Blocking Single-Stranded Transferred DNA Conversion to Double-Stranded Intermediates by Overexpression of Yeast DNA REPLICATION FACTOR A

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Agrobacterium tumefaciens delivers its single-stranded transferred DNA (T-strand) into the host cell nucleus, where it can be converted into double-stranded molecules. Various studies have revealed that double-stranded transfer DNA (T-DNA) intermediates can serve as substrates for as yet uncharacterized integration machinery. Nevertheless, the possibility that T-strands are themselves substrates for integration cannot be ruled out. We attempted to block the conversion of T-strands into double-stranded intermediates prior to integration in order to further investigate the route taken by T-DNA molecules on their way to integration. Transgenic tobacco (Nicotiana benthamiana) plants that overexpress three yeast (Saccharomyces cerevisiae) protein subunits of DNA REPLICATION FACTOR A (RFA) were produced. In yeast, these subunits (RFA1–RFA3) function as a complex that can bind single-stranded DNA molecules, promoting the repair of genomic double strand breaks. Overexpression of the RFA complex in tobacco resulted in decreased T-DNA expression, as determined by infection with A. tumefaciens cells carrying the β-glucuronidase intron reporter gene. Gene expression was not blocked when the reporter gene was delivered by microbacterium. Enhanced green fluorescent protein-assisted localization studies indicated that the three-protein complex was predominantly nuclear, thus indicating its function within the plant cell nucleus, possibly by binding naked T-strands and blocking their conversion into double-stranded intermediates. This notion was further supported by the inhibitory effect of RFA expression on the cell-to-cell movement of Bean dwarf mosaic virus, a single-stranded DNA virus. The observation that RFA complex plants dramatically inhibited the transient expression level of T-DNA and only reduced T-DNA integration by 50% suggests that double-stranded T-DNA intermediates, as well as single-stranded T-DNA, play significant roles in the integration process.

Genetic transformation of plant cells by Agrobacterium tumefaciens represents a unique case of transkingdom DNA transfer (Stachel and Zambryski, 1989; Tzifra and Citovsky, 2002; Lacroix and Citovsky, 2013). This bacterium creates crown galls by transforming plant cells with a part of its Ti plasmid, the transfer DNA (T-DNA), at the infection site. The T-DNA, which integrates into the plant genome, contains genes encoding for enzymes involved in the production, modification, and alteration of plant growth regulators and in the production of tumor-specific metabolites called opines. Expression of the integrated T-DNA, therefore, results in uncontrolled cell division and the formation of tumors (Gaudin et al., 1994; Das, 1998).

The route that takes the T-DNA from the bacterium to the plant cell nucleus occurs in three major steps. It begins inside A. tumefaciens, where the virulence proteins VirD1 and VirD2 are responsible for processing the single-stranded T-DNA. The T-strand with one VirD2 molecule covalently attached to its 5’ end (Herrera-Estrella et al., 1988; Young and Nester, 1988) is then exported, together with several other virulence proteins, through the bacterial type IV secretion system, where it is most likely coated with many VirE2 molecules, becoming the transported form of the T-DNA, the transport complex (Dumas et al., 2001). VirE2 is probably translocated to the plant cell independently of the VirD2-conjugated T-strand along with other A. tumefaciens effector proteins (Otten et al., 1984; Sundberg et al., 1996). Moreover, VirE2 binds to the T-strand in the plant and thus provides protection from nuclease attack inside the plant cells (Yusibov et al., 1994). Abu-Arish et al. (2004) and Citovsky et al. (1997) used the bacteriophage M13 single-stranded DNA (ssDNA) mixed with purified VirE2 molecules to gain direct insight into the transport complex structure. Electron microscopy and single-particle image-processing methods yielded a better understanding of the transport complex structure. During the last step on the way to the nucleus, VirD2 and...
VirE2 are thought to assist the import of the transport complex into the host cell nucleus (Howard et al., 1992; Rossi et al., 1993; Citovsky, 1994; Ziemienowicz, 2001), whereas phosphorylated VirE2 INTERACTED PROTEIN1 (VIP1; Djamei et al., 2007; Dafny-Yelin et al., 2008) functions as a molecular linker between VirE2 and KARYOPHERIN-α (KAPα; Tzfira and Citovsky, 2002), allowing VirE2 to use the host cell nuclear import machinery (Tzfira et al., 2001; Citovsky et al., 2004; Anand et al., 2007).

