	438/205	Catalytic subunit of the histone acetyltransferase complex (NuA4); acetylates histone H4 N-terminal tail and can acetylate histone H2A; required for regulation of autophagy
EXO70-38	43/74	Subunit of the exocyst complex; the exocyst mediates polarized targeting and tethering of post-Golgi secretory vesicles to active sites of exocytosis prior to SNARE-mediated fusion; PtdIns[4,5]P2-binding protein that localizes to exocytic sites in an actin-independent manner.
GPI8-ts	621/446	ER membrane glycoprotein subunit of the glycosylphosphatidylinositol transamidase complex that adds GPI anchors to newly synthesized proteins; human PIG-K protein is a functional homolog
HSP10-ts	810/436	Mitochondrial matrix co-chaperonin that inhibits the ATPase activity of Hsp60p, a mitochondrial chaperonin; involved in protein folding and sorting in the mitochondria; similar to *E. coli* groES
HYP2-1	63/98	Translation elongation factor eIF-5A; required for translation of proteins containing polyproline stretches; structural homolog of bacterial EF-P;
ILS1-1	781/104	Cytoplasmic isoleucine-tRNA synthetase, target of the G1-specific inhibitor reveromycin A
KOG1-1	962/141	Subunit of TORC1, a rapamycin-sensitive complex involved in growth control; contains four HEAT repeats and seven WD-40 repeats; may act as a scaffold protein to couple TOR and its effectors
MED7-163	58/238	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for transcriptional regulation
MPS3-1	735/249	Nuclear envelope protein; required for SPB insertion, initiation of SPB duplication and nuclear fusion.
MYO2-14	676/140	Type V myosin motor involved in actin-based transport of cargos; required for the polarized delivery of secretory vesicles, the vacuole, late Golgi elements, peroxisomes, and the mitotic spindle.
NSE4-ts2	307/92	Component of the SMC5-SMC6 complex; this complex plays a key role in the removal of X-shaped DNA structures that arise between sister chromatids during DNA replication and repair.
PKC1-4	971/290	Protein serine/threonine kinase essential for cell wall remodeling during growth; homolog of the alpha, beta, and gamma isoforms of mammalian protein kinase C (PKC)
POL5-2	46/94	DNA Polymerase phi; similar to the human MybBP1A; required for the synthesis of rRNA
PRP2-1	657/182	RNA-dependent ATPase in the DEAH-box family, required for activation of the spliceosome before the first transesterification step in RNA splicing; orthologous to human protein DHX16
PRP4-ts	1034/244	Splicing factor, component of the U4/U6-U5 snRNP complex
RPA190-1	305/99	RNA polymerase I largest subunit A190
RPN11-14	635/149	Metalloprotease subunit of 19S regulatory particle; part of 26S proteasome lid; couples the deubiquitination and degradation of proteasome substrates; involved, independent of catalytic activity, in fission of mitochondria and peroxisomes; response to DNA replication stress.
SEC8-6	449/148	Essential 121 kDa subunit of the exocyst complex; the exocyst mediates polarized targeting and tethering of post-Golgi secretory vesicles to active sites of exocytosis at the plasma membrane;
SEC13-1	381/100	Structural component of 3 distinct complexes; subunit of Nup84 nuclear pore sub-complex (NPC), COPII vesicle coat, and Seh1-associated (SEA) complex; homologous to human SEC13;
SEC22-3	482/318	R-SNARE protein; assembles into SNARE complex with Bet1p, Bos1p and Sed5p; cycles between the ER and Golgi complex; involved in anterograde and retrograde transport between the ER and Golgi; synaptobrevin homolog.
SEC23-1	198/59	GTPase-activating protein, stimulates the GTPase activity of Sar1p; component of the Sec23p-Sec24p heterodimer of the COPII vesicle coat, involved in ER to Golgi transport;
SEC26	358/140	Essential beta-coat protein of the COPI coatomer, involved in ER-to-Golgi protein trafficking and maintenance of normal ER morphology;
SEN2-1	338/212	Subunit of the tRNA splicing endonuclease; Sen2p contains the active site for tRNA 5' splice site cleavage and has similarity to Sen34p and to Archaeal tRNA splicing endonuclease
STU2-13	39/47	Microtubule-associated protein (MAP) of the XMAP215/Dis1 family; regulates microtubule dynamics during spindle orientation and metaphase chromosome alignment;
TSC11-7	805/287	Subunit of TORC2, a membrane-associated complex that regulates actin cytoskeletal dynamics during polarized growth and cell wall integrity; involved in sphingolipid metabolism;
VTI1-2	681/276	Protein involved in cis-Golgi membrane traffic; v-SNARE that interacts with two t-SNARES, Sed5p and Pep12p; required for multiple vacuolar sorting pathways
YEF3-F605S	448/231	(TEF3) Gamma subunit of translational elongation factor eEF1B; stimulates the binding of aminoacyl-tRNA (AA-tRNA) to ribosomes by releasing eEF1A (Tef1p/Tef2p) from the ribosomal complex;
YIP1-4	732/239	Integral membrane protein; required for the biogenesis of ER-derived COPII transport vesicles; interacts with Yif1p and Yos1p; localizes to the Golgi, the ER; homolog of human YIPF4

RNA recombination INHIBITORY function for the wt gene: red (34 factors)
STIMULATORY function: black (6 factors)
Previously identified in other screens: in gray box
Previously characterized (Ded1 and Rpn11) are underlined.

recombination, into the membrane-bound VRCs. Overall, we have identified a new function for the actin network in viral RNA recombination.

