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SHORT COMMUNICATION



An Arabidopsis protoplast isolation method reduces cytosolic acidification and activation of the chloroplast stress sensor SENSITIVE TO FREEZING 2

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ABSTRACT

Chloroplasts adapt to freezing and other abiotic stresses in part by modifying their membranes. One key-remodeling enzyme is SENSITIVE TO FREEZING2 (SFR2). SFR2 is unusual because it does not respond to initial cold stress or cold acclimation, instead it responds during freezing conditions in Arabidopsis. This response has been shown to be sensitive to cytosolic acidification. The unique lipid products of SFR2 have also been detected in response to non-freezing stresses, but what causes SFR2 to respond in these stresses is unknown. Here, we investigate protoplast isolation as a representative of wounding stress. We show that SFR2 oligogalactolipid products accumulate during protoplast isolation. Notably, we show that protoplast cytosol is acidified during isolation. Modification of the buffers reduces oligogalactolipid accumulation, while prolonged incubation in the isolated state increases it. We conclude that SFR2 activation during protoplast isolation correlates with cytosolic acidification, implying that all SFR2 activation may be dependent on cytosolic acidification. We also conclude that protoplasts can be more gently isolated, reducing their stress.

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Introduction

As sessile organisms, plants are unable to relocate to avoid unfavorable growing conditions and are often exposed to periods of intense abiotic stress. Freezing is one such stress, causing cells to lose up to 90% of their osmotically active water.^{1,2} Remodeling of membranes is one strategy plants employ to survive stressful periods including freezing. A key enzyme responsible for chloroplast lipid remodeling is SENSITIVE TO FREEZING 2 (SFR2), a galactosyltransferase. With a transmembrane domain in the outer envelope of the chloroplast and the remainder in the cytosol,³ SFR2 is in an optimal location to respond to both chloroplast and cytosol changes. SFR2 removes the galactose moiety from monogalactosyldiacylglycerol (MGDG) and adds it to another MGDG, creating digalactosyldiacylglycerol (DGDG). The released diacylglycerol is then converted into triacylglycerol (TAG) by other enzymes.⁴ SFR2 is also processive, meaning that the galactosyl removed from MGDG can be placed on product DGDG to generate trigalactosyldiacylglycerol (TGDG), or on TGDG to generate tetragalactosyldiacylglycerol, unique products of SFR2 together referred to as oligogalactolipids. Arabidopsis lacking SFR2 expression (*sfr2* T-DNA insertion lines) do not make oligogalactolipids and are unable to survive freezing temperatures (below 0°C) that wild-type plants can withstand.⁴ However, during normal and above-freezing growth conditions, *sfr2* mutants show no phenotypic effects.⁵

SFR2 protein is present at all temperatures.⁶ SFR2 products are not produced simultaneously with other responses to cold, which occur above freezing in Arabidopsis. Instead, oligogalactolipids only accumulate below freezing temperatures.⁷ We

previously showed that during freezing, the cytosol is acidified coincidentally with SFR2 activation and that forcing cytosolic acidification in the absence of freezing can activate SFR2.⁷ We concluded that SFR2 is responding to cytosolic acidification during freezing stress.

Oligogalactolipid accumulation has been observed in multiple species during multiple abiotic stresses at normal temperatures. In spinach, oligogalactolipid accumulation occurred due to ozone fumigation,^{8,9} and chloroplast isolation.^{10,11} In common bean, it was observed in response to protoplast isolation.^{12,13} In desiccation plants *Boea hygroskopica* and *Craterostigma plantagineum* under desiccation stress oligogalactolipids accumulated.^{14–16} Drought stress was also shown to cause oligogalactolipid accumulation in tomato, as was salinity.¹⁷ In Arabidopsis, *tgd* mutants that are compromised in lipid transport between the endoplasmic reticulum and chloroplasts accumulate oligogalactolipids.^{18–22} Similarly, a *dgs1* mutant allele that causes mitochondrial oxidation stress accumulates oligogalactolipids.²³ Other oligogalactolipid-accumulating stresses in Arabidopsis include wounding²⁴ and freezing.⁴ In Arabidopsis and tomato, the dependence of oligogalactolipid accumulation on SFR2 presence was confirmed, and SFR2 is conserved throughout all sequenced land plants,³ making oligogalactolipid accumulation likely to depend on SFR2 activation in all species and stresses. In Arabidopsis, SFR2 only provides an adaptive response to freezing stress.³ Conservation of stress genes is less likely after genetic divergence when they are not selected for,²⁵ raising the question of why SFR2 is conserved in species geographically constrained to non-freezing areas. We hypothesize that SFR2 is responding to the same signal, cytosolic

acidification. The multiple observations of the unique oligogalactolipid products of SFR2 and their dependence on a single gene gives us a unique opportunity to address this hypothesis.

