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A highly efficient agrobacterium-mediated method for transient gene expression and functional studies in multiple plant species

A Highly Efficient *Agrobacterium*-Mediated Method for Transient Gene Expression and Functional Studies in Multiple Plant Species

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ABSTRACT

Although the use of stable transformation technology has led to great insight into gene function, its application in high-throughput studies remains arduous. Agro-infiltration have been widely used in species such as *Nicotiana benthamiana* for the rapid detection of gene expression and protein interaction analysis, but this technique does not work efficiently in other plant species, including *Arabidopsis thaliana*. As an efficient high-throughput transient expression system is currently lacking in the model plant species *A. thaliana*, we developed a method that is characterized by high efficiency, reproducibility, and suitability for transient expression of a variety of functional proteins in *A. thaliana* and 7 other plant species, including *Brassica oleracea*, *Capsella rubella*, *Thellungiella salsuginea*, *Thellungiella halophila*, *Solanum tuberosum*, *Capsicum annuum*, and *N. benthamiana*. Efficiency of this method was independently verified in three independent research facilities, pointing to the robustness of this technique. Furthermore, in addition to demonstrating the utility of this technique in a range of species, we also present a case study employing this method to assess protein–protein interactions in the sucrose biosynthesis pathway in *Arabidopsis*.

Key words: transient expression, agro-infiltration, subcellular localization, protein-protein interaction

INTRODUCTION

Agrobacterium-mediated genetic transformation of plants, which delivers and integrates virulent DNA (transferred DNA, T-DNA) into plant cells, was initially regarded as a rare example of naturally occurring trans-kingdom DNA transfer (Chilton et al., 1977). Although later studies have suggested that horizontal gene transfer is considerably less rare than was previously thought (Keeling and Palmer, 2008), it was more recently demonstrated that agrobacterial infection of plant tissues (i.e., the expression of T-DNA harbored genes) could occur in either a transient or a stable manner (Janssen and Gardner, 1990; Krenek et al., 2015). Rapid transient expression in plants is suggested to predominantly occur from T-DNA copies that are not integrated into the host genome but exhibit high expression

levels (Lacroix and Citovsky, 2013; Krenek et al., 2015). In contrast, stable transformation normally requires several months to obtain the first generation of transformed plants. Drawing on these two instances, a new transient expression approach could achieve high levels of expression in infected tissues by delivering much higher numbers of T-DNA copies into plant cells than the one, or few, that are generally integrated into the host genome through stable transformation (Janssen and Gardner, 1990; Krenek et al., 2015). Although *Agrobacterium*-mediated stable transformation of plants has

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| Day1 | Transformed *Agrobacterium* AGL1 streaked on YEB-induced medium plate with appropriate antibiotics |

↓

| Day3 Morning | Resuspend cells in infiltration buffer (1/4 MS with 1% sucrose, 100μM Acetosyringone and 0,01% Silwet L-77)to OD_{600} to 0.5 |

↓

| Day3 | Infiltrate bacteria into *Arabidopsis* leaves (2 to 3 weeks young leaf) by 1mL plastic syringe, dry in the light (1 hour) and keep in the dark for 24 hours |

↓

| | Move to green house for plant recovery growth |

↓

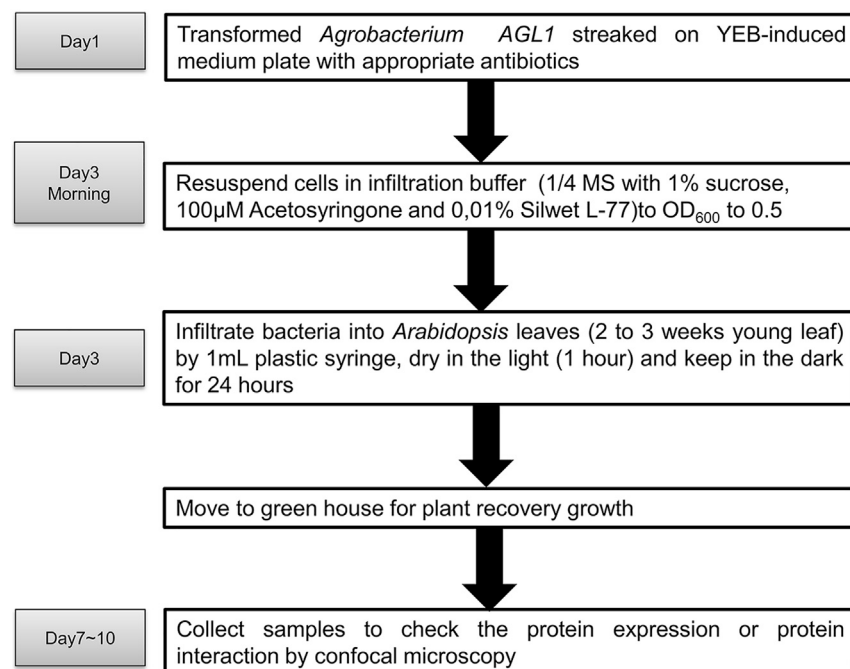| Day7~10 | Collect samples to check the protein expression or protein interaction by confocal microscopy |

Figure 1. Schematic Representation of the Timeline Required for the Generation of Transiently Transformed Plants Using Agro-Infiltration.

Briefly, *Agrobacterium* was streaked onto YEB agar plates containing acetosyringone and antibiotics. After washing, the transformed agrobacteria in infiltration buffer were injected into a plant leaf. Transiently transformed plants for all of the species mentioned could be obtained within 2–3 days after a single hour of infiltration.

been widely used in agriculture and is indeed indispensable for many aspects of plant science, recent attention has also been devoted to transient plant transformation (Wu et al., 2014; Krenek et al., 2015). Indeed the transient agro-infiltration method developed by Li et al. (2009) allows for rapid and scalable recombinant protein production, studies of protein subcellular localization and protein–protein interactions, as well as development of functional genomics assays.

