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LARGE-SCALE BIOLOGY ARTICLE

Screening a cDNA Library for Protein–Protein Interactions Directly in Planta[W]

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Screening cDNA libraries for genes encoding proteins that interact with a bait protein is usually performed in yeast. However, subcellular compartmentation and protein modification may differ in yeast and plant cells, resulting in misidentification of protein partners. We used bimolecular fluorescence complementation technology to screen a plant cDNA library against a bait protein directly in plants. As proof of concept, we used the N-terminal fragment of yellow fluorescent protein– or nVenus-tagged *Agrobacterium tumefaciens* VirE2 and VirD2 proteins and the C-terminal extension (CTE) domain of *Arabidopsis thaliana* telomerase reverse transcriptase as baits to screen an *Arabidopsis* cDNA library encoding proteins tagged with the C-terminal fragment of yellow fluorescent protein. A library of colonies representing ~2 × 10^5 cDNAs was arrayed in 384-well plates. DNA was isolated from pools of 10 plates, individual plates, and individual rows and columns of the plates. Sequential screening of subsets of cDNAs in *Arabidopsis* leaf or tobacco (*Nicotiana tabacum*) Bright Yellow-2 protoplasts identified single cDNA clones encoding proteins that interact with either, or both, of the *Agrobacterium* bait proteins, or with CTE. T-DNA insertions in the genes represented by some cDNAs revealed five novel *Arabidopsis* proteins important for *Agrobacterium*-mediated plant transformation. We also used this cDNA library to confirm VirE2-interacting proteins in orchid (*Phalaenopsis amabilis*) flowers. Thus, this technology can be applied to several plant species.

INTRODUCTION

The elucidation of protein–protein interactions is essential for understanding biological processes. Proteins frequently form transient or stable complexes with other proteins; these complexes mediate numerous aspects of cellular function. Scientists have developed numerous assays to detect and define protein–protein interactions. Although in vitro assays are useful, especially for quantifying biochemical parameters, in vivo assays present these interactions in their native milieu.

When known or suspected interacting partners have been identified, several techniques can be employed to define the nature and subcellular localization of complex formation. However, scientists frequently initiate their studies with a known protein and search for a protein(s) with which they form complexes. These searches can be conducted in vivo using biochemical approaches, such as tandem affinity purification tagging or coimmunoprecipitation, followed by identification of the interacting partners using analytical techniques (e.g., mass spectrometry).

Fields and Song (1989) described a genetic approach to define interacting proteins. This peptide complementation assay used two interacting proteins to bring together in yeast inactive transcription factor fragments to form a complex that would activate transcription of target reporter genes. The yeast two-hybrid system has subsequently become a routine technique to investigate protein–protein interactions. In addition to investigating interactions between two known proteins, one tagged with the transcription factor DNA binding domain (the bait protein) and the other with the transcription activation domain (the prey protein), the yeast two-hybrid system has been widely used to identify interacting prey proteins encoded by a cDNA library. Plant scientists have frequently taken advantage of this technique to identify novel proteins that interact with a known bait protein (Ballas and Citovsky, 1997; Bakó et al., 2003; Hwang and Gelvin, 2004; Tao et al., 2004).

Although peptide complementation (two-hybrid) assays have been developed in animal systems (Fearon et al., 1992; Shioda et al., 2000; Remy and Michnick, 2004; Ding et al., 2006), screening in yeast remains the favored route for identifying proteins that interact with a given bait protein. Screening in yeast is relatively rapid and simple, and a number of two-hybrid genetic systems have been developed for this organism (Bartel and Fields, 1997).

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However, screening plant proteins for interacting partners in yeast has several limitations. For example, some plant proteins may naturally interact in organelles, such as chloroplasts, absent from yeast. Some plant proteins may require posttranslational modification for interaction; such modifications may not occur in yeast. Conversely, protein–protein interactions that occur in yeast may not normally occur in plants because, for example, the proteins are naturally localized in different subcellular compartments and will not normally have the opportunity to interact. Thus, a rapid, convenient assay system to screen a bait protein against proteins encoded by a cDNA library directly in plants would provide an important addition to the arsenal of techniques used by plant biologists.

In this article, we describe a system that can be used to screen for protein interacting partners directly in planta. Although initially developed for use in *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) Bright Yellow-2 (BY-2) protoplasts, we show how this system can be employed in other plant species. We used this system to identify five novel *Arabidopsis* proteins important for *Agrobacterium tumefaciens*–mediated plant genetic transformation.

RESULTS AND DISCUSSION

Defining the System to Detect Protein–Protein Interactions in Planta

We chose bimolecular fluorescence complementation (BiFC) as a method to detect protein–protein interactions in planta. This technique was initially developed to detect interaction of transcription factors in animal cells (Hu et al., 2002). We (Citovsky et al., 2006; Lee et al., 2008) and others (Bracha-Drori et al., 2004; Walter et al., 2004; Bhat et al., 2006; Waadt et al., 2008; Gehl et al., 2009) subsequently adapted this assay system to investigate interaction of specific proteins in plant cells (reviewed in Ohad et al., 2007; Weinthal and Tzfira, 2009; Lee and Gelvin, 2012). However, scientists have not previously used this system to screen a cDNA library to identify unknown proteins, encoded by these cDNAs, that interact with a specific bait protein. BiFC is a peptide complementation assay in which nonfluorescent fragments of autofluorescent proteins, such as green fluorescent protein, yellow fluorescent protein (YFP), or their derivatives, are fused in frame with test proteins. Interaction of these test proteins may bring the YFP fragments into proximity such that they refold and restore fluorescence (fluorescence complementation; Hu et al., 2002).

BiFC is a sensitive assay that, after generating the bait and prey clones, requires only an efficient plant transformation system and a fluorescence imaging system, such as an epifluorescence or confocal microscope. As a transformation protocol, we initially chose the tape-*Arabidopsis*-sandwich leaf protoplast system (Wu et al., 2009). This protocol easily can be used to generate and transfect millions of high-quality mesophyll protoplasts from virtually any *Arabidopsis* genotype within a few hours. Samples are imaged for BiFC fluorescence the next day, permitting rapid screening of DNA from progressively more specific pools of cDNA clones. Supplemental Figure 1 online shows that this system is highly sensitive; a fluorescence signal is easily detected after transformation of <200 ng of a cloned

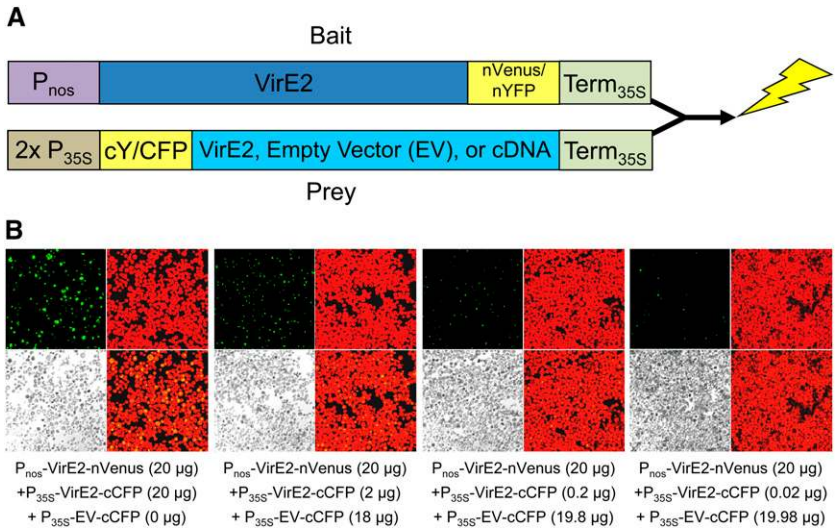

Figure 1. Sensitivity of the BiFC Reaction in *Arabidopsis* Leaf Protoplasts.

(A) Schematic representation of the constructions used in BiFC. Expression of VirE2-nVenus/nYFP is under the control of the relatively weak nopaline synthase (nos) promoter, whereas expression of VirE2-cCFP, cYFP-EV, or cYFP-cDNA protein is under the control of the strong CaMV double 35S promoter. $Term_{35S}$, CaMV 35S poly(A) addition signal. Interaction of VirE2-nVenus/nYFP with VirE2-cCFP or with a cYFP-cDNA–encoded protein may permit the two YFP-derived fragments to fold, generating yellow fluorescence.

