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ORIGINAL ARTICLE

# A highly efficient grapevine mesophyll protoplast system for transient gene expression and the study of disease resistance proteins

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**Abstract** Plant protoplasts constitute a versatile system for transient gene expression and have been widely used with several plant species for the functional characterization of genes and studies of diverse signaling pathways. However, such a system has not been developed for grapevine (*Vitis vinifera* L.) due to the challenges of large-scale isolation of viable grapevine protoplasts. Here, we report a simplified method for obtaining high yields and excellent viability of isolated protoplasts from young grapevine leaves. In addition, both the conditions for isolation and transfection of grapevine mesophyll protoplasts were modified, and the system was shown to be suitable for protein expression and studies of protein subcellular localization and protein–protein interactions. In addition, we heterologously and transiently expressed the *Arabidopsis thaliana* disease resistance protein RPW8.2, which has previously been reported to confer broad-spectrum resistance to several biotrophic pathogens in different plant families, as a fluorescent fusion protein in grapevine protoplasts. We observed that expression of the RPW8.2 fusion protein was induced in response to application of exogenous salicylic acid and following infection by the grapevine downy mildew pathogen, *Plasmopara viticola*. These results illustrate the potential of this highly efficient mesophyll protoplast system for transient gene expression and investigation of the activity of disease resistance proteins in grapevine.

**Keywords** Protoplasts · Grapevine mesophyll · Transient gene expression · RPW8.2 · Salicylic acid · Downy mildew

**Abbreviations**

| | |
|---|---|
| BiFC | Bimolecular fluorescence complementation |
| BSA | Bovine serum albumin |
| CDS | Coding sequence |
| EST | Expressed sequence tag |
| FDA | Fluorescein diacetate |
| GFP | Green fluorescent protein |
| MES | 4-Morpholineethanesulfonic acid |
| MAP | Mitogen-activated protein |
| MS | Murashige and Skoog |
| PEG | Polyethylene glycol |
| RPW8.2 | Resistance to powdery mildew 8.2 |
| SA | Salicylic acid |
| YFP | Yellow fluorescent protein |
| hpt | h post-treatment |
| hpi | h post-inoculation |

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## Introduction

Although the generation of transgenic lines represents a powerful research tool for characterizing plant gene function, low transformation efficiency and the time-consuming process of obtaining stable transgenic lines still limits the

utilization of this approach for large-scale, analyses of plant genes (Chen et al. 2006). Consequently, transient expression assays, which are characterized as being rapid and high-throughput, represent an attractive alternative (De Sutter et al. 2005; Marion et al. 2008). There are several methods for transiently expressing genes, including polyethylene glycol (PEG)-mediated protoplast transfection (Yoo et al. 2007), biolistic bombardment (Ueki et al. 2009) and *Agrobacterium tumefaciens*-mediated transient transformation (Manavella and Chan 2009). However, some of these transient expression assays have certain disadvantages. For example, although the bombardment approach has been successfully used to introduce DNA into plant calli (Chavez-Barcenas et al. 2000; Manavella and Chan 2009) or leaf tissue (Becker et al. 1994; Sheen et al. 1995; Zubko et al. 2004), low efficiencies have been reported with some species and the technique depends on the availability of relatively expensive equipment (Dekeyser et al. 1990). The *Agrobacterium*-mediated approach generally has a higher efficiency and is quite inexpensive, but it is often difficult to use for subcellular localization assays and other fluorescence-based analysis because of a high level of nonspecific auto-fluorescence (Dong et al. 2001; Li et al. 2009; Liu et al. 1992). In addition, the waxy cuticles of some plants organs can limit observations using a fluorescence microscope.

Alternatively, plant protoplasts provide a versatile cell-based experimental system for transient gene expression. Even though they lack a protective cell wall, their viability can be maintained and they can easily take up bacteria, cell organelles, and nucleic acids from outside the cell (Davey and Cocking 1972; Ohyama et al. 1972). Transient assays utilizing PEG-mediated protoplast transfection protocols have been established for several plant species, including *Arabidopsis thaliana* (Yoo et al. 2007), rice (*Oryza sativa*) (Chen et al. 2006; Zhang et al. 2011), *Populus trichocarpa* (Guo et al. 2012; Tan et al. 2013) and *Vitis vinifera* (Wang et al. 2015). Plant protoplasts have considerable potential for studying many aspects of plant biology such as somatic hybridization, protein activity, and light/chloroplast-related processes (Davey et al. 2005; Hong et al. 2012; Chen et al. 2015; Zhang et al. 2011). In this report, we provide another example through the development and application of grapevine protoplast transformation to characterize plant defense mechanisms.

Plant disease resistance (*R*) genes play crucial roles in plant defense processes, and to date, more than 100 *R* genes have been identified, together with associated downstream defense-related genes (Dodds and Rathjen 2010). However, the functions of most of these genes are unknown due to the lack of efficient systems for their detailed characterization. Plant protoplasts can provided a powerful and versatile system for high-throughput dissection of plant signal transduction pathways, including those regulating the expression of defense related genes. For example, *A. thaliana* mitogen-activated protein (MAP) kinase cascades were shown to be involved in oxidative stress and defense signaling pathways using a transient *A. thaliana* protoplast assay (Asai et al. 2002; Kovtun et al. 1998, 2000). Moreover, Chen et al. (2006) established a transient green fluorescent protein (GFP)-based reporter system in rice protoplasts to analyze the expression of defense-related genes, and confirmed that two rice defense-related genes, *PBZ1* and *chitinaseIII*, were induced by infection with *Magnaporthe grisea* (Chen et al. 2006). More recently, a maize protoplast system was established for studying programmed cell death-related processes, and transient expression of MADS29 in maize nucellus protoplasts was shown to increase the transcript levels of a cysteine protease (Chen et al. 2015).

The *A. thaliana R* gene, *RESISTANCE TO POWDERY MILDEW* 8.2 (*RPW8.2*), not only confers resistance to a broad spectrum of powdery mildews, but also enhances resistance to another biotrophic pathogen, *Hyaloperonospora parasitica* (Wang et al. 2007; Xiao et al. 2001). Accumulation of the RPW8.2 protein also increases in response to the application of exogenous salicylic acid (SA) (Wang et al. 2007; Xiao et al. 2001, 2003a, b). In this study, we addressed the hypothesis that *RPW8.2* might retain a similar function and exhibit similar characteristics when expressed in grapevine (*V. vinifera* L.), an important fruit crop due to its considerable nutritional, cultural and economic value (Myles et al. 2011). Despite substantial progress in grapevine transformation, it remains a labor intensive and time-consuming process (Kurth et al. 2012). Over the last decade, grapevine protoplasts have been isolated from different tissues or organs, including callus (Zhu et al. 1993, 1997; Skene 1974, 1975), cell suspension cultures (Brezeanu and Rosu 1984), mesophyll tissue (Barbier and Bessis 1990; Theodoropoulos and Roubelakis-Angelakis 1990), stems (Reustle and Natter 1994), pericarp (Mii et al. 1991) and berry mesocarp tissue (Fontes et al. 2010; Nunan et al. 1997; Wang et al. 2015). However, most of these studies focused on plant regeneration (Barbier and Bessis 1990; Reustle et al. 1995; Zhu et al. 1997), and only a few addressed cellular functions (Fontes et al. 2010; Wang et al. 2015). To date, as far as we are aware, there have been no reported studies of gene function, using a grapevine protoplast system.

Here, we report a simplified and highly efficient method for the isolation of mesophyll protoplasts from grapevine leaves, together with a protocol for their transfection. We also describe the use of genetically transformed grapevine mesophyll protoplasts to analyze the function of a plant defense gene. Lastly, we show that expression of the broad-spectrum disease resistance gene, *RPW8.2*, from *A.*

*thaliana*, when heterologously expressed in grapevine protoplasts, is induced by infection with the grapevine pathogen *Plasmopara viticola*, or in response to the application of exogenous SA. We propose that this grapevine protoplast isolation and transient gene expression system provides an excellent platform to study protein subcellular localization, protein–protein interactions and disease resistance gene expression.