An efficient tool to study the function of gene products, transient gene expression provides a convenient alternative to stable transformation. Although agro-infiltration has been widely used in *Nicotiana benthamiana* for the rapid evaluation of gene function and protein interaction studies (Sparkes et al., 2006), its use is limited to processes that naturally occur in this species. Thus, for the study of non-*N. benthamiana* appropriate, species-specific aspects of metabolism, development, or stress tolerance, use of this technique may be limiting (Krenek et al., 2015). At present, transient expression does not work efficiently in many other plant species/tissues, including *Arabidopsis* leaf tissue. *Arabidopsis* remains the most widely transformed species (predominantly by floral dipping-based transformation methods) (Provart et al., 2016), due to the wealth of genetic tools and resources available (Clough and Bent, 1998). Although stable transformation systems, such as floral dipping, have provided the potential for mutant complementation, as well as the ability to couple gene expression to marker proteins for examination of global gene expression (Chang et al., 1994), transient expression systems have provided a convenient alternative by virtue of time and labor efficiency (Krenek et al., 2015). *Agrobacterium*-mediated seedling transformation has been successfully used to express a wide variety of constructs driven by different promoters in cotyledons of *Arabidopsis* plants of diverse genetic backgrounds (Marion et al., 2008; Li et al., 2009; Wu et al., 2014; Wang et al., 2018). Furthermore, the transient transformation of *Arabidopsis* seedlings and

leaves either by biolistic bombardment (Ueki et al., 2008) or agrobacteria infiltration (Lee and Yang, 2006; Mangano et al., 2014; Rosas-Díaz et al., 2017) has been reported. However, neither method has become widely used due to special equipment requirements, use of expensive gold microparticles, and, most critically, due to extremely low transformation efficiency. Difficulties in *Arabidopsis* transient transformation have been attributed to the plant immune responses triggered by the perception of *Agrobacterium* (Mangano et al., 2014; Zhu et al., 2016; Rosas-Díaz et al., 2017). Given this, another transient transformation method was developed but was limited to transgenic plants expressing AvrPto under the control of a dexamethasone-inducible promoter (Tsuda et al., 2012). This background, however, is unlikely to be universally appropriate given that it is itself a mutant. Similar to *A. thaliana*, methods for stable transformation of *Brassica oleracea* (De Block et al., 1989), *Thellungiella halophila* (Li et al., 2007), *Solanum tuberosum* (Sheerman and Bevan, 1988; Zhang et al., 2014), and *Capsicum annuum* (Mahto et al., 2018) are well established, yet transient transformation methods are still lacking. Furthermore, neither transient nor stable transformation methods are well established in *Capsella rubella* and *Thellungiella salsuginea*. Because a method for reliable transient gene transformation in different species could significantly enhance our ability to evaluate protein function, localization, and interaction in a species-dependent or -independent manner, there is an urgent need for such a method to be developed.

Given that *Arabidopsis* has the advantage of having that harbours near-saturation level collections of T-DNA insertion mutants, we present a rapid and highly efficient transient expression assay for this species using simple infiltration of intact leaves with *Agrobacterium*. We additionally demonstrate its efficiency in *B. oleracea*, *C. rubella*, *T. salsuginea*, *T. halophila*, *S. tuberosum*, *C. annuum*, and *N. benthamiana*. In addition to demonstrating the utility of this method in a range of plant species, we also provide a case study of its utility in addressing protein–protein interactions. A recent paper has highlighted the utility of the recombination strategy for this purpose (Hu et al., 2019). Despite the great advances offered by this technique in aiding in the cloning of large genes, in *Arabidopsis* at least, it currently largely remains reliant on stable transformation techniques. Here, we demonstrate the capability of our transient expression system to investigate interactions of one of the key enzymes of sucrose biosynthesis in *Arabidopsis*.
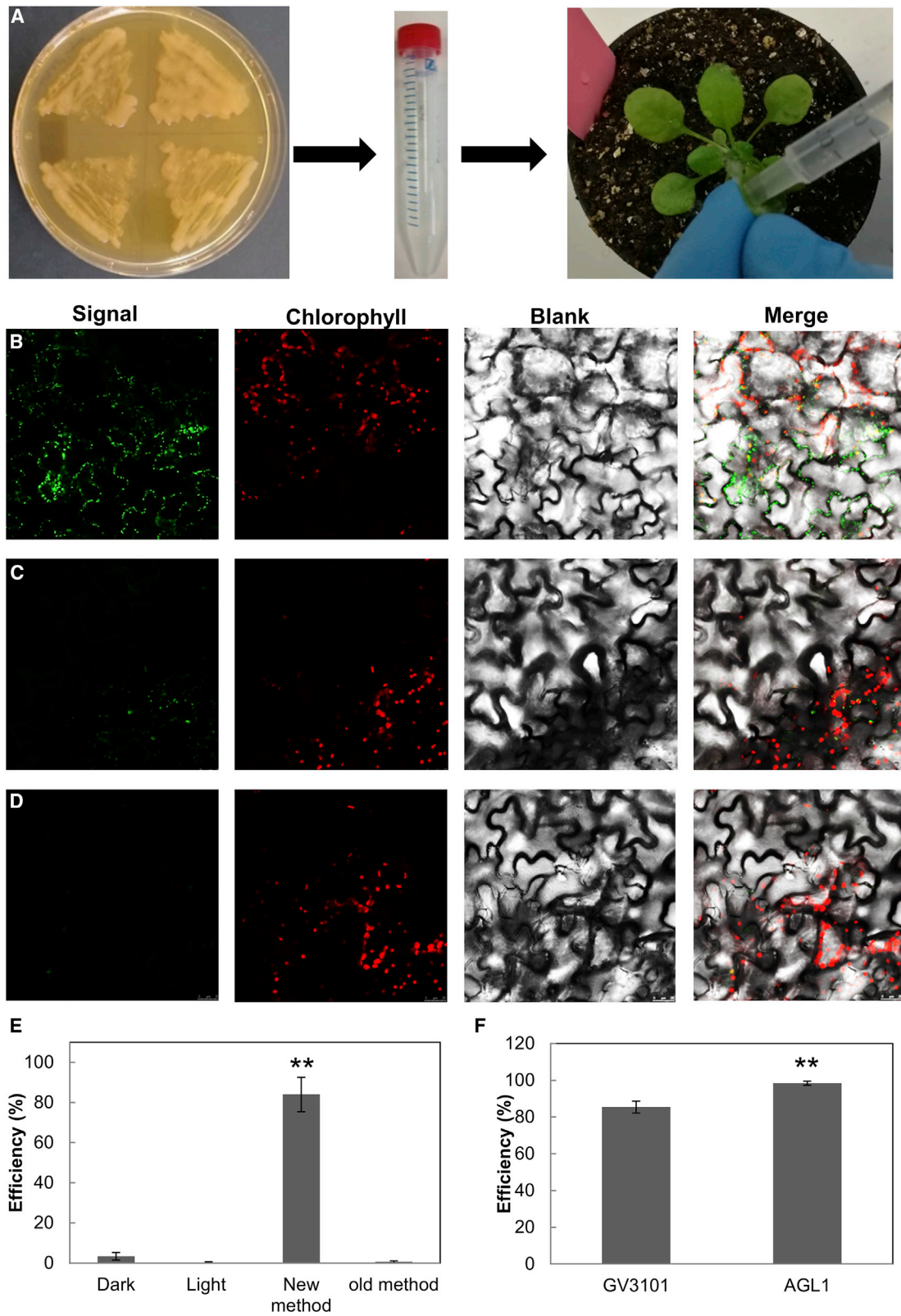
Figure 2. The High-Efficiency Agro-Infiltration of *Arabidopsis* Leaves.
(A) *Agrobacterium* was streaked on YEB-induced medium, diluted with infiltration medium, and injected into leaves of *Arabidopsis*.
(B) Mitochondria-targeted citrate synthase with C-terminal GFP (CYS4-GFP) was infiltrated into *Arabidopsis* leaves using the standard protocol described in this paper.