(B) Interaction of various quantities of a plasmid encoding VirE2-cCFP (diluted with the EV plasmid) and 20 µg of a plasmid encoding VirE2-nVenus. The three plasmids were cotransfected into *Arabidopsis* leaf protoplasts and visualized after 16 h by confocal microscopy. For each set of images: top left, YFP fluorescence; top right, chlorophyll autofluorescence; bottom left, differential interference contrast image; bottom right, merged yellow fluorescence and chlorophyll autofluorescence images.

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YFP gene under the control of a cauliflower mosaic virus (CaMV) double 35S promoter into 10^5 protoplasts.

We next tested the sensitivity of this system for BiFC using known protein–protein interactors. During the process of *Agrobacterium*-mediated transformation, VirE2 protein is secreted through a Type IV secretion system from the bacterium into plant cells (Vergunst et al., 2003). VirE2 proteins can form multimers that aggregate in the plant cytoplasm, often as perinuclear rings (Bhattacharjee et al., 2008; Gelvin, 2010). We observed that overexpression of genes by the CaMV double 35S promoter could generate the C-terminal fragment of cyan fluorescent protein (cCFP)- and nVenus-tagged peptides that generate nonspecific (i.e., prey protein-independent) fluorescence complementation (see below). We therefore expressed one of the VirE2 fusion protein partners as an nVenus fusion from the relatively weak nopaline synthase promoter (Figure 1A; P_{nos}-VirE2-nVenus; the bait protein) and the other VirE2 fusion protein partner as a cCFP fusion from the strong CaMV 35S promoter (P_{35S}-VirE2-cCFP; the prey protein). We transformed 20 μg bait protein DNA into 10^5 *Arabidopsis* mesophyll protoplasts along with 10-fold serial dilutions of prey protein DNA. However, in each transformation, we kept the total amount of prey vector DNA constant (20 μg) by adding empty vector (EV) prey DNA (P_{35S}-EV-cCFP). Serial dilution of specific prey DNA by EV DNA thus mimicked dilution of a specific interacting cDNA clone among a population of noninteracting cDNA clones and served as a reconstruction experiment to determine the extent to which we could detect a cDNA encoding an interacting protein among cDNAs encoding noninteracting proteins.

Figure 1B shows that we could easily detect interaction of VirE2-cCFP encoded by 20 ng of prey DNA with VirE2-nVenus encoded by 20 μg of bait DNA. These reconstruction experiments therefore indicate that we can detect an interacting protein encoded by <0.1% of transfected cDNAs. In addition, because the percentage of transfected protoplasts displaying a BiFC signal decreased with decreasing amounts of VirE2-cCFP prey DNA but increasing amounts of cCFP EV DNA, these experiments indicate that expression of the bait protein from a gene under the control of the nopaline synthase promoter does not significantly generate a nonspecific BiFC signal. We discuss below experiments to optimize BiFC fluorescence signals between interacting bait and prey proteins, while simultaneously minimizing BiFC signals between noninteracting bait and prey proteins or between a bait protein and an EV construction.

We repeated these reconstruction experiments using 10 μg VirE2-nVenus as the bait and an *Arabidopsis* cDNA clone encoding the VirE2-interacting protein At4g15390 (HSR201-like protein; see below). We diluted this cDNA with another cDNA (At5g45620) encoding a protein (the 26S proteasome regulatory subunit RPN9) that does not interact with VirE2 (Figure 2, panel marked "0"). To quantify transformation efficiency and to mark cell nuclei, we cotransfected a plasmid expressing monomeric red fluorescent protein (mRFP)-VirD2NLS (Citovsky et al., 2006). Figure 2 shows that we could detect a BiFC fluorescence signal from a reaction in which the interacting cDNA clone was diluted 3840-fold with a noninteracting cDNA clone.

Screening an *Arabidopsis* cDNA Library for Proteins That Interact with *Agrobacterium* VirE2 Using BiFC

Having defined the sensitivity of the system, we next used the tape-*Arabidopsis*-sandwich protoplast system to screen an *Arabidopsis* cDNA library against VirE2-nVenus as the bait protein. We recombined a normalized, full-length cDNA library, in the plasmid pDONR221, into the destination vector pSAT5-DEST-cEYFP-C1(B), which tags proteins encoded by the cDNAs at their N termini with the C-terminal fragment of yellow fluorescent protein (cYFP) (Figure 1A). Following amplification of the library in such a way as to maintain normalization of the

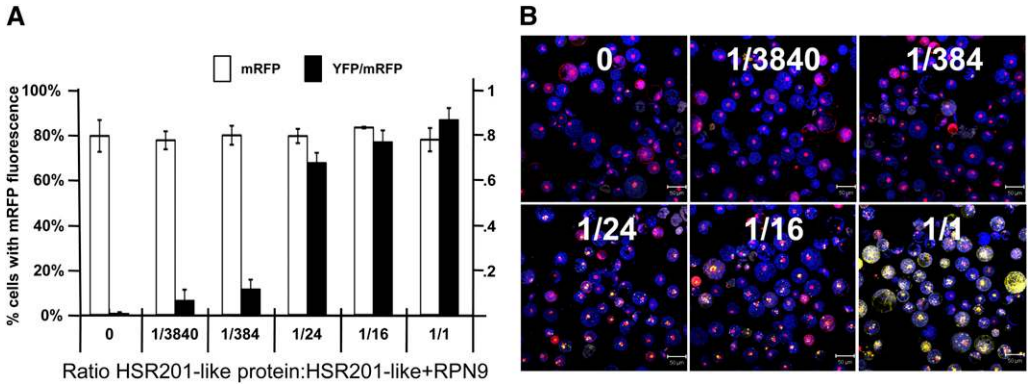

Figure 2. Reconstruction Reaction to Determine the Sensitivity of VirE2-cDNA Clone Interaction in BiFC.

(A) *Arabidopsis* leaf protoplasts were cotransfected with plasmids encoding mRFP-VirD2NLS (to mark transfected cells), VirE2-nVenus (10 μg; bait), and a total of 10 μg of a combination of a cDNA encoding a HSR201-like protein (At4g15390; the prey) plus cYFP-RPN9 (At5g45620; noninteracting protein) in various ratios (indicated on the abscissa). The cells were imaged by confocal microscopy after 16 h. The graph depicts the percentage of cells that were transfected (mRFP fluorescence) and the ratio of cells showing YFP:mRFP fluorescence. Note that the transfection efficiency was approximately the same in all experiments and that as the ratio of cYFP-HSR201-like protein:HSR201-like+RPN9 increases, a higher percentage of cells display YFP fluorescence.

(B) Merged confocal images of representative fields of transfected protoplasts. Ratios as in **(A)**. Red, mRFP fluorescence; yellow, YFP BiFC fluorescence; blue, false-color chlorophyll image. Bars = 50 μm.

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cDNAs (see below and Methods), we extracted DNA and cotransfected 20 μg of library DNA with 20 μg of a clone encoding VirE2-nVenus into *Arabidopsis* leaf mesophyll protoplasts. Using confocal microscopy, we detected, at low frequency, yellow fluorescent cells. A majority of these cells showed cytoplasmic, especially perinuclear fluorescence (Figure 3A), as previously detected in VirE2–VirE2 and VirE2–IMPa-1 interactions (Bhattacharjee et al., 2008; Lee et al., 2008). We occasionally detected nuclear fluorescence (Figure 3B), as previously seen with VirE2–IMPa-4 interaction (Bhattacharjee et al., 2008; Lee et al., 2008).

To identify clones encoding specific proteins that interact with VirE2, we arrayed ~10^5 cDNA colonies into 384 well microtiter plates. This initial arrayed library was used to screen for cDNAs encoding proteins that interact with VirE2 and VirD2 (see below) and is currently maintained at the Agricultural Biotechnology Research Center (Academia Sinica, Taipei, Taiwan). We extracted DNA from pools of 10 plates (~3800 individual cDNA clones) and used this DNA in a BiFC reaction with VirE2-nVenus. We additionally cotransfected the cells with a construction expressing the fusion protein mRFP-VirD2NLS (Lee et al., 2008). Red fluorescence generated by this clone indicated transfected cells and was used to calculate the ratio of YFP fluorescence (generated by the BiFC reaction) to red fluorescence (transfected cells). Although we could see some weak yellow fluorescence signals from many of the pools, DNA from pool number 1 yielded strong fluorescent signals (see Supplemental Figure 2 online), and we therefore concentrated our initial efforts on identifying VirE2 interacting cDNAs from this pool. To this end, we isolated DNA from each of the 10 plates comprising this pool and tested each of these DNA plate pools against a clone expressing VirE2-nVenus. Several plates contained clones encoding proteins that interacted with VirE2 to emit a weak yellow fluorescence signal. However, in initial experiments, DNA from plate 5 elicited a stronger BiFC signal than did DNA from other plates. We subsequently isolated DNA from rows and columns of this plate and repeated the procedure (see Supplemental Figures 3 and 4 online). Finally, we isolated DNA from specific colonies, tested these clones against the VirE2-nVenus–expressing plasmid (see Supplemental Figure 5 online), and sequenced individual cDNA clones. Supplemental Figure 6 online shows the results of these progressive enrichments for a particular clone expressing a protein that interacts with VirE2.