Results

Screening of the ts library of yeast identifies host genes that alter tombusvirus RNA recombination

To identify additional host factors affecting tombusvirus RNA recombination, here we have performed a novel screen with a library of temperature-sensitive (ts) mutants representing 497 essential yeast genes (out of 1101 essential yeast genes). This mutant library contains 787 mutant yeast strains, because many genes are represented by more than one ts mutants (Li et al., 2011). Please note that the ts-mutant is expressed as the only copy of a given gene in this haploid yeast system. We have transformed the yeast strains with TBSV expression plasmids, followed by testing TBSV RNA recombination at the permissive temperature (23 °C) and semi-permissive temperature [27–32 °C, depending on the particular strain (Li et al., 2011)]. The semi-permissive temperature was 4–5 °C below the nonpermissive temperature, and likely resulted in partial inactivation of the essential function of the mutated yeast protein (Li et al., 2011; Shah Nawaz-Ul-Rehman et al., 2013). We expressed the highly recombinogenic DI-AU-FP replicon (rep)RNA (Fig. 1A) together with the tombusvirus p33 and $p92^{pol}$ replication proteins to initiate TBSV repRNA replication and recombination in these yeast strains. The RNA recombinants in the TBSV system are generated via template-switching mechanism by the viral replicase using viral repRNA templates that are cleaved by cellular endo- and exoribonucleases (schematically shown in Fig. 1A) (Cheng et al., 2007, 2006; Jaag et al., 2011, 2010; Nagy, 2011; Serviene et al., 2006, 2005).

The high-throughput screening led to the identification of 40 yeast genes that affected TBSV RNA recombination at either permissive or semi-permissive temperatures in comparison with the wt yeast strain (Table 1). Among the genes identified, 34 mutant genes (85% of total number of genes) facilitated TBSV RNA recombination, while the remaining genes decreased TBSV recombination when mutated. Interestingly, 20 of the 40 genes have been previously identified in separate screens for TBSV (Table 1) (Li et al., 2008, 2009; Mendu et al., 2010; Panavas et al., 2005b; Serviene et al., 2006, 2005; Shah Nawaz-Ul-Rehman et al., 2013), thus, strengthening the relevance of these host proteins in TBSV replication and /or recombination.

Classification of the identified genes based on their known cellular/biochemical functions showed that the highest number of genes identified is involved in protein targeting/vesicle-mediated transport (10 genes, 25% of total number of genes, Table 1). Other genes identified function in RNA binding (5 genes), transcription/chromatin modification (5 genes), cytoskeleton (4 genes), ubiquitin/proteasomal degradation (4 genes), or DNA replication/repair (3 genes). Among the novel genes affecting TBSV recombination, there are protein chaperone-co-chaperones (2 genes), helicases (2 genes) or kinases (2 genes) or proteins involved in metabolism (2 genes).

Interestingly, the high-throughput screen has also led to the identification of yeast proteins involved in the actin network, including actin (Act1p), Arp3p, and Myo2p. Arp3p is a highly conserved protein in the Arp2/Arp3 complex involved in actin nucleation, while Myo2p is Type V myosin motor protein involved in actin-based transport of organelles, cargos and vesicles (Mishra et al., 2014). Since these yeast proteins have also been identified in previous screens (Shah Nawaz-Ul-Rehman et al., 2013), they might have important functions in TBSV replication/recombination. Therefore, we have decided to gain insights into the role of actin network in TBSV recombination.

Several Act1 ts mutants support high level of tombusvirus RNA recombination

We have individually tested seven Act1 ts mutants in our TBSV recombination assay in yeast at the semi-permissive temperature (32 °C) when the mutants were the only Act1 proteins expressed. The accumulation of TBSV repRNA (DI-AU-FP) was changed only moderately (up to ~40%) in case of 6 of the mutants, while repRNA level was increased by ~3-fold with $act1\text{-}121^{ts}$ (Fig. 1B). In contrast, 5 mutants supported vastly increased TBSV RNA recombination (up to ~6-fold increase), while one mutant (Act1-108) decreased and another mutant ($Act1\text{-}119^{ts}$) did not alter recRNA level in yeast (Fig. 1B). Interestingly, the amount of 5′-truncated viral repRNAs, called degRNAs (shown schematically in Fig. 1A), is also increased by ~2-to-6-fold in yeast expressing $act1\text{-}132^{ts}$, $act1\text{-}112^{ts}$, $act1\text{-}105^{ts}$, and $act1\text{-}121^{ts}$ mutants (Fig. 1B). The degRNAs represent partially degraded viral products, which are generated by cellular nucleases, and they serve as templates for viral RNA recombination (Fig. 1A) (Serviene et al., 2006, 2005). Thus, Act1p plays a role in formation and accumulation of both recRNAs and degRNAs. The tested Act1p mutants also supported altered level of TBSV RNA recombination at the permissive temperature (23 °C, Fig. 1C), albeit less efficiently than at 32 °C, suggesting that the mutants behave differently from wt Act1p in viral RNA recombination.