(legend continued on next page)

Washing Solution	
MgCl$_2$	10mM
Acetosyringone[a]	100μM
Infitration Solution (PH6,0, fresh!)[b]	
Murashige and Skoog medium (sigma M5519)	1.1g
Sucrose	1%
Acetosyringone[a]	100μM
Silwet L-77	0,01%

Table 1. The Infiltration Solution for the *Agrobacteria* Infiltration
[a]For Acetosyringone 100mM stock, dissolve 9.8mg in 0.5ml DMSO.
[b]Infiltration solution needs to freshly prepared, while MS with 1% sucrose (pH=6.0) could be prepared as stock.

RESULTS

Transient Expression in *Arabidopsis* Leaves

Here, we describe a robust transient expression method that is characterized by high efficiency, reproducibility, and suitability for a variety of functional *Arabidopsis* proteins with diverse biological activities and subcellular localizations (Figure 1). Two- to 3-week old *Arabidopsis* plants cultivated under 8-, 12-, or 16-h photoperiods were transformed before bolting and were used for proof-of-concept studies. For this purpose, the mitochondrial citrate synthesis 4 (CSY4) GFP fusion protein (Supplemental Tables 1–3) was transformed into the *Agrobacterium* stains GV3101 and AGL1, which contain vectors derived from the nopaline-type disarmed Ti-plasmids pTiC58 and pTiBo542, respectively, that harbor genes for rifampicin resistance (Lee and Gelvin, 2008). Unlike in *N. benthamiana*, infiltration of *Arabidopsis* leaves with a liquid *Agrobacterium* culture resulted in a much lower transformation efficiency in leaves (Figure 1). To circumvent this problem, the agrobacteria were streaked onto a YEB agar plate containing 200 μM acetosyringone for virulence gene induction, and 50 μM rifampicin and antibiotics for selection of the vector (Figure 2A and Supplemental Table 4). After 1 or 2 days of incubation at 28°C, it proved important to transfer agrobacteria (cultured to a high optical density at 600 nm [OD$_{600}$] > 12) into a wash solution, which diluted the antibiotics from the plate. The agrobacteria were then diluted in infiltration solution and injected into *Arabidopsis* leaves using a disposable plastic syringe (Figure 2A and Table 1). Because the leaves were full of water after infiltration, they were dried for 1 h at room temperature and then kept in the dark for 24 h. Following infiltration, plants were transferred to the greenhouse for 3 days. Protein expression levels were detected by western blotting and/or confocal microscopy. It is important to note that the presence of Silwet L-77 and keeping plants in the dark for 24 h greatly improved the efficiency of

transient transformation (Figure 2B–2D). Without Silwet L-77 or dark treatment after infiltration, the transformation ratio was as low as in previously published protocols (Mangano et al., 2014). Similarly, concentrations of Silwet L-77 that were too high and the lack of a leaf-drying step resulted in tissue damage leading to artificial fluorescence being observable under the confocal microscope.

For our case study, localization of the mitochondrial fusion protein was visualized by confocal microscopy. Infiltration with 0.005% Silwet L-77 resulted in 80%–90% of cells being transformed (Figure 2B and 2E), while only very few cells were transformed when plants were not kept in the dark for 24 h after infiltration (Figure 2C). No cells were transformed in the absence of Silwet L-77 (Figure 2D). In addition, the agrobacteria AGL1 strain resulted in higher transformation ratios of 95%–100% compared with the GV3101 strain (Figure 2F).

Transient Expression in the Leaves of Different Plant Species

To further test this method, we grew plants of several plant species commonly used in our research. Four Brassicaceae family species (*B. oleracea*, *C. rubella*, *T. salsuginea*, and *T. halophila*) were grown in the greenhouse under 8-, 12-, and 16-h photoperiods for 2–3 weeks. Younger leaves had higher transformation ratios compared with older leaves. Six-week-old *S. tuberosum Desiree*, *C. annuum*, and *N. benthamiana* were also cultivated in the greenhouse under 12-h photoperiods.

After growing AGL1 agrobacteria transformed with a mitochondrial CSY4 GFP fusion protein in induction medium (Supplemental Table 4) for approximately 2 days, the transformed agrobacteria were moved into wash solution (Table 1), diluted in infiltration solution, and injected into plant leaves using a disposable plastic syringe (Table 1). After drying the leaves and maintaining the plants in the dark for 24 h, the transformed plants were moved into the greenhouse for 2 days and examined under the microscope. *B. oleracea* transformation resulted in a success rate of only approximately 20% (Figures 3A and 4A), which is in contrast to the high success rates obtained for the other Brassicaceae family species used, where young leaves displayed transformation ratios of around 60%–80% (Figures 3B–3D and 4A). In *S. tuberosum* and *C. annuum*, a lower concentration of Silwet L-77 0.001% was used for infiltration, resulting in a transformation ratio of between 60% and 80% (Figures 3E and 3F and 4A), as it was observed that concentrations of 0.005%–0.01% Silwet L-77 could damage the leaves of these two species. In addition, the method we describe here also resulted in high levels of transiently expressed protein in *N. benthamiana* (Figure 3G), suggesting that it works efficiently in a broad range of species.

