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Paula Duque *Editor*

Environmental Responses in Plants

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Environmental Responses in Plants

Methods and Protocols

Edited by

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Preface

As sessile organisms, plants have evolved elaborate mechanisms to perceive and respond to a myriad of environmental cues, thereby increasing their chances of reproduction and survival. Their extraordinary phenotypic plasticity allows them to grow towards or away from stimuli, orchestrate their metabolism according to 24-h light–dark cycles, endure extremely harsh climatic and soil conditions, or defend themselves against pathogenic agents. Elucidating the mechanisms by which plant systems sense and respond to external signals is not only an interesting fundamental biological question in itself, but may also have attractive practical implications for agriculture by opening new avenues in the development of efficient strategies to improve the performance of crop plants.

Divided into four distinct parts—*Tropisms*, *Photoperiodism and Circadian Rhythms*, *Abiotic Stress Responses*, and *Plant-Pathogen Interactions*—this *Methods in Molecular Biology* volume describes different up-to-date methodological approaches, ranging from physiological assays to imaging and molecular techniques, to study a wide variety of plant responses to environmental cues. Aimed at plant physiologists, biochemists, or cell and molecular biologists, the book includes detailed protocols to investigate some of the many key biological processes underlying plant environmental responses, mostly in the model organism *Arabidopsis thaliana* but also in *Physcomitrella patens* and in different crop species such as rice, potato, barley, or tomato. It will hopefully be of great use to the numerous plant biologists worldwide interested in this exciting and fast-growing research topic.

Oeiras, Portugal ***Paula Duque***

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Part I

Tropisms

Chapter 1

Hydrotropism: Analysis of the Root Response to a Moisture Gradient

Regina Antoni, Daniela Dietrich, Malcolm J. Bennett, and Pedro L. Rodriguez

Abstract

Hydrotropism is a genuine response of roots to a moisture gradient to avoid drought. An experimental system for the induction of hydrotropic root response in petri dishes was designed by pioneering groups in the field. This system uses split agar plates containing an osmolyte only in a region of the plate in order to generate a water potential gradient. *Arabidopsis* seedlings are placed on the MS agar plate so that their root tips are near the junction between plain MS medium and the region supplemented with the osmolyte. This elicits a hydrotropic response in *Arabidopsis* roots that can be measured as the root curvature angle.

Key words Hydrotropism, Water potential gradient, Root curvature angle, Moisture gradient, Root growth, Sorbitol, ABA, *Arabidopsis*

1 Introduction

Drought is a major environmentalstress that affects plant growth and has a serious impact on agriculture. Plants have developed a range of mechanisms to overcome drought stress including stomatal closure, regulation of gene expression, accumulation of osmoprotectants or compatible solutes, and modulation of growth [1]. Hydrotropism forms part of the drought-avoidance plant response and is a mechanism by which roots change growth direction according to differences in the water potential of the soil. Elucidating the molecular mechanism of hydrotropism in roots is important both for understanding plant adaptation to soil moisture gradients and improving crop productivity. Root growth is strongly directed by gravity and also by other tropisms like phototropism, thigmotropism, or hydrotropism [2]. The study of the latter process is complicated by the fact that gravitropism and hydrotropism influence each other and make it difficult to observe hydrotropism in isolation. However, since roots of the pea mutant

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ageotropum are agravitropic but hydrotropic both sensing pathways must operate through independent mechanisms [3]. Additionally, the non-hydrotropic *Arabidopsis* mutants *no hydrotropic response* (*nhr1*) and *mizu-kussei* (miz1) show a normal root gravitropic response [4, 5]. Unlike gravitropism, where the central role of auxin in its regulation has been well described, the molecular mechanisms that regulate hydrotropism are still largely unknown. Screening procedures in model plant species are needed for genetic dissection of complex biological processes. Therefore, a protocol that allows the analysis of the hydrotropic response in *Arabidopsis* seedlings can help to isolate key genes involved in this response. Takahashi et al. [6] and Eapen et al. [4] described two methods to measure the hydrotropic response in *Arabidopsis* seedlings. Both methods are based on the same principle of challenging roots with a water potential gradient that will ultimately trigger root bending. Here we describe one of them based on the use of split agar plates that contain two media with different water potential. The use of these strategies has allowed the isolation of mutants that lack a normal hydrotropic response such as *miz1, miz2/gnom, ahr1*, and *nhr1* [6–8, 4], or the identification of mutants that show an enhanced hydrotropic response, such as the *pp2c* quadruple mutant impaired in the protein phosphatases type 2C that negative regulate ABA signaling [9]. As a result, a gene that plays an essential role in hydrotropism and is expressed in the columella cells of the root cap, MIZ1, has been identified [6]. It has been also found that GNOM-mediated vesicular trafficking and ABA signaling through PYR/PYL/RCAR receptors is required for hydrotropism in *Arabidopsis* [7, 9]. Finally, in addition to ABA [5, 9], cytokinins also play a role in root hydrotropism [8].

2 Materials

1. D-Sorbitol.
2. Half-strength (0.5×) Murashige and Skoog (MS) plates: 2.4 g/L MS medium (Duchefa Biochemie, Haarlem, NL) containing macroelements, microelements, and vitamins [10], 0.1 % MES and 1 % agar. The pH was adjusted to 5.7 with KOH before autoclaving.
3. 120 × 120 × 17 mm square polystyrene petri dishes.
4. 3 M Micropore surgical tape.
5. Growth chamber in darkness.
6. Imaging system (optional, if time course analysis is intended): IR camera system [11].
7. NIH image software ImageJ v1.37.

3 Methods

3.1 Growth of Arabidopsis Seedlings

1. Sterilize *Arabidopsis* seeds by treatment with 50 % sodium hypochlorite for 5 min followed by four washes with sterile distilled water and stratify them in the dark at 4 °C for 3 days.
2. Sow the seeds on plates containing 0.5× MS medium, pH 5.7 and solidified with 1 % agar (0.5× MS plates).
3. Seal the plates with 3 M Micropore surgical tape and put them vertically in a growth chamber with 24 h light at 22 °C for 5–6 days.

3.2 Preparation of Plates that Establish a Water Potential Gradient

1. The osmotic pressure (π) of an ideal solution can be approximated using the Morse equation: $\pi = iMRT$, where M is the molarity of the solute. Sorbitol is a common solute in the laboratory that lacks secondary effects and we use it to generate a water potential gradient in split agar plates. For instance, adding 0.4 M sorbitol as solute lowers the water potential by −1 MPa. Split agar plates are generated by filling the upper region of the square petri dish with medium containing 0.5× MS salts and the lower region with 0.5× MS salts + 0.4 M sorbitol (Fig. 1). Thus, a water potential gradient will be generated between the two different media. Root tips will be located at the border between both media and the root curvature will be measured within 12 h (Fig. 1). The water potential gradient between both media becomes smaller over time, but exists for a substantial portion of the experimental period (*see* **Note 1**).
2. The split agar plates must be prepared just before the experiment. We use a template placed below the plate in order to guide the diagonal cutting of the medium with a scalpel (Fig. 1). Next, we remove the 0.5× MS medium from the lower part of the dish and we replenish the empty part of the plate with 0.5× MS medium supplemented with 0.4 M sorbitol.
3. Use the same template to guide the transfer of *Arabidopsis* seedlings grown on vertical plates to the plates with a water potential gradient so that their root tips are 2–3 mm above the border between the two media (blue line in Fig. 1). Seedlings should be transferred only to the three central rectangles. According to the size of the square petri dishes, 5–6 seedlings of each genotype can be transferred to one of the rectangles in such a way that each plate contains up to three different genotypes (*see* **Notes 2** and **3**).
4. At least three plates per experiment are needed. In order to avoid a positional effect, the order of the genotypes on the plates should be varied. The two rectangles close to the edges

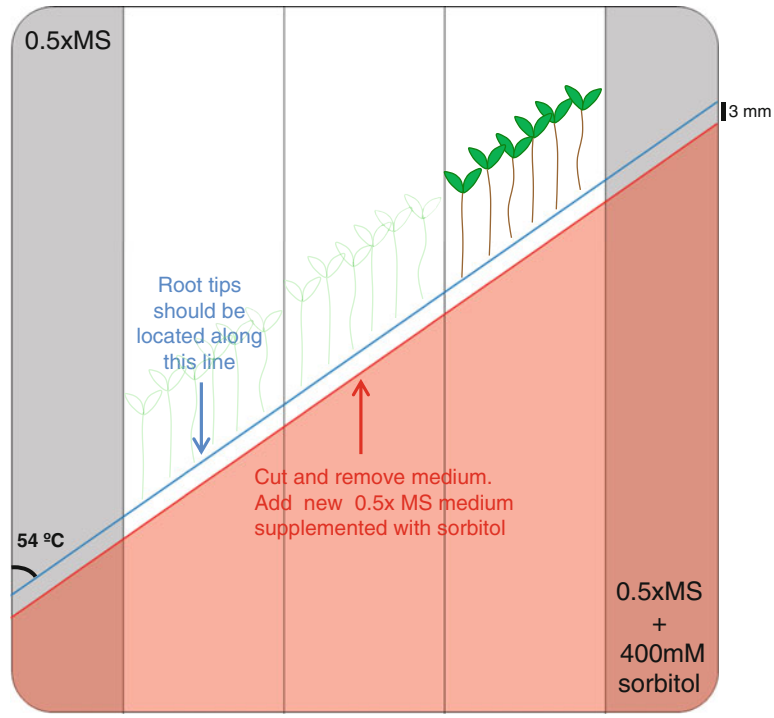

Fig. 1 Schematic representation of the split agar plate used for the analysis of the root hydrotropic response. The *upper part* of the plate contains 0.5× MS medium while the *lower part* contains 0.5× MS medium supplemented with 0.4 M sorbitol, which generates a water potential gradient. The plate is vertically divided in five *rectangles*. Seedlings should be located in the three *central rectangles*

of the plate should be left empty as diffusion of water and sorbitol could be affected by boundary effects.