Once inside the nucleus, the transport complex is the substrate destined for integration. Two different models have been suggested for the T-DNA integration, double-stranded break (DSB) repair and single-stranded gap repair (SSGR; Gheyseyn et al., 1991; Mayerhofer et al., 1991). The DSB model predicts that unwound ends of a double-stranded T-DNA molecule anneal with single-stranded overhangs of DSBs in the plant DNA, the residual 5' and 3' overhangs are removed, and the inserted T-DNA is ligated. Mutations in the Arabidopsis (Arabidopsis thaliana) RADIATION-SENSITIVE52 (RAD52) gene that plays an essential role in homology-dependent DSB repair and recombination (Symington, 2002) decrease the levels of intrachromosomal recombination compared with wild-type plants (Samach et al., 2011). Recent studies further supported the notion that the T-strand molecule becomes double stranded prior to integration. For example, a direct indication of the involvement of double-stranded integration intermediates came from the detection of T-DNA inserts within the sites of DSB repair in the host DNA, which suggested ligation of double-stranded T-DNA molecules to both sides of the break made using a rear-cutting restriction enzyme (Salomon and Puchta, 1998; Chilton and Que, 2003; Tzfira et al., 2003). Recent evidence shows that T-DNA molecules can be converted into double-stranded T-DNA molecules using T-strand priming (Liang and Tzfira, 2013), and while double-stranded T-DNA molecules may serve as substrates for integration (Chilton and Que, 2003; Tzfira et al., 2003), a recent study showed that double-stranded T-DNA molecules can circularize and may end up as a dead ends for the integration process (Singer et al., 2012). The SSGR model was established as the dominant model for T-DNA integration in plant cells based on two major observations. First, VirD2 is able not only to digest but also to rejoin single-stranded substrates in vitro (Pansegrau et al., 1993). This led to the suggestion that VirD2 may function as the T-DNA ligase in plant cells and, by implication, that T-DNAs integrate as single-stranded molecules. Second, specific mutations at the VirD2 putative integrase motif resulted in small deletions at the 5' end of the T-DNA molecules (Tinland et al., 1995). Therefore, Tinland et al. (1995) revisited the SSGR integration model to the microhomology-dependent integration model, suggesting that only short sequences of the T-DNA molecule actually anneal to preintegration genomic sites (Tzfira et al., 2004a; Windels et al., 2008; Ziemienowicz et al., 2008).

The DSB model suggests that the transport complex is first stripped of its attached protein in the nucleus and then arrives at the integration site as double-stranded DNA (dsDNA). However, in the SSGR model, the transport complex itself reaches the site of integration and then probably degrades during the integration process. Evidence for a physical connection between the transport complex and the chromatin was presented by Loyter et al. (2005), who found that VIP1 acts as a molecular linker between the transport complex and the histone constituents of the host chromatin. When the transport complex reaches the chromatin, VIP1 becomes poly-ubiquitinated by VirF or Skp1/Cullin/F-box protein VIP1-binding F-box [SCF(VBF); Zaltsman et al., 2010, 2013] containing a proteolysis complex, and after the protein complex is degraded, the single-stranded T-DNA remains naked, with only VirD2 attached to its right border (Magori and Citovsky, 2011).

We overexpressed the ssDNA-binding replication factor A (RFA) from yeast (Saccharomyces cerevisiae) in order to further characterize the route that takes the transport complex to the integration site through double- or single-stranded forms. Previous work shows that overexpression of an ssDNA-binding protein from Escherichia coli (i.e. the recombination protein A) can function in plant cells and can interact with or supplement the endogenous plant recombination machinery (Reiss et al., 1996).

RFA from yeast is a tightly associated complex composed of three subunits of 69, 36, and 13 kD, termed RFA1, RFA2, and RFA3, respectively (Longhese et al., 1994). Hays et al. (1998) suggested a role for RFA in recruiting DNA polymerase to the site of a DSB via simultaneous interactions with RAD52 and a repair polymerase, in order to protect the exposed single-stranded ends from cellular nucleases (Smith and Rothstein, 1995; Park et al., 1996; Hays et al., 1998). RFA heterologous proteins have been found in plants, but their function in T-DNA integration and their involvement in the DNA-repair mechanism are still unknown. In this study, we investigated the effect of the RFA complex from yeast on the T-DNA integration mechanism in the plant cell nucleus and report that overexpression of the RFA complex (Rex) in tobacco (Nicotiana benthamiana) plants almost completely blocks the T-DNA's transient and reduced stable transformation, probably by binding to single-stranded T-DNA, and prevents its expression and integration through the DSB pathway. Moreover, we show that the movement of Bean dwarf mosaic virus (BDMV; Begomovirus: Geminiviridae), a single-stranded virus, is inhibited in the Rex-transformed plant.