During non-freezing stresses, it is unknown if cytosolic acidification still correlates with oligogalactolipid accumulation. If it did, it would indicate that a subset of post-transcriptional responses to cytosolic acidification is consistent across divergent stresses and species. That would imply that cytosolic acidification links divergent stresses and could be a good target for engineering stress tolerance. To investigate this idea, we looked for similarities between the stress conditions that we could replicate in a model species. Wounding, chloroplast isolation, and protoplast isolation are similar in that they all involve cutting, grinding, or crushing of tissue. Wounding is a difficult stress to investigate because of its complexity. Responses are dependent on wound type, organ, and distance from the wound site. When we tried to determine if cytosolic acidification occurred during wounding accumulation of oligogalactolipids, cell crushing redistributed the fluorescent reporter confounding the results.²⁴ During chloroplast isolation the cytosol is removed making it too simplified a system to investigate the isolation process, though we did find that isolated chloroplast SFR2 responded to buffer acidification by producing oligogalactolipids.⁷ Protoplast isolation imposes a more consistent stress than wounding and includes the cytosol. In its current form, with enzymatic digestion of the cell wall, protoplast isolation has been used in plant biology since 1960.²⁶ Thus, we chose to use protoplast isolation as a well-defined, reproducible system to understand post-transcriptional stress-based activation of SFR2 during normal temperatures.

Protoplasting is a widely-used technique performed on multiple plant species and resulting in single cells that can be transiently transformed. It is often used to probe sub-cellular locations,^{27–29} microscopy of fluorescent proteins or intracellular dyes,^{30–33} a prelude to further cellular fractionation or cell sorting,^{34,35} and importantly, stress responses^{36–42} See Sheen 2001 for a review of protoplast applications and technology. If the stress of protoplast isolation could be defined and reduced, protoplasts would be a better model system.

Here, we show that the protoplast cytosol is acidified during isolation, correlating with oligogalactolipid production by SFR2. We develop a novel protoplast isolation method that reduces cytosolic acidification and oligogalactolipid accumulation.

Results and discussion

SFR2 activates during protoplast isolation

We used protoplast isolation as a convenient, controlled protocol to test if SFR2 activates in response to the same stimulus observed during freezing, cytosolic acidification. A commonly used protoplast isolation protocol that relies on leaf slicing and vacuum-based enzyme infiltration³⁷ resulted in the accumulation of TGDG. Oligogalactolipids were not detectable in protoplasts isolated from *sfr2* plants, confirming that oligogalactolipid production in Arabidopsis is due to the activity of SFR2. We tested the stage of protoplast isolation that allowed oligogalactolipid production. Protoplasts were isolated and incubated in typical MES pH 5.7 buffers (Table 1). Accumulation of oligogalactolipids was visualized by thin-layer chromatography (TLC)

Table 1. Composition of protoplast isolation buffers.

Buffer	Composition	pH
Enzyme Solution*	20mM MES* 20 mM KCl 0.4 M mannitol 1.5% cellulase R10 (wt/vol) 0.4% macerozyme R10 (wt/vol) 10mM CaCl ₂ 1mM β-mercaptoethanol ddH ₂ O	pH 5.7
W5 Solution*	2mM MES* 154 mM NaCl 125 mM CaCl ₂ 5mM KCl ddH ₂ O	pH 5.7
MMG Solution	4 mM MES 0.4 M mannitol 15 mM MgCl ₂ ddH ₂ O	pH 5.7
PEG Solution	20% (wt/vol) PEG 4000 0.2M mannitol 100 mM CaCl ₂ ddH ₂ O	
WI Solution	4 mM MES 0.5 M mannitol 20 mM KCl ddH ₂ O	pH 5.7

Composition of buffers used throughout the experiments. Asterisk indicates buffers in which MES, pH 5.7 was replaced with Tricine, pH 7.0 to create a neutral buffer. All other components stayed the same. Cellulase and Macerozyme was from Yakult Pharmaceutical Industry Co., Ltd. Japan.

and quantified with respect to the amount of DGDG, a major membrane lipid. Levels of accumulation varied between 4 and 8 percent relative abundance (Figure 1(a,b)). Results indicate oligogalactolipids have already accumulated in the first buffer following cell wall digestion, W5. This result is potentially consistent with activation because of physical removal of the cell wall or by cytosolic acidification because of the buffer pH.