Arp3 ts mutant also supports high level of tombusvirus RNA recombination

Arp3p (actin-related protein 3) and Arp2p form a complex that is required for actin nucleation and motility of actin patches (Mishra et al., 2014; Moseley and Goode, 2006). Arp2p/Arp3p complex initiates the formation of branched actin filaments from the mother filament. Arp3p was identified in the current screen affecting TBSV recombination (Table 1). To confirm that Arp3p has a function in TBSV recombination, we used a yeast strain with a single copy of mutant Arp3p to examine TBSV recombination. Arp3p mutant has increased the occurrence of recRNA by ~10-fold at the semi-restrictive temperature (Fig. 2, lanes 10–12 versus 7–9), and by ~8-fold at the permissive temperature (lanes 4–6). Therefore, we conclude that similar to Act1p protein, mutation in

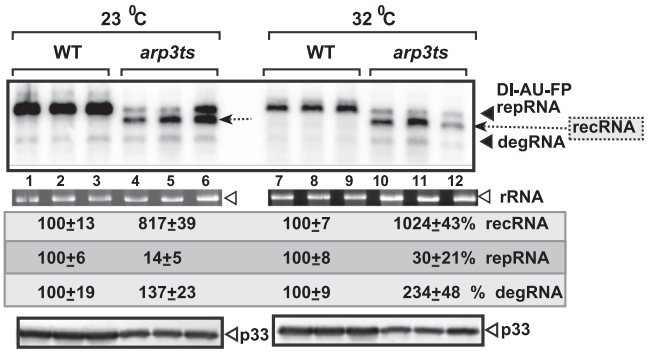

Fig. 2. Rapid accumulation of recRNAs in yeast expressing $arp3^{ts}$ mutant. Yeasts were grown at 32 °C (semi-permissive temperature) or at 23 °C (permissive temperature for yeast growth). Top panel: Northern blot analysis of the accumulation of TBSV DI-AU-FP repRNA, recRNAs and degRNA at the 24 h time point. Middle panel: The accumulation level of repRNA was normalized based on the ribosomal (r)RNA. Western blotting based measurement of the accumulation level of His_6-p33 (bottom panel). Each sample is obtained from independent yeast colonies. The experiments were repeated two times. See further details in Fig. 1.

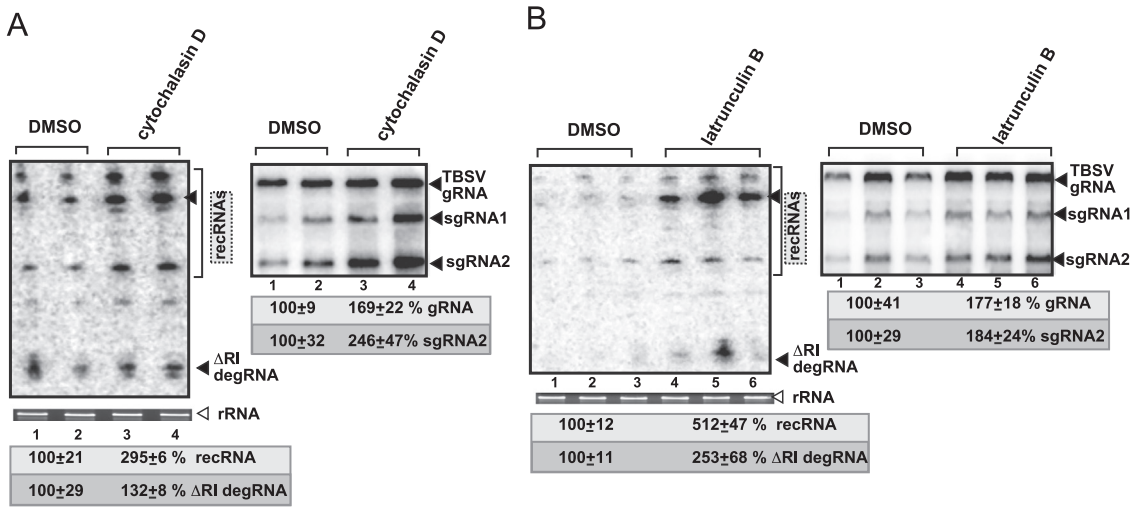

Fig. 3. Pharmacological inhibition of actin network promotes TBSV RNA recombination in plant cells. (A, B) *N. benthamiana* protoplasts were treated with Cytochalasin D (panel A) or Latrunculin B (panel B) or DMSO as a control. Then, 30 min later, the protoplasts were co-electroporated with DI-ΔRI degRNA (a highly recombinogenic RNA due to deletion of the 5′ RI domain of DI-72 repRNA) and TBSV helper virus RNA, which provides the replicase during infection. Total RNA was extracted from protoplasts 24 h after electroporation. The accumulation of DI-ΔRI degRNA and newly-formed recRNAs (left panel, denaturing PAGE) and the TBSV genomic and subgenomic RNAs (right panel, agarose gel) in *N. benthamiana* protoplasts was measured by Northern blotting (top panels). Note that the most abundant recRNAs (marked by an arrowhead) were used for quantification. The ribosomal RNA (rRNA) was used as a loading control and shown in PAGE stained with ethidium-bromide (bottom panel).

Arp3p also enhances TBSV recombination, confirming the role of actin network in viral recombination.

Pharmacological inhibition of actin network in N. benthamiana protoplasts enhances the accumulation of TBSV recRNAs

To test if the plant actin network has similar functions during tombusvirus recombination, we used Cytochalasin D and Latrunculin B inhibitors (Taylor et al., 2011) in *Nicotiana benthamiana* protoplasts, replicating the highly recombinogenic DI-ΔRI degRNA in the presence of TBSV that provided p33 and p92 *in trans* for DI-ΔRI degRNA replication. We observed ~3-fold increase in recRNA accumulation after the addition of Cytochalasin D (Fig. 3A) and ~5-fold higher level after Latrunculin B treatment (Fig. 3B). Interestingly, the replication of the TBSV helper virus was also increased by ~2-fold by the above treatments, suggesting that inhibition of the actin network also affects tombusvirus replication. Cytochalasin D treatment did not damage the plant cells (Holzinger and Blaas, 2016), which supported the formation of large number of tombusvirus virions as shown in electron microscopic image of a thin section of a plant leaf (Fig. 4). These inhibitors are known to inhibit actin polymerization and new actin filament formation (Taylor et al., 2011). Altogether, pharmacological inhibition of the actin network in plant cells has a similar stimulatory effect on TBSV recombination as the genetic mutations debilitating actin functions in yeast.