RESULTS

Overexpression of RFA from Yeast in the Transgenic Plant

We constructed transgenic tobacco plants overexpressing the yeast RFA subunits RFA1, RFA2, and RFA3 under three different strong constitutive promoters, Actin2 (Act2), nopaline synthase promoter (nosP), and tandem 35S, respectively (Fig. 1A), in order to study the route taken by the T-strand to integration. A total of three independently
transformed transgenic lines from *N. benthamiana* were produced and confirmed by PCR. Moreover, two independent transgenic lines that overexpress only the RFA3 subunit of the RFA complex were tested as a control. Figure 1B shows PCR analysis of the three tobacco transgenic lines Rex-A, Rex-B, and Rex-C in order to confirm the presence of the *Neomycin phosphotransferase II* (*nptII*) gene as well as the three RFA subunits. For a control, PCR analyses were performed on plasmid and on wild-type plants. Each transgenic line represents a different transformation event. In addition, Figure 1C shows northern-blot analysis of the total RNA obtained from wild-type, Rex-A, Rex-B, and Rex-C plants that detected a high level of the *nptII* transcript only in the Rex transgenic plants.

**The Yeast RFA Complex Accumulates in the Nucleus of the Rex Transgenic Plants**

In order to elucidate the function of the yeast RFA complex in planta and to examine its involvement in the T-DNA integration route, we first looked at the localization of each RFA subunit in the wild-type and Rex transgenic plants. Interestingly, fusion of RFA subunits with the reporter gene ENHANCED GFP (EGFP) showed that each subunit (RFA1, RFA2, and RFA3) was expressed in the nucleus and cytoplasm in wild-type plants (Fig. 2, B, E, and H, respectively). In the control experiment, the EGFP protein was expressed in the nucleus as well as in the cytoplasm (Fig. 2K), whereas VIP1-EGFP accumulated mainly in the nucleus (Fig. 2N). In all experiments, the position of the plant cell nucleus was confirmed by nucleus-specific staining with 4’,6-diamino-phenylindole (DAPI; Fig. 2, A, D, G, J, and M), and plant chloroplast autofluorescence was detected in red in the right column (Fig. 2, C, F, I, L, and O). The yeast RFA complex binds to ssDNA as a complex, with multiple functions in DNA replication, repair, and genetic recombination in the nucleus (Jeong et al., 2003). Figure 3A demonstrates that when the three different subunits were cloned and expressed in plasmids pGAD424 and pSST91, they did indeed interact with each other. However, in the yeast two-hybrid analyses, self-interaction was observed only for RFA1 and RFA3. Under the nonselective conditions (i.e., in the presence of His), all combinations of the tested proteins resulted in efficient cell growth (Fig. 3A, bottom). Moreover, microbombardment of the yeast subunits RFA1, RFA2, and RFA3 fused with EGFP onto a Rex-A transgenic plant showed that EGFP is expressed mainly in the nucleus (Fig. 3, C, F, and I, respectively). In all experiments, the position of the plant cell nucleus was confirmed by nucleus-specific staining with DAPI (Fig. 3, B, E, and H), and chloroplast autofluorescence was detected in red in the right column (Fig. 3, D, G, and J). Additional support for the activity of the yeast RFA complex in the Rex-transformed plant nucleus can be explained by the observation that overexpression of the complex in Arabidopsis resulted in decreased sensitivity to methyl methane sulfonate (MMS), a DNA-damaging agent. The wild-type plants were hypersensitive to 100 μL L⁻¹ MMS, whereas Arabidopsis Rex plants exhibited enhanced resistance to MMS and were only hypersensitive to 150 μL L⁻¹ MMS, indicating that heterologous and high expression of yeast RFA may interfere with the host DNA-repair machinery, probably by interacting with exposed ssDNA at genomic break sites in the nucleus.

**Homologous Recombination Is Reduced in Rex Plants**

We used a homologous recombination repair assay (Gherbi et al., 2001; Endo et al., 2006) based on the microhomology-dependent integration model in order to reveal the effect of the yeast RFA complex on recombination events in the plant cell. The constructs used for homologous recombination contained the 35S promoter (35SP) and the 5’ or 3’ region of the GUS gene followed by the 35S terminator (35ST). The two parts of the GUS gene share homology of over 700 bp, but the GUS gene can only be expressed after a homologous recombination event between the two constructs (Fig. 4A). Interestingly, since RFA functions in the homologous recombination pathway in yeast and mammalian cells (Park et al., 1996; Debruwère et al., 2001), plants infected with the two constructs demonstrated a decrease in recombination events, as reflected by the number of blue spots in Rex transgenic plant lines A, B, and C (Fig. 4, B, C, and D, respectively) relative to the wild type (Fig. 4E).