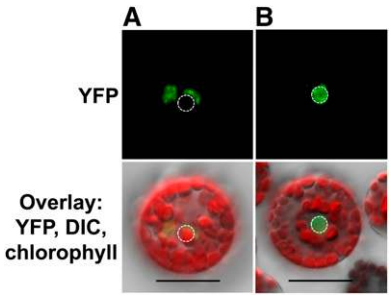

Figure 3. Subcellular Localization of YFP Fluorescence Generated by Interaction of VirE2-nVenus with Different cYFP-Tagged cDNAs.

Arabidopsis leaf protoplasts were transfected with 20 μg each of plasmids encoding VirE2-nVenus and a cYFP-tagged cDNA library. The cells were imaged by confocal microscopy 16 h later. Panels show examples of perinuclear **(A)** and nuclear **(B)** BiFC signals. White broken circle indicates the nucleus, which was identified visually from other images in the Z-stack. DIC, differential interference contrast. Bars = 20 μm.

These experiments indicated that plate 5 contains five clones encoding four different proteins that strongly interact with VirE2. Clone 5-2C (the plate number is denoted first, followed by the column and row numbers) corresponds to At1g08820 (annotated as a VAP33-like SNARE protein), clone 5-5E corresponds to At1g50320 (annotated as a thioredoxin), clones 5-9C and 5-23I correspond to At4g15390 (annotated as a HSR201-like protein), and clone 5-19G corresponds to At3g01120 (annotated as a cystathione γ-synthase). DNA sequence analysis of these clones indicated that they encode full-length open reading frames that are in frame with the N-terminal cYFP BiFC tag.

We next turned our attention to pool 1, plate 1. Protoplasts containing pooled cDNA clones from this plate also showed strong BiFC fluorescence signals when cotransfected with DNA encoding VirE2-nVenus. Successive rounds of purification identified two different cDNA clones, each encoding the same *S*-adenosyl-L-homocysteine hydrolase (At4g13940, *HOG1*). These clones similarly encode open reading frames in frame with the cYFP tag. HOG1 protein, also known as SAH1, is involved in DNA methylation and homology-dependent gene silencing (Rocha et al., 2005; Mull et al., 2006).

Screening an *Arabidopsis* cDNA Library for Proteins That Interact with *Agrobacterium* VirD2 Protein Using BiFC

Induction of the *Agrobacterium* virulence (*vir*) regulon by plant phenolic molecules results in the synthesis of VirD2, a protein essential for virulence. VirD2 nicks T-DNA border repeat sequences surrounding T-DNA and covalently attaches to the 5′ end of the resulting T-strand. VirD2 subsequently pilots T-strands through the type IV secretion apparatus into the plant and within the plant cell directs T-strands to the nucleus (reviewed in Gelvin, 2003). VirD2 may also play a role in T-DNA integration into the plant genome (Tinland et al., 1995; Mysore et al., 1998). Using yeast two-hybrid and plant-based BiFC systems, scientists have identified several plant proteins that interact with VirD2. Prominent among these plant proteins are members of the importin α family, which play a role in nuclear targeting of VirD2/T-strand complexes (Ballas and Citovsky, 1997; Bhattacharjee et al., 2008), and several different cyclophillin proteins whose role in transformation is not yet known (Deng et al., 1998; Bakó et al., 2003).

We used the N-terminal fragment of yellow fluorescent protein (nYFP)-VirD2 as a bait to interrogate the *Arabidopsis* cYFP-cDNA arrayed library. To set imaging parameters, we initially used importin α-3 (IMPa-3) protein (At4g02150; clone 126-6D) and the 26S proteasome regulatory subunit RPN9 (At5g45620; clone 17-9C) as positive and negative prey controls, respectively. Supplemental Figure 7 online shows confocal microscopy images of *Arabidopsis* protoplasts expressing nYFP-VirD2 and each of these prey proteins.

We next used nYFP-VirD2 as a bait to screen 14 pools of 10 384-well plates (~3800 cDNA clones/pool) for yellow BiFC fluorescence. For this analysis, we cotransfected protoplasts with mCherry, rather than mRFP-VirD2NLS, to determine transfection efficiency. Parallel experiments indicated that mRFP-VirD2NLS competed with

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VirD2 for interaction with prey proteins and was therefore unsuitable as a transfection efficiency control. mCherry localizes to both the nucleus and the cytoplasm (Citovsky et al., 2006; Lee et al., 2008).

Although some pools showed sporadic weak BiFC fluorescence signals, pool 13 (plates 121 to 130) showed numerous signals (see Supplemental Figure 8 online). Further analysis indicated that plates 126, 128, and 130 contained cDNA clones whose protein products strongly interacted with nYFP-VirD2. Clone 126-6D contains the IMPa-3 cDNA clone used previously as a positive control for VirE2 interactions. Bhattacharjee et al. (2008) previously showed that IMPa-3 interacts with both VirE2 and VirD2. This plate additionally contains an identical (likely sibling) IMPa-3 cDNA (126-17N). Plate 126 also contains a cDNA clone encoding the RNA binding protein 45A (clone 126-24A; At5g54900) that weakly interacts with VirD2. Plate 128 contains two cDNA clones whose encoded proteins interact with VirD2: The clone identified as 128-22O encodes a MYST-like histone acetyltransferase 2 (At5g09740), and 128-12K encodes a putative GTPase (At3g07050). BiFC analysis of DNA extracted from rows and columns of plate 130 ultimately identified two cDNA clones whose cDNAs encode proteins that interact with VirD2. The first of these (clone 130-14O) is cyclophilin ROC3 (At2g16600). Using yeast two-hybrid analyses, Deng et al. (1998) and Bakó et al. (2003) previously identified several *Arabidopsis* cyclophilins, including ROC3, as proteins that interact with VirD2. Finally, we identified the clone 130-6O whose protein product strongly interacts with nYFP-VirD2. Unexpectedly, this cDNA encodes *S*-adenosyl-L-homocysteine hydrolase (At4g13940; HOG1), the same protein that strongly interacted with VirE2. Thus, screening our cDNA library with VirD2 as a bait uncovered proteins (IMPa-3 and cyclophilin ROC3) previously identified to interact with VirD2 in yeast, several novel proteins that interact with VirD2, and a novel protein (*S*-adenosyl-L-homocysteine hydrolase) that interacts with both VirD2 and VirE2.

The results of sequential screening of the cDNA library for clones encoding VirD2-interacting proteins are shown in

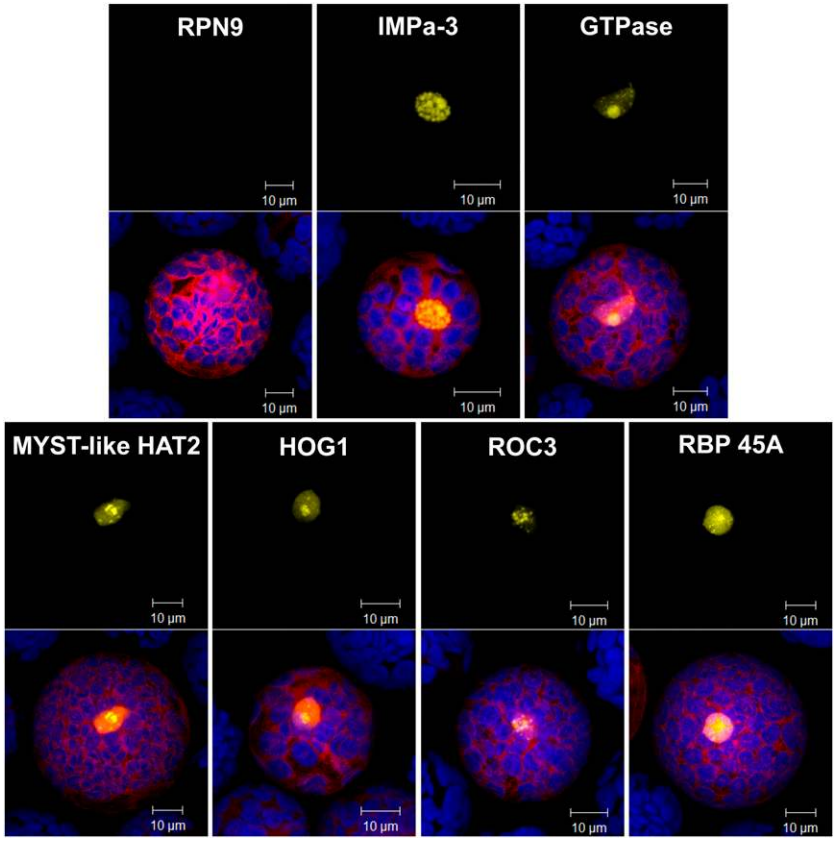

Figure 4. Interaction of nYFP-VirD2 with Various Proteins.