act1^{ts} mutant support high level of viral RNA recombination in vitro

To test if the act1 ts mutants support RNA recombination in vitro, we have isolated the membrane fraction of yeast replicating TBSV DI-AU-FP repRNA in the presence of co-expressed p33 and p92 proteins. The in vitro replication assay was performed in the presence of ^{32}P-labeled UTP to help visualizing the newly made RNA products. This assay revealed that the recRNAs were associated with the viral replicase and they were synthesized in vitro (Fig. 5A). Interestingly, *act1-121^{ts}* and *act1-132^{ts}* mutants supported recRNA accumulation by ~5-fold more efficiently than the WT preparation (Fig. 5A, lanes 7 and 2 versus 1). These two ts mutants could also support high level of viral RNA recombination in yeast (Fig. 1C).

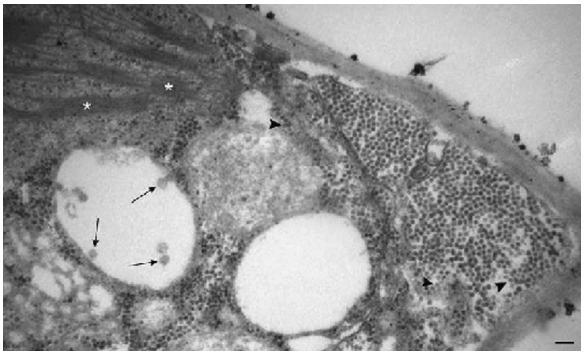

Fig. 4. Treatment with Cytochalasin D does not damage plant cells replicating a tombusvirus. A representative electron microscopic image of a stained ultra-thin section of a *N. benthamiana* cell. *N. benthamiana* leaves were inoculated with TBSV, and one day later, the same leaves were infiltrated with Cytochalasin D (80 μg/ml) or ethanol (as a control) using a syringe to inhibit actin polymerization. Sample preparations from the treated leaves were done three days after inoculation. The EM image shows a large number of TBSV virions (black arrowheads) in *N. benthamiana* cells treated with Cytochalasin D. An intact chloroplast (white asterisks) and the cell wall are visible, while tombusvirus-induced spherule-like structures are marked by black arrows. The bar represents 100 nm.

To test if the actin mutants could also support RNA recombination with the highly efficient TBSV DI-72 repRNA, which is much less recombinogenic than DI-AU-FP, we performed the in vitro assay with isolated membranes from yeast. We found that *act1-121^{ts}* and *act1-132^{ts}* mutants not only supported high level of TBSV repRNA replication (up to ~4-fold increase), but recRNA accumulation was also increased by 2.5-to-4-fold (Fig. 5B). Thus, mutations in the Act1p protein affect viral RNA replication and recombination with various template RNAs. Since the amount of the replication protein was normalized, the obtained data suggest that the relative activity of the tombusvirus replicase is enhanced by various mutations in the Act1p protein.

Act1p affects viral RNA recombination independent of Xrn1p exoribonuclease in yeast

The actin network affects multiple cellular pathways (Costanzo et al., 2010; McCurdy et al., 2001; Mishra et al., 2014), which might

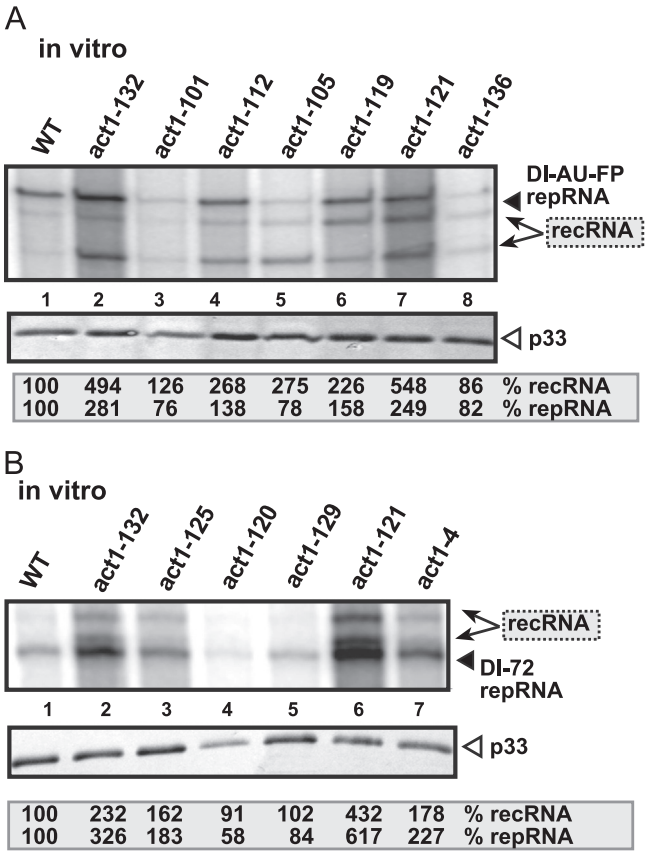

Fig. 5. In vitro replication/recombination assay supports a role for Act1p in TBSV RNA recombination. Membrane-enriched fractions from WT or various *act1*^{ts} mutant yeasts expressing His$_6$-p92 and His$_6$-p33 replication proteins and TBSV DI-AU-FP (+)repRNA (panel A) or TBSV DI-72 (+)repRNA (panel B) were assayed in vitro. Denaturing PAGE analysis of the ^{32}P-labeled TBSV RNA products obtained is shown. Note that comparable amounts of membrane-enriched fractions were used for the recombination assay. Each experiment was repeated three times.