**Transient Expression through the ssDNA Intermediate Is Blocked in Rex Plants**

We performed a transient expression assay with the 35SP-intron-GUS (Fig. 4F), which can be expressed...
only after splicing of the intron in the nucleus, in order to understand the cause of fewer homologous recombination events. GUS expression was suppressed in Rex transgenic lines A, B, and C (Fig. 4, G, H, and I, respectively) but not in RFA3 transgenic plants (over-expression of only the RFA3 subunit) lines A and B (Fig. 4, J and K, respectively) or in wild-type plants (Fig. 4L). T-DNA expression using *A. tumefaciens* infiltration was probably blocked, since the RFA complex bound to single-stranded T-DNA in the plant nucleus and prevented the conversion to dsDNA as well as its expression. In order to confirm this hypothesis, we bombarded the tobacco plant with 35SP-intron-GUS (as a dsDNA). Indeed, GUS was expressed in Rex lines A and B (Fig. 4, M and N, respectively) as well as in RFA3-A, RFA3-B, and wild-type plants (Fig. 4, O, P, and Q, respectively). These results confirmed that the RFA complex in the Rex plants does not interfere with double strand expression.

### Decreased Susceptibility of Rex Plants to *A. tumefaciens* Transformation

In order to further investigate foreign gene expression in Rex transgenic plants, they were tested for their susceptibility to *A. tumefaciens* infection and stable transformation by their ability to regenerate following genetic transformation with *A. tumefaciens*. Leaf discs derived from Rex line A and from the wild-type plants were inoculated with *A. tumefaciens* carrying the pBIGHYG plasmid with the marker genes for hygromycin resistance (hpt) and GUS enzymatic activity (uidA) on its T-DNA and cultured on regeneration medium in the presence of hygromycin, to allow regeneration and growth of the transgenic shoots. Rex transgenic plants produced fewer shoots than the wild-type plant (Fig. 5, A and B, respectively) 1 month after inoculation with *A. tumefaciens* (optical density at 600 nm = 0.25). The fresh weights of more than 26 leaf discs were quantified, and the average fresh weight per leaf disc (Fig. 5C) showed 46% inhibition in the Rex plants compared with the wild type. Interestingly, a higher concentration of *A. tumefaciens* culture of optical density at 600 nm = 0.45 resulted in only 20% inhibition, whereas a lower concentration of optical density at 600 nm = 0.1 resulted in the maximum inhibition of 61%. In order to confirm that shoot regeneration in the second transformation was not due to Rex plant resistance to hygromycin, we regenerated leaf discs of Rex and wild-type plants (without *A. tumefaciens* infection) on hygromycin regeneration medium. Indeed, as can be seen in Figure 5, H and G (Rex and wild-type plants, respectively), no shoots regenerated on the selection medium. Moreover, shoots from Rex and wild-type leaf discs (Fig. 5, A and B, respectively) were analyzed for...
GUS activity by histochemical staining of the explants and calli in order to confirm the presence of the uidA gene in the transgenic tissues. Figure 5, D and E, show that the hygromycin-resistant shoots regenerated from A. tumefaciens-infected Rex transgenic and wild-type plants, respectively, and efficiently expressed the uidA transgene, as reflected by blue staining of the entire callus and shoot. In control experiments, leaf discs from uninfected Rex and wild-type plants (Fig. 5, F and G, respectively) were grown on selection medium. However, no shoots were produced in the absence of A. tumefaciens infection, ruling out the possibility that RFA expression induced hygromycin resistance in the Rex plants and enabled them to regenerate untransformed shoots even on selective medium. An additional control experiment showed that shoots of Rex transgenic and wild-type plants are indistinguishable in the ability of an uninfected leaf disc to regenerate shoots on regeneration medium without any selection (Fig. 5, H and I, respectively).

**BDMV Movement Is Inhibited in Rex Plants**

The efficiency of RFA complex binding to single-stranded T-DNA molecules in Rex transgenic plants was also expected to affect the infection of an ssDNA virus. In order to test this hypothesis, Rex-A transgenic plants (*N. benthamiana*) were tested for their susceptibility to BDMV infection following replacement of the Coat Protein (CP) with the reporter gene EGFP. Transgenic and wild-type plants were bombarded with two mixed plasmids, BDMV DNA-A(EGFP) and DNA-B, which is responsible for the virus’s cell-to-cell movement. The spread of EGFP in the plant leaf was recorded 4 d after infection of the Rex-A and wild-type tobacco plants. As can be seen in Figure 6, the infected tissue in the Rex transgenic plant (Fig. 6, A and E) contained fewer clustered green fluorescent cells relative to the wild type (Fig. 6, H and K). The maximum number of fluorescent cells that could be detected in a few layers by confocal microscopy were counted in 34 and 18 clusters of Rex-A transgenic and wild-type plants, respectively, and the average ± SE is presented in Figure 6N, revealing a significant difference between them. In conclusion, BDMV movement was inhibited in 70% of the Rex plants relative to the wild type, probably due to RFA complex binding to the ssDNA form of the virus and prevention of its cell-to-cell movement.