Arabidopsis leaf protoplasts were transfected with 10 μg each of plasmids encoding nYFP-VirD2, specific cYFP-tagged cDNA clones, and mCherry. The cells were imaged by confocal microscopy 16 h later. Top panels from each set show yellow YFP fluorescence images. Bottom panels show overlay images of YFP fluorescence (yellow), mCherry fluorescence (red; marks both the cytoplasm and nucleus), and chloroplasts (blue pseudocolor). Individual plasmids used for interaction encode the following: RPN9 (26S proteasome regulatory subunit RPN9 [At5g45620]; negative control for a protein that does not interact with VirD2); IMPa-3 (At4g02150); GTPase (putative GTPase [At3g07050]); MYST-like HAT2 (MYST-like histone acetyltransferase 2 [At5g09740]); HOG1 (*S*-adenosyl-L-homocysteine hydrolase [At4g13940]); ROC3 (cyclophilin ROC3 [At2g16600]); and RBP 45A (RNA binding protein 45A [At5g54900]). Bars = 10 μm.

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Supplemental Data Set 1 online. Figure 4 shows representative pictures of these VirD2–protein interactions.

Identification of Proteins Important for *Agrobacterium*-Mediated Plant Transformation

Plant proteins that interact with the transferred *Agrobacterium* virulence effector proteins VirE2 and VirD2 may be important for plant genetic transformation. We therefore tested homozygous mutants of genes encoding four of the novel VirE2-interacting proteins for transformation susceptibility (Zhu et al., 2003). Exon insertions into At3g01120 (GABI_054C05; cystathione γ-synthase) or At1g08820 (Salk_047568; VAP33-like SNARE protein) did not alter transformation susceptibility (data not shown). However, Figure 5A shows that two independent insertions into At4g15390 (Salk_106653, an insertion into the 5′ untranslated region (UTR) and Salk_066311, an insertion into an exon; HSR201-like protein) resulted in reduced transformation susceptibility. Mutation of At4g13940, either by a T-DNA exon insertion (*hog1-4*) or by a missense mutation (*sah1*; Mull et al., 2006), resulted in decreased stable *Agrobacterium*-mediated transformation susceptibility but no difference in transient transformation susceptibility (Figure 5C). The differential effects of the these mutations on transient and stable transformation efficiencies suggest either that *HOG1* is important for T-DNA integration or that it is important for expression of integrated, but not nonintegrated, transgenes.

We also tested *Arabidopsis* mutants in genes encoding VirD2-interacting proteins. Two independent mutations in the 5′ UTR or an early exon of At5g09740, encoding a MYST-like histone acetyltransferase 2, resulted in transient, but not stable, transformation hypersusceptibility (Figure 5B). We previously identified other histone acetyltransferases that are important for transformation (Zhu et al., 2003; Crane and Gelvin, 2007). Mutation of At3g07050, encoding a putative GTPase, and two independent alleles of At5g54900, encoding the RNA binding protein 45A, reduced transformation susceptibility (Figure 5C).

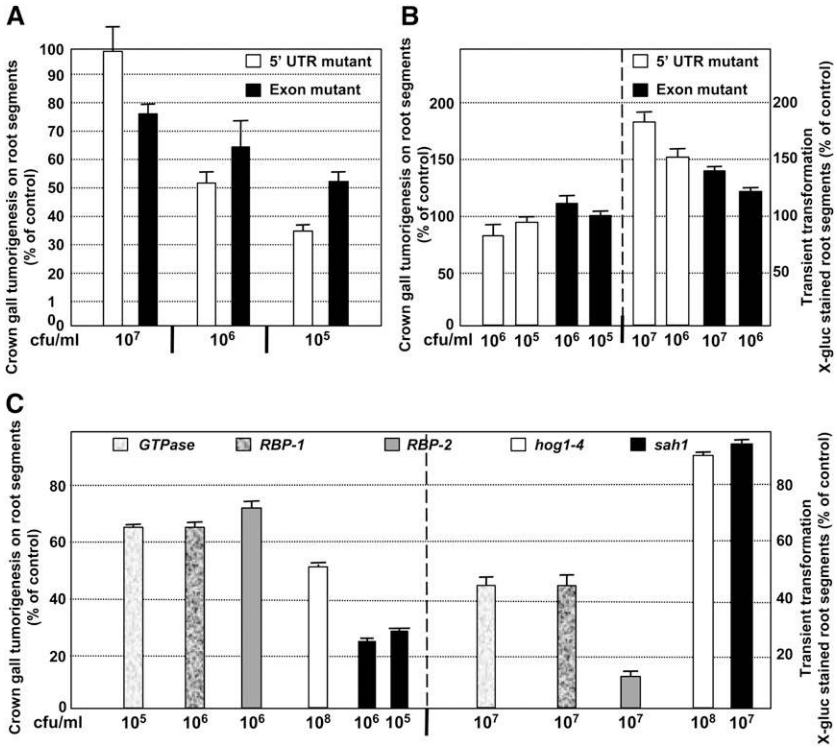

Figure 5. Mutation of Several VirD2- and VirE2-Interacting Proteins Results in Altered Susceptibility to *Agrobacterium*-Mediated Root Transformation.

Two independent alleles of *Arabidopsis* At4g15390, encoding a HSR-201–like protein that interacts with VirE2 **(A)**; two independent alleles of At5g09740, encoding a MYST-like histone acetyltransferase 2 that interacts with VirD2 **(B)**; At3g07050, encoding a putative GTPase that interacts with VirD2, two independent alleles of At5g54900, encoding the RNA binding protein 45A that interacts with VirD2, and two independent alleles of At4g13940, *hog1-4*, and *sah1*, encoding a *S*-adenosyl-L-homocysteine hydrolase that interacts with both VirD2 and VirE2 **(C)**. Root segments from wild-type *Arabidopsis* and various mutants were inoculated with the indicated concentrations of the tumorigenic strain *Agrobacterium* A208 (for stable transformation) or the disarmed strain At849 (for transient β-glucuronidase expression) as described (Tenea et al., 2009). The percentage (of control) of root segments generating crown gall tumors (**[A]** and the left side of **[B]** and **[C]**) was scored after 1 month of growth on MS medium lacking phytohormones for tumors. Transient β-glucuronidase activity (the right side of **[B]** and **[C]**) was measured 4 d after transfer of the root segments to callus-inducing medium. Error bars indicate the SE of five plates of 70 root segments/plate for tumorigenesis assays or >120 root segments/experimental point for transient β-glucuronidase assays. cfu, colony-forming units.

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Thus, the BiFC screen of the *Arabidopsis* cDNA library identified a total of five novel proteins involved in *Agrobacterium*-mediated transformation.

Screening an *Arabidopsis* cDNA Library for Proteins That Interact with the C-Terminal Extension of *Arabidopsis* Telomerase Reverse Transcriptase Using BiFC

We used the C-terminal extension (CTE) domain of *Arabidopsis* telomerase reverse transcriptase (At-TERT; At5g16850), tagged with nYFP, to screen the cYFP-tagged *Arabidopsis* cDNA library. Screening was conducted at the Department of Biological Sciences of Purdue University with a separately arrayed library of >110,000 colonies. Twenty micrograms of bait and prey DNAs from pools of 10 plates (10 μg from each plate, columns, or rows), along with a cDNA clone encoding mCherry, were used to transfect tobacco BY-2 protoplasts, and the resulting fluorescence was imaged by epifluorescence microscopy. Using a purification scheme similar to that described above, we ultimately identified interacting clones encoding two different proteins that were in frame with the N-terminal YFP tag. The first cDNA, corresponding to At4g33945, is annotated as an armadillo/β-catenin-like repeat-containing protein. Interaction of this protein with the CTE occurred predominantly in the cytoplasm of BY-2 cells. The second cDNA encodes a RNA recognition motif (RRM)-containing protein (At5g10350) that interacts with the CTE in nuclei. Figure 6 shows representative pictures of YFP fluorescence resulting from interaction of At-TERT (CTE) with these two proteins.

