Shedding light on the multiple functions of the geminivirus Replication initiator protein

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Chapter 6

Identification of novel tomato proteins that interact with TYLCV Rep using affinity purification

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ABSTRACT

Geminiviruses are plant infecting-viruses that reshape the intracellular environment of their host in order to create favorable conditions for viral replication and propagation. Most of these actions start by protein-protein interactions of virus-encoded proteins with host proteins. Identification of these virus-plant protein-protein interactions helps to understand how viruses manipulate the host machineries and may also generate novel sources of resistance against these pathogens by altering these host proteins into non-interacting versions. Several plant proteins have already been demonstrated as interactors for geminivirus proteins. Here, we use affinity purification and mass spectrometry analysis (AP-MS) to further expand the list of putatively interacting proteins from tomato with the replication initiator protein Rep from *Tomato yellow leaf curl virus* (TYLCV). The Rep protein has been demonstrated to be the only viral protein essential for viral replication and it is an integral part of the *viral replisome*, a complex of virus- and plant-encoded proteins responsible for viral DNA replication. Using our approach, 54 high-confidence tomato interactors of Rep were identified. For two of them, an EWS-like RNA-binding protein and the THO subunit 4A, a nuclear complex involved in the export of mRNA to the cytoplasm, protein-protein interaction with Rep could be confirmed *in planta* with an alternative technique, the bimolecular fluorescence complementation (BiFC). This work represents a first step towards characterization of novel additional host factors that are essential for virus infection by interacting with viral protein Rep and might thus be potential targets for the design of novel resistance strategies against geminiviruses.

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INTRODUCTION

Over the past 20 years, geminiviruses (family *Geminiviridae*) have emerged to become one of the most destructive families of plant viruses (Rojas et al. 2005), infecting a wide range of food and fiber crops, ornamental plants and weeds and causing significant yield losses of many economically important monocot and dicot crops (Mansoor et al. 2003; Mansoor et al. 2006). Geminiviruses are transmitted between plants by various species of leafhoppers, treehoppers and whiteflies and currently they are classified into nine genera on the basis of their insect vector and genome organization (Zerbini et al. 2017). Among them, *Begomovirus* represent the largest genus containing more than 200 species all being transmitted by the phloem sap-feeding whitefly *Bemisia tabaci*. Like other geminiviruses, begomoviruses have a wide host range naturally infecting plants in the tropical and subtropical regions, or anywhere the whitefly has established a foothold. Due to global climate change and the introduction of *B. tabaci* into new regions, these viruses have spread into more moderate climate zones (Navas-Castillo et al. 2011). Traditionally an effective strategy to control a begomovirus infestation in field conditions has been the rotation of insecticides to control whitefly populations combined with agricultural practices that reduce virus reservoirs in natural weeds. However, the success of these measures depends on the crop, cropping system, geographical region and knowledge of the virus-vector biology (Rojas et al. 2018). It would be of great help if the aforementioned measures could be complemented by virus resistance in crop plants. However, only a limited number of genetic resistance sources has, thus far, been identified against geminiviruses and moreover several of these genes have already been broken (Belabess et al. 2016; Butterbach et al. 2014; Verlaan et al. 2013). Hence, to increase options for plant breeders and help reduce insecticide spraying new strategies are required to control viral spread and to reduce damage in a sustainable manner. One strategy to create novel sources of resistance is to introduce mutations in so-called susceptibility (S) genes. S genes encode host factors essential for the viral reproduction cycle (van Schie and Takken 2014).

Geminiviruses are typified by their virus particle, which consists of a circular single stranded DNA molecule (ssDNA) packaged into a twinned capsid particle. Within the genus *Begomovirus*, the genome can be either monopartite (~2.8 kb) encoding six proteins that jointly control viral replication, movement, transmission, and pathogenesis, or bipartite with an 'A component' encoding five or six proteins and a 'B component' encoding two additional proteins (both components being 2.5-2.8 kb in size) (Jeske 2009). Begomoviruses normally infect plant cells that are in the G1 phase of the mitotic cell cycle or the G phase of the endocycle - a variation of the cell cycle that is characterized by increased ploidy number and cell expansion without

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cell division. In order to replicate the viral genome, the virus reprograms the host cell cycle to stimulate progression into S phase. Viral proteins are known to mediate this reprogramming (Hanley-Bowdoin et al. 2004; Hanley-Bowdoin et al. 2013). The only viral protein that is strictly essential for viral replication is Rep (Replication initiator protein). Rep is the most conserved viral protein of the *Geminiviridae* and it is critical for the assembly and recruitment of the *viral replisome*, a complex consisting of both viral proteins and a set of known and unknown host factors that control viral DNA replication (Rizvi et al. 2015; Ruhel and Chakraborty 2018). A second viral protein, REn (Replication enhancer protein) promotes viral DNA replication and is likely also part of the viral replisome (Sunter et al. 1990). Data supports that both Rep and REn interact with host factors to create a cellular environment favorable for virus replication by inducing e.g. the expression of plant genes that encode DNA polymerases and DNA replication accessory proteins (Gutierrez 2000a; Gutierrez 2000b). One of the key components of the viral replisome is the plant Proliferating nuclear antigen PCNA, that forms a DNA clamp and acts as a processivity factor of eukaryotic DNA polymerases. PCNA is highly conserved across eukaryotes and controls a variety of processes such as cell cycle regulation, DNA replication and DNA repair (Choe and Moldovan 2017). Both Rep and REn interact with PCNA (Bagewadi et al. 2004; Castillo et al. 2003) and Rep also manipulates the post-translational modification status of PCNA, thus inducing the switch between the different PCNA functions (Arroyo-Mateos et al. 2018) (Chapter 2).

The geminivirus-plant interaction is complex and involves a multitude of cellular pathways (Hanley-Bowdoin et al. 2013). To better understand how geminiviruses redirect these biochemical pathways, we thus need to characterize in detail the protein-protein interaction network of these viral proteins. This will aid to identify key steps for their replication. With this knowledge it will be possible to attain durable and broad geminivirus resistance by mutagenesis of these key factors to disrupt viral replication.