5. Seal the plates with 3 M Micropore surgical tape to allow gas exchange.

3.3 Root Curvature Measurement

1. Place the plates vertically in a growth chamber at 22 °C in darkness and take images of the plants after 8–13 h. Performing the experiment in darkness eliminates the effect of phototropism on root angle, although it has been described that the hydrotropic curvature of light-grown seedlings is notably higher than in dark-grown seedlings [12]. In wild type plants, the root curvature generated in response to the moisture gradient can be observed from the first hour onwards; however, after 8–13 h the angle values of root curvature will be higher, which will make it easier to distinguish between altered and normal hydrotropic response (Fig. 2a, b). If detailed analysis of the development of the hydrotropic root curvature over time

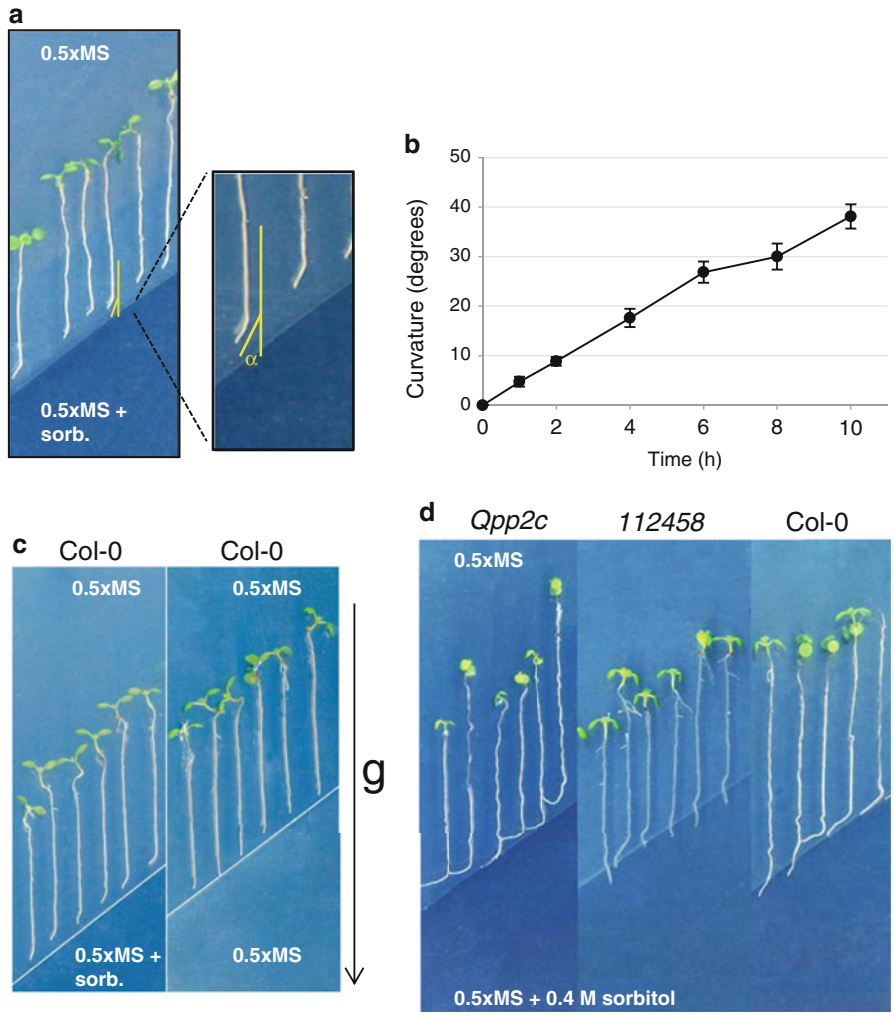

Fig. 2 Root hydrotropic response of 6-day-old *Arabidopsis* seedlings in plates where a water potential gradient has been established. (**a**) Measurement of the root curvature angle (α) after 10 h of transferring the seedlings to split agar plates to estimate the hydrotropic response. (**b**) Time course of the hydrotropic response in Col-0 *Arabidopsis* seedlings. (**c**) Root bending only occurs in Col-0 seedlings after transferring them to plates with a water potential gradient. Otherwise, they follow the gravity (g) vector. (**d**) Root hydrotropic response of the ABA-hypersensitive mutant impaired in four clade A PP2Cs (*abi1-2 hab1-1 pp2ca-1 abi2-2,* abbreviated as *Qpp2c*) and the ABA-insensitive mutant impaired in six PYR/PYL ABA receptors (*pyr1 pyl1 pyl2 pyl4 pyl5 pyl8*, abbreviated as*112458*). 6-day-old seedlings were transferred to plates with a moisture gradient and photographs were taken after 3 days. An enhanced hydrotropic response was observed in *Qpp2c*, whereas a lack of response was found in *112458* compared to Col-0 wt seedlings

is intended, images should be taken with an automated IR camera system (*see* **Note 4**).

2. Use Image J to measure the curvature angle of the primary root (Fig. 2a).

4 Notes

1. After the preparation of the plates, the water potential gradient will decrease over time [6]. For this reason, plates must be prepared just before the start of the experiment and angle measurements should be taken after 8–13 h. As temperature affects osmolyte diffusion and in order to keep the water potential gradient as steep as possible, cool down the 0.5× MS medium supplemented with sorbitol as much as possible before pouring.
2. While setting up the experiment, maintain plants in vertical orientation as much as possible. As roots tend to grow in the direction of gravity, keeping them in the same initial position will avoid unnecessary perturbations in root growth. Select seedlings of the same developmental stage, which should have similar root growth rates.
3. When preparing the plates take special care of working on a level surface to ensure that the level between the two media is similar. We use a spirit level to ensure the flow laminar hood where media are poured is really on the same level. Additionally take care to obtain a horizontal surface in the border between the two media that generate the water potential gradient. The transition between both media should be as flat as possible since irregular surfaces between both media could alter the root hydrotropic response.
4. It is necessary to include in the experiment control 0.5× MS plates lacking the water potential gradient. As shown in Fig. 2c, seedlings growing on control plates follow the gravity vector while those growing on plates that contain a water potential gradient bend and avoid the low water potential area. Once the measurement of root curvature is done, seedlings can be left growing for 2–3 days to observe if the response persists in time (Fig. 2d). Under our growth conditions (22 °C in darkness) Col-0 plants show a root curvature of 30–40° after 12 h. In addition to the wild type control, the use of mutants that lack or show enhanced hydrotropic response is strongly advisable (Fig. 2d).

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References

1. Verslues PE, Agarwal M, Katiyar-Agarwal S et al (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45:523–539
2. Roy R, Bassham DC (2014) Root growth movements: waving and skewing. Plant Sci 221:42–47
3. Jaffe MJ, Takahashi H, Biro RL (1985) A pea mutant for the study of hydrotropism in roots. Science 230:445–447
4. Eapen D, Barroso ML, Campos ME et al (2003) A no hydrotropic response (nhr1) root mutant that responds positively to gravitropism in *Arabidopsis*. Plant Physiol 131:536–546
5. Kobayashi A, Takahashi A, Kakimoto Y et al (2007) A gene essential for hydrotropism in roots. Proc Natl Acad Sci USA 104: 4724–4729
6. Takahashi N, Goto N, Okada K et al (2002) Hydrotropism in abscisic acid, wavy, and gravitropic mutants of *Arabidopsis thaliana*. Planta 216:203–211
7. Miyazawa Y, Takahashi A, Kobayashi A et al (2009) GNOM-mediated vesicular trafficking plays an essential role in hydrotropism of *Arabidopsis* roots. Plant Physiol 149:835–840
8. Saucedo M, Ponce G, Campos M et al (2012) An altered hydrotropic response (ahr1) mutant of *Arabidopsis* recovers root hydrotropism with cytokinin. J Exp Bot 63:3587–3601
9. Antoni R, Gonzalez-Guzman M, Rodriguez L et al (2013) PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. Plant Physiol 161:931–941
10. Murashige T, Skoog F (1962) A revised medium for rapid growth and Bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497
11. Wells DM, French AP, Naeem A et al (2012) Recovering the dynamics of root growth and development using novel image acquisition and analysis methods. Philos Trans R Soc B: Biol Sci 367:2245
12. Moriwaki T, Miyazawa Y, Fujii N et al (2012) Light and abscisic acid signalling are integrated by MIZ1 gene expression and regulate hydrotropic response in roots of *Arabidopsis thaliana*. Plant Cell Environ 35:1359–1368

Chapter 2

Assessing Gravitropic Responses in *Arabidopsis*

Richard Barker, Benjamin Cox, Logan Silber, Arash Sangari, Amir Assadi, and Patrick Masson

Abstract

Arabidopsis thaliana was the first higher organism to have its genome sequenced and is now widely regarded as the model dicot. Like all plants, *Arabidopsis* develops distinct growth patterns in response to different environmental stimuli. This can be seen in the gravitropic response of roots. Methods to investigate this particular tropism are presented here. First, we describe a high-throughput time-lapse photographic analysis of root growth and curvature response to gravistimulation allowing the quantification of gravitropic kinetics and growth rate at high temporal resolution. Second, we present a protocol that allows a quantitative evaluation of gravitropic sensitivity using a homemade 2D clinostat. Together, these approaches allow an initial comparative analysis of the key phenomena associated with root gravitropism between different genotypes and/or accessions.

Key words *Arabidopsis thaliana*, Gravitropism, Gravity perception, Time-lapse photography, Clinostat

1 Introduction

Gravity has been a constant factor in the evolution of life forms on earth, and organisms have evolved strategies to utilize this directional cue to their advantage. For instance, land plants have acquired the ability to utilize the gravity vector as a guide for organ growth (gravitropism), typically directing the roots downward into the soil for plant anchorage and water and nutrients uptake, and also guiding the shoots upward to access light for photosynthesis, exchange gases, and contribute to reproduction. This implies that each organ possess gravity-sensing machinery that allow them to identify changes in their orientation within the gravity field and respond by redirecting growth. In roots, gravitropism prevails amongst directional growth responses to the environment; competing tropic pathways triggered by light, touch, water gradients, salt, and/or oxygen, modulate the effectiveness of gravitropism when their contribution is needed most [1–3].

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Many years of experimentation have contributed to elucidating some of the mechanisms that mediate gravity sensing and signal transduction, including investigations on the role of amyloplast sedimentation within specialized gravity sensing cells (the statocytes) in the gravitropic responses of roots and shoots, studies of the molecular mechanisms that allow organs to redistribute the plant hormone auxin upon gravistimulation, identification and characterization of molecules that contribute to auxin transport and response (reviewed in Refs. [4–6]).

In this manuscript, we describe methods that are often used in the laboratory to evaluate: (1) the sensitivity of plant organs to gravistimulation; and (2) the kinetics of curvature response to gravistimulation. Indeed, these two procedures allow a careful evaluation of two critical components of gravitropism: (1) Gravisensing and (2) Properties of curvature response.

For many years, investigators have attempted to evaluate the ability of plant organs to respond to different doses of gravistimulation with distinct levels of curvature. One method has relied on rotating the plants to a defined angle from the gravity vector, and maintaining this orientation for distinct, short periods of time. At the end of each time period, the plants are transferred to a clinostat, which is a slowly rotating device that randomizes the orientation of the plant within the gravity field. Plants are allowed to grow on the rotating clinostat for a few hours, permitting the development of a tip curvature whose angle is a direct function of the gravistimulation dose (time × g) provided before clinorotation.