affect TBSV replication and RNA recombination. Interestingly, Act1p genetically interacts (Costanzo et al., 2010) with Xrn1p 5′-to-3′ exoribonuclease, which is a key enzyme in suppression of TBSV RNA recombination and in reduction of TBSV RNA stability in yeast (Cheng et al., 2007, 2006; Jaag and Nagy, 2009, 2010; Nagy, 2011). Therefore, we have tested the possibility that the above Act1p mutations affect TBSV recombination via controlling Xrn1p activity. We expressed a 5′-truncated TBSV DI-ΔRI degRNA (Fig. 6A), which goes through further ~70 nt 5′-truncations up to the RII(+)-SL hairpin structure, which then stops the nuclease activity of Xrn1p (Fig. 6A). However, in the absence of Xrn1p or when the function of Xrn1p is inhibited- for example due to the deletion of Met22p (Jaag and Nagy, 2010), then the 5′-truncation process with DI-ΔRI degRNA is weak in *xrn1Δ* or *met22Δ* yeast (Fig. 6B, lanes 1 and 2–5) (Jaag and Nagy, 2010). In *act1-132*^{ts} yeast, DI-ΔRI degRNA did not accumulate at a detectable extent due to rapid degradation (Fig. 6B, lanes 6–9), similar to wt yeast (Cheng et al., 2006; Jaag and Nagy, 2010). Importantly, the profile of recRNAs accumulating in *act1-132*^{ts} yeast was different from that in *xrn1Δ* yeast (Fig. 6B, lanes 6–9 versus 1). Therefore, it is unlikely that *act1-132*^{ts} affects TBSV RNA recombination via inhibition of exoribonuclease activity of Xrn1p.

Actin mutation leads to increased recruitment of the host eIF4AIII-like RH2 helicase into VRCs in yeast

One of the major groups of host proteins affecting TBSV recombination is DEAD-box helicases, such as DDX3-like Ded1p

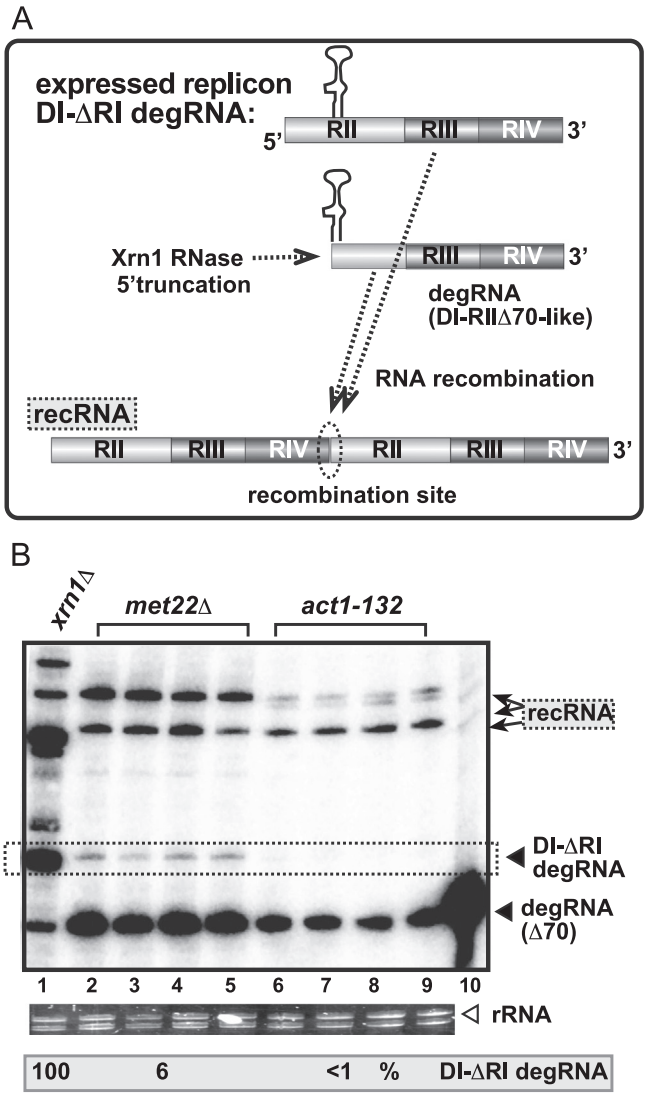

Fig. 6. The role of the actin network in viral RNA recombination is independent of the Xrn1p exoribonuclease pathway. (A) Schematic representation of the TBSV RNA recombination pathway in yeast. Plasmid-driven expression of the DI-ΔRI (highly recombinogenic due to deletion of the 5′ RI domain of DI-72 repRNA) in the presence of p33 and p92 replication proteins leads to partial 5′ truncations by the cellular Xrn1p 5′-to-3′ exoribonuclease generating a pool of DI-RIIΔ70-like degRNAs as shown. DI-RIIΔ70-like degRNAs then participate in RNA recombination as indicated. (B) Northern blot analysis shows the recombination profile of DI-ΔRI RNA in *act1-132*^{ts}, *met22Δ* or *xrn1Δ* yeasts. The original DI-ΔRI degRNA (boxed), DI-RIIΔ70-like degRNAs and the newly-formed recRNAs are depicted with arrowheads and arrows, respectively. Note that the DI-ΔRI degRNA is fully processed in *act1-132*^{ts}, unlike in *xrn1Δ* yeast.