**DISCUSSION**

*A. tumefaciens* is a unique organism that is capable of genetically transforming many types of eukaryotic cells. In order to achieve its goal of transferring its T-DNA molecule and integrating it into the host cell genome, the T-DNA in the plant cell is covered with bacterial proteins: VirD2 attaches to the T-DNA right border (Dürrenberger et al., 1989) and VirE2 binds to the ssDNA molecules with strong cooperativity, forming the T-DNA complex (Citovsky et al., 1989). The T-DNA complex is then transferred to the plant nucleus, recruiting the plant proteins VIP1 and KAPa (Tzifra et al., 2002; Djamei et al., 2007). In the nucleus, the transport complex interacts with H2A histone and CHROMATIN ASSEMBLY FACTOR1 (Mysore et al., 2000; Kirik et al., 2006), host proteins that have been suggested to facilitate its movement to points of integration (Bakó et al., 2003; Li et al., 2005) and its dissociation from VIP1 and VirE2 by the VirF-containing Skp1-Cdc53-cullin-F-box complex for...
proteolysis (for review, see Tzifra et al., 2004b; Dafny-Yelin et al., 2008). The last steps of the transformation process (i.e. the conversion of the T-DNA into a double-stranded molecule and its integration into the host cell genome or its integration as an ssDNA into a single-stranded gap) are probably affected exclusively by the host proteins and the host cell’s DNA machinery (Tzifra et al., 2004a). Nevertheless, the route for T-DNA integration in plants remains mostly unknown. In this study, we created transgenic plants that overexpress the

Figure 4. RFA transient gene expression decreases the susceptibility of Rex plants to A. tumefaciens infection. A to E, Leaves of tobacco wild-type and Rex plants were filtrated simultaneously with the nontumorigenic A. tumefaciens strain EHA105 carrying 35SP-GUS or (GUS-35ST (A). A recombination event between two regions of homology was visualized upon histochemical staining as a blue sector. B to D represent infected Rex transgenic lines A to C, respectively. E represents the wild-type plant. F to L. Leaves of wild-type and Rex plants were filtrated with the non-tumorigenic A. tumefaciens strain EHA105 carrying pBISN1 (containing the uidA intron gene within the T-DNA; F). G to I represent infected Rex transgenic lines A to C, respectively. J and K represent RFA3 transgenic plant lines A and B, respectively. L represents the wild-type plant. M to Q. Leaves of wild-type and Rex plants were microbombarded with the plasmid pBISN1. Recombination events between two regions of homology were visualized upon histochemical staining as blue sectors. M and N represent microbombarded leaf discs from the RFA transgenic plant lines A and B, respectively. O and P represent RFA3 subunit transgenic plant lines A and B, respectively. Q represents the wild-type plant. Restoration of transgene activity is visualized upon histochemical staining as blue spots. Bars = 1 mm.

Figure 5. RFA transgenes decrease the susceptibility of Rex plants to A. tumefaciens infection. A and B. Regeneration of stably transformed shoots. Shoots were photographed and weighed 1 month after infecting leaf discs with A. tumefaciens EHA105 carrying pBIGHYG (containing the uidA gene and the plant hpt gene). A. tumefaciens-infected discs from the RFA transgenic (A) and wild-type (B) plants were grown on hygromycin-containing selective medium. C. Quantification of leaf disc weight from Rex and wild-type (WT) plants. Average fresh weight (mg) is presented ± se. *P < 0.05; n, number of discs that were tested. D and E, GUS staining of shoots from A. tumefaciens-infected Rex (D) and wild-type (E) plants grown on hygromycin-containing selective medium for 1 month. F and G, Uninfected leaf discs from Rex transgenic (F) and wild-type (G) plants grown on hygromycin-containing selective medium. H and I, Shoots from uninfected RFA transgenic (H) and wild-type (I) plants regenerated without selection on regeneration medium.
yeast RFA complex (Rex plant) in order to elucidate the route by which T-DNA integrates into the plant genome through DSBs or SSGRs. The RFA subunits (RFA1, RFA2, and RFA3) function as a complex in yeast cells that is capable of binding ssDNA molecules as well as promoting the repair of DSBs (Firmenich et al., 1995).