Using this experimental setup, Larsen [7] demonstrated that a logarithmic (L) model provides an adequate fit to the data, linking angle of curvature to the logarithm of the dose of gravistimulation (defined as time of gravistimulation at 1 g under this experimental setup). He then proposed that the extrapolation of this model to the *X* axis (curvature angle = 0) is a good estimate of the gravisensitivity of the investigated organ, and called it the Presentation Time (minimal gravistimulation time needed to trigger a productive gravity signal transduction pathway). This parameter has often been used as estimate of organ gravisensitivity [reviewed in 8 and 9].

It should however be cautioned that a hyperbolic (H) model can be fitted equally well or better to the same experimental data linking the curvature response to the time of gravistimulation before clinorotation, as previously discussed in Perbal et al. [8]. In fact, in most cases, the H model will be more strongly correlated to the data than the L model [8]. Therefore, Dr. Perbal and his collaborators proposed to use this H model to fit the data, and suggested that the slope of the H curve at the origin is, in fact, a better estimate of gravisensitivity than the presentation time. Hence, in this case, one assumes that even infinitesimal times of gravistimulation are sufficient to trigger a minute response. Because recent investigations have shown that these models fit quite nicely

the data with similar, though not identical correlation values, most researchers use both the H and L models to estimate gravisensitivity. In the first section of this paper, we detail the method we use to estimate root gravisensitivity of different genotypes or accessions, using either *Arabidopsis thaliana* or *Brachypodium distachyon* as models.

Different genotypes (mutants or accessions) will develop distinct kinetics of gravitropic curvature upon gravistimulation, and a careful evaluation of the characteristics of these curvature-response curves provides important information on their distinct properties, such as: the latency period, the speed of curvature during the log phase; the ability to, or time needed by the organ to, resume growth at the gravity set point angle; the final angle of growth from the gravity vector after reorientation; the existence of discontinuities in the shape of the curve suggesting the involvement of distinct mechanisms over time [reviewed in Refs. 4 and 10]. In the second section of this paper, we describe a high-throughput method that allows us to investigate the kinetics of root gravitropism at high temporal resolution for many genotypes in parallel, using a robotic assembly with a mounted camera similar to that described in Brooks et al. and Wells et al. [11] and [12].

Together, these two protocols are being used quite effectively to implement genome-wide association studies (GWAS) of gravitropism using *Brachypodium distachyon* as a model.

2 Materials

2.1 General Materials

1. Square, non-gridded 100 mm petri dishes.
2. Agar Type E (Sigma-Aldrich, St Louis, MO).
3. ½ Strength Murashige and Skoog nutrient salts (Sigma-Aldrich).
4. 20 % Bleach (10 ml bleach, 40 ml H_2O, and 50 μl SDS) or 90 % (v/v) ethanol.
5. A standard controlled growth environment with the following abiotic conditions: Fluorescent light—225 μmol/m^2; Temperature—21–22 °C; Humidity—65–90 %; Long-day cycles: 16 h light and 8 h of darkness.

2.2 2D Clinostat Materials

1. Flatbed scanner (Epson Perfection V33).
2. Clinostat petri-dish holder: details available for download http://masson.genetics.wisc.edu/wp-content/uploads/sites/32/2014/07/Clinostat-Description1.pdf (Fig. 1).
3. Wheaton Roller Culture Apparatus.
4. Timer.

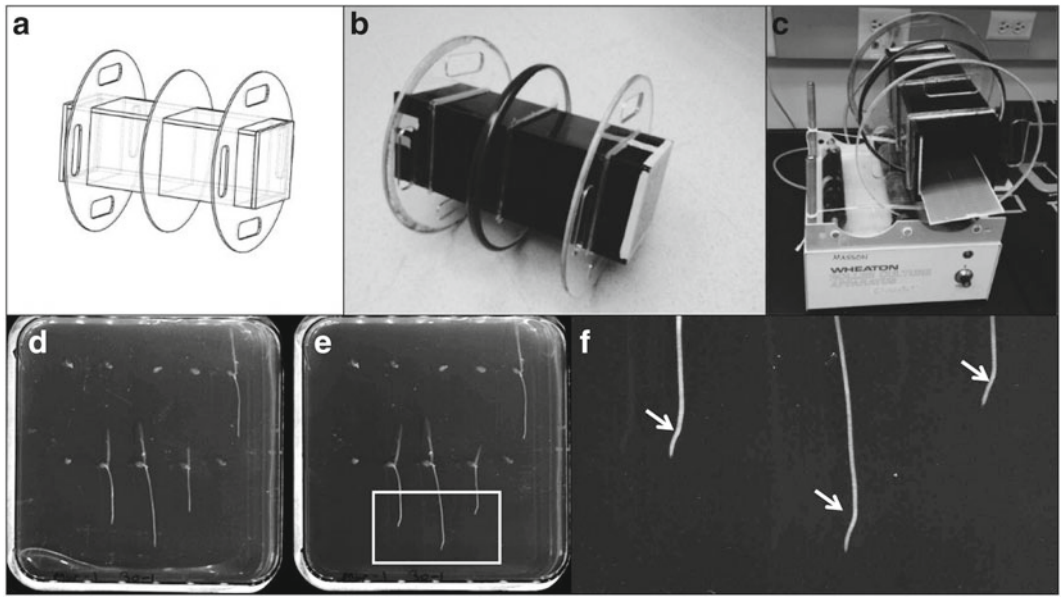

Fig. 1 (**a**) The AutoCAD design of the 2D clinostat petri dish holder. (**b**) This design allows 18 petri dishes to be rotated simultaneously. (**c**) Wires were used to ensure clinostat stayed on the center of the rotator in order to reduce friction with the roller. (**d**–**e**) Example image scanned before and after clino-rotation. (**f**) Schematic diagram highlighting the curvature induce by the gravitational observed 4 h after a clino-rotation

2.3 Time-Lapse Photography Robotic Assembly Materials

1. Camera (we use a Canon EOS rebel XSi with a Canon macro lens EF-s 60 mm 1:2:8 USM).
2. Two STAC6 applied motion stepper drivers and two NEMA 34 stepper motors that move the camera along an 80/20® Inc. aluminum stock track.
3. Custom robot and camera control software to determine the speed, timing, and distance at which the camera moves [13].
4. Four 120 V 2 ft lamps each containing T5 high emission growth light fixtures and 4 fluorescent bulbs illuminate the seedlings.
5. ROSCO E-colour+ #90 dark yellow green filter.
6. 64-bit Microsoft Windows 7 operating system running on a Dell PC with a 2.5 GHz Phenom™ II X4 905e Processor with 4GB of RAM to perform image analysis.
7. Adobe Bridge Camera Raw digital darkroom and Adobe Photoshop Creative Cloud.
8. ARRT software to measure images [14].

3 Methods

3.1 Measure Sensitivity to Gravity Using a 2D Clinostat Protocol

1. Pour molten 1 % agar containing ½ LS salts into petri plates in a laminar flow cabinet and allow it to harden.
2. Submerge *Arabidopsis* seeds for 15 min in 20 % bleach to surface sterilize them and then wash four times with distilled water to remove bleach solution (*see* **Note 1**).
3. Sow seeds horizontally on the agarose gel and seal plates (*see* **Note 2**).
4. Store the plates at 4 °C for 3 days in darkness provided by wrapping the plates in aluminum foil.
5. Place plates vertically in the controlled environment (CE) for 4 days.
6. Remove plates from the CE and scan the plates to create 600 dpi JPEG images (*see* **Note 3**).
7. Leave the plates vertical for 1 h in darkness.
8. Gravitationally stimulate the seedlings by rotating the plates 90°, and incubate in darkness for 10, 20, or 30 min (*see* **Note 4**).
9. Rotate the plates in the clinostat at 1 revolution per minute (RPM) for 4 h.
10. Remove from the clinostat then scan again.
11. Align the pre- and post-rotation scans next to each other using Adobe Photoshop or similar software.
12. Measure the angle of initial root tip curvature developed after transfer to the clinostat (*see* **Note 5** and Fig. 1).
13. For each gravistimulation time, quantify and plot the average angle of curvature with associated variance, fit the corresponding data with L and H model curves as defined in Perbal et al. [8], and use these models to calculate the presentation time (L model) and the sensitivity score (H model) (*see* **Note 6**).

3.2 Automated Time-Lapse Photography of Root Growth and Curvature Response to Gravistimulation

1. Submerge *Arabidopsis* seeds for 15 min in 20 % bleach to sterilize them and then wash four times with distilled water to remove bleach solution (*see* **Note 1**).
2. Sow seeds horizontally on 1 % agar containing ½ LS salts by placing the seeds on the surface of the agarose gel using a pipette or an autoclaved toothpick (*see* **Note 2**).
3. Store the plates at 4 °C for 3 days in darkness obtained by wrapping the plates in aluminum foil.
4. Place plates vertically in the controlled environment (CE) for 4 days.
5. Remove plates from CE and insert them into a petri dish holder that is 1 ft away from the lights, providing approximately

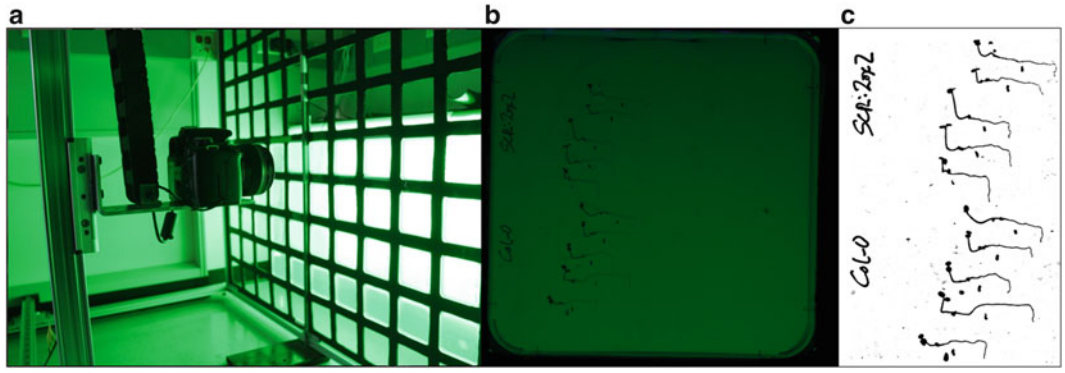

Fig. 2 (**a**) Semi-automated robotic photography system automates the time lapse photography of up to 40 petri dishes. (**b**) The plant growth parameters can be measured in the original photos by hand using ImageJ, Adobe Photoshop or other standard image analysis software. (**c**) Custom image enhancement scripts can digitize the images making it easier to analyze the whole time-lapse series using specific root tip tracking software

70 μmol m^{-2} s^{-1} of light. The room was kept between 22 and 24 °C and had a humidity between 50 and 60 % in front of the camera (*see* **Note 7**).