The CTE domain (corresponding to human TERT) contains regions important for intracellular trafficking of human TERT, including a nuclear export signal, 14-3-3, and CRM1 binding sites (Seimiya et al., 2000). The protein encoded by At4g33945 is a member of a structurally related group of armadillo repeat proteins with diverse cellular roles (Mudgil et al., 2004). The predominantly cytoplasmic localization of the At4g33945 BiFC signal suggests possible involvement in intracellular trafficking of telomerase and/or nontelomeric functions of TERT. The nuclear localization of a complex comprising CTE and a RRM-containing protein, encoded by At5g10350, suggests a possible telomeric function for this protein. Several previously characterized telomeric binding proteins from plants and other organisms contain a RRM (Petracek et al., 1994; Hirata et al., 2004; Kwon and Chung, 2004; Liu et al., 2009). Their specific binding to telomeric single-stranded DNA is mediated by a tandem pair of RRM located at the C terminus of the protein. The At5g10350 protein contains one RRM sequence and could function as a dimer, as is usual for many telomere binding proteins (Schrumpfová et al., 2004). Its predicted size (24 kD) corresponds to candidate telomeric single-stranded DNA binding proteins suggested to perform telomere end maintenance functions instead of At-POT1 (Shakirov et al., 2009). The physiological role of these *Arabidopsis* CTE binding proteins remains to be elucidated.

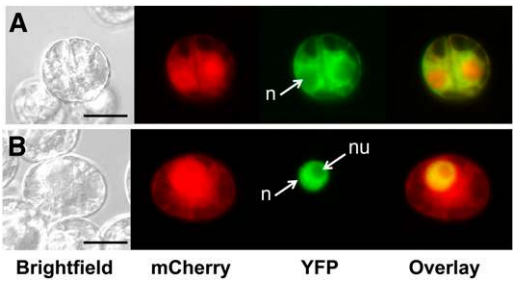

Figure 6. Interaction of AtTERT(CTE)-nYFP with Two Proteins as Detected by BiFC.

Interaction of AtTERT(CTE)-nYFP with an armadillo/β-catenin-like repeat-containing protein (encoded by At4g33945) **(A)** and an RRM-containing protein (encoded by At5g10350) **(B)**. Tobacco BY-2 protoplasts were transfected with 10 μg each of plasmids encoding mCherry, AtTERT(CTE)-nYFP, and either of the two interacting proteins. The cells were imaged 18 h later by epifluorescence microscopy. Red indicates mCherry fluorescence; green indicates BiFC YFP fluorescence; overlay indicates mCherry and YFP signals. n, nucleus; nu, nucleolus. The nucleus was identified by the intense mCherry red fluorescence. Bars = 25 μm.

Searching for Known VirE2 Interactors in the Arrayed cDNA Library

Our BiFC-based protein–protein interaction screening system identified some previously known VirE2- and VirD2-interacting proteins (e.g., several importin α proteins and the cyclophilin ROC3). However, we did not identify some previously known interacting proteins. For example, VirE2 protein interacts with all tested *Arabidopsis* importin α isoforms, as well as with VirE2 interacting protein1 (VIP1) and VIP2 (Tzfira et al., 2001; Anand et al., 2007; Bhattacharjee et al., 2008; Lee et al., 2008). Because

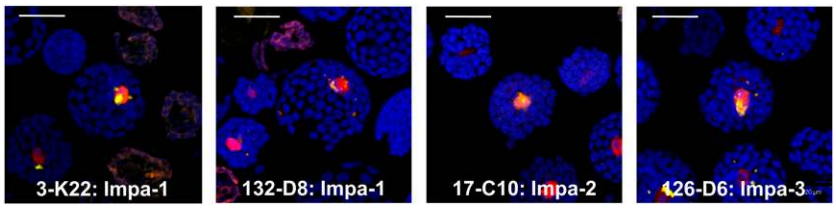

Figure 7. Interaction of VirE2-nVenus with cYFP-Tagged Importin α cDNA Clones.

Arabidopsis leaf protoplasts were cotransfected with 10 μg each of plasmids encoding mRFP-VirD2NLS (to mark the nuclei of transfected cells), VirE2-nVenus, and cDNA clones encoding in-frame fusions of cYFP with various importin-α proteins. The cells were imaged by confocal microscopy after 16 h. Red, mRFP fluorescence; yellow, YFP BiFC fluorescence; blue, false-color chlorophyll autofluorescence. Bars = 20 μm.

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Table 1. Presence and Organization of cDNA Clones Encoding Known cYFP-Tagged VirE2-Interacting Proteins

Gene	Presence in the Arrayed cYFP-cDNA Library	No. of Clones Identified	No. of Clones Analyzed	ORF in Frame with cYFP (n)	ORF out of Frame with cYFP (n)	Intervening Stop Codon(s) (n)
Impa1 (AtKapα) (At3g06720)	+	9	7	2	3	2
Impa2 (At4g16143)	+	6	4	1	1	2
Impa3 (At4g02150)	+	9	3	1	2	0
Impa4 (At1g09270)	+	2	2	0	2	0
Impa5 (At5g49310)	−	–	–	–	–	–
Impa6 (At1g02690)	+	3	3	0	3	0
Impa7 (At3g05720)	−	–	–	–	–	–
Impa8 (At5g52000)	−	–	–	–	–	–
Impa9 (At5g03070)	+	1	1	0	1	0
VIP1 (At1g43700)	+	1	1	0	1	0
VIP2 (At5g59710)	−	–	–	–	–	–

+, present in the cDNA library; −, not identified in the cDNA library; *n* = number of clones.

we did not identify cDNAs encoding these proteins in pool 1, plate 5 that we investigated in detail, we were curious as to whether these cDNAs existed in our BiFC library and, if so, whether they would express cYFP-tagged fusion proteins. We therefore conducted PCR analyses on DNA from various pools and individual plates using primers individually directed against each of the nine importin α genes, *VIP1*, and *VIP2*. These analyses identified cDNAs for Impa-1, -2, -3, -4, -6, and -9 and VIP1. We could not identify cDNAs for Impa-5, -7, and -8, nor could we identify a cDNA encoding VIP2. We previously failed to identify cDNAs for Impa-5 and Impa-8 from mRNA extracted from *Arabidopsis* plants (Bhattacharjee et al., 2008). These two importin α genes may therefore be pseudogenes or are expressed at extremely low levels or only in a few specialized cell types. Proteins encoded by the cYFP-tagged Impa-1, -2, and -3 cDNAs that we identified in our library interacted with VirE2-nYFP to form cytoplasmic aggregates that frequently were perinuclear. Occasionally, confocal microscopy revealed importin α–VirE2 interaction in the nucleus (Figure 7). These interactions were similar to those reported previously (Bhattacharjee et al., 2008; Lee et al.,

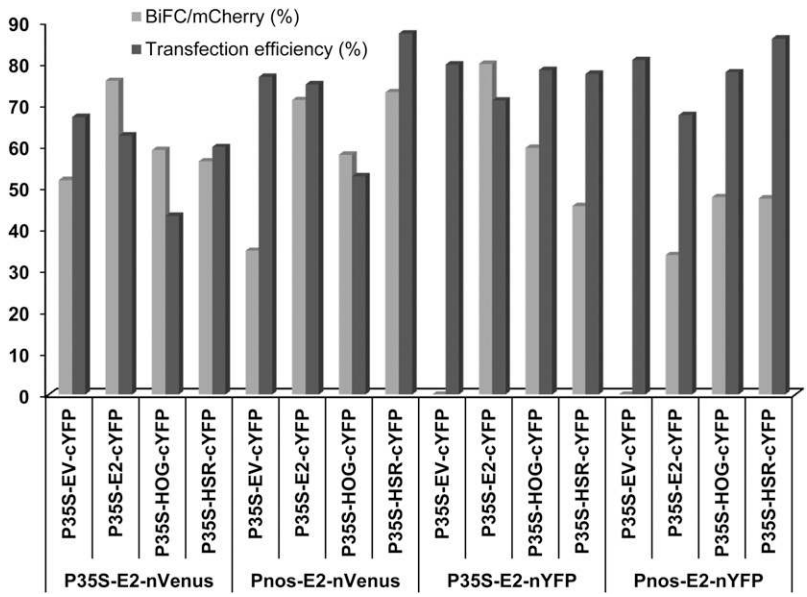

Figure 8. Interaction of P_{35S}-Prey-cYFP Proteins with Various VirE2 Bait Protein Constructs.