Protoplast cytosolic pH is decreased

We quantified the cytosolic pH of protoplasts during isolation using a ratiometric pH reporting GFP (*Pt-GFP*) in stably transformed Arabidopsis lines.⁴³ Protoplasts were isolated using typical MES pH 5.7 buffers.³⁷ Cytosolic pH was measured through ratiometric quantification of cytosolic *PtGFP* fluorescence.⁷ Representative results are shown in Figure 1(c), and are quantified in Figure 1(c). The cytosolic pH of protoplasts under all buffers during isolation was significantly lower than cells of intact plant tissues. The average cytosolic pH of intact plant cells was 6.99, while protoplasts were 6.47, 6.6, 6.5, and 6.5 for W5, MMG, PEG, and WI buffers respectively.

These results indicate an approximately 0.5 unit pH drop causes SFR2 activation and oligogalactolipid accumulation. Previous measurements of cytosolic acidification during freezing stress in Arabidopsis indicates a pH reduction of approximately one unit,⁷ suggesting that SFR2 is more responsive to pH than previously understood. Further, this discovery that cytosolic acidification co-occurs with TGDG accumulation during protoplast isolation suggests that the cytosol may be acidified during all stresses in which SFR2 activates.

Cytosolic acidification during protoplast isolation corresponds with literature discussing other cellular changes in isolated protoplasts. *Physcomitrella* protoplasts had multiple proteomic changes including several in the chloroplast.⁴⁴ This study also saw

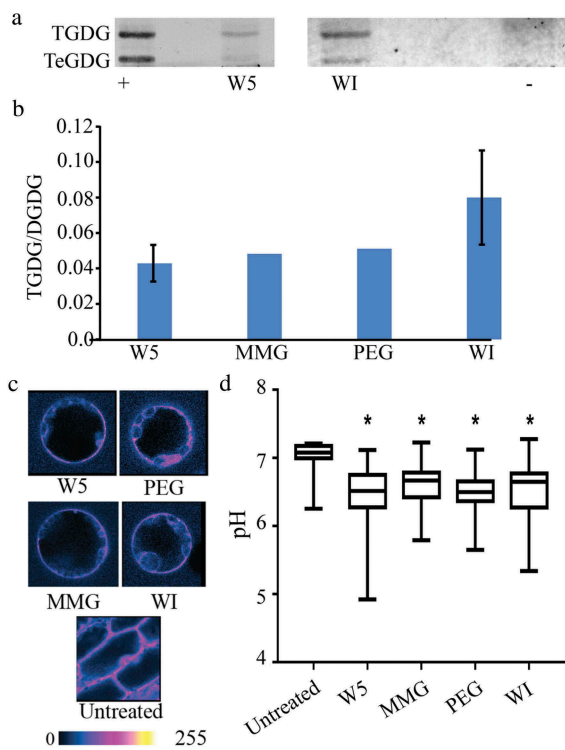


Figure 1. Oligogalactolipids accumulate and the cytosol is acidified in isolated protoplasts.

(a) Sugar-stained thin-layer chromatogram indicating the presence of both trigalactosyldiacylglycerol (TGDG) and tetragalactosyldiacylglycerol (TeGDG) oligogalactolipids after incubation for 1 hour in the initial (W5) and final (WI) buffers used for isolating protoplasts. W5 has 1.5 million protoplasts and WI has 2.1 million protoplasts. Positive and negative controls are acetic acid- and water-incubated whole *Arabidopsis* leaves, as described in.⁷ (b) Quantification of TGDG relative to DGDG in isolated protoplasts. For W5 and WI, $n = 6$, quantified twice. For MMG, and PEG, $n = 3$, quantified once. Error bars indicate standard deviation. (c) Representative confocal micrographs of protoplasts isolated from *Arabidopsis* stably transformed with pH reporting *Pt-GFP*. (d) Quantification of the protoplasts in C and three biological replicates. Standardization of the confocal microscope pH scale was published in 7. For untreated cells $n = 35$, W5 $n = 69$, MMG $n = 59$, PEG $n = 63$, WI $n = 60$. Statistical significance for each treatment compared to untreated cells was $p < .0001$ for all.