High-throughput protein-protein interaction studies based on affinity purification followed by mass spectrometry-based protein identification (AP-MS) form a well-suited technique to characterize protein complexes under 'native' conditions (Dunham et al. 2012; Gavin et al. 2011). In plants, AP-MS has recently been used to define e.g. the interactome of the NIa protein from the potyvirus *Tobacco etch virus* (Martínez et al. 2016) and to characterize the global landscape of interactions between the geminivirus *Tomato yellow leaf curl virus* (TYLCV) and one of its host plants *Nicotiana benthamiana* (Wang et al. 2017). Here we expanded the list of potential tomato proteins that reside in a protein complex with TYLCV Rep and PCNA. To this end, Rep was tagged with GFP and affinity purified for MS using a transient expression system based on tomato protoplasts. In addition, tagged

PCNA was transiently co-expressed in these tomato protoplasts. The MS data was used to compose a list of 'high-confidence' interactors of Rep and/or viral replisome consisting of 54 host proteins. For a small subset of these proteins their protein-protein interaction with Rep and PCNA was confirmed with an alternative technology. For two of these proteins, *(i)* an EWS-like RNA-binding, transcriptional repressor protein, and *(ii)* the THO subunit 4A, a nuclear complex involved in the export of mRNA to the cytoplasm, we confirmed with the bimolecular fluorescence complementation (BiFC) assay that they interact with both Rep and PCNA *in planta*. This work represents a first step towards characterization of novel host factors that interact with viral protein Rep and might be targeted for the design of novel resistance strategies against geminiviruses.

RESULTS

Identification of novel tomato proteins that interact with Rep[TYLCV] and PCNA

In order to identify novel interactors of Rep[TYLCV] that act as plant components in the viral replisome, we isolated protoplasts from tomato plants cultured *in vitro* and transfected them with a construct to express the Rep-GFP chimera alone or in combination with PCNA tagged with FLAG at its N-terminus (**Figure 1**). PCNA, a well-established interactor of Rep (Bagewadi et al. 2004; Castillo et al. 2003) and virus replication co-factor, was expressed together with Rep to have a positive internal control for protein-protein interactions and to promote the assembly of viral replisomes. Accumulation of both overexpressed proteins was confirmed in the protoplasts using fluorescence microscopy for Rep-GFP and immunoblotting for FLAG-PCNA chimera (**Figure S1**). Total protein fraction was extracted from three experimental biological replicates and subjected to affinity purification using anti-GFP beads followed by tryptic digestion. Identification of the released peptides was performed using nLC-MS/MS mass spectrometry followed by a MaxQuant analysis to identify and quantify the (co)purified proteins (both the tagged proteins and the associated host factors). Rep was successfully detected in every Rep-containing sample and the PCNA protein was enriched in the Rep-PCNA sample in comparison to the negative control (PCNA alone) in two out of three replicas. To obtain a list enriched for 'high-confidence' interactors, only those proteins were selected from the original list of 427 interactors, whose levels were the same or higher than PCNA levels after affinity purification in at least one biological replicate. Using this selection criterion, 54 tomato proteins were chosen (in addition to Rep and PCNA) (**Table S1**): 27 proteins were present in the sample expressing Rep-GFP alone, 40 in the sample

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in which Rep-GFP was expressed together with FLAG-PCNA, and 13 were in present in both samples (**Figure 2A**). Homologs of eight proteins from our list of tomato 'high confidence' interactors of Rep were also present in the earlier published list of putative *N. benthamiana* interactors of Rep (Wang et al. 2017) (**Figure 2B**).

Interactor network and functional analysis

In order to gain insight into the cellular processes affected by the over-expression of the viral protein Rep in tomato protoplasts, the putative function of the selected interactors was determined by gene ontologies (GO). The GO terms 'Biological process' and 'Cellular component' were assigned to each protein and the relative amount of plant proteins per GO category was calculated (**Table S2**). The largest biological process category was formed by proteins belonging to "RNA metabolism", in particular involved in spliceosome and ribosome assembly, regulation of RNA polymerase and mRNA/rRNA processing (**Figure 2B**). This class was then followed by the categories "protein metabolic processes", "photosynthesis', "metabolic processes", "translation" and "cellular component organization". Importantly, the
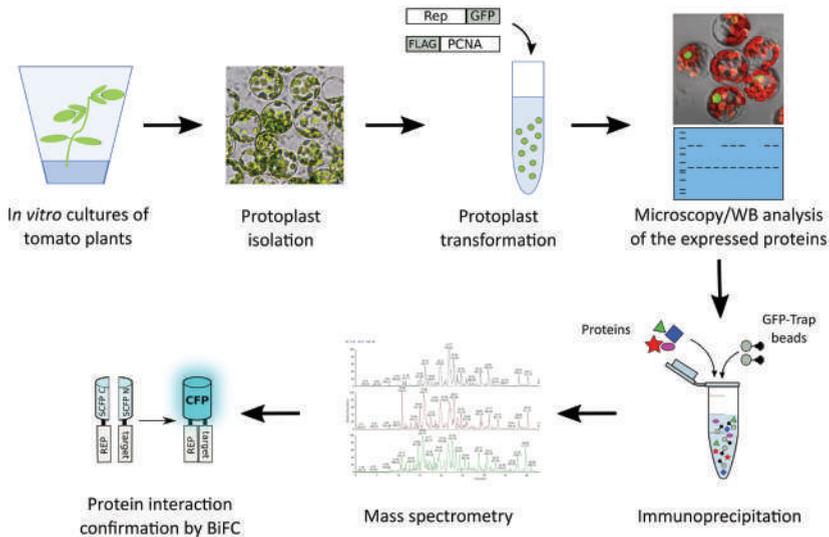