Ten micrograms of plasmid DNA expressing VirE2-cYFP or VirE2-nVenus as baits was cotransfected with 10 μg of DNA from plasmids expressing the prey proteins VirE2, the HSR201-like protein (HSR), an *S*-adenosyl-L-homocysteine hydrolase (HOG), or an EV into *Arabidopsis* protoplasts. Expression of the VirE2 bait constructs was directed by either a CaMV double 35S promoter (P35S) or by a nopaline synthase (Pnos) promoter. Expression of prey proteins was under the control of P35S. The cells were also transfected with 10 μg of a plasmid encoding mRFP-VirD2NLS to determine the transfection efficiency (dark bars). The cells were imaged after 16 h by confocal microscopy. Light-gray bars indicate the percentage of red fluorescent cells that also displayed BiFC yellow fluorescence.

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Table 2. BiFC Signal Strength and Background Levels Using Different Promoters and nYFP Derivatives

Bait	Prey	Transfection Efficiency (%)[a]	BiFC/mCherry (%)[b]	Yellow Fluorescence Signal/BiFC Protoplast[c]
P_{35S}-VirE2-nVenus	P_{35S}-EV-cYFP	67.0 ± 3.1	51.7 ± 6.2	6,765 ± 1,736
	P_{35S}-VirE2-cYFP	62.5 ± 4.1	75.7 ± 8.8	45,694 ± 9,283
	P_{35S}-HOG1-cYFP	43.1 ± 3.6	59.0 ± 8.1	3,037 ± 369.6
	P_{35S}-HSR201-cYFP	59.7 ± 1.4	56.3 ± 1.4	12,579 ± 1,929
P_{nos}-VirE2-nVenus	P_{35S}-EV-cYFP	76.7 ± 6.9	26.6 ± 12.8	4,043 ± 282
	P_{35S}-VirE2-cYFP	74.9 ± 2.6	71.1 ± 6.2	13,447 ± 1,705
	P_{35S}-HOG1-cYFP	52.7 ± 11.9	57.9 ± 9.4	2,428 ± 185
	P_{35S}-HSR201-cYFP	87.2 ± 4.6	73.0 ± 4.8	1,396 ± 103
P_{35S}-VirE2-nYFP	P_{35S}-EV-cYFP	79.7 ± 1.8	0 ± 0	0 ± 0
	P_{35S}-VirE2-cYFP	71.0 ± 2.9	79.8 ± 4.3	25,960 ± 2,755
	P_{35S}-HOG1-cYFP	78.4 ± 1.9	59.5 ± 3.6	832 ± 32
	P_{35S}-HSR201-cYFP	77.4 ± 3.2	45.4 ± 11.1	835 ± 282
P_{nos}-VirE2-nYFP	P_{35S}-EV-cYFP	80.8 ± 1.3	0 ± 0	0 ± 0
	P_{35S}-VirE2-cYFP	67.5 ± 4.8	33.6 ± 12.3	5,908 ± 1,138
	P_{35S}-HOG1-cYFP	77.8 ± 3.4	47.6 ± 7.2	728 ± 43
	P_{35S}-HSR201-cYFP	85.9 ± 2.6	47.3 ± 4.3	672 ± 110

[a]Percentage of protoplasts showing mRFP-VirD2NLS red fluorescence.
[b]Percentage of red fluorescent protoplasts showing yellow BiFC fluorescence signal.
[c]Average total yellow BiFC fluorescence pixel intensity/protoplast.

2008). Thus, a more extensive interrogation of our library identified many proteins previously known to interact with VirE2.

Characterization of the BiFC cDNA Library

In addition to not appearing in the cYFP-cDNA library, there are at least four reasons why a cDNA clone present in the library would not encode a fusion protein that interacts with a given nYFP-tagged bait protein to generate yellow fluorescence: (1) the cYFP peptide may be out of frame with the open reading frame (ORF) encoded by the cDNA; (2) the cYFP peptide may be in frame with the ORF encoded by the cDNA, but an in-frame stop codon in the 5′ UTR of the cDNA prevents fusion of the cYFP tag with the ORF; (3) the cYFP peptide may be in frame with a partial ORF such that the interaction domain of the ORF is not present; (4) the YFP peptide may be in frame with the ORF encoded by the cDNA, but interaction of the cYFP-tagged ORF with the nYFP-tagged bait protein does not permit correct

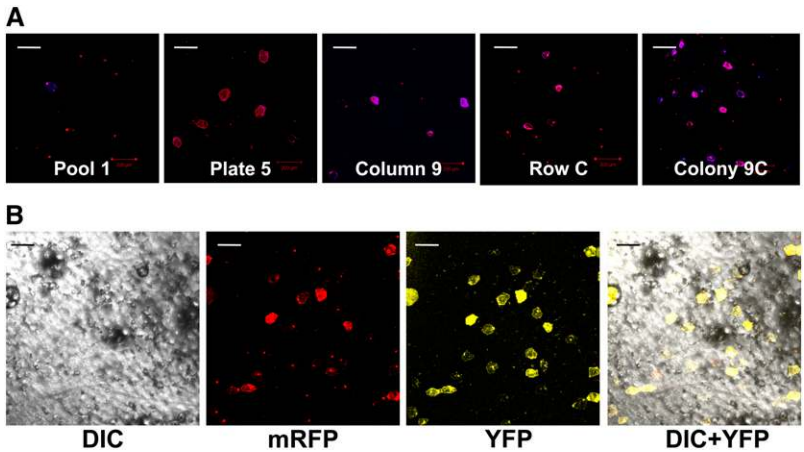

Figure 9. Interaction of VirE2-nVenus with cYFP-Tagged cDNA Clones in Orchid Flowers.

(A) Orchid flowers were bombarded with 2.5 μg each of plasmids encoding mRFP-VirD2NLS, VirE2-nVenus, and DNA from a cYFP-tagged cDNA library. The cells were imaged by confocal microscopy 16 h later. mRFP and YFP merged images show transformed cells lacking (red) or displaying (purple) BiFC signals. Bars = 200 μm.

(B) Orchid flowers were bombarded with 2.5 μg each of plasmids encoding mRFP, VirE2-nVenus, and DNA from plate 5, clone 9C (At4g15390, encoding an HSR 201-like protein). The cells were imaged by confocal microscopy 16 h later. DIC, differential interference contrast image. Bars = 200 μm.

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folding of the cYFP and nYFP peptides (i.e., fluorescence complementation cannot occur). The first three of these construction artifacts also apply to commonly used yeast two-hybrid systems. The fourth possibility is unique to BiFC systems.

Analysis of the cYFP-tagged cDNA library indicated that most identified importin α clones contained a full-length importin α ORF (data not shown). However, during cDNA synthesis, the 5′ UTR for a given cDNA may be of variable length. Therefore, cYFP-importin α clones may encode cYFP peptides either in frame or out of frame with the importin α ORF (Table 1). Similarly, several importin α clones encoded cYFP peptides in frame with the importin α ORF, but in-frame stop codons in the 5′ UTR precluded tagging the importin α ORF with cYFP. However, some importin α ORFs were preceded by a shortened 5′ UTR lacking this stop codon(s), resulting in a cYFP tag affixed to the importin α ORF (Table 1). Thus, although most queried cDNAs exist in our arrayed library, some may not encode proteins that can interact with a particular bait. We arrayed $\sim 2 \times 10^5$ tagged cDNA clones (~100,000 clones maintained at the Agricultural Biotechnology Research Center at the Academia Sinica and a slightly larger number independently arrayed and maintained at Purdue University). Because our primary tagged cDNA library contains 10^6 to 10^7 members, it is therefore likely that an in-frame cYFP-tagged cDNA exists for most expressed *Arabidopsis* genes.

We further sought to characterize the makeup of the cDNA library. Whole *Arabidopsis* plantlets were initially used for library construction. DNA sequence analysis of 739 random clones revealed that 678 (92%) could readily be assigned to known *Arabidopsis* ORFs. The great majority of the remaining clones correspond to other *Arabidopsis* sequences. Of these 678 annotated cDNAs, we identified 564 (83%) only once; just a few of the remaining cDNAs were identified more than twice. Only seven of the 678 identified cDNAs (~1%) encode photosynthetic proteins; five of these encode two different RBCS proteins. Of the sequenced cDNAs, four (0.6%) encode different ribosomal proteins. The underrepresentation of cDNAs encoding abundant proteins strongly suggests that the library has remained balanced (i.e., approximately equal representation of each cDNA) throughout its development and amplification.

We further analyzed the sequenced cDNA clones to determine what percentage encodes *Arabidopsis* ORFs in frame with the N-terminal cYFP tag. Thirty-five percent of the identified protein-coding sequences were in frame with the cYFP tag, very close to the expected 33.3%. Thus, the construction of our cDNA library is random with respect to reading frame, and the library remains balanced with respect to the representation of cDNA sequences.