increased abundance of proteins linked to reactive oxygen species detoxification and a reduction of electron transport chain components. *Citrus reticulata* var. Blanco isolated protoplasts had changes in 67 microRNA expression levels.⁴⁵ Also, high levels of antioxidant response machinery in protoplasts were discovered in multiple species.^{46–49} To these studies, we add that the protoplast cytosol is likely acidified, affecting the chloroplast membrane lipid composition, and having the potential to interfere with a wide swathe of protein functions.

Protoplast isolation with Tricine at pH 7 minimizes stress responses

The reason for the reduction in protoplast cytosol pH is unclear. It may directly result from wounding and be unavoidable, or from the low pH of the MES buffers. To test our hypothesis that low pH buffers were responsible for TGDG accumulation, we replaced MES with HEPES or Tricine at pH 7.0. HEPES is similar to MES in that it is a substituted taurine with similar properties and a more neutral buffering range.

Tricine buffers in the same pH range as HEPES but is chemically distinct as a substituted glycine.⁵⁰ Typical protoplast isolations with MES at pH 5.7 yielded 1 to 1.5 million protoplasts (Figure 2(a)). We expected that use of pH 7.0 buffers would reduce the effectiveness of cellulase and macerozyme, the cell wall digestive enzymes, as their pH optimum is acidic, usually reported as 3.5–7.⁵¹ Isolations buffered with HEPES did not yield any visible protoplast pellet during centrifugation. We assumed that HEPES was incompatible with the enzymes or the digestion process. Tricine-buffered digestion yielded approximately 250,000 protoplasts (Figure 2(a)), and we proceeded to optimize the Tricine-buffered protocol. We tested whether epidermal peeling, plant growth and the age of the plant affected yields. We found that peeling off the epidermis and allowing the entire leaf surface to contact the enzyme solution as in Wu, et al.,³⁰ was a more gentle procedure that resulted in a greater percentage of live protoplasts. Epidermal peeling was arduous using the small leaves of plate-grown plants, so we switched to using soil grown plants.

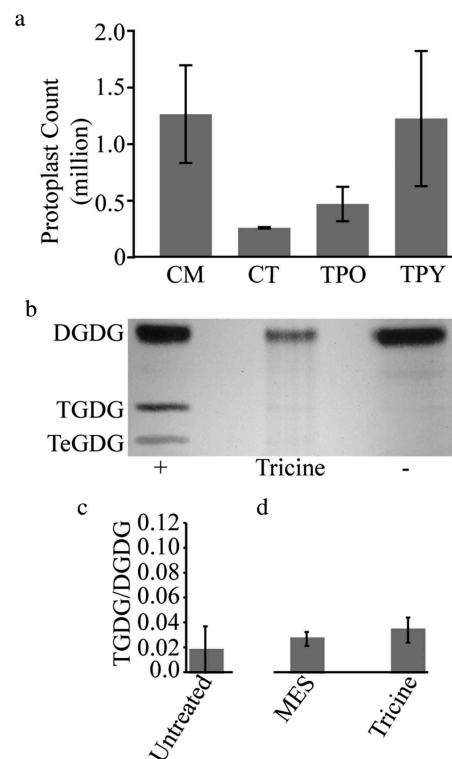


Figure 2. Protoplast isolation at neutral pH prevents the accumulation of oligogalactolipids.