6. Rotate plates 90° and set the camera to photograph every 30 min for 12 h (*see* **Note 8** and Fig. 2).
7. Optimize root contrast against agar using image enhancement software (*see* **Note 9** and Fig. 2).
8. Measure images using a semi-automated image analysis software package such as ARTT by Russino et al. [14] (*see* **Notes 10** and **11**).
9. Plot data using MS Excel or alternative software package (*see* **Note 12**).

4 Notes

1. Seed can also be sterilized by rinsing with 90 % ethanol three times, then allowed to air-dry onto sterile filter paper.
2. When sealing the plates use medical micro-pore tape before removing the plates from the laminar-flow cabinet to prevent microbial contamination. When working with *Arabidopsis*, it is important to carefully choose the taping material to prevent problems associated with ethylene accumulation [15]. If working with *Brachypodium* or rice submerge the end of the kernel where the radical emerges within the agar.
3. When scanning plates ensure they are parallel to the edge of the scanner. Set up three groups of plates: one for a 10 min stimulus, one for a 20 min stimulus, and another for a 30 min stimulus.

4. When providing the gravitational stimuli the plates should be inside the clinostat chamber as this will provide a dark environment reducing phototropic morphological changes, and it will also minimize the mechanical stimulation. The chamber should not be rotating during this period, of course.
5. Align the two images taken either before or after the clinorotation next to each other. First measure the root tip angle before the plants were gravistimulated. Then find the same location on post clinorotation and measure the new orientation that the root takes.
6. In the L-model ($\alpha = \lambda + \mu.\log10(d)$) where α corresponds to the gravitropic response and d is the dose of the stimulus, we plot the curvature at each time point on a log scale using Microsoft Excel. We then perform a linear regression to calculate the value where the logarithmic line of best fit intercepts the *X* axis. In the Hyperbolic model ($\alpha = a*d/(b+d)$), d is the time (min), α is the gravitropic response (angle of curvature), while a and b are parameters that we want to estimate. Therefore we are estimating two parameters; S = a/b and R^2 = 1- SSres/SStot. We use Wolframs Mathematica to analyze the clinostat results using the H-model running this code:

   In Wolframs Mathematica this will deliver the key parameters of the hyperbolic model

```
a, b, S and R^2wherer = sqrt(R^2)
d = {10, 20, 30};
alpha = {9.73, 13.933, 24.10};
SStot = Total[(Mean[alpha] - alpha)^2];
abOpt = NMinimize[Total[((a d)/(b + d) - alpha)^2], {a, b}];
d1 = 0;
d2 = 40;
alphamin = 0;
alphamax = 40;
Show[Plot[(a d)/(b + d) /. abOpt[[2]], {d, d1, d2},
PlotRange -> {{d1, d2}, {alphamin, alphamax}}],
ListPlot[Table[{d[[i]], alpha[[i]]}, {i, 1, 3}],
PlotMarkers -> {Automatic, 10}, PlotStyle -> Red,
PlotRange -> {{d1, d2}, {alphamin, alphamax}}]]
SSres = abOpt[[1]];
Print["SSres: ", SSres]
Print["SStot: ", SStot]
Slopes = Table[D[(a d[[i]])/(b + d[[i]]) /. abOpt[[2]]],
{i, 1, 3}]
Print["Slope of Fitted Curve at given times: ", Slopes]
Print["a: ", a /. abOpt[[2]]]
Print["b: ", b /. abOpt[[2]]]
Print["S: ", a/b /. abOpt[[2]]]
Print["R^2: ", 1 - SSres/SStot]
```

7. It is important to ensure the plates are filmed in an environment with appropriate temperature and humidity. These can be monitored using a Hobo® data logger inserted into the petri dish holder. In order to remove the negative phototropic effect of the backlight on root growth we use a ROSCO E-colour+ #90 dark yellow green filter to remove blue and red light. Alternatively an infrared camera and far red LED illumination can be used [12].
8. To ensure each plant is the same age when it is photographed each plate should be rotated 90° no more than 1 min before the first photo in the time series is taken.
9. Enhancing images using Adobe Photoshop: A custom image enhancement script was recorded using Adobe Photoshop to prepare the time series for quantification by ARTT [14]. The precise parameters will depend on the image acquisition parameters; these can be easily adjusted using either Adobe Bridge Camera Raw tool or automated batch processing scripts within Adobe Photoshop. Use a naming system that allows you to track the original image source so you can check the times between and add a 3-digit serial number so that the images stay in sequence. Select a new folder location to save them; this may take a long time depending on the number of images you are working with. Below is an example of a script designed to make roots slighty larger and bolder to aid with subsequnt analysis. The parameters to adjust using Adobe Photoshop or other digital darkrooms are adjusted in the following order: auto-tone, desaturate, median filter (2 pixels), brightness/contrast (brightness −50, contrast +100), gradient map (tailoring is system specific), shadow/highlight (tailoring is system specific), glowing edges (edge width 3, edge brightness 20, smoothness 1), and then median filter (Radius 3 pixels). This system will increase the contrast of the roots against the agar media, while the glowing edges and final median filter make the root easier to track as it is larger.
10. ARTT software pre-tracking: Open the software from the shortcut on the desktop or start menu. Select the directory where your time-lapse series has been saved. You can choose which photos from the time series you measure using the drop-down menu. Go to the parameters tab, this has another sub-tab called settings that allows you to tell the software size of the root (preset to 50), the max orientation displacement (preset to 30) and maximum forward prediction (preset to 20). These values should be tailored to the distance your root tip was displaced which depends on its speed of growth and the length of time between each photo. We have never seen any effect associated with the Gaussian filter size so ignore that setting. Above this there is a tick box called “Apply inclusion

filter". If measuring the root tip we recommend this option as it stops the software measuring the movement of seeds or leaves. If measuring the Coleoptile tip, do not add the inclusion filter, this will cause the software to track all moving tips within the image series. There will be multiple moving parts tracked that will require discarding after the tracking has been completed, but this will allow you to select just the coleoptile for measuring. The measuring process in ARTT can take some time depending on the number of images in the time series but is usually really reliable and not prone to crashing.

11. ARTT software post-tracking analyses validation: After tracking has been completed, you should notice a drop-down menu labeled view in the top left hand corner; click on this and select analysis view. This will open a new window that will allow you to view the tracings made by the software. Reject any tracings the software made that are incorrect using the trace list in the bottom left hand corner, right click on the root and select delete. If two root paths cross each other the software can lose its tracking, but it may also regain it after the paths have crossed. If this occurs you can merge these two tracings into one by right clicking on the root name in the Trace list and selecting the merge option. If the software has lost the root tip you can move the tracking point by changing the X and Y values. If you are happy with your selection of roots then go to the top left corner and click on the single file option, this will ensure all the measurements are saved in one file instead of one file per root. Choose a destination directory and then click on the save data button.
12. Data processing in spreadsheet: Open the CSV data file, select all, copy and then paste it into Excel. This file contains the X and Y coordinates of the root tip, the time (which is the number of the image), how much it was displaced with its velocity, direction and orientation. The time column is actually the image number so should be converted to minutes, hours and/or days depending on the time scale of the experiment. This can be done by calculating the length of time between the first 2 photos and then extrapolating the rest of the time points. Displacement in ARTT is measured in pixel so should be converted to mm or cm. The accumulative displacement can be used to give you the root growth kinetics; this is achieved by adding together all the measurements from previous images. When the software fails to measure the root tip orientation it delivers either a "0" or "nan", these should be removed to prevent them skewing the final results. The root tip orientation can have the mean and standard error calculated averages and standard errors of each mutant/ecotype/treatment can be calculated and plotted on a graph.

References

1. Galvan Ampudia C, Julkowska M, Darwish E, Gandullo J, Korver R et al (2013) Halotropism Is a response of plant roots to avoid a saline environment. Curr Biol 23:204450
2. Massa G, Gilroy S (2003) Touch modulates gravity sensing to regulate the growth of primary roots of *Arabidopsis thaliana*. Plant J 33:43545
3. Takahashi N, Yamazaki Y, Kobayashi A, Higashitani A, Takahashi H (2003) Hydrotropism interacts with gravitropism by degrading amyloplasts in seedling roots of *Arabidopsis* and radish. Plant Physiol 132(805):10
4. Baldwin K, Strohm A, Masson P (2013) Gravity sensing and signal transduction in vascular plant primary roots. Am J Bot 100(1):126–142
5. Strohm A, Baldwin K, Masson P (2012) Molecular mechanisms of root gravity sensing and signal transduction. Wiley Interdiscip Rev Dev Biol 1:276–285
6. Strohm A, Baldwin K, Masson P (2012) Multiple roles for membrane-associated protein trafficking and signaling in gravitropism. Front Plant Sci 3:274
7. Larsen P (1962) Orthogravitropism in roots. In: Ruhland W (ed) Encyclopedia of plant physiol, Vol 17, Part 2, physiology of movements. Springer-Verlag, Berlin, pp 153–199
8. Perbal G, Jeune B, Lefranc A, Carnero-Diaz E, Driss-Ecole D (2002) The dose-response curve of the gravitropic reaction: a re-analysis. Physiol Plant 114:336–342
9. Young L, Harrison B, Murthy N, Moffatt B, Gilroy S, Masson P (2006) Adenosine kinase modulates root gravitropism and cap morphogenesis in *Arabidopsis thaliana*. Plant Physiol 142:564–573
10. Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T et al (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature 482(7383):103–106
11. Brooks T, Miller N, Spalding E (2010) Plasticity of *Arabidopsis* root Gravitropism throughout a multidimensional condition space quantified by automated image analysis. Plant Physiol 152(1):206–216
12. Wells D, French A, Naeem A, Ishaq O, Traini R, Hijazi H, Bennett M, Pridmore T (2012) Recovering the dynamics of root growth and development using novel image acquisition and analysis methods. Philos Trans R Soc B: Biol Sci 367(1595):1517–1524
13. Sangari A, Ohme-Takagi M, Mitsuda N, Assadi A (2012) Automated imaging & high-throughput phenotyping, massively parallel image acquisition, analysis and modeling: mapping plant genotype-phenotype relation. Cold Spring Harbor, Cold Spring Harbor, NY
14. Russino A, Ascrizzi A, Popova L, Tonazzini A, Mancuso S, Mazzolai B (2013) A novel tracking tool for the analysis of plant-root tip movements. Bioinspir Biomim 8:025004
15. Buer CS, Wasteneys GO, Masle J (2003) Ethylene modulates root-wave responses in *Arabidopsis*. Plant Physiol 132:1085–1096

Chapter 3

Physiological Analysis of Phototropic Responses in *Arabidopsis*

Mathias Zeidler

Abstract

Plants utilize light as sole energy source. To maximize light capture they are able to detect the light direction and orient themselves towards the light source. This phototropic response is mediated by the plant blue light photoreceptors phototropin1 and 2 (phot1 and phot2). Although fully differentiated plants also exhibit this response it can be best observed in etiolated seedlings. Differences in light between the illuminated and shaded site of a seedling stem lead to changes in the auxin-distribution, resulting in cell elongation on the shaded site. Since phototropism connects light perception, signaling, and auxin transport, it is of great interest to analyze this response with a fast and simple method.