(C) Agro-infiltration protocol with transformed plants kept in darkness for 2 days.
(D) Agro-infiltration protocol without the addition the 0.005% Silwet L-77.
(E) Transformation efficiency of wild-type *Arabidopsis* plants. Dark method, CYS4-GFP was infiltrated into *Arabidopsis* leaves and plants were kept in the dark for 2 days. Light method, CYS4-GFP was infiltrated into *Arabidopsis* leaves without 0.005% Silwet L-77 and plants were kept in the light. New method, CYS4-GFP was infiltrated into *Arabidopsis* leaves using the standard protocol described in this paper. Old method, CYS4-GFP was infiltrated into *Arabidopsis* leaves using a previously published method (Mangano et al., 2014). The transformation efficiency was calculated from eight different replicates.
(F) Both agrobacteria strains, GV3101 and AGL1, were transformed into *Arabidopsis* leaves. Agro-infiltration of AGL1 into *Arabidopsis* leaves was achieved with high efficiency agro-infiltration of leaves.
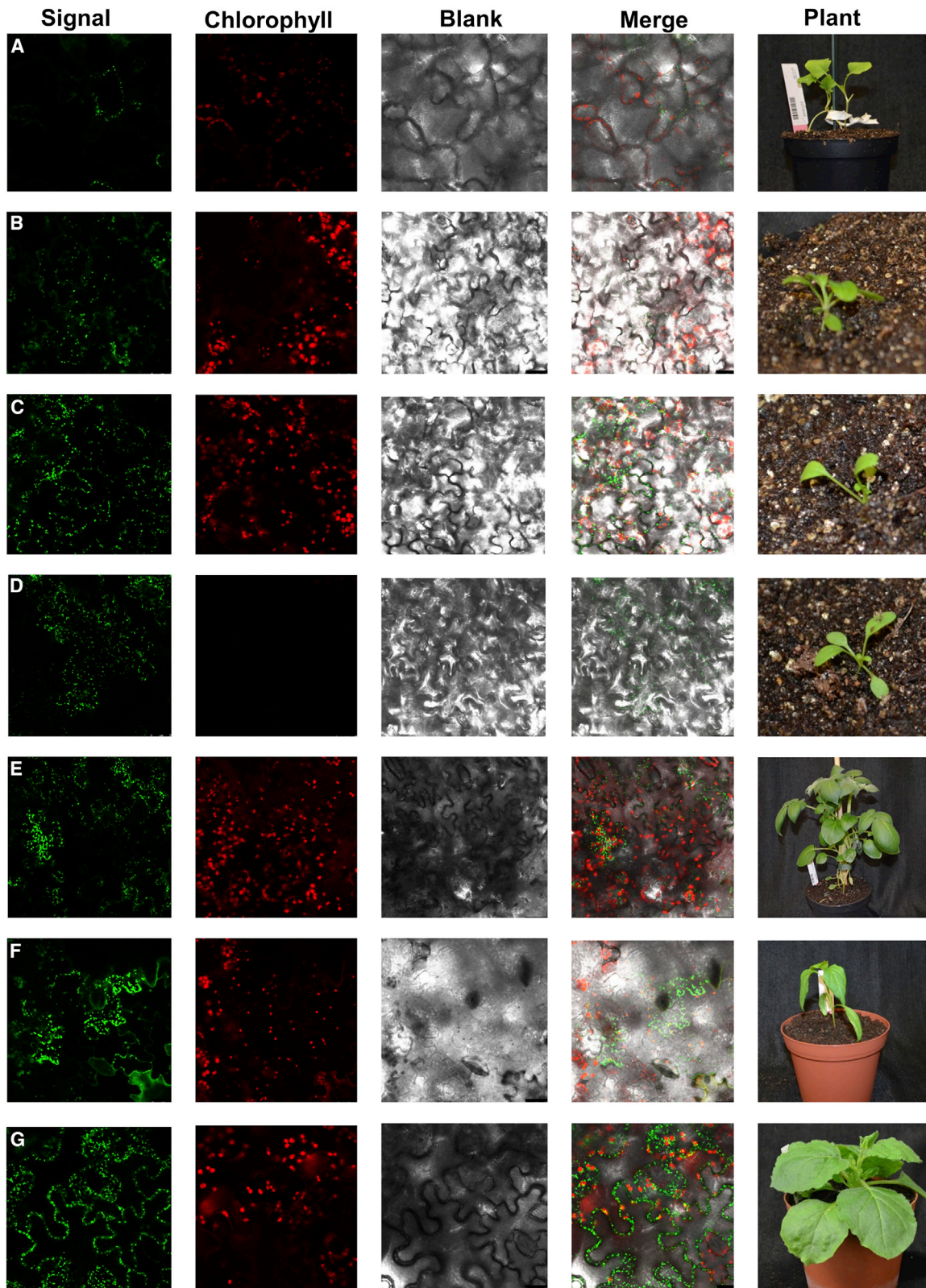
Signal	Chlorophyll	Blank	Merge	Plant

Figure 3. The High-Efficiency Agro-Infiltration of the Leaves of Different Plant Species.

The standard *Arabidopsis* transient transformation protocol described above was used without modifications for the transformation of *Brassica oleracea* **(A)**, *Capsella rubella* **(B)**, *Thellungiella salsuginea* **(C)**, *Thellungiella halophila* **(D)**, *Solanum tuberosum* **(E)**, *Capsicum annuum* **(F)**, and *Nicotiana benthamiana* **(G)**.
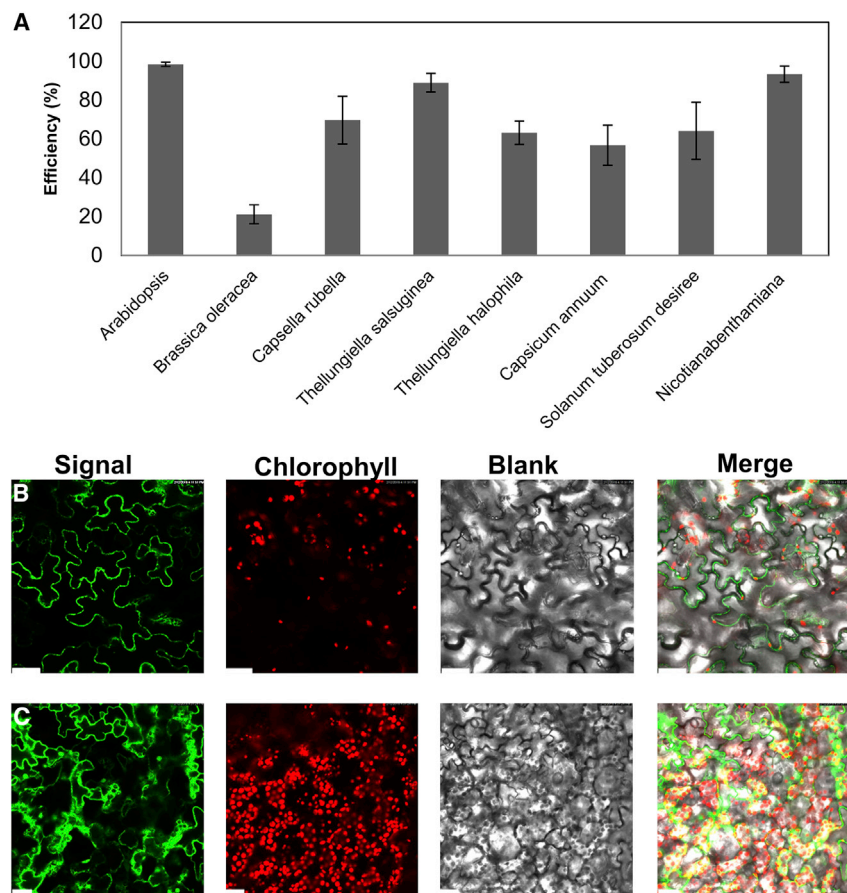
Figure 4. Efficiency and Case Study of the Transient Transformation Method for Assessing Protein–Protein Interactions.