Figure 1. Schematic representation of the strategy followed to identify interactors of Rep
Workflow to obtain novel interactors of TYLCV Rep using affinity purification followed by mass spectrometry. Rep fused to GFP was expressed with and without PCNA tagged with FLAG in tomato protoplasts upon plasmid transfection. The next day, the total protein fraction was extracted, analyzed for the presence of the overexpressed proteins using immunoblotting (WB) and fluorescence microscopy and subjected to affinity purification using an anti-GFP resin. The co-purifying proteins were identified after tryptic digestion by tandem mass spectrometry (MS/MS). For a subset of interactors their interaction with Rep was then confirmed using bimolecular fluorescence complementation (BiFC).
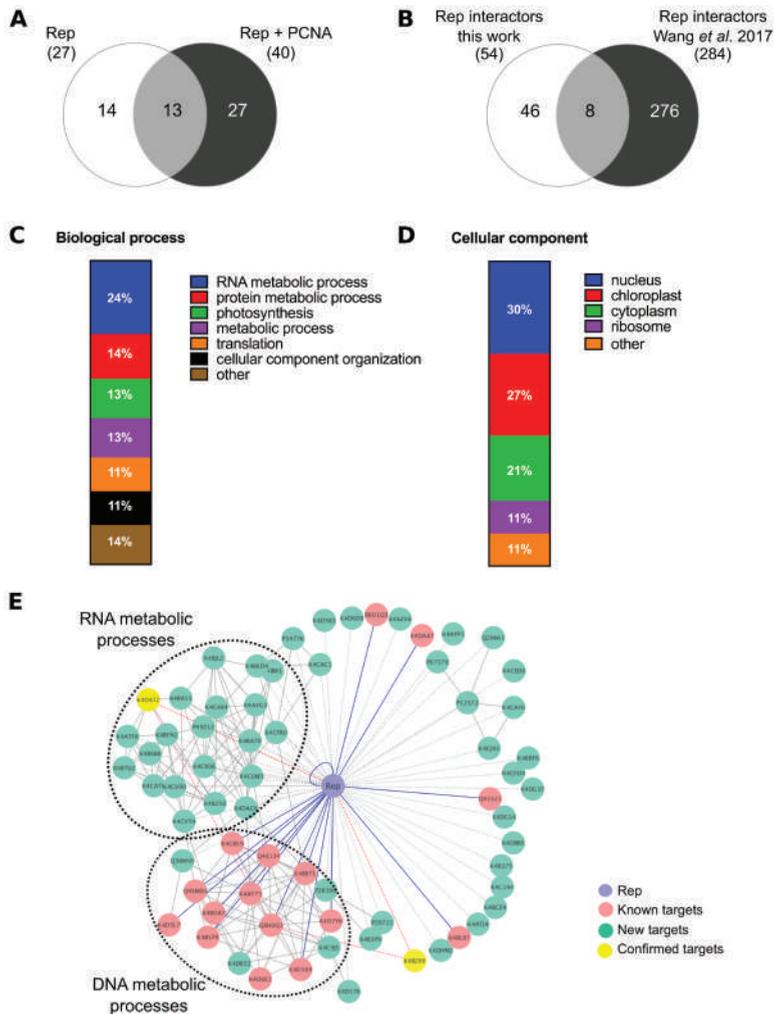
Figure 2. Functional annotation of the 'Rep' and 'Rep+PCNA' tomato interactomes

(A) Venn diagram representing the number of proteins that co-purify with 'Rep alone' and/or 'Rep+PCNA' samples. **(B)** Venn diagram representing the number of interactors of Rep identified in our work and in Wang et al. 2017 work. **(C)** Bar chart depicting the percentage of proteins in different GO categories "biological process" for tomato interactors of Rep. **(D)** Bar chart showing the percentage of proteins per cellular component GO category for the two samples. **(E)** Protein network of the Rep tomato interactome identified in this work (green nodes) and retrieved from the literature (pink nodes). Rep (blue node) was connected manually to the host proteins and host proteins were connected together automatically using Cytoscape. Yellow nodes represent targets whose interaction with Rep was confirmed in this work. Blue lines, known interactions with host proteins; dotted lines, putative interactions with host proteins; red lines, novel interactions; grey solid lines, protein-protein interactions between plant proteins.

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only GO terms that resulted over-represented, with a *p*-value <0.05, were "RNA metabolic processes" and "metabolic processes". The majority of the identified proteins (30%) were localized in the nucleus, while the rest of them were predicted to localize in the chloroplast, cytoplasm, ribosome and other cellular compartments (**Figure 2C**).

In order to visualize the Rep-interactome, we plotted the 54 putative tomato targets identified by AP-MS in a protein-protein network representation (**Figure 2D**) with the nodes representing *(i)* the newly identified host proteins (green and yellow), *(ii)* 16 Rep-interactors derived from previous studies (pink) (reviewed by Ruhel and Chakraborty 2018) and *(iii)* Rep (blue). 70 interactions between Rep and the host targets were manually introduced (blue edges and dotted edges) and 148 tomato protein-protein interactions (grey edges) were derived from the STRING database. These tomato protein-protein interactions helped to determine host complexes that are potentially targeted (or assembled) by Rep. In particular, we identified two major sub-clusters: *(i)* one comprising (mostly known) Rep-interactors involved in DNA metabolism, such as DNA replication and repair and *(ii)* another one formed by newly-identified candidates implicated in RNA metabolic processes.

The MS screen identifies novel Rep interactors involved in viral DNA and RNA propagation

The list of novel interactors of TYLCV Rep contained a number of proteins that were known to be involved in viral replication and pathogenesis. For example, Elongation factor 1-alpha (EF1A) participates in many cellular functions connected to translation, nuclear export, transcription and apoptosis in virus infected cells (reviewed in Sasikumar et al. 2012). EF1A is also involved in the propagation of numerous positive-strand RNA viruses, as it can interact with the viral RNA and with the virus-encoded RNA-dependent RNA polymerase (Abbas et al. 2015). Another viral target present in our list is Nucleolin, an abundant protein in the nucleolus that plays an important role in regulating chromatin structure, processing of pre-RNA and assembly of ribosome particles (Ginisty et al. 1999). A plethora of animal and plant viruses or virus –encoded proteins reside in the nucleolus interacting with nucleolar proteins and certain viral proteins co-localize with, reorganize and re-distribute nucleolar proteins such as Nucleolin (Hiscox 2002; Kim et al. 2004; Malik 2018). The THO subunit 4A is another protein involved in host-virus interactions. As component of the complex implicated in nuclear export of spliced and unspliced mRNA, THO is targeted by different viral proteins to redirect and control the viral mRNAs translocation (Schneider and Wolff 2009; Yarbrough et al. 2014). Finally, in the list of candidates we found a poorly-characterized tomato RNA-binding protein,

whose human homolog, the RNA-binding protein EWS, functions as transcriptional repressor and plays a role in tumorigenic processes (Ohno et al. 1994). The above-mentioned proteins (marked in bold in **Table S1**) were selected for further analysis.