(RH20 in plants) and the eIF4AIII-like RH2 (Chuang et al., 2015; Prasanth et al., 2015). While Ded1p suppresses viral RNA recombination, RH2 can enhance RNA recombination when overexpressed. Based on these features, we wondered if the actin mutations, such as *act1-132*^{ts}, affect the recruitment of these cellular helicases for viral replication. To test if *act1-132*^{ts} facilitates the recruitment of the cellular Ded1p and RH2 helicases into the tombusvirus VRCs, we affinity-purified the tombusvirus VRCs after solubilization of the membrane-bound p33: p92^{pol} complexes. This was then followed by Western blotting to measure the co-purified Ded1p and RH2 proteins in the replicase complex. These experiments revealed that RH2 was present in ~2-fold higher amount in the tombusvirus VRCs from *act1-132*^{ts} yeast (Fig. 7, lane 2 versus 1), while Ded1p helicase was co-purified with the tombusvirus replication proteins in similar amounts from wt and

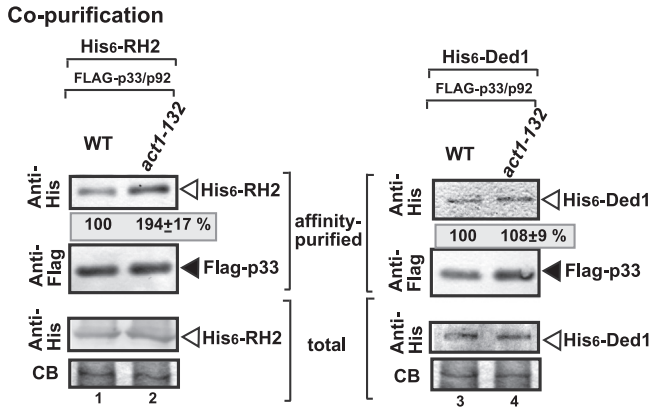

Fig. 7. Act1p mutation affects the recruitment of RH2 DEAD-box helicase into the tombusvirus replicase in yeast. Co-purification of *Arabidopsis* RH2 and yeast Ded1p DEAD-box helicases with the p33 and p92 replication proteins from WT or *act1-132^{ts}* yeasts at the semi-permissive temperature (27 °C). The FLAG-tagged p33 and FLAG-p92 were co-purified from solubilized membranous fraction of yeast extracts using a FLAG-affinity column. Top panel: Western blot analysis of the co-purified 6xHis-tagged AtRH2 or 6xHis-Ded1p with anti-His antibody in the FLAG-affinity-purified preparations. Middle panel: Western-blot analysis of the same samples as in the top panel, but using anti-FLAG antibody. Bottom panels: Western blot analysis of 6xHis-AtRH2 or 6xHis-Ded1p with anti-His antibody in the total protein extracts from yeasts expressing the shown proteins. CB: Coomassie-stained SDS-PAGE of total protein extracts. Each experiment was repeated two times.

act1-132^{ts} yeast (Fig. 7, lane 4 versus 3). These data strongly suggest that *act1-132^{ts}* mutation facilitates the recruitment of cellular RH2 helicase into the tombusvirus VRCs. The increased amount of RH2 helicase in the VRCs could promote TBSV RNA replication (Kovalev and Nagy, 2014; Kovalev et al., 2012b) and RNA recombination (Chuang et al., 2015) in *act1-132^{ts}* yeast, although we cannot exclude that other factors and conditions might also be involved.

Discussion

Systematic genome-wide screens performed with TBSV in yeast surrogate host (Serviene et al., 2006, 2005), are powerful to identify cellular factors with key roles in viral RNA replication or RNA recombination (Cheng et al., 2007, 2006; Jaag et al., 2011, 2010; Jaag and Nagy, 2009, 2010; Nagy, 2011). However, in spite of the major efforts, it is likely that the list of host factors identified in previous screens is incomplete. Indeed, screening of the ts essential gene library of yeast here has led to the identification of 40 host factors affecting TBSV recombination including 20 novel factors (Table 1). The 20 host factors, which were also identified in various TBSV screens earlier, also show that combinations of screens are getting closer to identifying all the cellular factors affecting TBSV replication and/or recombination. Altogether, the current list of host factors affecting TBSV RNA recombination is ~80 yeast proteins (Nagy, 2011), indicating that many different cellular processes could influence viral RNA recombination. The above ts gene library-based screen seems to be very valuable in identification of host factors with roles in viral recombination, since in addition to the current results with Act1p and Arp3p actin network proteins, we have already characterized the functions of Ded1p RNA helicase and Rpn11p proteasomal metalloprotease in viral RNA recombination based on ts mutants (Chuang et al., 2015; Prasanth et al., 2015). However, the roles of most of the identified cellular factors in viral RNA recombination have not yet been determined.

The actin network affects tombusvirus RNA recombination

This work has demonstrated the unexpected role for the essential actin network in tombusvirus recombination. Act1p inhibits tombusvirus recombination based on the reduced level of recRNA accumulation in wt yeast in comparison with ts mutants, such as *act1-132^{ts}* and *act1-121^{ts}* (Fig. 1). Pharmacological inhibition of the actin network with Cytochalasin D or Latrunculin B also led to increased level of viral RNA recombination in plant cells, suggesting that the actin network plays a comparable role in RNA recombination in plant cells to the role dissected with genetic mutants in yeast.