In this study, we report that Arabidopsis Rex plants were more sensitive to the DNA-damaging agent MMS than wild-type plants, indicating that high expression of the yeast RFA complex in plants may interfere with the host DNA-repair machinery, probably by interacting with exposed DNA strands at genomic break sites. Interestingly, overexpression of the yeast RFA complex in tobacco did not affect plant sensitivity to MMS but had a dramatic effect on T-DNA integration. The yeast RFA complex plays a role in DNA repair and T-DNA integration through the homologous recombination pathway (Firmenich et al., 1995; Nagaraj and Norris, 2000; Debrauwère et al., 2001; van Gent et al., 2001). Here, we showed, using a divided *uidA* gene that was expressed only following homologous recombination between a shared sequence and regeneration of the GUS sequence, that GUS expression is inhibited in the Rex-transformed plants. Moreover, the GUS intron’s transient expression following delivery to the plant by *A. tumefaciens* infiltration was dramatically reduced in the Rex plant. These results suggest that the yeast RFA complex probably does not function in T-DNA recombination in the Rex plant. Moreover, it prevents the expression of the T-DNA, probably by binding to single-stranded T-DNA and preventing the synthesis of the complementary strand. Indeed, GUS expression was prevented, and no differences were observed in GUS expression between Rex and wild-type plants after delivering the construct as dsDNA by microbombardment of the pBIHYG plasmid. To further support the conclusion that the Rex plants inhibit ssDNA, we tested the movement of an ssDNA

![Figure 6. Inhibition of BDMV cell-to-cell movement in Rex plants. A to L, Leaves of Rex transgenic (A–F) and wild-type (G–L) plants were bombarded with the mixed plasmids: pRCS2 1.8 \times BDMV-B and pRCS 1.9 \times BDMV-A/CP/EGFP (where the CP was replaced with EGFP). Confocal images were taken 4 d after bombardment. The left column shows EGFP signal (green), the middle column shows chloroplast autofluorescence signal (red), and the right column shows merged images. Bars = 20 μm. N, Quantification of the number of green cells (from EGFP signal) in each cluster from Rex and wild-type (WT) plants. The average of green cells in more than 17 clusters is presented ± s; n, number of clusters tested.](image-url)
virus, the BDMV. Geminivirus DNA replication occurs in two stages. First, the ssDNA is converted into dsDNA with the involvement of cellular factors, and the dsDNA serves as a template for viral gene expression. Second, the dsDNA initiates the rolling-circle phase, with the participation of viral and cellular factors, to produce new ssDNA products that can reenter the DNA replication pool or spread in the plant tissue as ssDNA (Gutierrez, 2000). Indeed, the cell-to-cell movement of the BDMV, as observed 4 d after infection with the modified virus (replacing the CP with the EGFP reporter gene), was inhibited dramatically (70% inhibition).

We fused the RFA subunits to GFP in order to elucidate where ssDNA blockage occurs. As can be seen, each subunit is expressed separately in the cytoplasm and nucleus and can bind to ssDNA, whereas the RFA1 subunit binds stably to ssDNA on its own, and RAF2 and RFA3 contain a single ssDNA-binding site but do not bind stably (Philipova et al., 1996). However, since the RFA is a trimer that is expressed mainly in the plant nucleus as a complex, as indicated after micro-bombardment when the RFA subunits fused to GFP on the Rex plants, we propose that the main function of the yeast RFA complex is in the plant nucleus.

Transient expression through *A. tumefaciens* infiltration was dramatically inhibiting. In order to examine the effect of the yeast RFA complex on stable transformation, we infected leaf discs with different concentrations of *A. tumefaciens* carrying the binary plasmid pBIGHYG. Inhibition of explant formation is reduced as the initial concentration of the *A. tumefaciens* culture is increased. These results indicate that the capability of the RFA complex to block *A. tumefaciens* is limited to the number of RFA units that exist in the Rex plant’s tissue. Since transient GUS expression was blocked almost completely, whereas its stable transformation was inhibited by less than 50% (*N. benthamiana*), we suggest that T-DNA integration can occur in Rex plants, mainly through ssDNA intermediates, whereas in the wild-type plants, it can occur through both ssDNA and dsDNA.

In the wild-type plants, the single-stranded T-DNA can integrate into the genome as an ssDNA with the help of plant factors involved in DNA repair (Tinland et al., 1994, 1995) and chromatin structures (Endo et al., 2006), or it can be converted from naked single-stranded T-DNA into double-stranded T-DNA (De Neve et al., 1997; De Buck et al., 1999; Magori and Citovsky, 2011) with the help of a DNA or RNA primer and DNA polymerase (Liang and Tzfira, 2013). The model we propose in Figure 7 is that the RFA complex finds the naked T-DNA and binds to it before its conversion into dsDNA (Fig. 7A) while integration of the T-DNA as an ssDNA is not disturbed (Fig. 7B) and can even be promoted by the yeast RFA complex, which could assist the integration process with the help of RAD (Samach et al., 2011; Fig. 7C). The possibility of RFA