Localization of the GFP-tagged candidates in *N. benthamiana* cells

Next, the CDS of the selected putative Rep-interactors were amplified from tomato cDNA, their identity was confirmed by sequencing and these CDS were cloned as fusion with the gene coding for the Green fluorescent protein (GFP). The GFP-fusion proteins were transiently expressed after DNA transfection in *N. benthamiana* leaf cells by agroinfiltration and their subcellular localization was assessed by confocal microscopy. As expected, GFP-EF1A localized to the cytoplasm and GFP-Nucleolin localized exclusively to the nucleoli of the transformed cells (**Figure 3**). The EWS protein was observed in speckle-like structures in the transfected cell nuclei. GFP-THO chimeric protein was distributed uniformly in the nucleoplasm and occasionally in some nuclear speckles. All the GFP-fusion proteins accumulated at the expected protein mass in *N. benthamiana* based on immunoblots of total protein extracts probed with anti-GFP antibody (**Figure S2**).

Confirmation of the interactions between Rep and the putative interactors using BiFC

After verifying that the selected candidates could be transiently expressed in plant cells, we aimed to confirm our MS results, i.e. their interaction with Rep, using the bimolecular fluorescence complementation (BiFC) assay. To this end, the N-terminal half of the super Cyan fluorescent protein (SCFP^N) was fused to the N-termini of the candidate interacting proteins and these fusion proteins were expressed in *N. benthamiana* epidermal cells together with Rep or PCNA fused to the C-terminal half of SCFP (SCFP^C). As negative control, the SCFP^N-fusion interactors were co-expressed together with SCFP^C-tagged GUS (β-Glucuronidase from *E. coli*). No BiFC signal was detected for any of the negative controls in any cellular compartment (**Figure 4A**). Also, no or very weak reconstitution of the SCFP signal was observed in the nucleus when SCFP^N-EF1A and SCFP^N-Nucleolin were co-expressed with Rep or PCNA. In contrast, the BiFC couple EWS-Rep showed a strong and specific fluorescence reconstitution in distinct (large) nuclear bodies (NBs). Also, the BiFC pair EWS-PCNA resulted in strong fluorescence in nuclei but the signal was typically evenly distributed across the nucleoplasm or in small NBs (**Figure 4B**). Finally, THO subunit 4A interacted also with Rep in the nucleoplasm and with PCNA in both the cytoplasm and nucleoplasm. BiFC experiments thus confirmed the mass
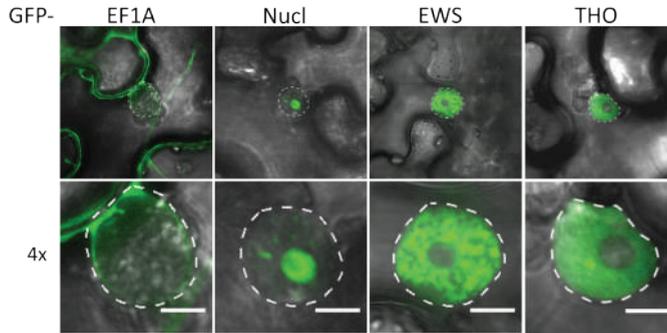
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Figure 3. Candidate Rep-interacting proteins EF1A, Nucleolin, EWS and THO localize in different cellular compartments in *N. benthamiana* epidermal cells

Subcellular localization of GFP-tagged tomato EF1A, Nucleolin, EWS and THO proteins in *N. benthamiana* epidermal cells upon transient overexpression. Image shown represents a typical cell (top) and a 4x zoom showing only its nucleus (bottom); dotted lines encircle the nucleus and scale bars represent 5 μm.
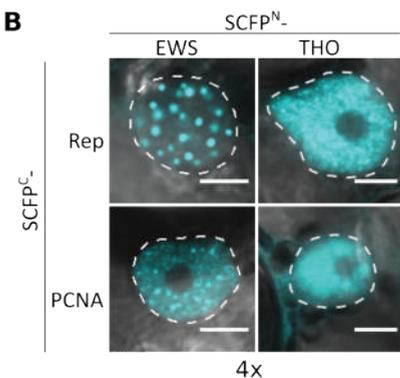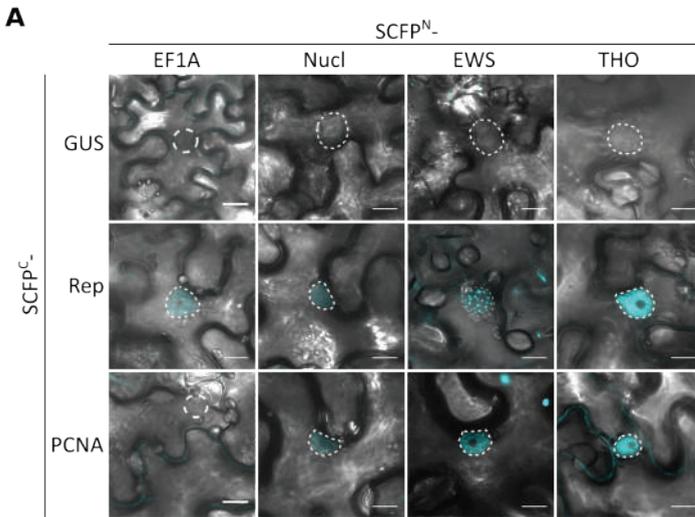
Figure 4. BiFC assay indicates that EWS and THO interact with Rep and PCNA in the nuclei of plant cells

(A) In a BiFC assay, EF1A and Nucleolin do not seem to interact with Rep nor PCNA, whereas EWS and THO proteins show reconstitution of the SCFP signal in combination with Rep and PCNA in the nuclei of the transfected cells. Scale bar is 20 μm. **(B)** 4x zoom of the nuclei of the positive BiFC interaction pairs shown in (A). Scale bar is 5 μm. Dotted lines outline the nucleus.

spectrometry data for EWS and THO subunit 4A, confirming their interaction with Rep (and PCNA) *in planta*.

EWS is an RNA-binding protein with a conserved Zinc finger domain.