(A) The transformation efficiency achieved using AGL1 agrobacteria for the transient transformation of the species described. Data presented are mean ± SD (n = 8).

(B) Transient expression of the *Arabidopsis* sucrose phosphate synthase 1 (AtSPS) gene in *Arabidopsis* leaves.

(C) Transient expression of free GFP in *Arabidopsis* leaves as a control.

(D) Using the transiently transformed *Arabidopsis* leaves to analyze protein–protein interactions by AP-MS.

D

Preys	Name of preys	Fold change
AT5G11110	ATSPS2F	101060666
AT5G56010	Heat shock protein 90	13398666
AT1G78570	UDP-L-Rhamnose synthase	16
AT5G16070	T-complex protein 1	7
AT3G63460	Protein transport protein SEC31	6
AT2G39770	Mannose-1-phosphate guanylyltransferase 1	6
AT1G01800	NAD(P)-binding Rossmann	6
AT1G78300	14-3-3OMEGA	5
AT1G51160	SNARE-like superfamily protein	4

MS) experiments have been widely used to generate meaningful interaction networks (Puig et al., 2001; Bürckstümmer et al., 2006; Morris et al., 2014; Zhang et al., 2017b, 2018, 2019), it follows that they could be used to produce information-rich data concerning both within-pathway and extra-pathway protein–protein interactions. Such interactions would aid in the characterization of interacting protein function, provide detailed catalogs of proteins involved in forming protein complexes, and reveal networks of biological processes at local and proteome-wide scales (Morris et al., 2014).

The success of AP-MS greatly depends on tagged protein expression for AP, and the efficiency of trypsin digestion and the recovery of tryptic peptides for MS analysis (Zhang et al., 2017b, 2019). Unlike in digestion-based AP-MS, in which a gel is cut into pieces for several independent trypsin digestions (Morris et al., 2014), we used a proteomics-based in-solution digestion method to directly digest proteins on the beads that they were bound following AP (Zhang et al., 2017b, 2018). Thus, in an AP-MS experiment a single sample is used for the liquid chromatography-mass spectrometry (LC-MS) measurement. In subsequent data analysis, normalized signal intensities were processed to determine fold change in abundance (FC-A) scores using the SAINT algorithm embedded within CRAPome software (Choi et al., 2012; Morris et al., 2014). A total of 22 protein–protein interactions were obtained, displaying in excess of four-fold changes in abundance (Supplemental Table 3). We considered only the protein pairs for which the intensity scores were in the top 2% compared with GFP, which corresponded to FC-A values of at least 4 in a minimum of three of the four replicates as positive interactions. Because of the possible mis-targeted subcellular localization of AtSPS following high-level overexpression, only the cytosol localized preys were selected for network generation. Screening of the SUBA4 database

Using the Agro-Infiltration Method to Test for Novel Protein–Protein Interactions

In addition, AtSPS-GFP and GFP (Supplemental Table 2) were transformed into *Arabidopsis* leaves to further test the use of this method for the discovery of novel protein–protein interactions. Both GFP and AtSPS-GFP could be expressed to a high level in *Arabidopsis* leaves (Figure 4B and 4C). For each construct, four plants were used for infiltration and kept in the greenhouse for 6 days to produce approximately 2–3 g of plant material for affinity purification (AP). Because AP-mass spectrometry (AP-
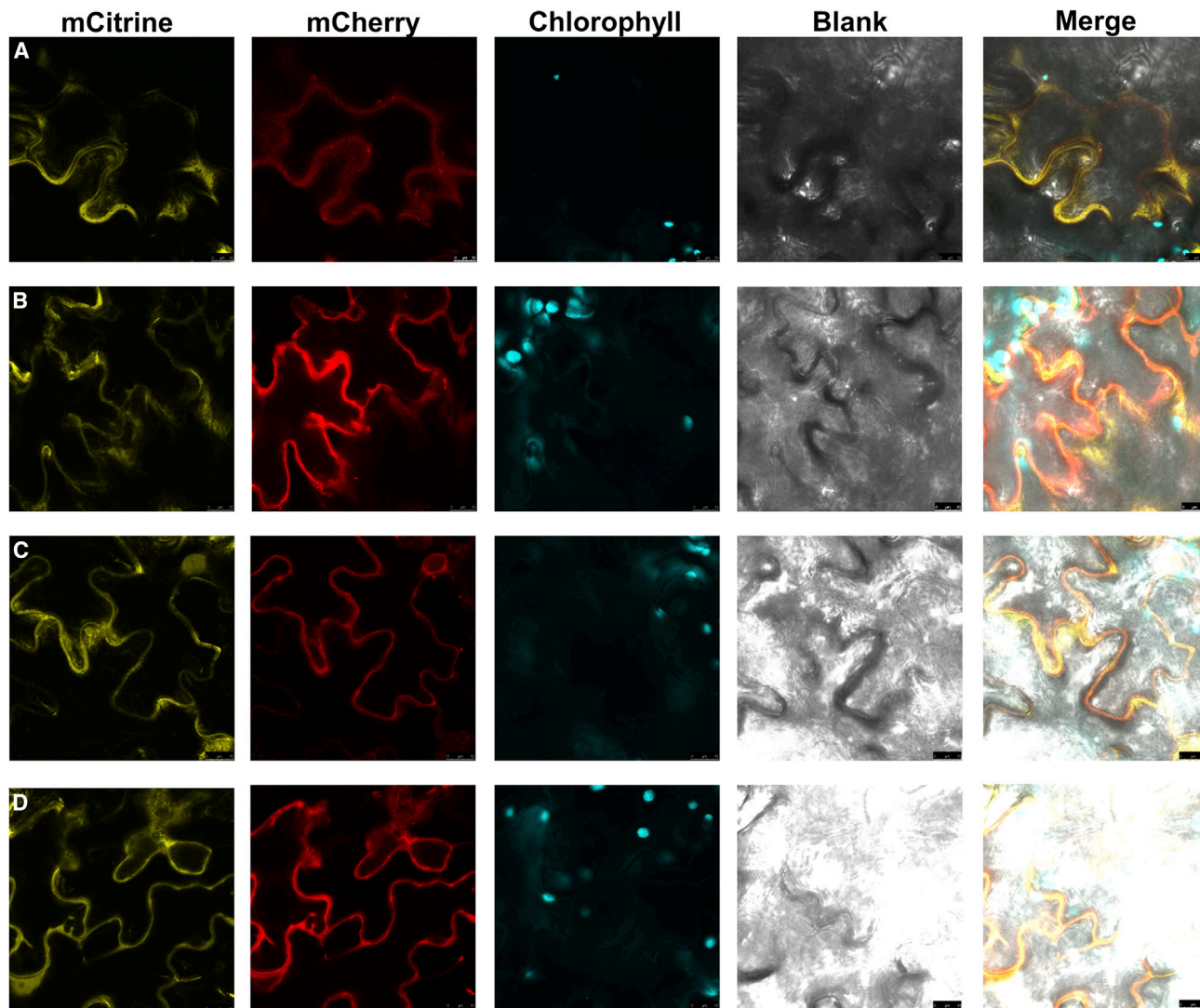
Figure 5. Subcellular Co-localization of Four AtSPS1 Interactors.
(A) AtSPS2F-mCitrine/AtSPS1-mCherry.
(B) AtCYT1-mCitrine/AtSPS1-mCherry.
(C) AtRHM1-mCitrine/AtSPS1-mCherry.
(D) 14-3-3OMEGA-mCitrine/AtSPS1-mCherry. The panels from left to right show the mCitrine fluorescence, mCherry fluorescence, autofluorescence, blank, and the merged image, respectively. AtSPS2F (AT5G11110) is an isoform of sucrose phosphate synthase. AtCYT1 (AT2G39770) is GDP-mannose pyrophosphorylase/mannose-1-pyrophosphatase. AtRHM1 (AT1G78570) is a UDP-L-rhamnose synthase. 14-3-3OMEGA (AT1G78300) is a 14-3-3 protein.