Choice of nYFP Derivatives to Use for cDNA Library Screening: Mitigating False-Positive Interactions

BiFC analyses currently employ several YFP derivatives, including enhanced (EYFP) and the high-fluorescent autofluorescent protein Venus (Shyu et al., 2006). Several of these are currently used in plants systems (Bracha-Drori et al., 2004; Bhat et al., 2006; Citovsky et al., 2006; Ohad et al., 2007; Lee et al., 2008; Lee and Gelvin, 2012). One of the limitations of BiFC is the self-assembly of complementary autofluorescent protein fragments (Shyu and Hu, 2008; Kodama and Hu, 2010). Interaction of N- and C-terminal protein fragments may occur when strong promoters are used to generate high concentrations of these fragments in the cell and/or when highly fluorescent YFP derivatives (such as Venus) are used, thus increasing fluorescence detection sensitivity. Fluorescence may be visualized in the absence of bait and prey protein interaction (e.g., when one of the peptide complementation fragments is not linked to another protein—the EV control). Self-assembly can thus confound interpretation of BiFC data, yielding false-positive interactions.

Although our initial reconstruction experiments indicated that self-assembly was not a major problem (Figures 1B and 2), we wished to determine the extent of self-assembly and, if present, mitigate it. We thus quantified fluorescence signals derived from interaction of VirE2 affixed to different nYFP derivatives as bait proteins, with cYFP-tagged proteins or EV proteins as preys. We expressed the prey proteins from a strong CaMV double 35S promoter to mimic the situation with tagged cDNA prey clones. We then altered the promoter (either a strong CaMV double

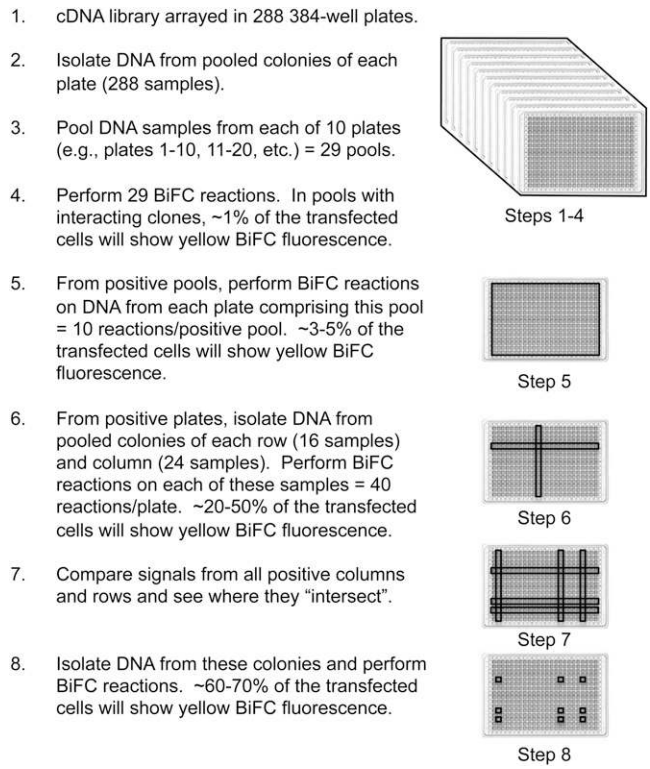

Figure 10. Flow Chart of cDNA Library Screening for Interacting Proteins.

The steps in the screening process are presented, starting with DNA isolation from mixed colonies of plates to identification of individual cDNA clones encoding interacting proteins. For each step, a plasmid expressing an mRFP or mCherry reporter protein is cotransfected with the bait and cDNA library plasmids to evaluate transformation frequency (percentage of total cells that fluoresce red). Black boxes encircling plates, rows, columns, or individual colonies indicate which cDNA library plasmids are evaluated for each step of the process. The entire screening process takes 4 to 8 weeks.

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35S or a relatively weaker nopaline synthase [*nos*]) and the N-terminal fragment of YFP affixed to the VirE2 bait. Preliminary studies investigated the mutant nYFP derivative nYFP^{I152L}. This derivative shows a high signal-to-noise ratio when used for BiFC reactions in animal cells (Kodama and Hu, 2010). However, use of this mutant nYFP form, when affixed to VirE2 (VirE2-nYFP^{I152L}), resulted in extremely weak fluorescence signals, even when paired with the strongly interacting protein VirE2-cYFP (data not shown). We thus discontinued using this nYFP derivative for our further investigations.

Figure 8 and Table 2 show the results of these investigations. The transformation frequency, measured by the percentage of protoplasts displaying red fluorescence from cotransfected DNA encoding mRFP-VirD2NLS, was similar in all experiments. Using the CaMV double 35S promoter to express the bait VirE2 construct resulted in stronger BiFC signals than when the *nos* promoter was used. Tagging the VirE2 bait protein with nVenus generated stronger BiFC signals than when VirE2 was tagged with nYFP. However, nVenus-tagged VirE2 also produced a (relatively weak) yellow fluorescence signal when paired with an empty cYFP bait vector. This signal disappeared when VirE2 was tagged with nYFP, essentially eliminating false-positive signals resulting from self-assembly of the complementing autofluorescent protein fragments. We thus recommend that, to eliminate false-positive background fluorescence signals, nYFP rather than nVenus be used as the bait tag. We obtained similar results regardless of whether VirE2, HOG1, or the HSR201-like protein was used as prey protein. We repeated these experiments three times; each time, we examined at least four fields containing >400 red fluorescent protoplasts for each construction.

Screening the cDNA Library in Other Species Using BiFC

An important feature of this cDNA screening system is that it is independent of the plant species and cDNA library used. We initially used protoplasts from either *Arabidopsis* leaves or tobacco BY-2 cells for screening. To assess the utility of this system in other plant species, we bombarded flower petals of orchid (*Phalaenopsis amabilis*) with plasmids expressing mRFP (to mark transformed cells), VirE2-nVenus, and DNA from the cYFP-tagged *Arabidopsis* cDNA library and examined the petals for fluorescence the next day. As a proof of concept, we used pooled cDNA clones from pool 1, plate 5, column 9, row C and the single clone 5-9C (At4g15390; HSR201-like protein), previously identified as interacting with VirE2 in *Arabidopsis* protoplasts. Figure 9 shows that a similar BiFC cDNA screening system works well in orchid flower petals when DNA was delivered by particle bombardment; BiFC fluorescence signals appeared with increasing frequency and strength as the interacting clone was enriched among the samples. Thus, this BiFC cDNA library screening system is applicable to plant species and systems other than *Arabidopsis* and tobacco protoplasts.

Conclusions

We developed a system to screen for protein–protein interactions using a cDNA library and a bait protein directly in planta. The system uses BiFC as a readout for protein–protein interactions. This system has a number of advantages over traditional protocols in which interacting proteins are first identified using a yeast two-hybrid system, followed by confirmation of interaction in plants: (1) Because screening is conducted directly in plant cells, it eliminates the necessity of screening a cDNA library against a bait protein first in yeast, then recloning the cDNA into a plant expression vector for confirmation in plants. It is thus more rapid and efficient. (2) Protein–protein interactions occur in their native plant cell environment and can thus be localized to specific subcellular domains or organelles. (3) The screening system is easily adapted to use cDNA libraries from any plant species for testing in that (or other) plant species. Thus, scientists can rapidly evaluate protein–protein interactions in any plant cell type that is amenable to transformation. Figure 10 shows a flow chart of how we have used this system to identify cDNAs encoding proteins that interact with specific bait proteins. Once bait protein expression plasmids have been constructed, screening the cDNA library to identify and confirm individual cDNA clones takes 4 to 8 weeks. We deposited the Gateway cDNA BiFC expression vectors, the various bait vectors, and the mixed cDNA library in the ABRC stock center at The Ohio State University. We expect that this system will facilitate experimentation to define the nature and site of protein–protein interactions in plants and to develop protein–protein interaction networks important for understanding gene function.