Since the recRNAs are replicated after their generation, it is possible that recRNAs accumulate more efficiently than the original DI-AU-FP RNA in the *ACT1* mutant yeast strains. However, our in vitro replication assay with the most efficient template, DI-72 repRNA, resulted in recRNAs when the tombusvirus replicase was obtained from the *ACT1* mutant yeast strains (Fig. 5B), suggesting that the frequency of recombination must be higher in the *ACT1* mutant yeast strains than in the wt yeast.

The actin network might affect replicase assembly and the extent of host factor subversion that influence viral RNA recombination

Based on the results presented in this paper, it seems that the actin network is involved in VRC assembly or formation of viral factories. Accordingly, the relative in vitro recombination activity of the tombusvirus replicase is enhanced by various mutations in the Act1p protein (Fig. 5), suggesting that Act1p influences VRC functions. We observed that the amounts of host RNA helicases recruited to the VRCs are altered in Act1p mutant yeast. Namely, the eIF4AIII-like RH2 DEAD-box helicase is co-opted more efficiently in the Act1p mutant yeast, while Ded1p DEAD-box helicase was present in comparable amounts in wt and mutant yeasts (Fig. 7). We have shown previously that changing the ratio and/or amounts of RH2 helicase with recombination promoting activity versus Ded1p with viral recombination suppressor activity leads to changes in the frequency of TBSV RNA recombination (Chuang et al., 2015; Prasanth et al., 2015). Thus, the increased level of RH2 versus Ded1p in the VRCs could explain the enhanced recombination activity of the viral VRCs in Act1p mutant yeast (Fig. 8).

The actin network is known to affect many aspects of viral infection cycles, both in plants and animals (Heinlein, 2015; Matthews et al., 2013; Taylor et al., 2011). The actin network could be involved in delivering viral and cellular proteins and lipids to the sites of viral replication. The recruitment of these factors is critical for virus replication and could affect viral RNA recombination as well.

Materials and methods

Yeast strains and expression plasmids

S. cerevisiae strains BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*), *xrn1*Δ, and *met22*Δ were obtained from Open Biosystems (Huntsville, AL, USA). The library of temperature-sensitive (ts) mutants of yeast was kindly provided by Charles Boone (University of Toronto) (Li et al., 2011). Temperature-sensitive yeast strains were transformed and cultured in 96 deep-well plate format as described earlier (Shah Nawaz-Ul-Rehman et al., 2013).

Each strain in the ts library was co-transformed with plasmids UpGBK-Hisp33-Adh/CUP1-DIAU and LpGAD-Hisp92-CUP1 (Jaag and Nagy, 2010; Jaag et al., 2010) The transformed yeast strains were grown at 23 °C in SC-UL$^-$ (synthetic complete media without uracil and leucine) medium with 2% glucose for 12 h at 23 °C. The

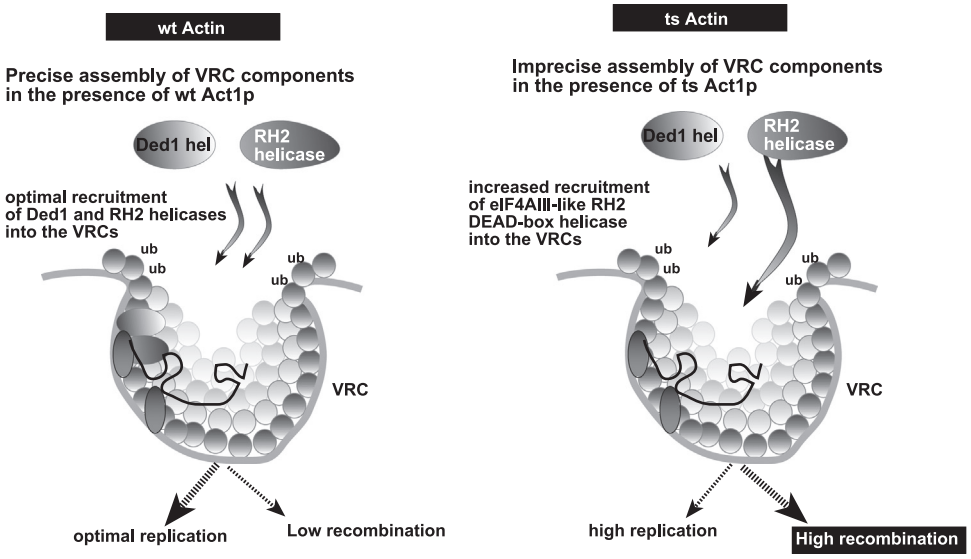

Fig. 8. Models showing the proposed function of the actin network in TBSV RNA recombination. The actin network is proposed to facilitate the balanced recruitment of the cellular DDX3-like Ded1 and the eIF4AIII-like RH2 DEAD-box helicases together with the viral p33 and $p92^{pol}$ replication proteins into the VRCs. This allows the proper VRC assembly resulting in low level RNA recombination. When Act1p is mutated, then recruitment of RH2 helicase is highly efficient into the VRCs. The increased level of RH2 versus normal level of Ded1p helicases in the VRCs leads to enhancement of template-switching type viral RNA recombination. Altogether, these models propose key host factor recruitment and VRC assembly functions for the actin network during TBSV replication and recombination.

liquid culture from each yeast culture plate was divided into two separate 96-deepwell plates, centrifuged; the liquid was replaced with fresh SC-UL$^-$ medium with 2% glucose containing 50 μM $CuSO_4$. One plate was grown at 23 °C (permissive temperature) while the second plate was grown either at 27 °C or 32 °C (semi-permissive temperature depending on the nature of temperature-sensitive yeast mutant) (Li et al., 2011). After 24 h culturing, yeasts were harvested for RNA or protein analysis.