(Hooper et al., 2016) revealed a total of 11 interactions between cytosol-localized AtSPS1 and cytosol target proteins, which comprise the interaction network (Figure 4D). Of these 11 interactions, 2 have been previously reported: ATSPS2F (Bahaji et al., 2015) and 14-3-3OMEGA (Toroser et al., 1998). These two previously known and nine novel interactions are shown in Figure 4D.

Colocalization of Selected Interaction Pairs and Confirmation of Interactions by BiFC

As the FC score from the AP-MS analysis is indicative of the inter-action intensity (Mellacheruvu et al., 2013), we randomly selected two protein interactors with a high FC score (AtSPS2F and

AtRHM1) and two interactors with a low score (AtCYT1 and 14-3-3OMEGA) to analyze the protein interaction network (Figure 4C). Colocalization refers to the observation of spatial overlap between two different fluorescent labels (mCitrine and mCherry), each having a separate emission wavelength, to see if the different "targets" are located in the same area of the cell or very near to one another, which provides support for their interaction. Because these proteins have the same suggested subcellular localization in SUBA4, we used two fluorescence signals to analyze the colocalization of these interaction pairs (Figure 5). In our experiment, both AtSPS1 and the four identified interactors were localized to the plasma membrane, suggestive of possible interactions. Moreover, we used two vectors co-infiltrated into *Arabidopsis* leaves with a previous
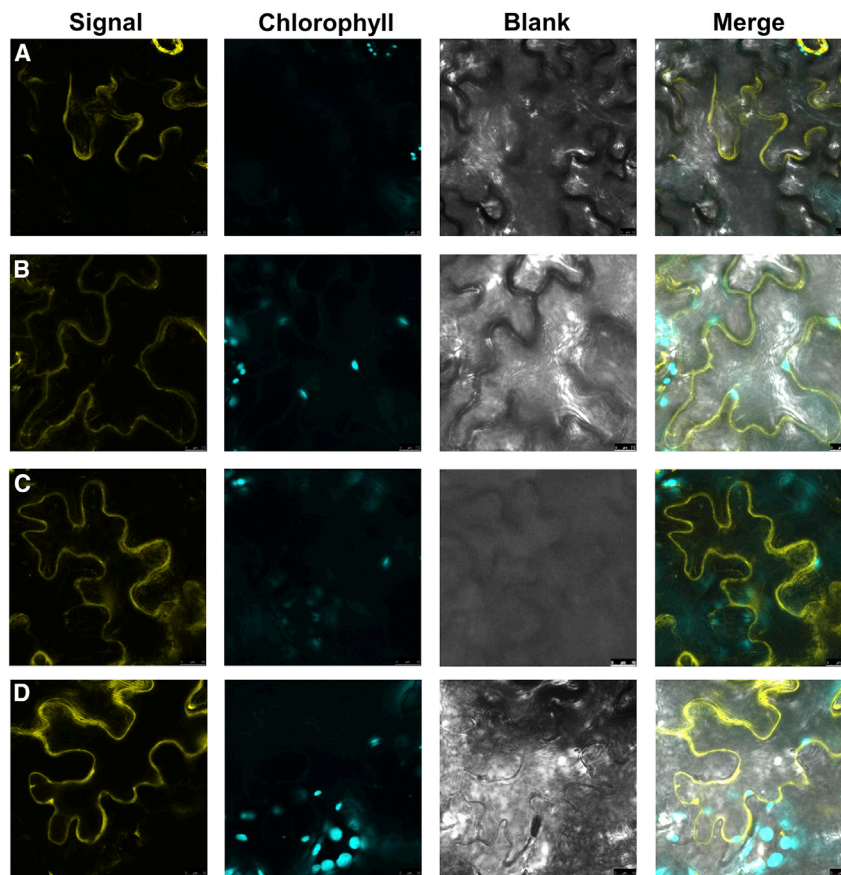
diverse types of proteins and experimental designs. We expect that this method can be readily adopted for other types of functional assays both in wild-type and mutant *Arabidopsis* plants. Importantly, this method nicely complements commonly used *Arabidopsis* mesophyll–protoplast transformation methods, *Agrobacterium* plant cell culture transformation, and *Agrobacterium*-mediated infiltration of *N. benthamiana* leaves (Sparkes et al., 2006) and would seem likely to be a great boon for researchers wanting to functionally complement studies in a medium- to high-throughput manner.