![Figure 7](image-url). *A. tumefaciens* T-DNA integration model. A, In unstable transformation in the nucleus, VirE2 and VIP1 are degraded, probably with the help of VirF or SCF(VBF), from the T-DNA complex. The stripped T-DNA, with the help of DNA or RNA primer and DNA polymerase, is converted into dsDNA and expressed. In the Rex plant, the naked T-DNA cannot be expressed, probably because the RFA complex is bound to the naked single-stranded T-DNA, thereby blocking its conversion into dsDNA and its ability for expression. B to D, Microhomology-dependent integration model in the Rex plant. According to this model, integration begins with the annealing of the T-strand’s 3’ end to the host genome at a single-stranded gap. The yeast RFA complex in the Rex plant either cannot be attached to the single-stranded T-DNA that is protected by the bacterial protein (B) or be bound to the naked single-stranded T-DNA and recruit a RAD complex to promote the integration (C). D presents a third option: RFA binds to the single-stranded T-DNA prior to integration and prevents integration into the ssDNA gap. It should be noted that this option is unlikely; if ssDNA were the only way of integration, we would not obtain any transformation events.
binding to ssDNA before integration, thereby preventing it (Fig. 7D), does not seem likely; if integration through ssDNA were the only means of integration, we would not see any stable transformation events in the transgenic plants. Moreover, integration via dsDNA intermediates probably occurs via naked T-DNA being converted into dsDNA, whereas integration through an ssDNA intermediate into a single-stranded gap occurs step by step, and the T-DNA is covered with bacterial proteins or plant proteins but does not remain unprotected as an ssDNA. The exact role of the plants’ RFA-like proteins in the integration process is still unknown. However, data suggest that REPLICA PROTEIN A (RPA) may participate in the DNA replication of Mung bean yellow mosaic India virus (Singh et al., 2007) and somatic DNA repair (Chang et al., 2009). The large RPA subunit family has been implicated in having a role in DNA repair (Aklilu et al., 2014). Since plants code for multiple RFA1, RFA2, and RFA3 subunits (Ishibashi et al., 2006; Shultz et al., 2007; Aklilu et al., 2014), further analysis of the T-DNA integration process is required in order to determine the precise role of specific plant proteins in this process.

MATERIALS AND METHODS

Construction of the prCS2-nptII[RFA1][RFA2][RFA3]

Binary Plasmid

For coexpression of the nptII gene and the three RFA subunits, we first produced the plasmids pSAT1(A).ocsAocsP.nptII.ocsT and pSAT4.35SP.yRFA3.35ST, pSAT5(A).ocsP.RFA2.ocsT, and pSAT5(A).act2P.yRFA1.agsT, from pSAT2 nosP.RFA2 nosT (A as an AsclI fragment. The yRFA1 expression cassette was then transferred as a 1-Coi fragment from pSAT5(A), act2p.yRFA1.agsT. Finally, we transferred the yRFA3 expression cassette as a 1-Scl fragment from pSAT4.35SP.yRFA3.35ST.

Production and Analysis of Transgenic Plants

Binary plasmids were introduced into Agrobacterium tumefaciens strain EHA105 using the calcium chloride transformation protocol (Tzfira et al., 1997). The resulting bacterial cultures were used to genetically transform tobacco (Nicotiana benthamiana) leaf discs according to Horsch et al. (1985). Transgenic plants were produced using the pRCS2[KAN][RFA1][RFA2][RFA3] binary plasmid and selected in the presence of 25 μg mL⁻¹ kanamycin. Plants were maintained in tissue culture on basal Murashige and Skoog medium (Murashige and Skoog, 1962) with no exogenous growth regulators. Selected lines were cultivated in commercial compost in a regular growth chamber.

PCR analyses were performed to confirm transgenic plants using specific primers to each gene: nptII, 5'-ATGATGGAACAAAGTTATGC-3' and 5'-TCGAGAGCTCTGCAAGAAG-3'; RFA1, 5'-AGAGGAGCTTGACC-TTCGA-3' and 5'-TTAATCTACAACACCAACTGGAT-3'; RFA2, 5'-AGCGAACATCACAACATACAAC-3' and 5'-CTCATGGCCAAAGATGATGTC-3'; and RFA3, 5'-AGGCCACGAAACACAAAG-3' and 5'-CTTATATATTCTGGGTATT-3'.