Here we describe a method to analyze the phototropic response of *Arabidopsis* seedlings. With numerous mutants available, its fast germination and its small size *Arabidopsis* is well suited for this analysis. Different genotypes can be simultaneously probed in less than a week.

Key words Phototropism, *Arabidopsis*, Phototropin

1 Introduction

The first scientific observations of phototropic plant responses date back to the sixteenth century (reviewed in Ref. [1]). Darwin discovered that the perception of the stimulus and the site of action could be separated [2] which later led to the discovery of Auxin [3–5]. The responsible photoreceptors were identified with the help of molecular-genetics in the model plant *Arabidopsis thaliana* and later named after their major response: PHOTOTROPINS (phot) [6, 7]. *Arabidopsis* has two phots (phot1 and phot2) which are also involved in the regulation of chloroplast movement and stomatal opening. They are light-activated kinases. A sensory part with two LOV (light, oxygen, voltage) domains inactivates the kinase domain in the dark. After activation the sensory domain releases the kinase domain and phots can phosphorylate target proteins [8]. Phot1 regulates the phototropic response to a wide range

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of blue light intensities while phot2 mainly mediates the response to high-intensity blue light [9].

Other photoreceptors modulate phot mediated phototropism, mainly the blue light sensing CRYPTOCHROMEs (cry1 and cry2; [10]) as well as the red light sensing phytochromes, especially PHYTOCHROME A (phyA) [11–14]. While crys and phyA appear to enhance the phototropic response to blue light, cryptochromes also seem to be responsible for residual phototropism in the *phot1phot2* double mutant [15]. Additionally phyA has been shown to mediate the enhancement of phototropism by a red light pretreatment [11, 16, 17]. Consequently a *cry1cry2phyA* triple mutant has a severely disturbed phototropic response [18]. Signaling factors for phototropism downstream of the phots have also been identified, among them NONPHOTOTROPIC HYPOCOTYL 3 (NPH3), ROOT PHOTOTROPISM 2 (RPT2) [19, 20], and the PHYTOCHROME KINASE SUBSTRATE (PKS) family with four members (PKS1–4). PKS1, PKS2, and PKS4 are associated with the plasma membrane and interact with phot1, phot2, and NPH3 [21–23]. PKS1, 2, and 4 as well as NPH3 have been shown to influence auxin distribution and auxin-dependentgene expression [24, 25]. Further downstream auxin transport facilitators are involved in phototropism. Among them are the PIN-FORMEDs (PINs), ATP-binding-cassette B-type transporter (ABCB), and the AUXIN RESISTANT (AUX) 1—LIKE-AUX (LAX) family proteins [26–31].

Many different techniques have been described to analyze phototropism in *Arabidopsis* seedlings from which the growth on vertical oriented agar plates is the most common and easiest, and therefore this technique is described here. Others, like continuous video documentation of the seedlings [32] or microbeam irradiation on single seedlings [33] have also been used successfully to measure hypocotyl bending in unilateral illumination conditions. They require a more sophisticated setup and data management but deliver time resolved information. Nevertheless, for the routine test of few lines the technique described here is easy and requires no specific instruments or software. Results can be obtained in less than a week.

2 Materials

1. 20–40 *Arabidopsis* seeds per line you want to investigate.
2. ½ MS solid medium: 2.15 g/l MS (Murashige–Skoog salts, without vitamins; *see* **Note 1**) in H_2O (Milli-Q). Adjust pH to 5.7 with KOH, add 0.7 % agar, and autoclave. Pour in sterile square petri dishes (12.5 × 12.5 × 1.5 cm).

3. Sterilization solution: 2.5 % (v/v) sodium hypochlorite in H_2O (Milli-Q) with 0.1 % (v/v) Triton X-100. Prepare fresh and be careful because the solution is quite corrosive.
4. Plating Solution: 0.1 % agarose (*see* **Note 2**) in H_2O (Milli-Q), autoclaved.
5. A narrow band width light source (full width at half height; FWHH < 30 nm) with the option to adjust different fluence rates is necessary. Usually a slide projector with an interference filter can be used. Much better are high power LEDs that are easily available. For unilateral blue light we use 450 nm Luxeon high power LEDs (Roithner Lasertechnik, Austria), which are mounted on a 2-mm thick aluminum plate for cooling and connected to a power supply. Fluence rates between 1 and 100 μmol m^{-2} s^{-1} can be easily obtained by changing the current, and for lower fluence rates semitransparent paper is brought into the light path.
6. Black paper.
7. Book stands or black wooden boxes in which up to 6 square petri dishes will fit (inner dimensions 10 × 13 × 13 cm one side open).
8. Computer with the ImageJ software (National Institutes of Health) installed.

3 Methods

3.1 Seed Preparation

1. You will need 1–2 petri dishes per genetic line to investigate. Take the plates with the solidified media, turn them bottom-up and draw a line on the bottom of the petri dishes. The start point should be 5 mm before the top-midpoint of the plate and the end point 5 mm after the bottom midpoint of the plate if it lies on a table in front of you (Fig. 1a; *see* **Note 3**). Turn the plates back to the normal position (bottom-down).
2. Sterilize the seeds by mixing them with 500 μl of sterilization solution in 1.5-ml reaction tubes and let the suspension incubate for 10 min at room temperature (*see* **Note 4**). Centrifuge briefly. From this point onwards work on a sterile bench. Discard the sterilization solution quickly and wash the seeds three times with 1 ml of sterile water. After the last wash, discard the water, add 100 μl of 0.1 % agarose, and mix.
3. Pipette some of the seeds in 0.1 % agarose on the drawn line on the MS medium (Fig. 1a). Move the single seeds with a sterile inoculation loop. Make sure that the distance between each seed is approximately 3–5 mm (*see* **Note 5**). Let the surface of the medium dry briefly on the clean bench so that the seeds do not shift when the plates are placed in the vertical

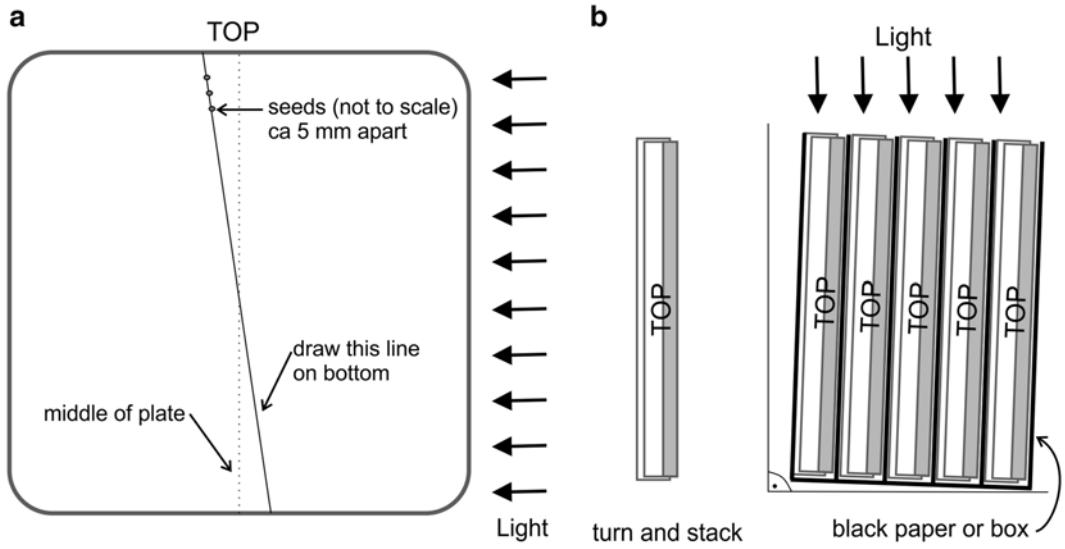

Fig. 1 Schematic drawings of plate layout and light path to the plate stack. (**a**) View of open plate with the line on the bottom slightly rotated in relation to the center of the plate. Seeds are separately arranged on this line with a distance of 3–5 mm each. (**b**) Plate is closed and placed vertically so that the TOP (see also **a**) is on the upper side. Plates are stacked with black paper in between them as well as on the outside and on the back of the stack. The stack is slightly turned so that the light falls with a 0.5–1° angle on the agar surface

position. Close the petri dishes and label them on the bottom with the genotype of the seeds they contain.

4. Stratify your seeds for 3–4 days wrapped in aluminum foil at 4 °C, already in the vertical position. Mark the top side of the plates.

3.2 Germination and Illumination

1. Unwrap the plates and expose 2 h to white light to synchronize germination.
2. Place the plates back in standing position and insert a black paper between each separate plate to minimize reflection. Also put a black paper at the back of the plate stack.
3. The plates should now be in a stack in which all the plates are standing, with black paper on both sides of the plates and on the back of the cube-like stack (Fig. 1b; *see* **Note 6**).
4. Let seeds germinate and the emerging seedlings grow negatively gravitropically for 2–3 days in darkness at 20–25 °C. Ideally, the plates should already be in front of the irradiation setup. The irradiation setup and position of the plate stack should be prepared in a dark room or in a completely dark cabinet.
5. After the germination and negative gravitropic growth, switch the light on to irradiate the seedlings from one side with blue light of the desired fluence rate for 12 h (*see* **Note 7**). The plates should be slightly moved out of the direct straight line

of the light path so that the light falls obliquely onto the agar surface (Fig. 1b). Depending on the light source, different fluence rates can be adjusted in the light field by shading the front side of the petri dish cubes with semitransparent paper.

6. Document the phototropic bending angle by digital photography. Place a piece of millimeter paper next to the seedlings to obtain a scale.

3.3 Measuring the Bending Angle with ImageJ

1. Install ImageJ from http://rsb.info.nih.gov/ij/ and start the program.
2. Open your image: File>Open.
3. Make sure the plate is aligned with the border of the window: Image>Transform>Rotate.
4. Change the image to 8-bit greyscale: Image>Type> 8-bit.
5. Click on the angle-measuring button in the tools menu.
6. Click once on the base of the hypocotyl, once on the midpoint of the bend and last on the upper part of the hypocotyl.
7. Press M on the keyboard to save the measurement in the results windows.
8. Go back to **step 6**.
9. When finished with one biological sample, copy the data to Excel or a similar program to plot the angles (*see* **Note 8**).