However, use of this method is not restricted to *Arabidopsis*, with data presented here revealing high-efficiency transformation of close *Arabidopsis* relatives, including *C. rubella*, *T. salsuginea*, and *T. halophilia*. Although a high transformation efficiency could not be obtained for *B. oleracea*, increased use of the other three Brassicaceae species tested here (Tohge et al., 2016, 2017, 2018) suggests that availability of a transient expression system for them will likely be highly useful to many researchers. Beyond Brassicaceae, we also demonstrate that the protocol could be used for transient expression in *S. tuberosum*, *C. annum*, and *N. benthamiana*. Although the latter is commonly used for transient expression (Martin et al., 2009; Li et al., 2013), the fact that our protocol also works in this species may prove useful in instances where it is preferable to assess the effect of transient expression in several species. Although potato was one of the earliest stably transformed plants (Fernie and Willmitzer, 2001; Hofius et al., 2001) using virus-induced gene silencing (VIGS) (Brigneti et al., 2004; Faivre-Rampant et al., 2004), only a single report of transient overexpression has been, to date, reported in this species (Mba'u et al., 2018). This situation is even more severe in *C. annum*, with only a handful of laboratories even reporting successful stable transformation to date (Manoharan et al., 1998; Kumar et al., 2012). Therefore, having a functional transient expression system will likely be a great boon for this species and for many others. Indeed both potato and *C. annum* have advanced genetic resources available, including high-quality genomes (Potato Genome Sequencing Consortium, 2011; Qin et al., 2014) and ample transcriptomics datasets (Li et al., 2016), and are relatively well characterized at the proteomic and metabolomics levels (Palma et al., 2018; Shekhar et al., 2016).

high efficiency of transformation. In addition, we also used bimolecular fluorescent complementation (BiFC) assays for analysis of these binary protein interactions (Figure 6). All four selected interactions were independently validated by BiFC assays, which enabled qualitative but highly sensitive detection of protein–protein interactions with subcellular localization information. Importantly, no BiFC signal was detected for any of the enzyme pairs that were not defined in our AP-MS interaction network.

DISCUSSION

We have developed a novel transient transformation method that enables unprecedentedly high transformation efficiency in a range of plant species, including *Arabidopsis* Col-0 seedlings. We believe a good transient expression system needs to fulfill several criteria: namely it should be highly reproducible in different labs, offer considerable time savings in comparison with stable transformation methods, and have a high transformation efficiency. Finally, it should achieve this without producing significant detrimental effects or artifacts and should be broadly applicable to different types of functional assays. Generally, the overall transformation efficiency achieved by agro-infiltration depends on the presence of mild concentrations of Silwet L-77 (Kim et al., 2009), streaking agrobacteria on agar plates, and keeping *Agrobacterium*-treated plants in the dark for 24 h. This method is optimized for transient expression of genes in *Arabidopsis* leaves, and routinely results in transformation of up to 70%–90% of cells. Furthermore, our method is applicable to very

Furthermore, both mapping populations and a global panel of accessions for both species have been generated or collected allowing quantitative trait loci and genome-wide association studies, respectively, to be performed. Thus the transient expression method we describe here will likely also find great utility as a tool for the rapid validation of candidate genes. Although VIGS is one strategy for transient expression, as we mention above this system is not currently developed for *C. annum*. We should note here that the method proved successful for every species tested, with the exception of *B. oleracea*, suggesting that its range and hence its utility likely exceeds that we report here.

In addition to the development of the technique, we describe one experiment designed to provide rapid insight into *Arabidopsis* biology. For this purpose, we investigated the protein binding partners of sucrose phosphate synthase. We chose this protein because of a hypothesis raised following the elucidation of the crystal structure of an enzyme from the sucrose synthesis pathway, sucrose phosphate phosphatase (from cyanobacteria), which suggested that sucrose phosphate synthase and sucrose phosphate phosphatase may operate in tandem to channel sucrose phosphate (Fieulaine et al., 2005). We recently demonstrated that plants harbor the canonical glycolysis metabolon (Zhang and Fernie, 2018) as well as a novel plant-specific channel within the TCA cycle (Zhang et al., 2017a; Fernie et al., 2018). Furthering this investigation, here we adopted the same approach but used transient rather than stable transformation of *Arabidopsis* cultures to assess the protein–protein interactions of sucrose phosphate synthase. We were unable to find an association between sucrose phosphate synthase and sucrose phosphate phosphatase, suggesting that it is highly unlikely that they channel sucrose 6-phosphate. However, 11 other interacting proteins were found with a similar subcellular location. These sucrose phosphate synthase interactors include a different isoform of sucrose phosphate synthase (Bahaji et al., 2015) and the known regulatory protein 14-3-3OMEGA (Toroser et al., 1998), in addition to nine other proteins with various functions, including metabolic enzymes, transport proteins, and chaperones, as well as proteins with little known functions. This study was able to rapidly evaluate the hypothesis that sucrose 6-phosphate is channeled in plants and also to provide a list of candidate regulatory proteins that may aid in the assembly or stability of sucrose phosphate synthase. We identified a similar set of proteins when assembling the extra-pathway TCA cycle interactome (Zhang et al., 2018), and feel that this may prove to be a common feature in metabolism-associated protein–protein interaction networks.

In summary, this technical report presents a protocol for transient expression in *Arabidopsis* that can also be applied to a range of other species. In addition, we demonstrate its utility in protein localization and protein–protein interaction studies as well as proving its usefulness as a means to rapidly test previous biological postulates. We strongly believe that this protocol will prove highly useful for a wide number of laboratories since it improves greatly on existing protocols for *Arabidopsis* as well as opening up new avenues of research in several other species of ecological, environmental, and agronomic importance. Although the tremendous interest and development of CRISPR-based strate-gies is rendering genome editing possible in an ever-increasing range of species (Schmidt et al., 2019), the use of transient systems currently have clear advantages for addressing several questions. It is envisaged that future development of this approach, in addition to CRISPR, will widen the range of species that can be transformed.

METHODS

cDNA Cloning and Vector Construction

All the *Arabidopsis* proteins used in this study are listed in Supplemental Table 1. Full-length coding sequences of these proteins were cloned from a cDNA pool generated from 2-week old *A. thaliana* Col-0 ecotype plants by PCR-based Gateway BP cloning using the pDONR207 Donor vector (Thermo Fisher Scientific, Waltham, MA). Expression vectors for AP-MS and BiFC were constructed using the Gateway LR reaction with pK7FWG2 (Karimi et al., 2002), pDEST–^{GW}VYNE, and pDEST–^{GW}VYCE (Gehl et al., 2009). mCitrine and mCherry (Grünberg et al., 2013) were sub-cloned with the related gene into pDONR207 using the In-Fusion (Clontech) method, and then sub-cloned into pK7WG2 (Karimi et al., 2002) by means of LR reaction (Thermo Fisher Scientific) for the co-sublocalization assays (Supplemental Table 2).