## Materials and methods

### Plant materials

For tissue culture plantlets, *V. vinifera* cv. Rizamat and the wild Chinese grapevine *V. pseudoreticulata* accession Baihe-35-1 were propagated by tissue culture on MS medium (Murashige and Skoog, Phyto Technology Laboratories, USA). Two-month-old plantlets were then transplanted into a plastic pot (diameter: 14 cm, height: 10 cm) containing soil mix (perlite: vermiculite: peat, 0.5: 0.5: 4, v/v/v) and grown in a controlled environment chamber with temperatures ranging from 18 to 22 °C and a relative humidity ranging from 55 to 65 %. Leaves from the plantlets were used to isolate protoplasts. For greenhouse plantlets, *V. vinifera* cv. Rizamat and the wild Chinese grapevine *V. pseudoreticulata* accession Baihe-35-1 were grown in the greenhouse, with temperatures ranging from 22 to 27 °C and a relative humidity ranging from 70 to 93 %, without supplemental lighting.

### Protoplast isolation

The first fully expanded leaf of tissue-cultured or greenhouse-grown plants was used for protoplast isolation. Grapevine mesophyll protoplast isolation was based on a protocol for the preparation of *A. thaliana* protoplasts (Yoo et al. 2007) with some modifications. Enzyme digestion time and enzyme concentration are important factors affecting the yield and quality of protoplasts. Briefly, grapevine leaves (~100 mg) were sliced into 0.5–1.0 mm strips using a razor blade and transferred into 5 ml of a freshly prepared and sterilized cell wall degrading enzyme solution consisting of 20 mM MES (4-morpholineethanesulfonic acid, pH 5.7), 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 0.1 % (w/v) bovine serum albumin (BSA), 1.5 % (w/v) cellulase R-10 and 0.4 % (w/v) macerozyme R-10. The solution was first sterilized by passing through a 0.45 µm filter. To infiltrate tissue strips with the enzyme solution, a vacuum (0.07–0.08 M Pa) was applied for 30 min and the infiltrated tissue strips were incubated in the dark for 12 h at 26 °C. After incubation, an equal volume of W5 solution [2 mM MES (pH 5.7), 154 mM NaCl, 5 mM glucose, 125 mM $CaCl_2$ and 5 mM KCl] was added to stop the reaction. Protoplasts were filtered through a 75 µm nylon mesh and pelleted by centrifuging at 200*g* for 3 min at room temperature before being resuspended in W5 solution. After one wash with W5 solution, the pellets were resuspended in MMG solution [4 mM MES (pH 5.7), 0.4 M mannitol, and 15 mM $MgCl_2$]. The protoplast yield was measured immediately after purification under visible light using a hematocytometer. The viability of the freshly produced protoplasts was evaluated under UV-light after staining with 0.05 % fluorescein diacetate (FDA) and incubation in the dark for 5 min (Widholm 1972). Finally, cells were diluted to 2–3 $\times$ $10^5$ protoplasts $ml^{-1}$ for subsequent transient expression assays.

### Plasmid construction

The recombinant plasmids and primers used in this study are listed in Supplementary Table S1. The pBI221 vector (Clontech, Beijing, China) containing the CaMV 35S promoter was used to test the transfection efficiency of the grapevine mesophyll protoplasts. To express the cabbage *BolABI5* gene, the full-length coding sequence (CDS) of *BolABI5* was inserted into the *BamH*I and *Sal*I sites of the Cam-35S-GFP vector, resulting in the expression vector of GFP-BolABI5 fusion protein (BolABI5-GFP) (Zhou et al. 2013).

Total RNA of wild Chinese grapevine *V. pseudoreticulata* accession Baihe-35-1 leaves was extracted using the E.Z.N.A Plant RNA kit (Omega, Guangzhou, China) according to manufacturer's instructions. First-strand cDNA was synthesized from 2 µg total RNA using PrimeScript Rtase (Takara, Dalian, China). To examine protein subcellular localization, the coding sequence (CDS) of several grapevine genes, including *VpCDPK2*, *VpCDPK9* and *VpCDPK11* were amplified by high-fidelity Taq HS-mediated (Takara, Dalian, China) PCR using cDNA from the leaves of wild Chinese grapevine *V. pseudoreticulata* accession Baihe-35-1. The amplified PCR products were digested with *Xba*I and *Xho*I and inserted into the corresponding sites of the pBI221 vector in-frame with the sequence encoding GFP, resulting in the pVpCDPK2-GFP, pVpCDPK9-GFP, and pVpCDPK11-GFP plasmids (Zhang et al. 2015).

The constructs used for bimolecular fluorescence complementation (BiFC) were made by sub-cloning the CDS of the wild type canola *BnaABF4* and *BnaCBL4* genes without their stop codons into the 35S-SPYCE (M) vector (Waadt et al. 2008; Zhang et al. 2014a, b), resulting in the fusion constructs *BnaABF4-YFP*$_C$ and *BnaCBL4-YFP*$_C$, respectively. The CDS of the wild type canola *BnaCPK4* and *BnaCIPK10* genes with their stop codons were sub-

cloned into the 35S-SPYNE (R) 173 vector, resulting in the fusion constructs *$YFP_N$-BnaCPK4* and *$YFP_N$-BnaCIPK10*, respectively (Waadt et al. 2008; Zhang et al. 2014a, b).

To test the activity of the *A. thaliana* disease resistance gene *RPW8.2* in grapevine protoplasts, the plasmid *NP::RPW8.2-YFP*, containing *RPW8.2* driven by its native promoter (NP) and fused to yellow fluorescent protein (YFP), was generously provided by Dr. Shunyuan Xiao (Institute for Bioscience and Biotechnology Research, University of Maryland, USA). More detailed information of the construct has been previously described (Wang et al. 2007).

### Protoplast transfection and fluorescence microscopy

Protoplast transfection was performed essentially as previously described with slight modifications (Yoo et al. 2007). Briefly, for each assay, different amounts of plasmid DNA (5, 10, 20, 40, 80, and 160 µg) were added to 100 µl of isolated protoplast solution (about 2–3 × $10^5$ protoplasts $ml^{-1}$), to which an equal volume of freshly prepared PEG solution [40 % PEG 4000 (w/v), 0.4 M mannitol and 100 mM $CaCl_2$] had been added. After incubation in PEG for 5 min at room temperature, 440 µl W5 solution was slowly added to stop the reaction. The resulting solution was mixed gently by inverting the tube, and protoplasts were pelleted by centrifugation at 200 *g* for 2 min. The protoplasts were re-suspended in 200 µl W5 solution and incubated for 20–25 h at room temperature in the dark before examination by fluorescence microscopy. Leaf mesophyll protoplasts were observed and images captured using an Olympus BX-51 fluorescence microscope (Olympus, Japan). GFP and YFP fluorescent signals were acquired using 450–490 nm excitation (Ex) wavelengths, while chlorophyll auto-fluorescence was monitored using 540–580 nm excitation (Ex) wavelengths. All florescence experiments were independently repeated at least three times with similar results.

### Salicylic acid (SA) treatment and pathogen inoculation of grapevine with downy mildew

The first fully expanded leaves of *V. vinifera* cv. Rizamat, grown in a controlled environment chamber, were used for PEG-mediated transient protoplast expression. After protoplasts had been transfected with 20 µg of *NP::RPW8.2-YFP* plasmid DNA, SA was added to a final concentration of 0, 0.5, 1 or 5 mM. The *RPW8.2*-transfected protoplasts treated with SA were observed for presence of fluorescent signals at 24 and 48 h post-treatment (hpt).

The grapevine downy mildew pathogen, *P. viticola*, was collected from heavily infected leaves of *V. vinifera* cv. Pinot Noir, which was grown in the grapevine germplasm resource orchard of the Northwest A&F University, China. *P. viticola* spores (~2×$10^5$ spores $ml^{-1}$) were added to the protoplasts transfected with *NP::RPW8.2-YFP* and fluorescent signals were measured at 24, 48 and 72 h post-inoculation (hpi).

### Statistical analysis

Statistical analysis was performed using the Duncan's multiple range test of IBM SPSS statistics 20 software. Least significant differences were calculated at the 5 or 1 % level of probability. All florescence experiments were independently repeated at least three times with similar results.