Tomato EWS protein is a poorly characterized protein. To obtain more information about this protein a database search was performed. The most likely homolog for tomato EWS protein in Arabidopsis is At4g28990, a predicted 395 amino acid protein and putative member of the Interpro TAF15/EWS/TLS family (IPR034870) (Law et al. 2006). Proteins belonging to this family are RNA-binding proteins and are indicated to be involved in transcription, (alternative) splicing and mRNA transport and DNA repair (Kovar 2011). The Zinc finger domain, RanBP2-type (ZnF_RBZ), was predicted in position 197-233 of the *At*EWS protein (**Figure 5A**). RanBP2-type ZnFs are found in organisms as diverse as fungi, plants and mammals and they have been shown to bind DNA, RNA and protein and regulate the RNA transport between nucleus and cytoplasm (Nguyen et al. 2011). In summary, EWS is a nuclear protein that contains a Zinc finger domain potentially involved in nuclear transport of RNA, transcription and DNA repair. However, the specific function of EWS protein *in planta* still remains to be established.

DISCUSSION

The manuscript describes the use of a transient expression system to affinity purify Rep from tomato protoplasts followed by AP-MS to identify novel host proteins that (directly or indirectly) interact with Rep and are potentially part of the *viral replisome.* This approach yielded a novel set of Rep interactors comprising 54 proteins of which 40 were detected in cells overexpressing Rep together with *Sl*PCNA and 2 were confirmed to bind to both Rep and PCNA using the BiFC system. The majority of the identified interactors are involved in RNA biogenesis, such as spliceosomal complex assembly, regulation of DNA-dependent RNA polymerase, maturation of rRNA and RNA binding, or in protein metabolic processes like catabolic activity,

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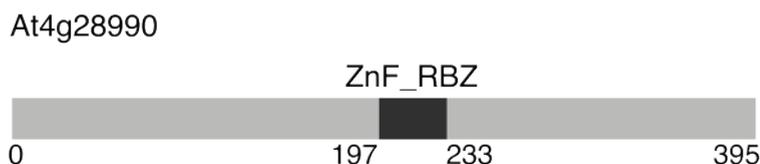At4g28990

ZnF_RBZ

| 0 | 197 | 233 | 395 |

Figure 5. EWS protein contains a conserved Zinc finger domain
Diagram of the closest homologue in Arabidopsis (At4g28990) of the tomato protein EWS showing its predicted RanBP2-type Zinc finger domain.

enzyme regulation and proteasome complex (**Figure 2**). These findings align in part with the results obtained by Wang and collaborators who found an enrichment of functional GO terms linked to 'protein catabolic processes' but not for 'RNA metabolism' amongst their interactors of TYLCV Rep obtained from *N. benthamiana* (Wang et al. 2017). Moreover, eight of the tomato Rep-interactors were also present in the list of putative *N. benthamiana* interactors of Rep described by Wang et al. 2017. The validity of the current findings is supported by three arguments. First, (overexpressed) PCNA levels were higher in two out of three of the Rep affinity purifications (replicas) compared to the negative control, demonstrating that the conditions used were suitable to identify interactors of Rep. In addition, some of the AP-MS hits found were previously described to interact with Rep, e.g. Histones 2A and 2B (Kong and Hanley-Bowdoin 2002). Second, a high percentage of putative Rep-interactors are predicted to localize to the nucleus (including the nucleolus). This is in agreement with the known subcellular localization of both Rep and the viral replisome (Kushwaha et al. 2017) (Chapter 5). Third, we were able to confirm the interaction between Rep and two selected candidates *in planta* using the BiFC system and to localize the complex to the nucleus (**Figure 4**). These two proteins also interacted with PCNA in our BiFC, suggesting that they might be part of the same complex which may be the viral replisome. Future studies should reveal if these interactions are also crucial to promote viral replication.

Despite the successful identification of novel interactors of Rep in tomato, the limitations of the followed approach should be taken in consideration. For example, chloroplastic proteins implicated in photosynthesis and proteins involved in ribosome assembly and biogenesis were also detected, suggesting the presence of false positives in the list with candidates. To date, no relation has been reported between Rep and photosynthesis activity, and given the difference in compartmentalization of these processes this does not seem obvious. At the same time, photosynthesis-related and ribosomal proteins are highly abundant plant proteins and common contaminants picked up with protein-protein interaction methods such as AP-MS (Smaczniak et al. 2012). Hence, their identification as Rep-interactor should be taken with caution and always confirmed individually with alternative methods. At the same time, certain true interactors may have been missed due to the stringency of our selection, low protein abundance in non-dividing mesophyll cells, weak or transient interactions with Rep or the presence of the tag to Rep, which all could prevent the detection of these protein-protein interactions. Another point to consider is that the experimental approach used searches for proteins bound to multiprotein complex to which the viral protein also binds. Hence, the identified host interactors may bind directly or indirectly to the bait protein. Such indirect binding cannot be excluded for the two candidate proteins Elongation factor 1A and Nucleolin-like

protein that were found in the pull-down study, but the interaction with Rep could not be confirmed by reconstitution of the fluorescence signal using BiFC (**Figure 4**). This notion is corroborated by the observation that, in our interaction network, EF1A is connected to two known interactors of Rep, Ubiquitin-conjugation enzyme E2 2 and Histone 3 (**Figure 2D**). The last aspect to consider is that our experimental results derive from the analysis of epidermal tomato protoplasts that express Rep in the absence of viral infection. To have a clearer picture of the dynamics of host-virus interactions during the infection, interactions of host proteins with Rep should be studied in virus-infected tissues.

Interestingly, two RNA-binding proteins were confirmed as interacting proteins of both Rep and PCNA. The first one is EWS, a conserved RNA-binding protein that contains a Zinc finger domain of the RanBP2 type. The human EWS is implicated in cellular transformation in Ewing's sarcoma tumours and it acts as a transcriptional repressor (Owen et al. 2008). Interestingly, in Arabidopsis the *EWS* gene is mostly expressed in the shoot apex and floral tissue (data available on ePlant, http://bar. utoronto.ca/eplant/), where meristematic cells are dividing, suggesting that EWS has potentially a role in cell division/differentiation in plants. Moreover, Rep-EWS BiFC pair interacts and localizes in discrete nuclear bodies (NBs). Co-localization studies with markers of different NBs should elucidate the nature of these EWS-containing NBs to reveal the function of this Rep-EWS interaction.