(a) Isolated protoplast yields by multiple methods as follows: slicing and vacuum in MES (CM, $n = 13$), slicing and vacuum in HEPES (SH, $n = 1$), none detected ($n = 0$), slicing and vacuum in Tricine (CT, $n = 2$), peeling in Tricine, 4- to 5-week-old soil-grown plants (PTO, $n = 5$), peeling in Tricine, 3-week-old plants (PTY, $n = 10$). (b) Representative sugar-stained thin-layer chromatogram of 1.5 million protoplasts from five pooled protoplast isolations are shown. Lack of SFR2 activation in these protoplasts can be observed by the absence of trigalactosyldiacylglycerol (TGDG) and tetragalactosyldiacylglycerol (TeGDG) present in the positive control. Positive and negative controls are acetic acid- or water-incubated whole *Arabidopsis* leaves, as described in.⁷ (c, d) Quantification of TGDG relative to DGDG in protoplasts isolated by peeling in Tricine, (PTO and PTY). In (c) lipids were extracted immediately after counting and washing (Untreated). In (d) counted and washed protoplasts were incubated in MES pH 5.7 or Tricine pH.

When relatively young, 3-week-old soil-grown plants were used with epidermal peeling, yields rebounded to approximately 1 million protoplasts (Figure 2(a)). To test the levels of oligogalactolipids present when protoplasts were isolated by this method, lipid extracts from at least three isolations were pooled to reach at least 1.5 million protoplasts, as this was a similar value to tests in Figure 1. Oligogalactolipid accumulation varied between 0.4 to 3 percent relative abundance (Figure 2(b,c)).

Our modified protoplast isolation method using younger soil-grown plants, Tricine buffers at a neutral pH, and epidermal peels resulted in less than half of the oligogalactolipid accumulation observed using the slicing protocol (compare Figures 1(b and c)). This suggests that the low pH buffers used for protoplast isolation cause abiotic stress signaling sensed on the outside of the chloroplast by SFR2. Additionally, the newly developed protocol is ideal for studies that use protoplasts immediately for downstream processes such as microscopy, metabolic measurements, or proteomics/transcriptomics.^{52–54}

Protoplast incubation post-isolation causes TGDG accumulation

Some uses of protoplasts involve post-isolation incubation of the protoplasts. To test whether protoplasts are sufficiently stressed during post-isolation incubations to accumulate TGDG, we treated isolated protoplasts with MES, pH 5.7 W5 or with Tricine, pH 7.0 W5 for at least one hour. This post-isolation treatment was done in a 6-well plate pre-treated with fetal bovine serum and with a final W5 volume of 200,000 protoplasts per mL, mimicking a post-transfection incubation.³⁷ TGDG accumulation occurred independently of the buffer acidity (Figure 2(d)), suggesting incubation stress depends on other factors.

By comparing stresses known to cause oligogalactolipid accumulation and those known to cause cytosolic acidification, we can learn more about the nature of protoplast stress during incubation. Oligogalactolipid accumulation has been reported in response to salinity and drought,¹⁷ desiccation,^{14–16} ozone fumigation,^{8,9} wounding,²⁴ chloroplast isolation,^{10,11} and oxidative stress.²³ Acidification of the cytosol has been reported during pathogen hypersensitive response,⁵⁵ fungal defense responses,⁵⁶ salinity stress in salt-sensitive species,^{57,58} and anoxic stress.^{59–61} Protoplast incubation may mimic one or more of these stress states. A common denominator between the stresses may be oxidative stress, which would be consistent with incubation of cells under buffer. We caution against uses of protoplasts to investigate the above stresses, particularly oxidative stresses without further improvement of the isolation protocol.

In this work, we tested whether cytosolic acidification co-occurred with oligogalactolipid accumulation during protoplast isolation. We then devised a method of protoplast isolation that minimized stress, as determined by production of oligogalactolipids. We hypothesized that SFR2 responded to the same signal in multiple stresses, cytosolic acidification. We showed that cytosol acidified and oligogalactolipids accumulated in all buffers used in protoplast isolation (Figure 1). This substantiates our hypothesis, because SFR2 activation has now been shown to co-

occur with cytosolic acidification in response to freezing and protoplast isolation, and implies that SFR2 may be responding to all stresses through cytosolic acidification. This may be the rationale behind retention of the SFR2 gene and other cytosolic acidification-responsive stress genes. Cytosolic acidification has only been associated with a subset of the stresses to which oligogalactolipids are known to accumulate, providing new avenues of investigation. Secondly, we amended traditional protoplast isolation methods, resulting in half as much accumulation of oligogalactolipids after isolation (Figure 2(a,b)), providing a new resource for protoplast applications.