Transient Expression Experiments

For transient expression, attached leaves were infected with A. tumefaciens EHA105 (A 500 = 0.45) carrying pBSNI, a GUS intron-expressing binary vector (which expresses the GUS gene only after splicing in plant cells), by microbombardment for 24 h and stained with 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide. In the homologous recombination assay, attached leaves were infected with A. tumefaciens EHA105 harboring pRCS.35SP.GUS (containing the 5' region of the GUS gene) and pRCS.(G)US.35ST (containing the 3' region of the GUS gene): the two constructs share an approximately 700-bp homologous region. pRCS.35SP.GUS was constructed by cloning the expression cassette from pSAT4(A).35SP.GUS (A as a 1-Coi fragment in the plasmid pRCS. G)US.35ST was constructed by cloning the cassette from pSAT4(A).GUS.35ST as a 1-Coi fragment into pRCS. The GUS fragment was amplified with the primers 5'-GAATATCCGTCGTCCTGAGAANCC-3' and 5'-GAATATCCGTCGTCCTGAGAANCC-3', cut with EcoRI and KpnI, and cloned into pSAT4(A).35SP.mcs as an EcoRI-KpnI fragment. The GUS gene was amplified with primers 5'-GAATATCCGTCGTCCTGAGAANCC-3' and 5'-CGGATCCCTGCTGAGAANCC-3', cut with EcoRI and BamHI, and cloned into pSAT4(A).mcs.35ST as an EcoRI-KpnI fragment.

Stable Expression Experiments

The binary plasmid pBIGHYG containing the uidA gene and the plant hygromycin resistance gene was introduced into A. tumefaciens strain EHA105 using the calcium chloride transformation protocol (Tzfira et al., 1997). The resulting bacterial cultures were used to genetically transform leaf discs from N. benthamiana wild-type and Rex transgenic plants according to Horsch et al. (1985). Transgenic plants were produced carrying pBIGHYG and selected in the presence of 20 μg mL⁻¹ hygromycin. The leaf discs were photographed, weighed at 26 d after transformation, and stained with 5-bromo-4-chloro-3-indolyl-ß-D-glucuronide.

RNA Gel-Blot Analysis

Total RNA was isolated from approximately 200 mg of leaf tissue using Tri-Reagent (Sigma). RNA samples (10 μg lane⁻¹) were electrophoresed on a 1.2% formaldehyde/agarose gel, blotted onto Hybond N+ membranes, and probed with a digoxigenin-labeled nptII probe followed by autoradiography using standard hybridization and detection protocols. Ribosomal RNA within the analyzed RNA preparation was detected by ethidium bromide staining of agarose gels and served as an internal control for equal loading of the lanes.

Construction, Expression, and Localization of RFA Subunits

The coding region of yeast RFA1, RFA2, and RFA3 was amplified by PCR and inserted into plasmid pSAT6-EGFP-C1 (Dalny-Yelin and Tzfira, 2007), from pSAT2 nosP.RFA2 nosT(A) as an AsclI fragment. The yRFA1 expression cassette was then transferred as a 1-Coi fragment from pSAT5(A), act2p.yRFA1.agsT. Finally, we transferred the yRFA3 expression cassette as a 1-Scl fragment from pSAT4.35SP.yRFA3.35ST.
allowing in-frame cloning of RFA1, RFA2, and RFA3 upstream of EGFP and providing the fusion proteins RFA1-EGFP, RFA2-EGFP, and RFA3-EGFP. Transient expression of EGFP fusions in tobacco leaves was evaluated following bombardment using the Helios Gene Gun (Bio-Rad) at a pressure of 120 psi. EGFP was detected 24 h after bombardment using a Leica confocal microscope (TCS, SP5).

**Yeast Two-Hybrid Assays**

Yeast strain L40 [MATa his3A200 trp1-901 leu2-3,112 ade2-1 lys2-801 URA3: (lexAop)2, Uas2 LYS2:(lexAop)2 HIS3] (Hollenberg et al., 1995) was grown in yeast extract/peptone/dextrose or in the appropriate selective minimal medium under standard conditions (Kaiser et al., 1994). Plasmids were introduced into yeast cells using a standard lithium acetate protocol (Kaiser et al., 1994). The yeast RFA1, RFA2, and RFA3 were cloned into pSST91 (Ballas and Citovsky, 1997) and transformed into yeast. Interactions between pairs of proteins were examined by a second transformation with pGAD424 (LEU3+; Clontech; Ballas and Citovsky, 1997) that contains the yeast RFA1, RFA2, or RFA3. The double-transformed yeast was selected on His/Trp/Leu-deletion medium (with the amino acid), whereas pSST91-RFA2 required 5 μM 3-amino-1,2,4-triazole to prevent self-activation.

**BDMV-EGFP Movement**

The plasmids pRCS2 1.8 × BDVM-B and pRCS 1.9 × BDVM-ACP-EGFP (where the CP was replaced with EGFP) were mixed, and the transient expression of EGFP in wild-type and Rex-transformed tobacco leaves was evaluated following bombardment using the Helios Gene Gun (Bio-Rad) at a pressure of 120 psi. EGFP was detected 4 d after bombardment using a Leica confocal microscope.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers: RFA1 (X59748), RFA2 (X59749), and RFA3 (X59750).

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