## Results

### Isolation of protoplasts from grapevine leaves

To establish an efficient protocol for grapevine protoplast isolation, a pool of all the leaves of 2-month old sub-cultured *V. vinifera* cv. Rizamat plantlets were initially chosen as the source material, and a protocol developed for *A. thaliana* protoplast isolation (Hoffman et al. 1994; Sheen 2001; Yoo et al. 2007) was followed with some modifications. As shown in Fig. 1a, protoplasts could be isolated from different amounts of tissue. However, 20 mg $ml^{-1}$ in the pre-digestion solution resulted in the highest yield (approximately 3.0 × $10^4$ protoplasts $ml^{-1}$), and clear green fluorescent signals were observed in almost all the protoplasts after staining with FDA (Fig. S1a), indicating that the isolated protoplasts were intact and viable.

To determine whether the concentration of enzymes influenced the quantity and quality of protoplasts, different concentration of enzymes including cellulase and macerozyme were used to isolate protoplasts from the leaves of 'Rizamat' plantlets. The highest yield (approximately 3.4 × $10^5$ protoplasts per gram fresh weight) was obtained by combining 1.5 % cellulase with 0.4 % macerozyme (Fig. 1d; Fig. S2). To establish the optimal digestion time, the optimal enzyme concentration, described above, was used to digest leaves for periods ranging from 6 to 18 h. The number of isolated intact protoplasts increased gradually with time, reaching a peak after 14 h (Fig. 1b; Fig. S1b). In addition, 0.4 M mannitol resulted in a higher protoplast yield and vitality (Fig. 1c; Fig. S1c).

We next investigated whether the position of the leaf, the species of the grapevine, or the growth conditions also affected the protoplast yield and quality. The 1st, 2nd, 3rd, and 4th position leaves from *V. vinifera* cv. Rizamat and the wild Chinese grapevine *V. pseudoreticulata* accession Baihe-35-1 grown in MS medium, in the greenhouse or in an environment-controlled chamber were used for protoplast isolation. The results indicated that, regardless of the

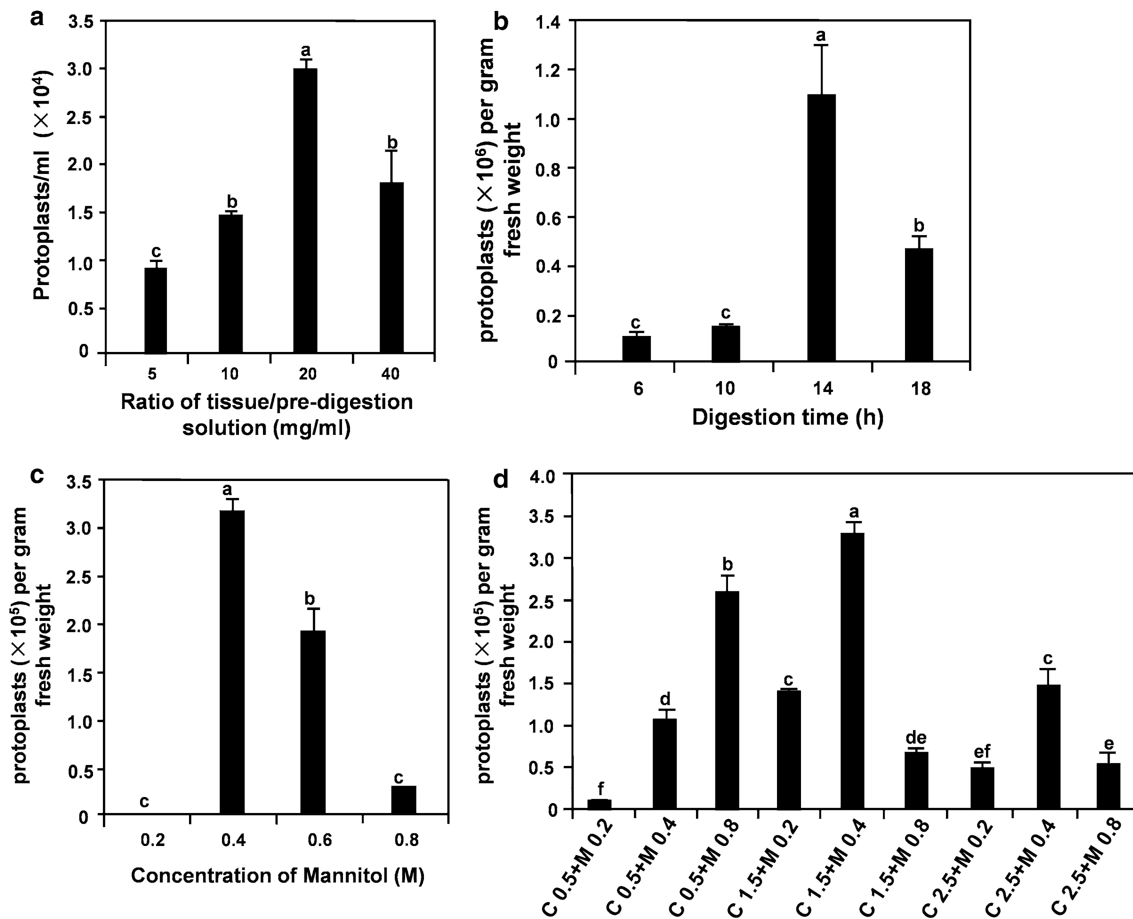


**Fig. 1** Isolation of grapevine (*Vitis vinifera* cv. Rizamat) leaf mesophyll protoplasts from tissue cultured plantlets. **a** The effect of the amount of tissue used on protoplast production. Yield of leaf-derived protoplasts when using different enzyme digestion times (**b**), mannitol concentrations (**c**) and enzyme concentrations (**d**). *C* cellulase R-10, *M* macerozyme R-10; Values represent mean standard errors (SEs). Values followed by *different letters* in a column are significantly different at $P \leq 0.01$ according to Duncan's multiple range tests

grapevine species and growth conditions, the yield from the first leaf was the highest (approximately 3–5.7 × $10^6$ protoplasts per gram fresh weight), and the isolated protoplasts were intact and viable, which is significantly higher than that from leaves of other positions (Fig. S3a, b). This highlighted the importance of using young tissue for protoplast isolation.

In summary, it was found that for grapevine mesophyll protoplast isolation, the optimal conditions were: 20 mg $ml^{-1}$ of tissue from the first position leaves in the pre-digestion solution and digestion for 14 h with 1.5 % cellulase R-10/0.4 % macerozyme R-10 in a solution containing 0.4 M mannitol. Other steps are similar to the protoplast isolation procedure used for *A. thaliana* (Yoo et al. 2007). Using this optimized protocol, an average yield of 3.3 × $10^6$ protoplasts per gram fresh weight was routinely obtained from the leaves of 2-month-old transplanted plants, and the viability of the protoplasts was up to 96 %, as judged by the FDA staining assay (Fig. 2a–d).

## Transfection efficiency of grapevine mesophyll protoplasts

PEG-mediated protoplast transformation has been widely used in plants (Bart et al. 2006; Marion et al. 2008; Yoo et al. 2007). We next tested whether the concentration of plasmid DNA, the density of grapevine protoplasts, and the incubation time with PEG affected the transfection efficiency. After introducing a 35S::GFP plasmid into grapevine mesophyll protoplasts (2–3×$10^5$ protoplasts $ml^{-1}$) using a PEG-mediated transfection approach and incubating for 18–22 h, a GFP signal was clearly detected throughout the protoplast (Fig. 3d). The transfection efficiency was calculated by counting the ratio of transfected cells to non-transfected cells under a fluorescence microscope. As shown in Fig. 3a, d, the efficiency increased significantly and in proportion to the concentration of plasmid DNA, but reached a stable level with when using 20–80 μg plasmid. Thus, 20 μg plasmid DNA was then