Methods

Plant material and growth

For protoplast isolation and following lipid analysis or confocal microscopy reported in Figure 1, we followed,³⁷ using *Arabidopsis thaliana* Columbia ecotype grown on plates. pH-reporting lines were *Pt-GFP* stably transformed *Arabidopsis thaliana* of the Columbia ecotype and were a gift from Christoph Plieth and were grown as above. Seeds were sterilized and planted on Murashige and Skoog medium (Caisson Laboratories) with 1% sucrose, 0.5% MES (2-morpholinoethanesulfonic acid), pH 5.7, and 6% AgarGel (Sigma). Seeds were vernalized at 4°C for two days in the dark and subsequently grown at 22°C under 16-hour day/8-hour night conditions for approximately four weeks before isolating protoplasts.

For protoplast isolation using epidermal peels adapted from³⁰ and reported in Figure 2, *Arabidopsis* of the Columbia ecotype were grown on Sungrow Propagation Mix combined with Turface. Growing conditions were 22°C under 16-hour day/8-hour night conditions for approximately three weeks before isolating protoplasts

Protoplast isolation

Protoplast isolations using the slicing protocol and reported in Figure 1 were performed identically to.³⁷ Briefly, 25 leaves were sliced and put into enzyme digestion solution. Following 30 minutes of vacuum infiltration, they were incubated in the dark for 3 hours. Leaves were then swirled to release the protoplasts and residual leaf material was removed by filtration before pelleting protoplasts at 100 × g for 2 minutes. Cells were then washed, quantified, and treated with buffers as described. Buffer compositions are given in Table 1.

Protoplasts isolated after leaf disruption by epidermal peel followed by neutral pH isolation and reported in Figure 2 were performed similarly to³⁰ using buffers from.³⁷ As mentioned above under plant growth, soil-grown plants were used to increase leaf size for epidermal peels. Protoplasting of leaves without an epidermis did not require vacuum infiltration or dark incubation. Instead, shaking at 50 RPM for 1 hour was used to enhance protoplast release from the leaves. To achieve a neutral pH, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) or Tricine (N-(Tri(hydroxymethyl)methyl)glycine) at pH 7 was used in place of MES pH 5.7 in the enzyme solution, W5, MMG, and WI solutions at concentrations of 20 mM, 2 mM, 4 mM, and 4 mM respectively.

Lipid analysis of SFR2 activation

To analyze SFR2 activation, at least 1.5 million protoplasts were pelleted by centrifugation at $100 \times g$ for two minutes. Protoplast lipids were extracted from the pellet using a modified Bligh and Dyer method.⁶² Lipids were resolved by thin layer chromatography (TLC) on Silica Gel 60 plates (Millipore). TLC plates were pre-baked at 120°C for 30 minutes then briefly cooled before loading lipids and resolving with a liquid phase of chloroform:methanol:acetic acid:water (85:20:10:4, v/v/v/v). Oligogalactolipids were detected using α -naphthol, which detects galactose moieties.⁶²

Cytosolic pH measurement using *Pt*-GFP

Protoplasts were isolated from stably transformed Arabidopsis expressing *Pt*-GFP. A single protoplast preparation was used and subdivided four ways and treated with the individual buffers utilized in Yoo, et al.³⁷ W5 treatment was performed by isolating, washing and counting and then incubating in W5 for one hour. MMG treatment was done by replacing the W5 buffer with MMG for one hour after counting. PEG treatment was employed by suspending the isolated and counted protoplasts in MMG and adding 110 μ L PEG solution for every 20,000 protoplasts, incubated for 5 minutes, and pelleted. They were then incubated in W5 for one hour, mimicking a transformation protocol.³⁷ Lastly, WI treated cells were resuspended in WI for one hour after counting and removing W5 buffer. Confocal microscopy images and cytosolic pH measurements were taken as in Barnes, et al.,⁷ using the same pH standard curve.

MES and Tricine treatment

After isolating protoplasts with epidermal peels and Tricine at a neutral pH, they were exposed to W5 made with Tricine, pH 7.0 or W5 made with MES, pH 5.7 at a concentration of 2×10^5 protoplasts per mL of buffer for one to three hours in a 6-well dish pre-treated with Fetal Bovine Serum.

Author Contributions

RR, AB, and CE conceived and designed experiments; AB and CE performed experiments and analyzed the data; AB wrote the article; RR and CE edited the article

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