The other confirmed interactor is a subunit of the THO complex. The biological function of the THO/TREX complex is well characterized in yeast and human cells, where it is recruited to nascent mRNA and functions in transcriptional elongation and mRNA export (Jimeno et al. 2002; Strässer et al. 2002). This complex is also essential for the nuclear export of the Kaposi's sarcoma-associated herpes virus mRNAs and for viral DNA replication (Boyne et al. 2008). In plants, the THO/TREX complex has been shown to influence the production of trans-acting small interfering RNAs (Yelina et al. 2010) and a component of this complex appears to be involved in plant disease resistance against powdery mildew pathogen (Pan et al. 2012). As Rep is known to act as suppressor of RNA silencing (Liu et al. 2014), additional studies are needed to elucidate the mechanism and biological consequence of this Rep-THO interaction.

The identified TYLCV Rep-interacting host proteins can potentially play an essential role in the viral infection and therefore may be considered as susceptibility genes that produce proteins that are essential for successful virus infection of the host. If indeed this is the case, such proteins are good targets to explore for novel resistance strategies against geminivirus based on generating specific alterations of these genes (van Schie and Takken 2014). Manipulation or hijacking of the host targets is often critical for virulence and modest changes in these genes could provide durable and

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broad 'non-host' resistance. Identification of the protein domains or even amino acid residues via which Rep interacts with EWS or THO followed by functional studies that disrupt these interactions might prevent or reduce viral replication and/or spread inside the host plant.

MATERIALS AND METHODS

Tomato protoplast isolation and transfection

Protoplasts were isolated from *in vitro* shoot cultures of tomato according to (Shahin 1985; Tan et al. 1987) and 4×10^7 protoplasts per sample were subjected to Polyethylene-glycol (PEG4000) mediated transformation (Negrutiu et al. 1987) with the following plasmids: (i) pK7FWG2 (Karimi et al. 2002) containing Rep from TYLCV fused to enhanced GFP (EGFP) (referred to as Rep-GFP), (ii) Rep-GFP + pJL-TRBO (Lindbo 2007) containing tomato (*Sl*)PCNA fused to FLAG tag at its N-terminus, referred to as FLAG-PCNA, (positive control for interaction) and (iii) FLAG-PCNA as a negative control. Transfected protoplasts were incubated in Gamborg B5 [Duchefa] liquid medium overnight at 25°C in the dark. Three biological replicates were prepared and used for AP-MS analysis.

Protein extraction and immunoprecipitation

Protoplasts were collected in 1.5 ml eppendorf tubes by centrifugation (5 minutes at 85x g) and resuspended in 1 ml per 10^7 protoplasts of Triton X-buffer (20 mM Tris-HCl pH 7.5, 10 mM KCl, 10% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF, and 1 mM NaF). Protoplast mixtures were incubated for 30 min on ice and sonicated twice for 15 seconds. NaCl 2M solution was added to the protoplast suspension to a final concentration of 420 mM and tubes were incubated for 1 h in ice with occasional mixing. Samples were centrifuged at 10,000g for 30 min at 4°C and the supernatant, containing the extracted proteins, was transferred into a fresh tube. Expression of FLAG-PCNA chimeric protein was confirmed by immunoblotting according to procedures described in Chapter 2. For mass spectrometry-based identification of the proteins that co-purify with Rep-GFP, 1 ml of total protein extract from each sample was incubated for 1 h with 25 µl (50% slurry) of GFP-Trap_M® beads (Chromotek) at 4°C. After incubation, beads were magnetically separated and washed 3 times in 0.5 ml of washing buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 1 mM PMSF protease inhibitor).

Triptic digestion of the immunopurified proteins

The affinity purified proteins were subjected to on-bead tryptic digestion. Briefly, the GFP-Trap_M beads were washed twice with 400 μl of 50 mM ammonium bicarbonate buffer (pH 8), after which the beads were incubated for 1 h at 60°C with 50 mM Dithiothreitol (DTT) in 50 mM sodium bicarbonate buffer pH 8. Subsequentely, 50 mM iodoacetamide in 50 mM sodium bicarbonate buffer pH 8 was added and beads were incubated at room temperature in the dark for 1 h. Next, 50 mM cystein was added and then the proteins were digested by adding 1 μl of a trypsin (Roche, sequencing grade) solution (0.5 μg/μl in 1 mM HCl) overnight at 20°C. Digestion was terminated by adding a droplet of a 10% trifluoroacetic acid solution to acidify the tryptic digest to pH 3. Peptides were concentrated and cleaned using μColumns (Ishihama et al. 2002). These μColumns were prepared by stacking in a 200 μl tip two 3M™ Empore™ C18 extraction disks and LiChroprep® RP-18 50% (Merck Millipor) slurry in methanol. The μColumns were washed with 100% methanol and equilibrated with 0.01% formic acid. Protein samples were added and eluted through to the μColumns. μColumns were washed with 1 ml/l formic acid and the bound peptides were eluted by adding 1:1 v/v mixture of acetonitrile and 1 ml/l formic acid in water. The acetonitrile content was reduced by putting the samples in a Speed-Vac concentrator at 45°C for 2 hours.

Mass spectrometry analysis and data processing

Digested peptides were analyzed using nanoLC-MS/MS as previously described (Gawehns et al. 2015). The MaxQuant software (Cox and Mann 2008; Hubner et al. 2010) was used to analyze the raw data from the LTQ-Orbitrap (Thermo Fisher) for protein identification and label-free quantification (LFQ). The Uniprot proteome database of tomato (UP000004994) and an *in-house* made contaminants database (Peng et al. 2012) were included in the Andromeda search engine (Cox et al. 2014). Data filtering from the MaxQuant output was carried out with Perseus 1.5.8.5 (http://www.perseus-framework.org). Only the LFQ values of the proteins that were identified with at least two tryptic peptides, of which one should be unique and one unmodified, were log10 transformed for further analysis. Proteins that showed a ΔLFQ (log10 LFQ in the sample – log10 LFQ in the control) equal or higher than the ΔLFQ of FLAG-PCNA (*internal control*) in at least 1 out of 3 biological replicates were annotated as 'putative interactors' of Rep and included for further analysis.