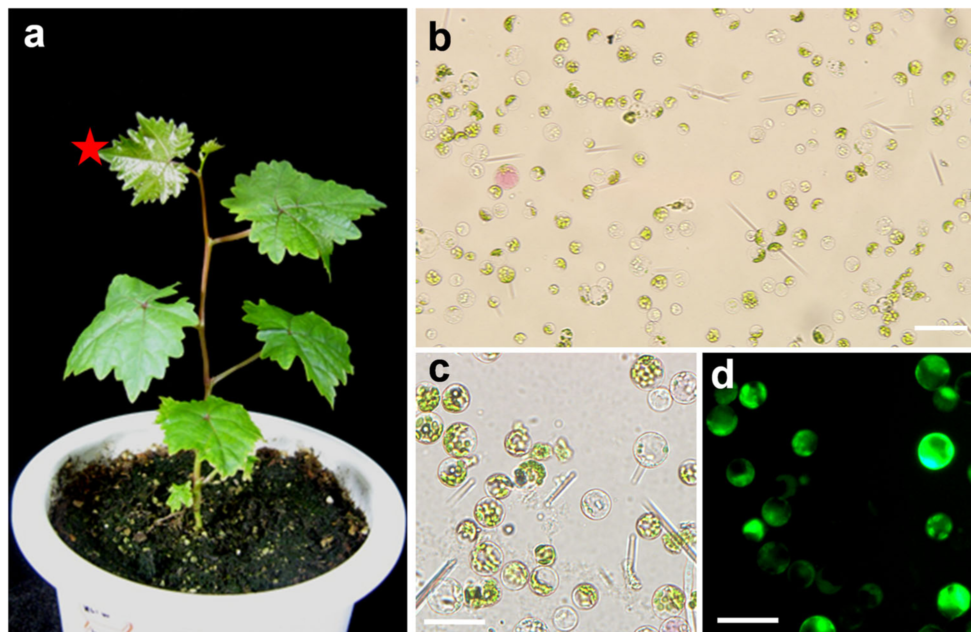


**Fig. 2** Grapevine protoplast isolation and fluorescein diacetate (FDA) staining. **a** A healthy 2-month old *V. vinifera* cv. Rizamat plant grown in a controlled environment chamber for use in protoplast isolation. *Star* indicates the optimal leaf (*first*) for protoplast isolation. Freshly isolated grapevine protoplasts were imaged using bright field conditions with a fluorescence microscope and a ×20 objective lens (**b**) or ×40 objective lens (**c**). **d** Freshly isolated protoplasts were stained for viability with FDA and visualized as above with a ×40 objective lens. *Scale bars* represent 3 cm (**a**) and represent 50 µm (**b**–**d**)

used for optimizing the transfection efficiency of different protoplast concentration. The highest transfection efficiency (~94 %) was achieved when using $6 \times 10^4$ protoplasts $ml^{-1}$, which was 1.4-fold and 2.5-fold greater than what was achieved using $2.4 \times 10^5$ and $4.8 \times 10^5$ protoplasts $ml^{-1}$, respectively (Fig. 3b; Fig. S4a). It should be noted that when the concentration of protoplasts was $<6 \times 10^4$ protoplasts $ml^{-1}$, they were not detectable with the naked eye (data not shown) due to discard supernatant while easily discarding the protoplast, which can be a disadvantage. Finally, we performed a time course analysis, monitoring the transfection efficiency with different PEG incubation times. As shown in Fig. 3c and Fig. S4b, the transfection efficiency increased significantly with incubation time, such that the transfection efficiency at 2 min reached 81 %, before peaking at ~86 % with a 5 min incubation. The efficiency with 20 and 40 min incubation times decreased significantly and we concluded that the optimal incubation time was approximately 2–5 min.

## Highly efficient transfection of different sized constructs in grapevine mesophyll protoplasts

In previous studies, the size of plasmids used for transfection was found to affect transfection efficiency (Bart et al. 2006; Hong et al. 2012; Zhang et al. 2011). In order to determine whether this was also the case for the grapevine system, 20 µg of a 4.6 kb plasmid (empty pBI221 vector) and of an 11 kb plasmid (BolABI5-GFP plasmid) was used in separate transfections of the 'Rizamat' mesophyll protoplasts, using the optimized protocol described above. GFP fluorescence was clearly detected throughout the protoplasts transfected with the pBI221 vector plasmid (Fig. 4c), while the 11 kb binary plasmid, BolABI5-GFP, containing the transcription factor BolABI5 fused to GFP, resulted in a nuclear GFP signal, in accordance with typical transcription factor characteristics (Zhou et al. 2013) (Fig. 4c). A transfection efficiency of 86 % were obtained using the small sized 4.6 kb plasmid, compared to a 61 % transfection efficiency with the 11 kb plasmid (Fig. 4b), consistent with the suggestion that plasmid size affects transfection efficiency.

## Subcellular protein localization in grapevine mesophyll protoplasts

The above results indicated that the isolation and transfection efficiency was sufficiently high to be used for high-throughput screening and systematic gene function characterization. Three calcium-dependent protein kinase (CDPK) genes were cloned from wild Chinese *V. pseudoreticulata* (*VpCDPK2*, *VpCDPK9* and *VpCDPK11*), fused to the GFP coding sequence and used for transfection of 'Rizamat' mesophyll protoplasts (Zhang et al. 2015). As shown in Fig. 5b, the VpCDPK2-GFP fusion protein

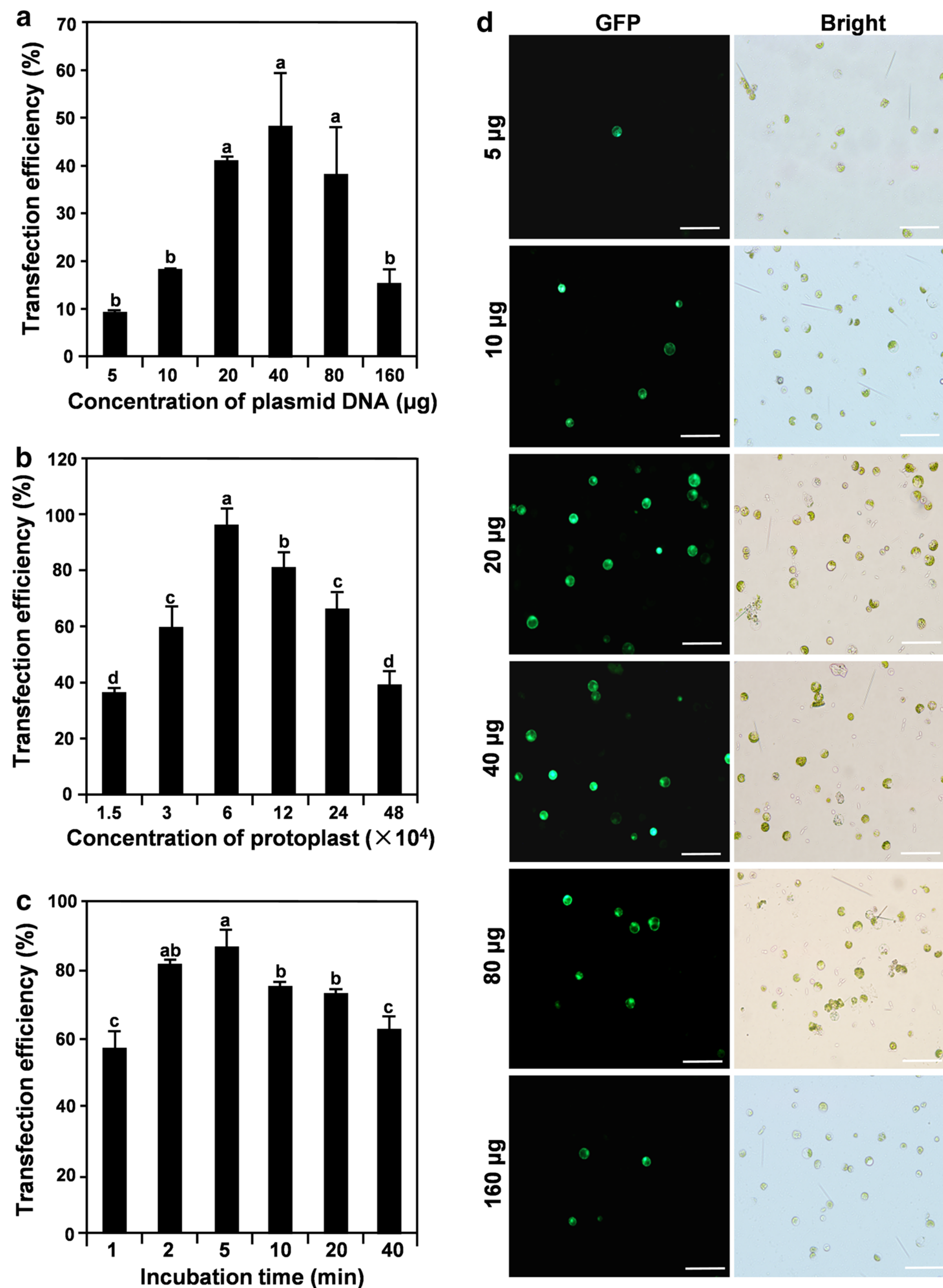


**Fig. 3** Transient gene expression in grapevine protoplasts. **a**, **d** Effect of concentration of plasmid DNA on the efficiency of grapevine protoplast transfection. Fluorescent (*left panel*) and bright field (*right panel*) microscopic images were taken using a fluorescence microscope with a ×20 objective lens (**d**). **b** Effect of protoplast concentration on transfection efficiency. **c** Effect of incubation time on polyethylene glycol (PEG)-mediated transfection of grapevine protoplasts. *Scale bars* 50 μm. *Different letters* indicate a statistical difference at $P \leq 0.01$ (**a**, **b**) or $P \leq 0.05$ (**c**) among samples according to Duncan's multiple range tests