METHODS

Plant and Bacterial Growth Conditions

Arabidopsis thaliana ecotype Columbia plants were grown in a mixture of vermiculite, perlite, and peat moss (1:1:1) in an environmentally controlled chamber with a long photoperiod (16 h light/8 h dark) at 22°C. Tobacco (*Nicotiana tabacum*) BY-2 cells were maintained as previously described (Tenea et al., 2009). Flowering orchid (*Phalaenopsis amabilis*) plants were obtained from a local grower (Yung Hsin Orchid Nursery). *Escherichia coli* DH10B was grown at 37°C in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin.

cDNA Library Generation, Colony Arraying, At-TERT(CTE) Construct Preparation, and Plasmid DNA Extraction

A full-length, normalized *Arabidopsis* cDNA library (Invitrogen Life Technologies; catalog number 12210-030) was introduced into the destination expression vector pSAT5-DEST-cEYFP-C1 (Gelvin laboratory stock number E3130; The Arabidopsis Information Resource stock number CD3-1097) using the LR recombinase reaction (Invitrogen). The final library, containing >10^6 individual cDNA clones at a titer of 6×10^8 colony-forming units/mL, was stored at −80°C. For colony arraying, the cDNA library was plated on LB-amp medium in 22 × 22-cm plastic plates at a density of ~1000 colonies/plate. After overnight growth at 37°C, colonies were picked and arrayed in 384-well plates containing 50 μL liquid LB-amp medium using a Q-bot robot (Genetix). After overnight growth at 37°C, the plates were stored at −80°C. For DNA isolation, colonies were grown such that the cDNA library would remain normalized, as follows: Colonies from 384-well microtiter plates (either 10 plates for pools or individual plates) were stamped onto 22 × 22-cm plastic plates (pools) or 8 × 12-cm pipette tip box lids (individual plates) containing LB medium supplemented with 100 μg/mL ampicillin. After overnight growth at 37°C, the colonies were washed off the plates in prewarmed (37°C) LB

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medium and grown for a further 4 h in 500 mL liquid LB-amp medium. Cells were harvested and DNA was isolated using plasmid midi-preparation kits (Bio-Genesis). A plasmid encoding the full-length *Arabidopsis* TERT was used as a template for PCR amplification of the CTE domain using Phusion HF DNA polymerase (Finnzymes). The amplified DNA fragment was gel purified, digested with *Kpn*I and *Bam*HI (Takara Bio Europe), ligated into the vector pSAT1-nEYFP-C1 (Gelvin laboratory stock number E3075), and transformed into One Shot TOP10 chemically competent *E. coli* cells (Invitrogen Life Technologies).

Protoplast Preparation, Transfection, and Microscopy

Arabidopsis leaf protoplasts were prepared and transfected according to Wu et al. (2009). DNA (10 or 20 μg of each construction) was introduced into 1×10^5 protoplasts. Following 8 h incubation in the dark and 8 h in the light at 22°C, the cells were imaged with a Zeiss LSM510 META laser scanning confocal microscope using a ×10/0.45 Plan-Apochromat or a ×20/0.8 Plan-Apochromat and ×100/1.4 Oil Plan-Apochromat objectives in multitrack channel mode. Excitation wavelength and emission wavelength ranges were 514 nm/band-pass filter 520 to 555 nm for YFP, 561 nm/meta-detector 576 to 629 nm for mRFP, and 514 nm/meta-detector 650 to 704 nm for chloroplast autofluorescence. The gain value was adjusted to a level that would eliminate fluorescence imaging of intrinsic autofluorescence and nonspecific BiFC signals. All images were captured at the same gain value. For measurement of the transfection efficiency, four fields were acquired (>400 cells). The positive and negative cells were judged by eye, and the numbers were counted using Zeiss AxioVision version 4.8 software. We used Zeiss confocal software version 4.2 for fluorescence intensity measurements.

DNA was introduced into tobacco BY-2 protoplasts by polyethylene glycol (PEG)–mediated transfection. Protoplasts were isolated as previously described (Tenea et al., 2009) and resuspended at 1×10^6/mL in MMg medium (0.4 M mannitol, 15 mM $MgCl_2$, and 4 mM MES, pH5.7). Protoplasts (100 μL) were gently mixed with all DNA samples (in a 10 μL final volume) in a polypropylene tube, followed by drop-wise addition of 110 μL PEG solution (40% PEG [Fluka], 0.4 M mannitol, and 0.1 M $CaCl_2$). The mixture was incubated at room temperature for 5 min, followed by the addition of 2000 μL W5 solution. After gently mixing, the protoplasts were pelleted by centrifugation at 190*g* for 3 min. The protoplasts were washed again with 2000 μL W5 solution and pelleted by centrifugation. The protoplasts were suspended in 0.6 mL incubation solution (BY-2 medium [per l, 4.33 g Murashige and Skoog salts, 370 mg KH_2PO_4, 1.0 mg thiamine, 2.0 mg 2,4-D, and 30 g Suc, pH 5.7] plus 0.4 M mannitol) and the protoplasts distributed into wells of a BSA-coated plastic 24-well plate. The protoplasts were incubated at room temperature overnight and then observed for fluorescence using a Nikon Eclipse E600 microscope equipped with YFP-, RFP- (HcRed), and UV-specific filters.

DNA (2.5 μg/plasmid) was introduced into orchid flowers (Chiou et al., 2008) by particle bombardment (PDS1000/He Biolistic) using the following parameters: 1-μm gold particle size, 1350 p.s.i., 27-mm Hg vacuum, and 9-cm distance.

Transformation Assays

Homozygous T-DNA insertion lines were obtained from The Ohio State University *Arabidopsis* stock center. Plants were grown axenically and root segments were transformed as described (Tenea et al., 2009) using the tumorigenic strain *Agrobacterium tumefaciens* A208 for stable transformation or *A. tumefaciens* At849 for transient transformation at inoculum concentrations ranging from 10^5 to 10^8 colony-forming units/mL. For each data point, root segments from 5 to 10 plants were pooled, and data from a minimum of four pools were averaged.

PCR Conditions

PCR was conducted using a Fast-Run Taq Master Kit (Protech Technology) and the following reaction conditions: 95°C for 3 min (95°C, 30 s; 55°C, 30 s; and 72°C, 1 min) for 30 cycles; 72°C for 5 min. Primers are listed in Supplemental Table 1 online.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: At1g02690, IMPa-6; At1g08820, VAP33-like SNARE protein; At1g09270, IMPa-4; At1g43700, VIP1; At1g50320, thioredoxin; At2g16600, cyclophilin ROC3; At3g01120, cystathione γ-synthase; At3g05720, IMPa-7; At3g06720, IMPa-1 (AtKapα); At3g07050, putative GTPase; At4g02150, IMPa-3; At4g13940, *S*-adenosyl-L-homocysteine hydrolase (*SAH1*; *HOG1*); At4g15390, HSR201-like protein; At4g16143, IMPa-2; At4g33945, armadillo/β-catenin-like repeat-containing protein; At5g03070, IMPa-9; At5g09740, MYST-like histone acetyltransferase 2; At5g10350, RRM-containing protein; At5g16850, telomerase reverse transcriptase; At5g45620, 26S proteasome regulatory subunit RPN9; At5g49310, IMPa-5; At5g52000, IMPa-8; At5g54900, RNA binding protein 45A; and At5g59710, VIP2.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Sensitivity of Detecting YFP Fluorescence in Transfected *Arabidopsis* Protoplasts.

Supplemental Figure 2. Screening Pools of cYFP-Tagged cDNA Clones for Interaction with VirE2-nVenus.

Supplemental Figure 3. Screening Rows from Plate 5 of cYFP-Tagged cDNA Clones for Interaction with VirE2-nVenus.

Supplemental Figure 4. Screening Columns from Plate 5 of cYFP-Tagged cDNA Clones for Interaction with VirE2-nVenus.

Supplemental Figure 5. Screening Individual Colonies from Plate 5 of cYFP-Tagged cDNA Clones for Interaction with VirE2-nVenus.

Supplemental Figure 6. Progressive Enrichment of a Clone Encoding a cYFP-Tagged Protein That Interacts with VirE2-nVenus.

Supplemental Figure 7. Positive and Negative Controls to Set Imaging Parameters for Interrogating the cYFP-cDNA Library with nYFP-VirD2.

Supplemental Figure 8. Screening Pools of the cYFP-cDNA Library with nYFP-VirD2.

Supplemental Table 1. PCR Primers Used in This Study.

Supplemental Data Set 1. Sequential Screening of the cDNA Library for Clones Encoding VirD2-Interacting Proteins.

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AUTHOR CONTRIBUTIONS

L.-Y.L., E.S., S.B.G., and C.-S.L. designed the research. L.-Y.L., F.-H.W., C.-T.H., S.-C.S., H.-Y.Y., D.-C.L., M.-J.F., N.-T.L., Y.-C.Y., and L.D. performed research. L.-Y.L., F.-H.W., S.B.G., and C.-S.L. analyzed data. L.-Y.L., E.S., S.B.G., and C.-S.L. wrote the article.

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