Computational analysis

Gene ontology (GO) terms for biological process and cellular component were assigned to the host interactors using Panther (Mi et al. 2017) and QuickGO (EMBL-

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EBI, https://www.ebi.ac.uk/QuickGO) tools. GO terms enrichment in every sample was represented in bar graphs using Prism 7.0v (GraphPad) software. Venn diagram was drawn using InteractiVenn (Heberle et al. 2015), http://www.interactivenn.net/). The protein-protein interaction network was constructed with Cytoscape (Shannon et al. 2003). For that, interaction networks of every putative host interactors and known Rep-interactors, as reported by (Ruhel and Chakraborty 2018) were imported from the STRING protein database (https://string-db.org/) and connected automatically. The node corresponding to Rep was connected manually to each target. Nodes were arranged according to the force-directed layout. The protein sequence of the gene model At4g28990 was analyzed using the InterPro database (https://www.ebi.ac.uk/interpro/) to identify functional protein domains.

Construction of clones used for confocal microscopy

All molecular DNA cloning techniques were performed using standard methods (Sambrook et al. 2001). The *E. coli* strain DH5α was used for subcloning. The CDS of *Solanum lycopersicum* EF1A (XM_004251106; Solyc11g069700.1.1), Nucleolin like 2 (NM_001319854.1; Solyc02g014310.2.1), RNA-binding protein EWS (XM_004239224; Solyc05g018340.2.1) and THO complex subunit 4A (NM_001347950.1; Solyc10g086400.1.1) were amplified from tomato cDNA by PCR with the Phusion DNA polymerase (Thermo Fisher) and the primers listed in **Table S3** that contained *attB1* and *attB2* recombination sites for Gateway-directed cloning. The resulting PCR products were recombined with the Gateway vector pDONR207 (Thermo Fisher) using the BP Clonase II (Thermo Fisher) reaction and confirmed by DNA sequencing. The cDNA clones were then introduced into the destination vectors pGWB452 (Nakamura et al. 2010) and pDEST-SCYNE[GW] (Gehl et al. 2009) using the Gateway LR Clonase II reaction (Thermo Fisher). All plasmids generated and used in this work are listed in **Table S4**.

Transient expression in *N. benthamiana* by agroinfiltration and confocal microscopy

The binary constructs were introduced in *Agrobacterium tumefaciens* GV3101 strain and for transient expression *in planta* agroinfiltrated in *N. benthamiana* plants according to procedures described in Chapter 3. The cellular localization of the GFP-tagged proteins and the reconstituted SCFP fluorophore were detected by confocal microscopy. Detailed procedures are described in Chapter 3.

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SUPPLEMENTARY DATA
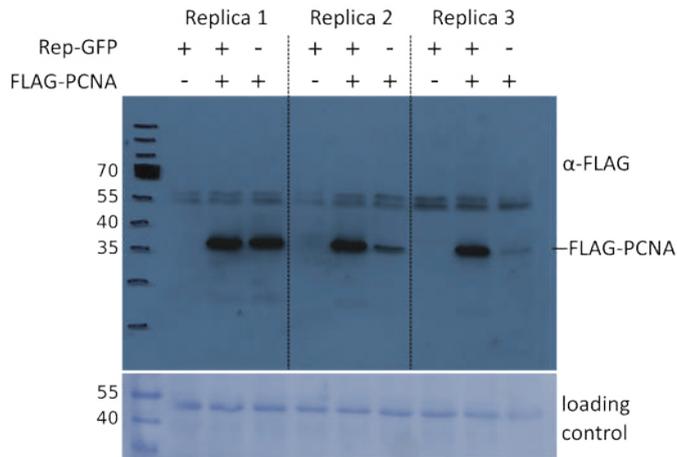
Figure S1. Western blot analysis of the protoplast protein extracts
Accumulation of FLAG-tagged PCNA in the total protein extracts from transfected tomato protoplasts, which were used for affinity purification and mass spectrometry analysis. The protein membrane was stained with Coomassie Brilliant Blue to confirm equal protein loading for the samples

Figure S2. Protein accumulation of the GFP-tagged putative interactors expressed *in planta*
Immunoblot showing the accumulation of the GFP-fusions using an anti-GFP antibody. Asterisks indicate the expected bands at the correct size for every sample. As control for equal protein loading, the membrane was stained with Ponceau S staining.

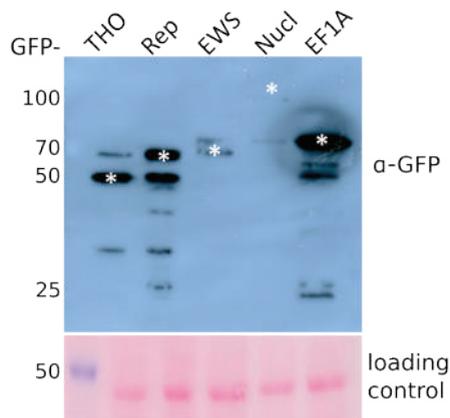| Protein ID	Functional description	Rep	Rep-PCNA
K4CJ02	Photosystem I reaction center subunit		+
K4DB32	Histone 2A	+	
Q2MIA1	P700 chlorophyll a		+
K4DG37	Glycosyltransferase	+	
K4B6B8	**Nucleolin-like**		+
K4ATJ4	Peptidylin-prolyl cis-trans isomerase	+	
K4D6D0	Putative prohibitin-3		+
K4CVT4	MAR-binding protein		+
K4BCZ4	Protein CROWDED NUCLEI 4		+
K4BFN2	Small nuclear ribonucleoprotein Sm D3		+
K4DAC6	**Elongation factor 1-alpha**		+
P12372	Photosystem I reaction center subunit II		+
K4BPK3	Actin-41		+
K4B256	Glycine-rich RNA-binding protein RZ1A	+	
K4D472	**THO complex subunit 4A**		+
P26300	Enolase		+
K4C2T0	Chlorophyll a-b binding protein		+
K4CFD4	Aconitate hydratase		+
K4DHM2	Serine hydroxymethyltransferase	+	
K4CV90	Small nuclear ribonucleoprotein Sm D2		+
K4BXD4	Serine/threonine-protein phosphatase	+	+
K4B375	Prosequence protease 1		+
K4D383	NDR1/HIN1-like protein	+	
K4BTU2	Serine/arginine-rich splicing factor SC35	+	
K4AZV6	Rhodanese-like domain-containing protein 4		+
K4C9J5	Histone H2B	+	+
K4CAF0	Photosystem I reaction center subunit XI		+
K4BJL2	50S ribosomal protein L29	+	+
K4ATF8	Pre-mRNA cleavage factor Im		+
K4C144	Malic enzyme		+
K4BRF6	Putative methyltransferase	+	
P49212	60S ribosomal protein L37	+	+
K4BA51	H/ACA ribonucleoprotein complex subunit 4		+
K4BZ89	**RNA-binding protein EWS**	+	
K4BBI1	50S ribosomal protein L12	+	
K4AYG3	50S ribosomal protein L1	+	+
P54776	26S proteasome regulatory subunit 6A		+
K4CAT6	Small nuclear ribonucleoprotein Sm D1		+
K4CUW3	60S ribosomal protein L23	+	+
K4C4X4	60S ribosomal protein L36	+	+
K4BA70	40S ribosomal protein S15		+
K4B3P9	Fructose-bisphosphate aldolase	+	
K4D576	Protein RNA-directed DNA methylation 3	+	+
K4AYP1	Dynamin-related protein 1E		+
Q2MIB8	30S ribosomal protein S16	+	+
K4CED0	Uncharacterized protein		+
K4DBB5	Zinc finger CCCH domain-containing protein	+	+
P07370	Chlorophyll a-b protein 1B		+
K4CX06	Ribosome biogenesis protein NSA2 homolog	+	
K4CAC1	26S proteasome regulatory subunit 6B	+	+