showed two different subcellular locations: homogeneous distribution throughout the cytosol, or in vesicles, which could be either lipid bodies or peroxisomes. The VpCDPK9-GFP fusion protein showed three different localization patterns: (1) the cytosol, as small fluorescent spots resembling lipid bodies or peroxisomes; (2) the cytoplasm and the nucleus; or (3) the endomembrane system, most likely on the endoplasmic reticulum (ER), as well as in vesicles. The VpCDPK11-GFP fusion protein was present at the cell periphery, consistent with localization in the plasma membrane. These results demonstrated that the grapevine protoplast transient expression system is suitable for subcellular localization assays.

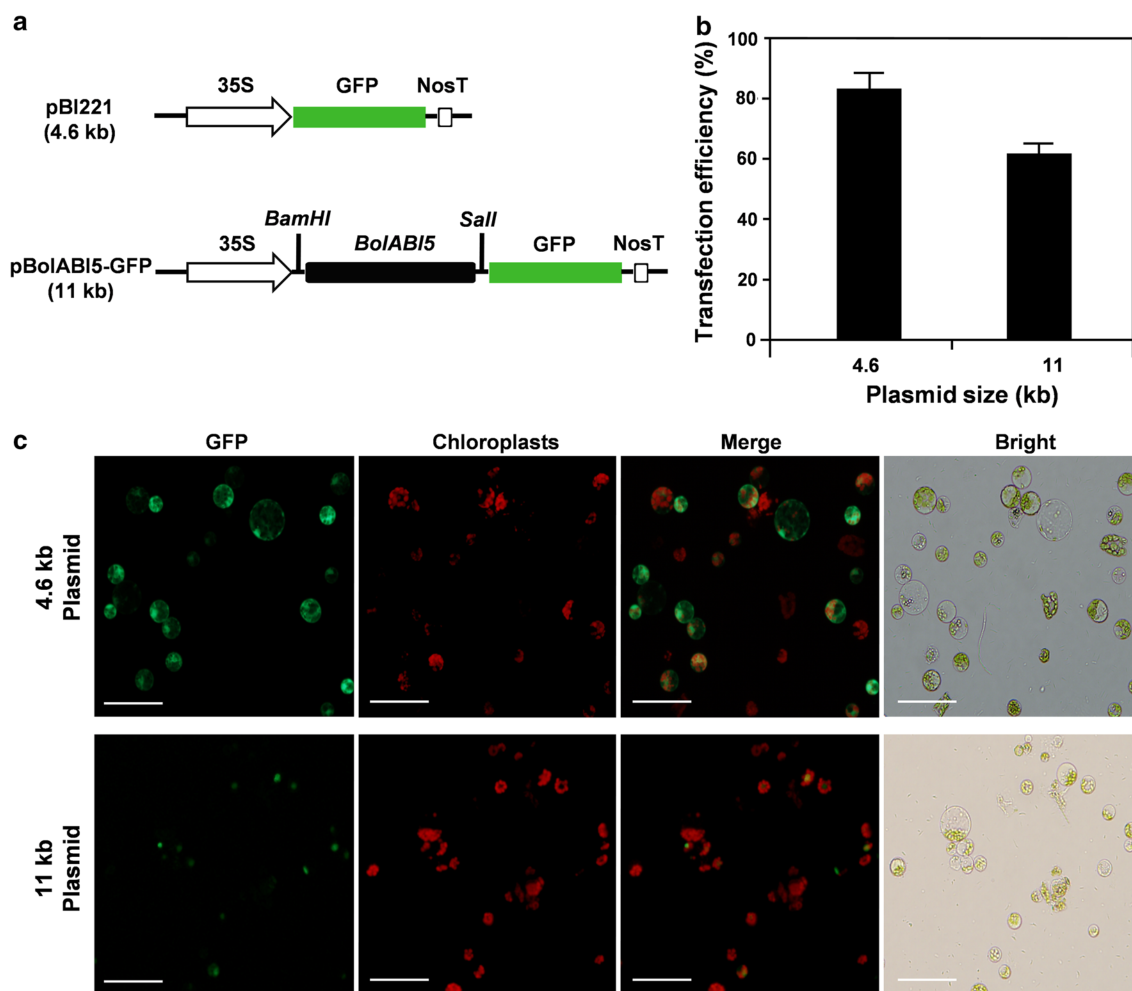


**Fig. 4** Transient expression efficiency of different sized plasmids in grapevine leaf protoplast. **a** The schematic illustration of the vectors pBI221 and BolABI5. *Black filled boxed* indicate BolABI5 gene. *Green filled boxed* indicate green fluorescence protein (GFP). **b** Transfection efficiency of a 11 kb plasmid compared with that of a 4.6 kb plasmid, expressed as the ratio of GFP-posotive cells to the total number of protoplasts (n ≥ 100). Values are means, with standard errors indicated by *error bars*, representing at least 3 replicates. **c** A 4.6 kb plasmid and a 11 kb plasmid were transiently expressed in protoplasts. Individual and merged images of GFP and chlorophyll autofluorescence (Chl) as well as bright field images of protoplasts are shown. *Scale bars* 50 µm

## Detecting protein–protein interactions in grapevine mesophyll protoplasts

The grapevine mesophyll protoplast system was next used to investigate protein–protein interactions using a BiFC assay. CPK proteins are known to play important roles in many aspects of plant growth and development, as well as response to a broad variety of abiotic and biotic stresses (Zhang et al. 2014a). Previous studies have shown that BnaCPK4 interacts with ABF (ABA responsive cis-element binding factor protein) in both a yeast two-hybrid and a BiFC assay (Choi et al. 2005; Zhang et al. 2014a), and we used these two proteins to test protein–protein interaction in the grapevine protoplast system. As shown in Fig. 6b, co-expression of the $YFP_N$-*BnaCPK4* and *BnaABF4*-$YFP_C$ fusion constructs resulted in clear YFP signals in the nucleus of the protoplasts, consistent with previous results using transformed tobacco leaves (Zhang et al. 2014a). For negative controls, we used co-expression of the $YFP_N$-*BnaCPK4* and empty $YFP_C$ vector, *BnaABF4*-$YFP_C$ and empty $YFP_N$ vector, $YFP_N$-*BnaCIPK10* and empty $YFP_C$ vector, and empty $YFP_C$ vector and $YFP_N$ vector, and these combinations did not produce any fluorescent signals. To further validate the system, we used another set of interacting proteins, calcineurin B-like proteins (CBL) and CBL-interacting protein kinase (CIPK), which have been demonstrated to play crucial roles in plant development and responses to various environmental stresses (Zhang et al. 2014b). When $YFP_N$-*BnaCIPK10* and *BnaCBL4*-$YFP_C$ fusion constructs were co-expressed in grapevine protoplasts, YFP signals were detected in the cytoplasm (Fig. 6c), consistent with previous data indicating that