4 Notes

1. Adjust the amount of MS salts according to the recommendations of the supplier. The number here refers to MS-salts from Genaxxon (Germany).
2. Agarose is used here and not agar. The low concentration agarose has a similar density as the seeds and allows them to float. This makes pipetting and manipulating the seeds much easier.
3. A slightly slanted line is intended so that the seedlings do not disturb each other when growing negatively gravitropically.
4. It is important not to exceed these 10 min because then the seeds suffer damage and germination rates drop. On the other hand, a much shorter time does not sterilize the surface sufficiently. Therefore 10 min should be observed closely (+/− 1 min).
5. This may be difficult at the beginning because the seeds tend to stick to each other in the drops of liquid. Therefore, less liquid is better. Try first to position a few seeds and when you are done, take more out of the tube onto the agar surface.
6. Care should be taken that the plates are exactly upright. This can be achieved by constructing wooden boxes or by using bookstands on both sides of the upright stack.

7. For standard tests a fluence rate of 1 μmol m^{-2} s^{-1} should result in a good response of the wild type. Fluence rates should be determined with a PAR meter or similar device at the point where the seedlings will be positioned.
8. Usually it is not conclusive to take a mean value of the angles, especially if plants are not behaving normally (for instance showing a positive or negative reaction towards the light source). It therefore is better to take classes of angles (0–10°, 11–20° etc.) and sort all the measured values into these classes, so that a table is generated which comprises numbers of seedlings per class. This table needs to be normalized to the number of seedlings by translating the numbers per class to percentage of all seedlings per class. This dataset contains more information than the mean (Fig. 2).

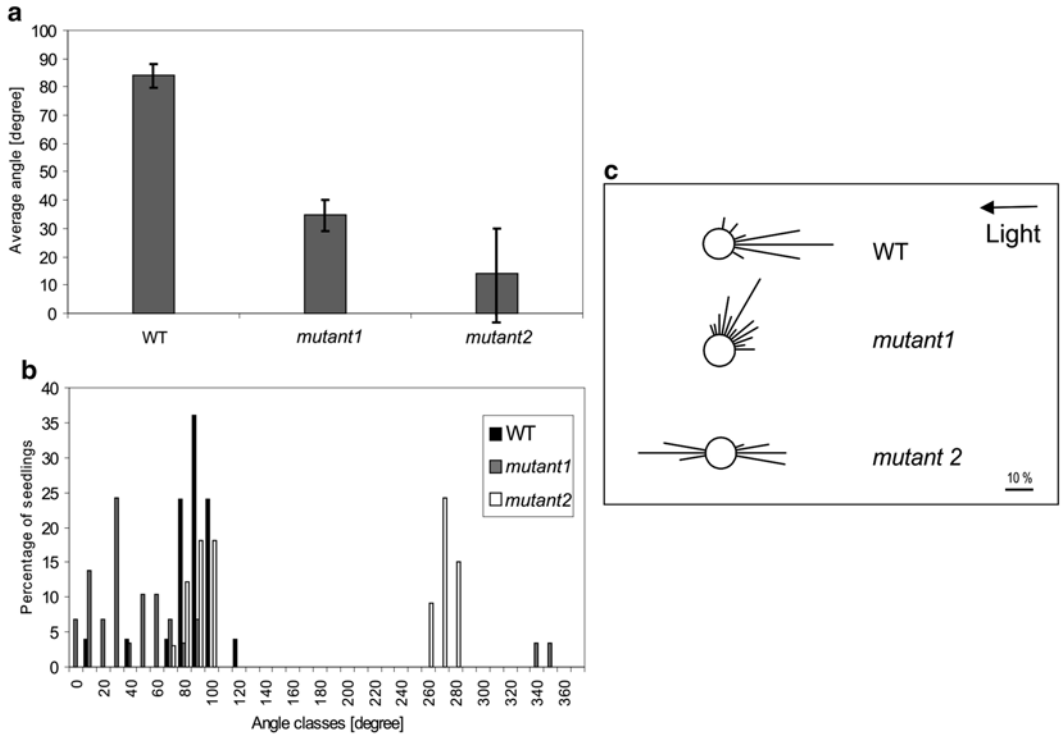

Fig. 2 Different presentations of the same bending angle data. Three artificial data sets, representing a normal distribution, a wide distribution, and an avoidance component in the bending response were plotted with three different methods. (**a**) An average does not differentiate between *mutant1* and *mutant2* (positive and negative phototropism) nor does it reflect the avoidance reaction. *Error bars* represent standard errors of the mean. (**b**) All seedlings were sorted into classes and normalized to the total amount of seedlings from each genotype. This is a much better representation of the response and the differences between the different genotypes. In (**c**) the same data were converted into circular histograms (*wuschel*-diagrams). Here the length of a line represents the percentage of seedlings in this specific angle class. This diagram, also based on the same data set as in **b**, is more informative. The light was coming from the right hand side as indicated by the *arrow* and the scale bar is 10 % of the seedlings represented

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References

1. Whippo CW, Hangarter RP (2006) Phototropism: bending towards enlightenment. Plant Cell 18(5):1110–1119. doi:10.1105/tpc.105.039669
2. Darwin C (1880) The power of movements in plants. John Murray, London
3. Went FW (1926) On growth accelerating substances in the coleoptile of Avena sativa. Proc K Akad Wet 30:10–19
4. Cholodny N (1927) Wuchshormone und Tropismen bei den Pflanzen. Biol Zentralbl 47:604–626
5. Kögel F, Haagen-Smit AJ (1931) Mitteilung über pflanzliche Wachstumsstoffe. Über die Chemie des Wuchsstoffes. Proc K Ned Akad Wet 34:1411–1416
6. Huala E, Oeller PW, Liscum E, Han IS, Larsen E, Briggs WR (1997) *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. Science 278(5346):2120–2123
7. Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K (2001) *Arabidopsis* nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci U S A 98(12):6969–6974
8. Okajima K, Matsuoka D, Tokutomi S (2011) LOV2-linker-kinase phosphorylates LOV1-containing N-terminal polypeptide substrate via photoreaction of LOV2 in *Arabidopsis* phototropin1. FEBS Lett 585(21):3391–3395
9. Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M (2001) *Arabidopsis* NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science 291(5511):2138–2141
10. Ahmad M, Jarillo JA, Smirnova O, Cashmore AR (1998) The CRY1 blue light photoreceptor of *Arabidopsis* interacts with phytochrome A in vitro. Mol Cell 1(7):939–948
11. Parks BM, Quail PH, Hangarter RP (1996) Phytochrome A regulates red-light induction of phototropic enhancement in *Arabidopsis*. Plant Physiol 110(1):155–162
12. Lariguet P, Fankhauser C (2004) Hypocotyl growth orientation in blue light is determined by phytochrome A inhibition of gravitropism and phototropin promotion of phototropism. Plant J 40(5):826–834
13. Rösler J, Klein I, Zeidler M (2007) *Arabidopsis fhl/fhy1* double mutant reveals a distinct cytoplasmic action of phytochrome A. Proc Natl Acad Sci U S A 104(25):10737–10742
14. Rösler J, Jaedicke K, Zeidler M (2010) Cytoplasmic phytochrome action. Plant Cell Physiol 51(8):1248–1254
15. Ohgishi M, Saji K, Okada K, Sakai T (2004) Functional analysis of each blue light receptor, cry1, cry2, phot1, and phot2, by using combinatorial multiple mutants in *Arabidopsis*. Proc Natl Acad Sci U S A 101(8):2223–2228
16. Hangarter RP (1997) Gravity, light and plant form. Plant Cell Environ 20(6):796–800
17. Janoudi AK, Gordon WR, Wagner D, Quail P, Poff KL (1997) Multiple phytochromes are involved in red-light-induced enhancement of first-positive phototropism in *Arabidopsis thaliana*. Plant Physiol 113(3):975–979
18. Tsuchida-Mayama T, Sakai T, Hanada A, Uehara Y, Asami T, Yamaguchi S (2010) Role of the phytochrome and cryptochrome signaling pathways in hypocotyl phototropism. Plant J 62(4):653–662
19. Motchoulski A, Liscum E (1999) *Arabidopsis* NPH3: a NPH1 photoreceptor-interacting protein essential for phototropism. Science 286(5441):961–964
20. Sakai T, Wada T, Ishiguro S, Okada K (2000) RPT2. A signal transducer of the phototropic response in *Arabidopsis*. Plant Cell 12(2):225–236
21. Lariguet P, Schepens I, Hodgson D, Pedmale UV, Trevisan M, Kami C, de Carbonnel M, Alonso JM, Ecker JR, Liscum E, Fankhauser C (2006) PHYTOCHROME KINASE SUBSTRATE 1 is a phototropin 1 binding protein required for phototropism. Proc Natl Acad Sci 103(26):10134–10139
22. de Carbonnel M, Davis P, Roelfsema MR, Inoue S, Schepens I, Lariguet P, Geisler M,

Shimazaki K, Hangarter R, Fankhauser C (2010) The *Arabidopsis* PHYTOCHROME KINASE SUBSTRATE2 protein is a phototropin signaling element that regulates leaf flattening and leaf positioning. Plant Physiol 152(3):1391–1405

23. Demarsy E, Schepens I, Okajima K, Hersch M, Bergmann S, Christie J, Shimazaki K, Tokutomi S, Fankhauser C (2012) Phytochrome kinase substrate 4 is phosphorylated by the phototropin 1 photoreceptor. EMBO J 31(16):3457–3467
24. Haga K, Takano M, Neumann R, Iino M (2005) The Rice COLEOPTILE PHOTOTROPISM1 gene encoding an ortholog of *Arabidopsis* NPH3 is required for phototropism of coleoptiles and lateral translocation of auxin. Plant Cell 17(1):103–115
25. Kami C, Allenbach L, Zourelidou M, Ljung K, Schutz F, Isono E, Watahiki MK, Yamamoto KT, Schwechheimer C, Fankhauser C (2014) Reduced phototropism in pks mutants may be due to altered auxin-regulated gene expression or reduced lateral auxin transport. Plant J 77(3):393–403. doi:10.1111/tpj.12395
26. Friml J, Wisniewska J, Benkova E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature 415(6873):806–809. doi:10.1038/415806a
27. Ding Z, Galvan-Ampudia CS, Demarsy E, Langowski L, Kleine-Vehn J, Fan Y, Morita MT, Tasaka M, Fankhauser C, Offringa R, Friml J (2011) Light-mediated polarization of the PIN3 auxin transporter for the phototropic response in *Arabidopsis*. Nat Cell Biol 13(4):447–452. doi:10.1038/ncb2208
28. Noh B, Murphy AS, Spalding EP (2001) Multidrug resistance-like genes of *Arabidopsis* required for auxin transport and auxin-mediated development. Plant Cell 13(11): 2441–2454
29. Nagashima A, Uehara Y, Sakai T (2008) The ABC subfamily B auxin transporter AtABCB19 is involved in the inhibitory effects of N-1-naphthyphthalamic acid on the phototropic and gravitropic responses of *Arabidopsis* hypocotyls. Plant Cell Physiol 49(8):1250–1255. doi:10.1093/pcp/pcn092
30. Christie JM, Yang H, Richter GL, Sullivan S, Thomson CE, Lin J, Titapiwatanakun B, Ennis M, Kaiserli E, Lee OR, Adamec J, Peer WA, Murphy AS (2011) phot1 inhibition of ABCB19 primes lateral auxin fluxes in the shoot apex required for phototropism. PLoS Biol 9(6):e1001076. doi:10.1371/journal.pbio.1001076
31. Stone BB, Stowe-Evans EL, Harper RM, Celaya RB, Ljung K, Sandberg G, Liscum E (2008) Disruptions in AUX1-dependent auxin influx alter hypocotyl phototropism in *Arabidopsis*. Mol Plant 1(1):129–144. doi:10.1093/mp/ssm013
32. Whippo CW, Hangarter RP (2003) Second positive phototropism results from coordinated Co-action of the phototropins and cryptochromes. Plant Physiol 132(3):1499
33. Yamamoto K, Suzuki T, Aihara Y, Haga K, Sakai T, Nagatani A (2014) The phototropic response is locally regulated within the topmost light-responsive region of the *Arabidopsis thaliana* seedling. Plant Cell Physiol 55(3):497–506. doi:10.1093/pcp/pct184