P20721	Low-temperature-induced cysteine proteinase	+	+
K4CFR0	26S proteasome non-ATPase regulatory subunit 2	+	
K4DG14	ATP synthase delta chain	+	
Q38MV0	Tubulin beta chain	+	+

Table S1. List of the putative interactors of Rep identified by affinity purifications/MS analysis. Proteins in bold were selected for further protein-protein interaction studies.

Biological process		
GO ID	**GO term**	**Proteins**
GO:0016070	RNA metabolic processes	K4CVT4, K4CAT6, K4BFN2, K4CV90, K4BZ89, K4D576, K4ATF8, K4CX06, K4AYG3, K4BA51, K4BTU2, K4D472, K4DBB5
GO:0019538	protein metabolic processes	K4B375, K4CFR0, P54776, K4CAC1, P20721, K4BXD4, K4DAC6
GO:0015979	photosynthesis	K4CJ02, K4CAF0, P12372, Q2MIA1, A0A0J9YZP9, P07370, K4DG14
GO:0008152	metabolic processes	K4DG37, K4CFD4, K4C144, K4B3P9, P26300, K4DHM2, K4AZV6
GO:0006412	translation	P49212, K4BJL2, Q2MIB8, K4CUW3, K4BBI1, K4C4X4
GO:0016043	cellular component organization	K4BPK3, K4BCZ4, K4BA70, K4B6B8, K4AYP1, Q38MV0
GO:0006259	DNA metabolic process	K4CED0, K4DB32, K4C9J5
_	Other	K4ATJ4, K4BRF6, K4D383, K4D6D0, K4B256
Cellular component		
GO ID	**GO term**	**Proteins**
GO:0005840	ribosome	K4BJL2, K4BBI1, K4BA70, K4CUW3, P49212, K4C4X4,
GO:0005634	nucleus	K4CED0, K4CFR0, K4C9J5, K4ATF8, K4D576, K4CAT6, K4B-FN2, K4CV90, K4BZ89, K4BTU2, K4B6B8, K4B256, K4BXD4, K4BCZ4, K4CX06, K4CVT4, K4DB32
GO:0005737	cytoplasm	K4AYP1, K4BRF6, K4DAC6, Q38MV0, P26300, K4BA51, K4CFD4, P20721, K4DHM2, K4B6B8, K4B256, K4BXD4
GO:0009507	chloroplast	K4C144, Q2MIB8, K4AYG3, P07370, K4DG14, A0A0J9YZP9, Q2MIA1, K4CJ02, P12372, K4B375, K4B3P9, K4ATJ4, K4D472, K4BCZ4, P20721
_	Other	K4DBB5, K4AZV6, K4D383, K4D6D0, K4CAF0,K4DHM2

Table S2. List of the gene ontology terms categories assigned to the identified proteins.

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Number	Name	Sequence (5′ to 3′)
7522	EF1A attB1 Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTAAA-GAGAAGGTTCA
7523	EF1A attB2 Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCATTTTTTCTTCT-GAGCAG
7567	Nucl_064 attB1 Fw	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTAAATCTAT-CAAGAAG
7568	Nucl_064 attB2 Rv	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTACTCGTCACTAAAG-GTAGTC
7614	Fw_EWS_X2_attB	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGTTCGCGT-GAGAAGG
7615	Rv_EWS_X2_attB	GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAATATGCACCTC-GTCCA
7692	Fw_THO4A_attB	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGTCAAATCTTGAT-GTATC
7693	Rv_THO4A_attB	GGGGACCACTTTGTACAAGAAAGCTGGGTCTTAGTTTGTCTGCAT-GGCTT

Table S3. Sequences of the primers used in this study. The primer numbers refer to the interal database

Identifier	Name	Vector	Tag	Template/source
bgIFP5568	EF1A	pENTR207	-	PCR cloning (primers 7522/7523)
pFP1696	Nucleolin	pENTR207	-	PCR cloning (primers 7567/7568)
bgIFP5569	EWS	pENTR207	-	PCR cloning (primers 7614/7615)
bgIFP5570	THO4A	pENTR207	-	PCR cloning (primers 7692/7693)
pFP1194	35S-G3GFP-gw	pGWB452	GFP-	(Nakamura et al. 2010)
pFP1412	pDEST-SCYNE(R)-gw		SCFP[N]-	(Gehl et al. 2009)
bgIFP5560	TYLCV Alb13 Rep-GFP	pK7FWG2	-EGFP	Keygene N.V. (Karimi et al. 2002)
bgIFP5571	FLAG-PCNA	pJTRBO	FLAG-	Chapter 2
bgIFP5368	TYLCV Rep-SCFP[C]	p-[GW]-SCYCE	-SCFP[C]	Chapter 3
bgIFP5401	PCNA-SCFP[C]	p-[GW]-SCYCE	-SCFP[C]	Chapter 2
bgIFP3944	SCFP[C]-GUS	pSCYCE-[GW]	SCFPC-	

Table S4. Clone information

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