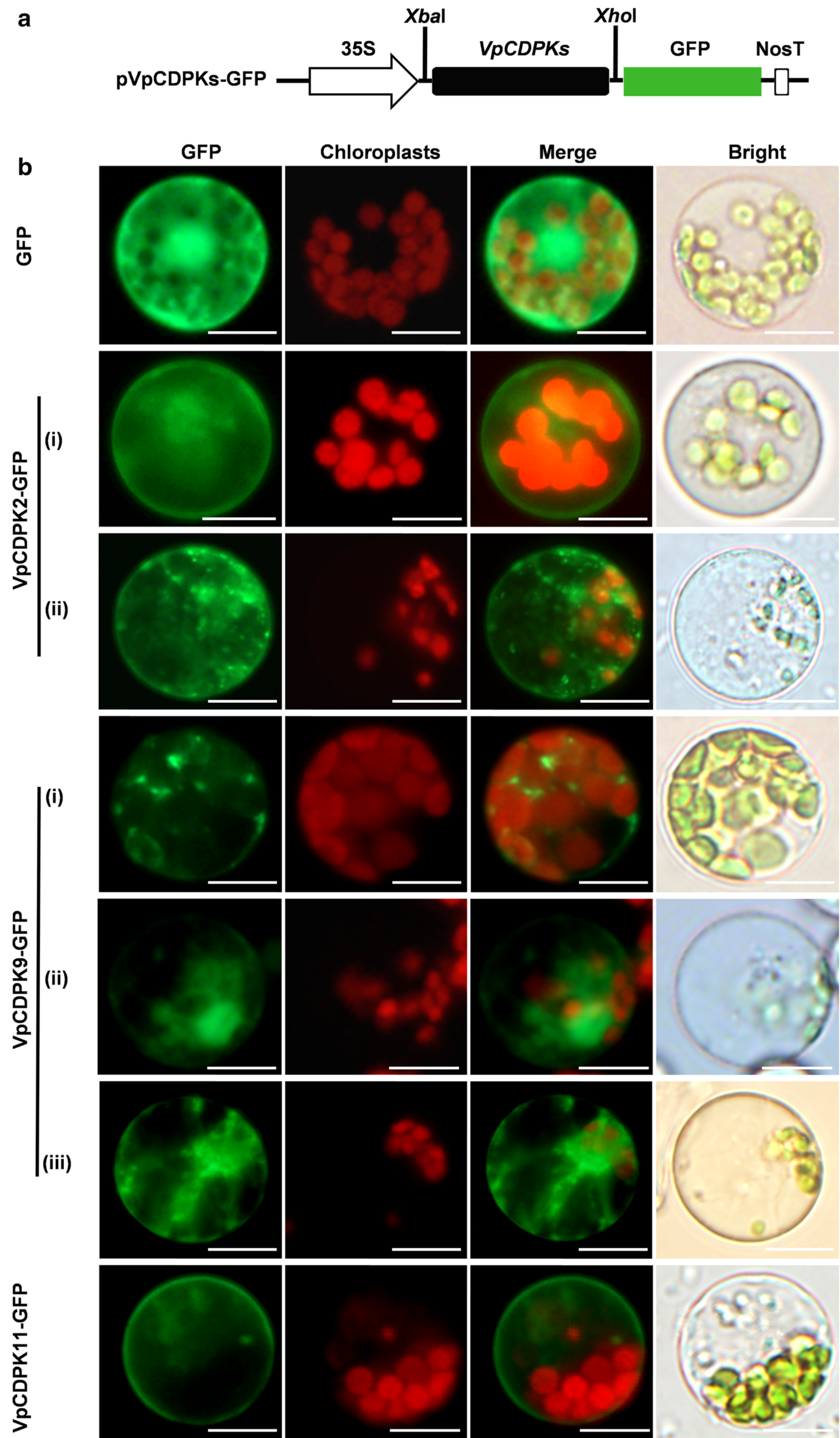


**Fig. 5** Subcellular localization analysis using grapevine mesophyll protoplasts. **a** The schematic illustration of VpCDPKs-GFP constructs. **b** Three VpCDPK-GFP constructs (VpCDPK2-GFP, VpCDPK9-GFP and VpCDPK11-GFP), as well as a control 35S::GFP construct were transiently expressed in grapevine protoplasts. Individual and merged images of GFP and chlorophyll auto-fluorescence as well as bright field images of protoplasts are shown. *Scale bars* 10 μm

these two proteins indeed interact in the cell cytoplasm (Zhang et al. 2014b). Again, the negative controls did not produce any fluorescent signals. These results further illustrated the value of the grapevine protoplast transient expression system as a tool for studies of protein–protein interactions.

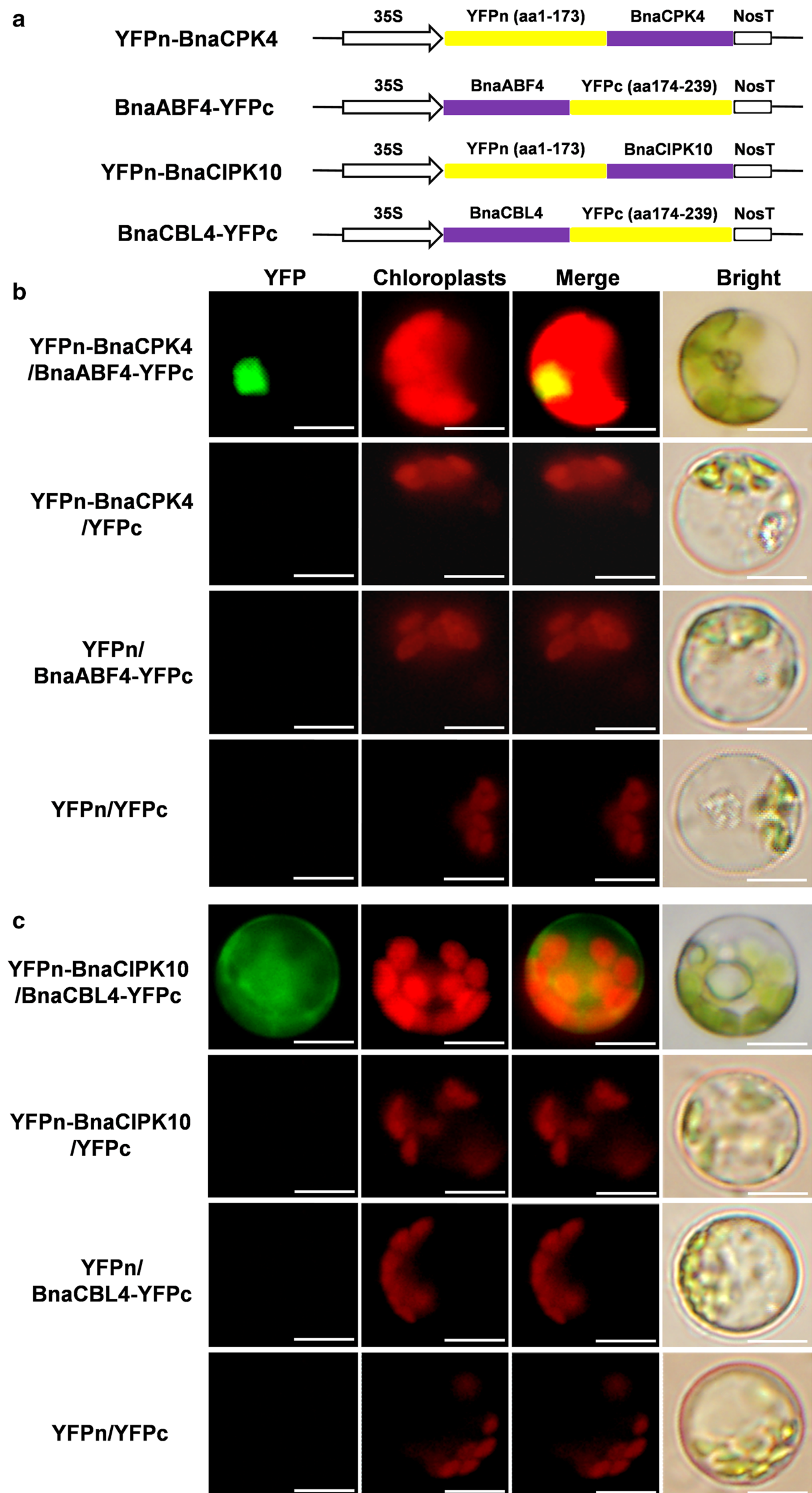


**Fig. 6** Protein–protein interaction assays in grapevine protoplasts. **a** The schematic illustration of BiFC constructs used in the transfections. **b** Protein–protein interaction analysis using bimolecular fluorescence complementation (BiFC). Construct pairs of $YFP_N$-BnaCPK4 and BnaABF4-$YFP_C$, $YFP_N$-BnaCPK4 and empty $YFP_C$ vector, BnaABF4-$YFP_C$ and empty $YFP_N$ vector, and empty $YFP_N$ vector and empty $YFP_C$ vector were transiently co-expressed in grapevine protoplasts. **c** Construct pairs of $YFP_N$-BnaCIPK10 and BnaCBL4-$YFP_C$, $YFP_N$-BnaCIPK10 and empty $YFP_C$ vector, BnaCBL4-$YFP_C$ and empty $YFP_N$ vector, and empty $YFP_N$ vector and empty $YFP_C$ vector were transiently co-expressed in grapevine protoplasts. BiFC fluorescence was indicated by a YFP signal. Individual and merged images of YFP and chlorophyll auto-fluorescence as well as bright field images of protoplasts are shown. *Scale bars* 10 µm