For detailed analysis, yeast strains (BY4741 and Act1 and Arp3 ts mutants) were co-transformed with UpGBK-Hisp33-Adh/CUP1-DI-AUFP and LpGAD-Hisp92-CUP1 (Jaag et al., 2010), using a culture tube format. The transformed yeast strains were grown at 23 °C in SC-UL$^-$ medium with 2% glucose for 12 h at 23 °C. Then, yeast cultures were re-suspended in SC-UL$^-$ medium with 2% glucose containing 50 μM $CuSO_4$. The yeast cultures were grown for additional 24 h at 23 °C, 27 °C or 32 °C before sample collection for total RNA extraction and Northern analyses.

In vitro replication and recombination assay

BY4741 yeast or Act1 ts mutant strains carrying UpGBK-Hisp33-Adh/CUP1-DIAU and LpGAD-Hisp92-CUP1 plasmids were pre-grown in SC media supplemented with 2% glucose at 23 °C for 12 h. Then, the yeast cultures were transferred to new SC media with 2% glucose and 50 μM $CuSO_4$ and incubated at either 27 °C or 32 °C for 24 h. Yeast cultures were collected by centrifugation and cells were broken with glass beads in a Genogrinder to obtain membrane-enriched fractions containing the active tombusvirus replicase complexes including the repRNA as described (Barajas et al., 2009). Comparable amounts of replicase preparations (based on p33 levels by Western blots) were used in the in vitro assays. The reactions were performed in 100 μl containing 25 μl of the normalized membrane-enriched fraction preparations, 50 mM Tris–Cl pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 0.2 μl RNase inhibitor, 1 mM ATP, 1 mM CTP, 1 mM GTP, and 0.1 μl of α^{32}P UTP (3000 Ci/mmol). Reaction mixtures were incubated for 2 h at 25 °C and then the viral RNAs were obtained by phenol/chloroform extraction and isopropanol/amonium acetate (10:1) precipitation. The α^{32}P UTP-labeled repRNA products were separated on 8% acrylamide/8M urea gels and analyzed using a phosphorimager.

DI-ΔRI RNA recombination profile in act1-132^{ts} yeast

Yeast strains *xrn1*Δ, *met22*Δ, and *act1-132^{ts}* were co-transformed with HpGBK-Hisp33-CUP1, LpGAD-Hisp92-CUP1, and UpYC2-DIΔRI-Gal1 (Prasanth et al., 2015). The transformed yeast cultures were grown at 23 °C for 12 h in SC-ULH$^-$ medium supplemented with 2% glucose. Yeast cultures were collected by centrifugation and dissolved in SC-ULH$^-$ medium supplemented with 2% galactose and 50 μM $CuSO_4$, followed by additional culturing at 23 °C for 2 days before sample collection for RNA analysis.

Treatment of N. benthamiana protoplasts with actin inhibitors

Protoplasts were isolated from *N. benthamiana* callus as previously described (Panaviene et al., 2003). Freshly prepared protoplasts were treated with 100 μg/ml DMSO/Cytochalasin D or 40 μM of DMSO/Latrunculin B before electroporation with 1 μg of TBSV gRNA+ DI-ΔRI transcripts. Protoplasts were incubated in the dark for 24 h at room temperature and the total RNA was extracted for Northern hybridization.

Co-purification of cellular proteins with the tombusvirus replicase from act1-132^{ts} yeast

Yeast strains *act1-132^{ts}* and BY4741 were transformed with plasmids pGBK-HIS-Cup-Flag33/Gal-DI-72 (Kovalev and Nagy, 2013) expressing Flag-tagged p33 of Cucumber necrosis virus (CNV) and the TBSV DI-72 repRNA, pGAD-Cup-Flag92 (Barajas et al., 2009) expressing Flag-tagged CNV p92 and either pYC(Ura)-Gal-DED1 (Chuang et al., 2015) or pYES(Ura)-Gal-RH2 (Kovalev and Nagy, 2014). The transformed yeast cells were pre-grown in SC-ULH$^-$ media containing 2% glucose and 100 μM BCS at 23 °C. Then, yeast cells were centrifuged at 2000 rpm for 3 min, washed with SC-ULH$^-$ media containing 2% galactose and resuspended in SC-ULH$^-$ media containing 2% galactose and 100 μM BCS. After growing for 24 h at 27 °C, the media was changed to the ULH$^-$ media containing 2% galactose and 50 μM $CuSO_4$. After growing for 6 h at 27 °C, yeast cells were pelleted and the viral replicase was purified according to a previously described method (Kovalev et al., 2012b; Panaviene et al., 2004). Total fraction from broken yeast cells (balancing was done based on total proteins

analyzed by SDS-PAGE) and the purified fraction from anti-FLAG M2-agarose affinity resin column (balancing was done based on Flag-p33 amount in Western blot) were analyzed for the presence of His_6-tagged DED1 or His_6-RH2. Purified Flag-p33 and Flag-p92 were detected by Western blot using anti-Flag antibody, while the co-purified His_6-tagged host proteins were detected with anti-His_6 antibody. Primary detection was followed by anti-mouse antibody conjugated to alkaline phosphatase. Colorimetric detection was performed with NBT and BCIP (Kovalev et al., 2012b; Panaviene et al., 2004).

Acknowledgments

We thank Drs. Judit Pogany, and C. Chuang for their comments on the manuscript. The authors also thank Dr. Charles Boone (University of Toronto) for yeast ts mutants. This work was supported by NSF (MCB 1517751).

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