Chapter 4

Automatic Chloroplast Movement Analysis

Henrik Johansson and Mathias Zeidler

Abstract

In response to low or high intensities of light, the chloroplasts in the mesophyll cells of the leaf are able to increase or decrease their exposure to light by accumulating at the upper and lower sides or along the side walls of the cell respectively. This movement, regulated by the phototropin blue light photoreceptors phot1 and phot2, results in a decreased or increased transmission of light through the leaf. This way the plant is able to optimize harvesting of the incoming light or avoid damage caused by excess light. Here we describe a method that indirectly measures the movement of chloroplasts by taking advantage of the resulting change in leaf transmittance. By using a microplate reader, quantitative measurements of chloroplast accumulation or avoidance can be monitored over time, for multiple samples with relatively little hands-on time.

Key words Phototropin, *Arabidopsis*, Chloroplast movements, Microplate reader

1 Introduction

Plants growing in the natural environment need to monitor and respond to environmental changes constantly to optimize growth and development. The intensity of the incoming light is important because if too high, the light can cause damage to the chloroplasts [1], and when too low it does not provide sufficient energy for the plant to grow [2]. However, plants are able to react to these changes by relocating their chloroplasts within the cell to increase (chloroplast accumulation) or decrease (chloroplast avoidance) their exposure to the incoming light [3]. This movement, towards low light and away from high light intensities, has been shown to be regulated by the phototropin blue light photoreceptors (phot1 and phot2 in *Arabidopsis*). While phot2 is required for avoidance [4], both phot1 and phot2 regulate the accumulation response [5].

An array of techniques can be used to measure the movement of chloroplasts [6], including the simple "band assay" which was successfully used in a mutant screen to identify *phot2* [4], and microbeam

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experiments tracking the movement of single chloroplasts within the cell [7]. However, as the chloroplast movement ultimately alters the light absorbance of the leaf, accumulation and avoidance responses can also be measured indirectly by probing the change in light absorbance/transmittance of the leaf. Since the phototropin photoreceptors are in general solely responsible for the response, blue light is used to stimulate the movement while a red light beam (that will not trigger movement) is used to probe the leaf transmittance. Methods utilizing these properties were first developed four decades ago [8] and have since then been used by others with modifications [9–11]. With the use of commonly available programmable microplate readers, quantitative and automatic time course data of chloroplast movements can be obtained with ease. In this chapter, we describe this method, including the setup of the measuring equipment, preparation of samples and data analysis.

2 Material

1. Plate reader: Should be able to be programmed to eject and insert a 96-well plate at any given time to measure absorbance of 660 nm and 800 nm. We use the Synergy 2 plate reader (BioTek, USA).
2. Blue light source: Any narrow peak light source with an equal light intensity over the area of the plate will be sufficient. We prefer high power luxeon LEDs, mounted on a cooling panel with peak emission at 470 nm (30 nm FWHH; Roithner Lasertechnik, Austria).
3. Liquid ½ MS media: Dissolve ½ of the recommended weight of MS salts in 100 ml of dH_2O. Set the pH to 5.7 using KOH and autoclave for 20 min at 120 °C. Let cool to room temperature.
4. ½ MS media with 0.6 % Phytagel: Prepare as the liquid ½ MS media, but add 0.6 g of Phytagel (Sigma-Aldrich) prior to autoclaving.
5. Clear 96-well microplate with flat bottom.
6. Adhesive seal for 96-well plate.
7. Cork borer: 6 mm in diameter with "push rod".
8. 2–4-week-old *Arabidopsis* plants grown on soil (*see* **Note 1**).

3 Methods

3.1 Setup of Plate Reader and Light Source

1. Place the microplate reader in a dark and temperature controlled room or cabinet. Connect the computer, preferably in a different room/outside the cabinet to avoid possible light contamination from the screen (Fig. 1).

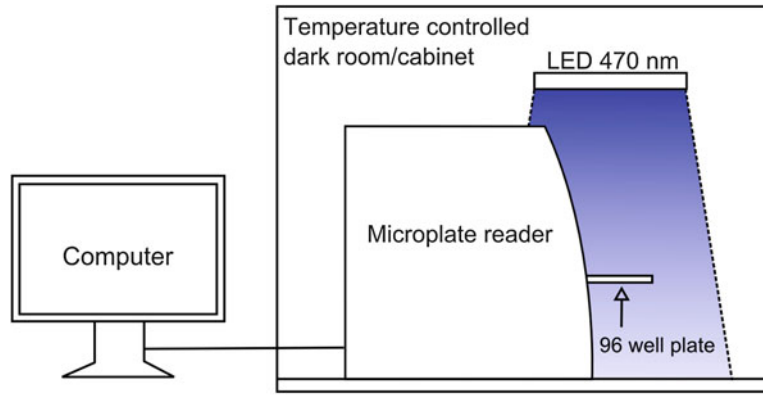

Fig. 1 Schematic representation of the general setup of microplate reader, computer, and LED array

Table 1
A general example of the different actions that need to be programmed in the microplate reader

Step #	Action	Step #	Action	Step #	Action
1	Measure 660 nm	8	Measure 800 nm	15	Measure 660 nm
2	Measure 800 nm	9	Delay 10 min	16	Measure 800 nm
3	Delay 10 min	10	Measure 660 nm	17	Plate Out
4	Measure 660 nm	11	Measure 800 nm	18	Delay 10 min
5	Measure 800 nm	12	Plate Out	19	Plate In
6	Delay 10 min	13	Delay 10 min	20	Measure 660 nm
7	Measure 660 nm	14	Plate In	21	Measure 800 nm

Here, four measurements are taken in darkness (in the machine), followed by two measurements after exposure to the blue light

2. Place the blue LED array above the 96-well plate ejected from the microplate reader (Fig. 1; *see* **Note 2**).
3. Program the plate reader to take a measurement every 10 min at both 660 nm (*see* **Note 3**) and 800 nm (*see* **Note 4**). For the first 30 min (four measurements) the plate stays in the machine. After the fourth absorbance reading, the program should continue to measure every 10 min (*see* **Note 5**). However, now the plate should be ejected directly after the measurement and stay ejected for the light treatment until the next measurement (Table 1).

3.2 Preparation of 96-Well Plate and Leaf Disks

1. Prepare the ½ MS-media containing 0.6 % Phytagel and autoclave (*see* **Note 6**).
2. While still warm, pipette 100 µl of media into each well and leave the plate on a flat surface to settle.

3. Detach a rosette leaf from a 2–4-week-old *Arabidopsis* plant using a scalpel. Stamp out a leaf disk of 6 mm in diameter using the cork borer against a normal sheet of (ca. 20 × 30 cm) paper folded twice in the middle (*see* **Note 7**). Place the leaf disk adaxial side up in a Petri dish filled with liquid ½ MS medium. Continue this procedure until you have all disks needed for the experiment.
4. Gently transfer the leaf disks to the 96-well plate using a pair of tweezers and taking care not to damage the disks (*see* **Note 8**).
5. Add 20 µl of liquid ½ MS media to each well to avoid dehydration of the leaf during measurements.
6. By holding the plate against a light source, you might now notice air bubbles trapped under some leaves. Carefully remove these by gently pressing the leaf disk with the back side of the tweezers or another blunt object (*see* **Note 9**).
7. Close the plate with the adhesive seal. Wrap the plate in aluminum foil and place in a dark room/cabinet overnight (*see* **Note 10**).

3.3 Starting of Measurements and Data Analysis

1. Working in darkness, or using green safe light, remove the aluminum foil and the adhesive seal and place the 96-well plate in the microplate reader.
2. Start the absorbance measurements using the predefined program.
3. Turn on the blue LED source and leave the program to run for as long as required (*see* **Note 11**).
4. When done, export the absorbance data (660 and 800 nm) to Excel or an equivalent program.
5. To account for light scattering in the 660 nm absorbance data, subtract the 800 nm value for each well and time point from the 660 nm value (*see* **Note 4**).
6. Transform absorbance (Abs) to % transmittance (%T) by $\%T = 10^{(2-Abs)}$.
7. **Steps 5** and **6** can be performed simultaneously using the equation: $\%T = 10^{((2-(Abs_{660}-Abs_{800}))}$.
8. To normalize the data, subtract the transmittance data of time point 0 (the fourth measurement) from all other time points to generate Δ Transmittance (%).
9. Calculate the mean value and standard deviation of the samples at each time point. The mean value and standard deviation of time point 0 (fourth measurement) should read 0.
10. Finally, create a graph plotting Δ Transmittance (%) over time (min) (Fig. 2).