### Grapevine mesophyll protoplasts as a transient and heterologous reporter system for studying the expression of the *A. thaliana* disease resistance gene, *RPW8.2*

The grapevine mesophyll protoplast system that was established here, represents a versatile means to for characterize gene functions and activities. To provide a specific example we used the system to monitor the expression activity of a disease resistance gene in response to pathogen infection. Specifically, a plasmid harboring *RPW8.2-YFP* driven by the *RPW8.2* native promoter was transfected into grapevine protoplasts, which were then inoculated with the grapevine pathogen, *P. viticola*, the causal agent of downy mildew. As shown in Fig. 7b, a YFP signal was not observed at 24 hpi; however, the transfected protoplasts inoculated with *P. viticola* clearly showed a YFP signal at 48 and 72 hpi, and the signals were notably brighter at 72 hpi than at 48 hpi.

It has previously been reported that the RPW8.2 protein levels increase in response to application of exogenous SA (Xiao et al. 2003a). To determine whether SA also induces *RPW8.2* expression in the transfected protoplasts, they were treated with different concentrations of SA for either 24 or 48 h (Fig. 8). YFP signals were only observed in the protoplasts treated with 5 mM of SA at 24 hpt, while no signal was observed for the 0.5 and 1 mM SA treatments (Fig. 8). The control sample, where the transfected protoplasts were not treated with SA, also showed no signal. In contrast, when the transfected protoplasts were treated with SA for 48 h, all the three tested concentrations induced RPW8.2-YFP protein expression, with the YFP signal resulting from the 5 mM SA application being brightest (Fig. 8). Again, no signal was observed in the control.

## Discussion

Plant protoplasts constitute a powerful and versatile system for dissection of gene function; however, although reports over the past decade have described the isolation of grapevine protoplasts from different tissues and organs, most have focused on their use for plant regeneration (Reustle et al. 1995; Zhu et al. 1997). Additionally, there are limitations, such as low yield and the large time investment, associated with the existing protoplast protocols developed for grapevine callus, pericarp, stem and berry mesocarp tissue (Fontes et al. 2010; Nunan et al. 1997; Reustle et al. 1995; Reustle and Natter 1994; Zhu et al. 1997; Mii et al. 1991), which limit their efficiency for functional genomic studies. In this report, we describe a simplified method for obtaining high yields of protoplast with excellent viability from young grapevine leaves, together with a protocol for their transient PEG-mediated transfection and a targeted analysis of a heterologously expressed disease resistance gene.

Our protocol was based on a previously published methodology developed for *A. thaliana* (Yoo et al. 2007). While young leaves are often used (Chen et al. 2006; Hong et al. 2012; Zhang et al. 2011), there are very few reports describing the effect of age and physiological state of the leaves used for protoplast isolation (Theodoropoulos and Roubelakis-Angelakis 1990). We found that regardless of the species or growth conditions, using the first position leaf as the starting material improved both the quantity and quality of the protoplasts compared to using leaves from other positions (Fig. S3). Interestingly, although there is a significant difference in the optimal digestion time needed for protoplast isolation from grapevine or *A. thaliana*, the same concentration of enzymes supplemented with mannitol gave the highest yield with both species (Fig. 1b–d, Yoo et al. 2007).

We found that three factors were particularly important to achieve maximum transfection efficiency. Firstly, it has been reported that the optimal concentration of plasmid DNA varies depending on the plant species being transformed and the cell types in question (Bart et al. 2006; De Sutter et al. 2005; Hong et al. 2012; Tan et al. 2013). For example, the optimum concentrations for *Populus* and *Brachypodium* have been reported to be 10 and 20 µg, respectively (Hong et al. 2012; Tan et al. 2013). We found that the optimum concentration of plasmid DNA in this study was 40 µg (Fig. 3a, d), which is similar to the grapevine (Wang et al. 2015). Secondly, our results indicated that a protoplast density of $6 \times 10^4$ protoplasts $ml^{-1}$ yielded the best transfection efficiency (Fig. 3b; Fig. S3a). Thirdly, the most effective PEG incubation time for transfection was about 2–5 min (Fig. 3c; Fig. S3b), which is similar to the equivalent times for *Brachypodium* (Hong et al. 2012) and potato (Craig et al. 2005; Nicolia et al. 2015). Interestingly, there were significantly declined transformation efficiencies with 20 and 40 min incubation (Fig. 3c), which maybe due to accumulation of PEG on the grapevine protoplasts. It was reported that the use of a lower concentration of PEG and a shorter incubation time retained the potato protoplast vitality with high reproducibility (Craig et al. 2005). In addition, the transfection was more effective with a smaller plasmid than with a larger plasmid (Fig. 4), which is consistent with previous studies of rice (Bart et al. 2006; Zhang et al. 2011) and *Brachypodium* (Hong et al. 2012).

The subcellular localization of a protein can provide insights into its function (Zhang et al. 2015) and protoplasts can provide a useful cell-based experimental system in this regard (Lee et al. 2009; Swanson et al. 1998; Zhang et al. 2011). For example, *A. thaliana* and tobacco leaves are

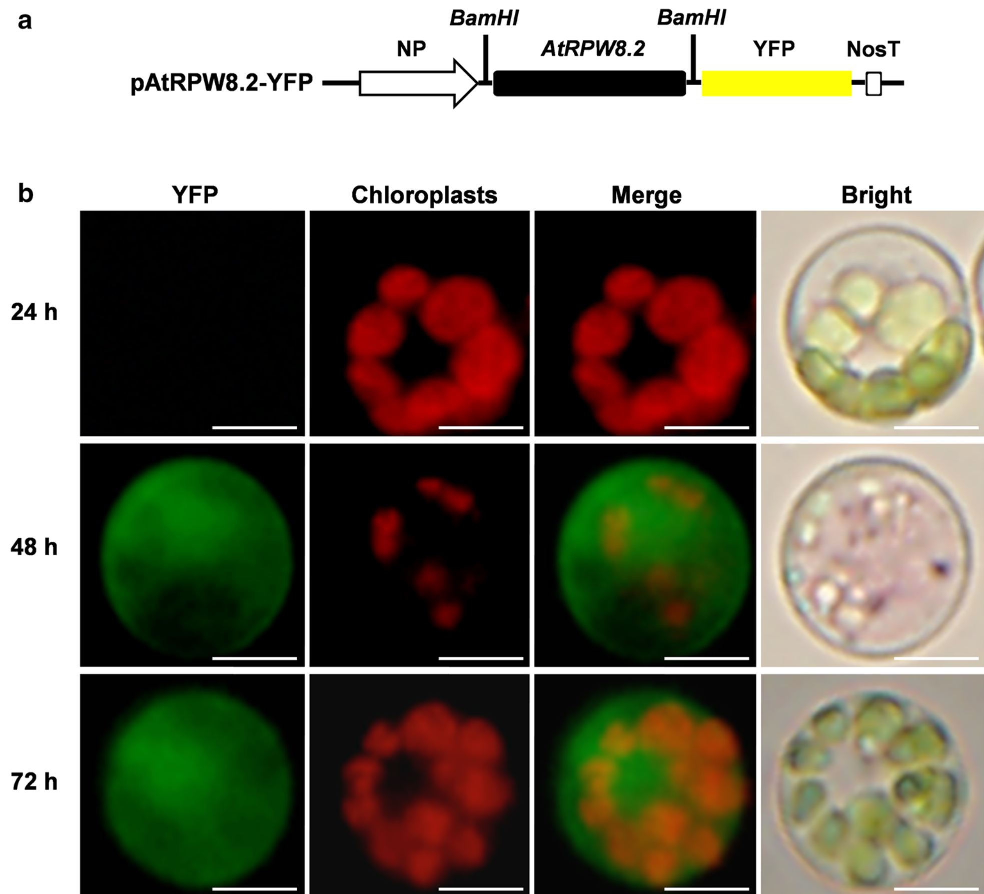


**Fig. 7** Transient expression assay of the *A. thaliana* disease resistance gene *RPW8.2* in grapevine protoplasts, when induced by grapevine downy mildew. **a** The schematic illustration of AtRPW8.2-YFP constructs. **b** Fluorescence was detected at 24, 48 and 72 h after transfection with *RPW8.2-YFP*. Merged and individual images of YFP and chlorophyll auto-fluorescence as well as bright field images of protoplasts are shown. *Scale bars* 10 µm