Plant Growth

Arabidopsis plants were grown in a greenhouse for 2–3 weeks under 8-, 12-, and 16-h photoperiods. *B. oleracea*, *C. rubella*, *T. salsuginea*, and *T. halophila* were grown in the greenhouse under 8-, 12-, and 16-h photoperiods for 2–3 weeks. Six-week-old *S. tuberosum* cv. Desiree, *C. annuum*, and *N. benthamiana* were also cultivated in the greenhouse under 12-h photoperiods.

Agrobacteria Transformation

Frozen stocks of AGL1 were added to YEB medium with Carb+ (carbenicillin 25 mg/l) and Rif+ (rifampicin 20 mg/l) and incubated at 28°C overnight. The agrobacteria were centrifuged for 30 s at 20 000 g, 4°C and washed with 1 ml and 500 µl ice-cold water. The cells were finally resuspended in 200 µl of water (hereafter referred to as agrobacteria-competent cells). Around 1–5 µl of expression plasmid was added into a 2-ml tube with 45 µl of agrobacteria-competent cells on ice for 5 min. The solution was electrically shocked in cuvettes and kept at 28°C with 1 ml YEB medium. After shaking at 250 rpm for 1–2 h at 28°C, the cells were plated on a YEB plate (Carb+, Rif+, and appropriate antibiotics) and incubated at 28°C for 2–3 days.

Agrobacterium Culture Preparation and Infiltration

The agrobacteria were grown on YEB-induced medium plates (Supplemental Table 4) at 28°C for 24–36 h. The cells were scraped and resuspended in 500 µl washing solution (10 mM $MgCl_2$, 100 µM acetosyringone). After briefly vortexing, 100-µl resuspended agrobacteria were diluted 10 times to measure the OD_{600} (should be equal to or greater than 12). The agrobacteria were finally diluted to an OD_{600} of 0.5 in infiltration solution ($^1/_4$MS [pH = 6.0], 1% sucrose, 100 µM acetosyringone, 0.005% [v/v, 50 µl/l] Silwet L-77). The agrobacteria were infiltrated into *Arabidopsis* leaves using a 1-ml plastic syringe, kept in the light to dry the leaves (1 h), and then subsequently kept in the dark for 24 h at room temperature. The transformed plants were then transferred back to the greenhouse for another 2–3 days before sample collection/microscopy. The efficiency was calculated by observing all cells under a confocal microscope in the same focal plane with four biological replicates.

Affinity Purification-Mass Spectrometry

AP-MS was conducted by expressing target proteins fused with a C-terminal GFP tag in *Arabidopsis* leaves using the above transformation protocol. Tandem GFP was used as a negative control. Expression and

localization of the tagged proteins were evaluated by visualizing GFP fluorescence using confocal microscopy. The transformed plant materials were collected 6 days after their transfer to the greenhouse. After grinding tissue into a fine powder using a ball mill (MM301, Retch, Haan, Germany), proteins were extracted by mixing 2 g of material with 2 ml extraction buffer (25 mM Tris–HCl [pH7.5], 15 mM MgCl₂, 5 mM EGTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Following removal of cell debris by repeated centrifugation at 22 000 g, 4°C for 5 min, the supernatant was mixed with 25 μl of GFP-Trap_A slurry (ChromoTek, Martinsried, Germany) equilibrated with extraction buffer and incubated for 1 h at 4°C with rotation. The beads were collected by centrifugation at 3000 g, 4°C for 3 min and washed three times each with extraction buffer containing different concentrations of NaCl (0, 250, and 500 mM) (Zhang et al., 2019).

The proteins remaining on the beads were subsequently subjected to proteolysis via in-solution digestion with LysC and trypsin and the resulting peptides were purified (Wiśniewski et al., 2009). LC-tandem MS (LC-MS/MS) analysis was performed on a Q Exactive Plus (Thermo Fisher Scientific) machine. Quantitative analysis of MS/MS measurements was performed with Progenesis IQ software (Nonlinear Dynamics, Newcastle, UK). Proteins were identified from spectra using Mascot (Matrix Science, London, UK). Mascot search parameters were set as follows: TAIR10 protein annotation, requirement for tryptic ends, one missed cleavage allowed, fixed modification (carbamidomethylation [cysteine]), variable modification (oxidation [methionine]), peptide mass tolerance ± 10 ppm, MS/MS tolerance ± 0.6 Da, allowed peptide charges of +2 and +3. A decoy database search was used to limit the false discovery rate to 1% at the protein level. Peptide identifications below rank 1 or with a Mascot ion score below 25 were excluded. Mascot results were imported into Progenesis QI, quantitative peak area information extracted, and the results exported for data plotting and statistical analysis. These intensities were filtered against the experiment control and normalized using the spectral index using the algorithms embedded in the CRAPome website (Mellacheruvu et al., 2013). Finally, the possible interactions were assigned a fold change-A score (FC-A) calculated by the SAINT algorithm (Choi et al., 2012; Mellacheruvu et al., 2013). All of the FC-A scores of the detected peptides are presented in Supplemental Table 3. Finally, the interaction pairs with an FC score above 4 were selected and analyzed using SUBA4 (Hooper et al., 2016) to restrict defined interactors to those proteins that are co-localized to the cytosol (Zhang et al., 2019).

BiFC

Confocal images were taken using a DM6000B/SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). BiFC and MitoTracker fluorescence were imaged with 488 and 555 nm laser excitation, and emission fluorescence was captured by 500–520 and 560–580 nm band-pass emission filters, respectively.

Co-subcellular Localization Analysis

Constructs (Supplemental Table 2) were transiently expressed in *Arabidopsis* leaves by agro-infiltration as mentioned above for the protein co-sublocalization analysis. Confocal images were taken using a DM6000B/SP8 confocal laser scanning microscope.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

Y.Z. and M.C. designed the experiments. Y.Z. and M.C. developed the protocol for the *Arabidopsis* agro-infiltration. M.C., M.R.T., Y.J., V.S., and Y.S. independently tested the protocol. Y.Z and B.S. finished the AP-MS measurement and analysis. Y.Z, and A.R.F. wrote the manuscript.

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