commonly to study protein subcellular localization, when used as a heterologous system this approach may result in artifacts (Bai et al. 2014; Marion et al. 2008). Indeed, the VpCDPK2-GFP fusion protein has been reported to be localized in the nucleus and cytosol in *A. thaliana* protoplasts (Zhang et al. 2015), but we observed that it accumulated not only in the cytosol, but also in lipid bodies or peroxisomes in grapevine mesophyll protoplast (Fig. 5b). These results underline the importance of carefully the appropriate expression system, or systems. We also showed that grapevine protoplasts may be used to investigate protein–protein interactions, specifically using a BiFC analysis to study two pairs of fusion proteins: $YFP_N$-BnaCIPK10 with BnaCBL4-$YFP_C$ and $YFP_N$-BnaCPK4 with BnaABF4-$YFP_C$. A clear YFP signal was observed in the cytoplasm after co-expressing $YFP_N$-BnaCIPK10 and BnaCBL4-$YFP_C$, or in the nucleus after co-expressing $YFP_N$-BnaCPK4 and BnaABF4-$YFP_C$ (Fig. 6b, c). These results are consistent with previous results using tobacco leaves (Zhang et al. 2014a, b).

Transient expression systems can enable a rapid and high-throughput analysis of plant gene function and avoid the more time consuming process of stable plant transformation plants. For example, an *A. thaliana* mitogen-activated protein (MAP) kinase cascades have been characterized using an *A. thaliana* protoplast transient assay systems (Asai et al. 2002; Kovtun et al. 1998, 2000). Similarly, protoplasts have been used to evaluate defence-related genes function (Chen et al. 2006, 2015). In the present study, we transfected the *A. thaliana* broad-spectrum disease resistance gene, *RPW8.2*, into grapevine protoplasts and found that expression of an RPW8.2-YFP fusion protein was induced in response to application of exogenous SA or by downy mildew infection (Figs. 7, 8), which is consistent with a previous study in *A. thaliana* transgenic plants (Wang et al. 2009; Xiao et al. 2003a, b). Our results indicate that *RPW8.2* retains its activity in a heterologous species, and demonstrate the utility of a transient fluorescent protein-based reporter system for the study of defence-related genes in grapevine.

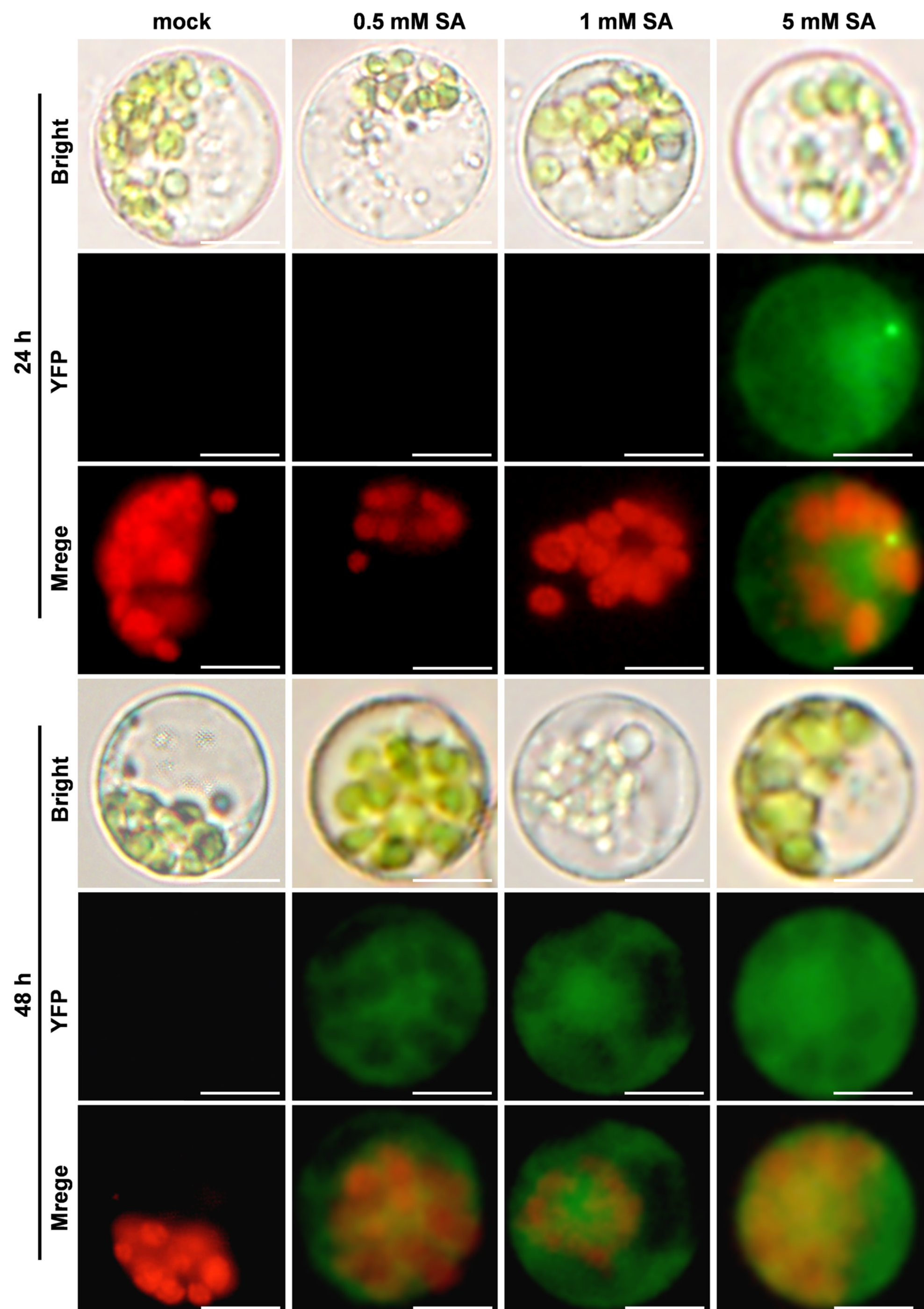


**Fig. 8** Transient expression assay of the *A. thaliana* disease resistance gene *RPW8.2* in grapevine protoplasts, when induced by salicylic acid (SA). Fluorescence was detected at 24 and 48 h after treatment with SA. Merged and individual images of YFP as well as bright field images of protoplasts are shown. *Scale bars* 10 µm

In conclusion, we have established a simple and versatile protocol for grapevine protoplast isolation and transformation, and we describe a range of associated applications involving transient expression, including the evaluation of protein subcellular localization and protein–protein interactions.

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**Author contributions** Y.Q.W. conceived the research. F.L.Z. performed all treatments with assistance of Y.H., Y.J.L., Y.R.G., X.W.Z., and Q.D. Y.H. and Y.R.G. carried out partly subcellular

localization experiments. Y.J.L. prepared all plant materials. Y.Q.W., F.L.Z. and Y.H. analysed and interpreted the data. Y.J.W. contributed with consultation. F.L.Z. wrote the manuscript and Y.Q.W. revised it. All authors read and approved the final manuscript.

**Compliance with ethical standards**

**Conflict of interest** All these authors declare that they have no conflict of interest.

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