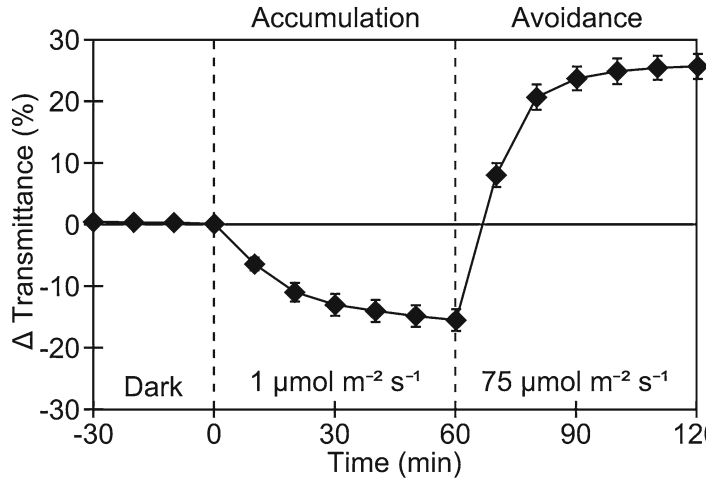

Fig. 2 Example of chloroplast movements in response to low and high fluence rates of blue light as measured by a microplate reader

4 Notes

1. We generally grow our plants in short day conditions (8 h light, 16 h dark). Although we have no indication that chloroplast movement is circadian regulated, the possibility should be kept in mind when designing an experiment.
2. It is very important that the light source produces a homogenous light field over the area of the 96-well plate as the chloroplast accumulation/avoidance is strictly dependent on the fluence rate of blue light. The light intensity should be tested using a PAR-meter or equivalent. However, we have found that a good way of determining the homogeneity of the light field is to conduct a test experiment using a full 96-well plate of WT leaf disks and search for any trends in the data that correlates with the positioning of the sample well.
3. Although leaf absorbance generally can be measured using red light without affecting the results in higher plants, it should be kept in mind that chloroplast relocation of some ferns and mosses are dependent on both blue and red light [12, 13]. In these cases, it would be important to set up a control experiment to in order to determine any effects of the red light pulses given during measurements.
4. Looking at the published literature, the 800 nm measurement is not an absolute requirement. However, we believe the addition of this measurement is beneficial to correct for light scattering effects.

5. Depending on the type and speed of the microplate reader and the number of samples used, the intervals between the measurements could be reduced. We aim at keeping the samples exposed to blue light for at least 90 % of the total time.
6. If the ½ MS, 0.6 % Phytagel media are allowed to settle after the autoclaving, reheating in the microwave is greatly aided by breaking up the gel, using a spatula prior to reheating. Although contamination is a concern, the leaf disks later put on top of the media in the wells are not sterile and generally not a problem.
7. It is important to use leaves of similar size and age. Furthermore, the disks should be stamped out from the same area of each leaf. We use the middle of the leaf, with the midvein crossing each leaf disk.
8. To counter act any potential problems related to the homogeneity of the light field, it is recommended to "mix up" the different genotypes on the microplate. Thus, do not put for example the WT samples in well A1-H8 and the mutant in A9-H12. In addition, it is important to remember that this method does not measure the movement of chloroplasts, but the increase or decrease of leaf transmittance. Thus, it is important to consider that an unrelated phenotype of a mutant (different shape/size of palisade cells) might also have an effect.
9. Although it is preferable to not have any air bubbles trapped under the leaf disks, we have no indication that this actually constitutes a problem. Therefore, this potential problem needs to be weighed against possible damage to the leaf disks during the removal of the trapped air.
10. In order to avoid any sudden changes in temperature, it is recommended to store the 96-well plate overnight in the room/cabinet where the measurements will take place. Shorter incubation times can also be used. However, if too short (<3 h), the initial four measurements in darkness are likely to show some minor increase or decrease of transmittance.
11. To observe a strong chloroplast accumulation response in WT leaf disks, we generally use a blue light fluence rate of 1 μmol m^{-2} s^{-1}. In order to observe a strong chloroplast avoidance response, a fluence rate of >50 μmol m^{-2} s^{-1} is recommended. In our experience, the measured avoidance/accumulation generally reaches a plateau after being exposed to light for 1 h (Fig. 2), but this might depend on the leaf tissue and fluence rate of blue light used.

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References

1. Kasahara M, Kagawa T, Oikawa K, Suetsugu N, Miyao M, Wada M (2002) Chloroplast avoidance movement reduces photodamage in plants. Nature 420(6917):829–832. doi:10.1038/nature01213
2. Trojan A, Gabrys H (1996) Chloroplast distribution in *Arabidopsis thaliana* (L.) depends on light conditions during growth. Plant Physiol 111(2):419–425
3. Kong SG, Wada M (2014) Recent advances in understanding the molecular mechanism of chloroplast photorelocation movement. Biochim Biophys Acta 1837(4):522–530. doi:10.1016/j.bbabio.2013.12.004
4. Kagawa T, Sakai T, Suetsugu N, Oikawa K, Ishiguro S, Kato T, Tabata S, Okada K, Wada M (2001) *Arabidopsis* NPL1: a phototropin homolog controlling the chloroplast high-light avoidance response. Science 291(5511):2138–2141. doi:10.1126/science.291.5511.2138
5. Sakai T, Kagawa T, Kasahara M, Swartz TE, Christie JM, Briggs WR, Wada M, Okada K (2001) *Arabidopsis* nph1 and npl1: blue light receptors that mediate both phototropism and chloroplast relocation. Proc Natl Acad Sci U S A 98(12):6969–6974. doi:10.1073/pnas.101137598
6. Wada M (2013) Chloroplast movement. Plant Sci 210:177–182. doi:10.1016/j.plantsci.2013.05.016
7. Kagawa T, Wada M (2000) Blue light-induced chloroplast relocation in *Arabidopsis thaliana* as analyzed by microbeam irradiation. Plant Cell Physiol 41(1):84–93
8. Inoue Y, Shibata K (1973) Light-induced chloroplast rearrangements and their action spectra as measured by absorption spectrophotometry. Planta 114(4):341–358. doi:10.1007/BF00387947
9. Berg R, Koniger M, Schjeide BM, Dikmak G, Kohler S, Harris GC (2006) A simple low-cost microcontroller-based photometric instrument for monitoring chloroplast movement. Photosynth Res 87(3):303–311. doi:10.1007/s11120-005-9012-1
10. DeBlasio SL, Mullen JL, Luesse DR, Hangarter RP (2003) Phytochrome modulation of blue light-induced chloroplast movements in *Arabidopsis*. Plant Physiol 133(4):1471–1479. doi:10.1104/pp. 103.029116
11. Kodama Y, Suetsugu N, Kong SG, Wada M (2010) Two interacting coiled-coil proteins, WEB1 and PMI2, maintain the chloroplast photorelocation movement velocity in *Arabidopsis*. Proc Natl Acad Sci U S A 107(45):19591–19596. doi:10.1073/pnas.1007836107
12. Kadota A, Sato Y, Wada M (2000) Intracellular chloroplast photorelocation in the moss *Physcomitrella patens* is mediated by phytochrome as well as by a blue-light receptor. Planta 210(6):932–937
13. Kawai H, Kanegae T, Christensen S, Kiyosue T, Sato Y, Imaizumi T, Kadota A, Wada M (2003) Responses of ferns to red light are mediated by an unconventional photoreceptor. Nature 421(6920):287–290. doi:10.1038/nature01310

Chapter 5

Microscopic and Biochemical Visualization of Auxins in Plant Tissues

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Abstract

Auxins are a particularly notable class of phytohormones in that they regulate plant growth and development at sites of synthesis, and via a regulated polar transport system comprising PIN, ABCB, and AUX/LAX transport proteins. In order to fully understand auxin-regulated physiological processes, it is therefore essential to be able to determine where indole-3-acetic acid and related compounds are being synthesized, where they are transported to, and how much IAA is accumulating in any given tissue. Auxin may be visualized either indirectly, through the use of auxin responsive promoters; directly, through the use of radiolabelled auxin or fluorescent auxin analogs; or biochemically through extraction and mass-spectrometric quantification of auxin and auxin metabolites from target cells or tissues. Here we focus on the use of the DR5::GUS synthetic auxin promoter reporter construct, fluorescent auxin analogs, and confirmatory biochemical (high-pressure liquid chromatography tandem mass-spectrometry) visualization of auxin and auxin metabolites.

Key words Auxin, DR5::GUS, Fluorescent auxin analogs, LC-MS/MS

1 Introduction

The phytohormone auxin is essential for normal plant growth. Auxin regulates programmed developmental processes, such as meristem organization, and plastic growth responses, including tropisms and differential lateral root growth. While the primary auxin found in most plants is indole-3-acetic acid (IAA), the terms "auxin" or "auxinic compound" are also applied to a range of structurally related molecules either synthesized by plants or algae, including indole-3-butyric acid and indole-3-propionic acid, or through industrial processes, such as 2,4-dichlorophenoxyacetic acid [1, 2]. Auxins are particularly interesting as hormones because they regulate plant development not only at the site of synthesis, but are also transported to more distal sites of action. Transport of auxins occurs through both the phloem ("bulk flow"), as well as through cell-to-cell movement. Auxin transport streams are

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organ-specific and, at the tissue and cell levels, are carefully defined and controlled through the action of both influx permeases (i.e., AUX/LAX proteins) and efflux transporters, primarily members of the pin-formed (PIN) major efflux facilitator or ATP-binding cassette B family (ABCB) (reviewed in Blakeslee et al.; Peer et al.) [3, 4]. Mis-regulation or inhibition of either auxin synthesis or transport (through either chemical or genetic means) results in moderate to severe disruptions in normal organ growth and development, leading to loss of root or shoot apical meristem identity, abnormal stem or shoot growth (e.g., *Arabidopsis pin1* and maize *br2* mutants), altered lateral root or root hair growth, or decreased seed production [5–8].

Given the importance of synthesis and transport in auxin physiology, it is crucial to determine sites of auxin accumulation and to be able to trace auxin movement in intact plants and tissues. As a result, considerable energy has been devoted to developing microscale techniques to visualize and quantify auxin movement in plant tissues. These techniques fall into two broad categories: indirect methods, in which the presence of auxin is inferred through either the location of transport proteins or the response of auxin-response elements; and direct methods, in which auxin is visualized either through the use of radioisotopes, fluorescently labeled analogs, or direct biochemical detection (primarily mass-spectrometry). This chapter provides a review of three of the methods most commonly used to visualize auxin and auxinic compounds in plant tissues: the DR5::GUS synthetic auxin reporter construct (an indirect method of visualization); fluorescent auxin analogs, which allow direct visualization of auxin accumulation and limited visualization of auxin transport, both at the cellular level; and the use of high-pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) to visualize and quantify auxin and auxin analogs in plant tissues.

1.1 Visualization of Auxin Using the DR5::GUS Synthetic Reporter System

To date, the majority of published work investigating auxin physiology and biochemistry has relied on the use of indirect methodology to visualize auxin transport and accumulation. In these studies, accumulations of auxin in target tissues have been inferred by demonstrating that proteins involved in transport of auxins exhibit a subcellular localization, which would result in auxin being funneled into the target tissue [6, 9–11]. While these methods provide a very good indication of the directionality of the vector of auxin transport, they do not allow a quantitative measurement of the amount of auxin being moved through the transport stream and into target cells or tissues. Auxin responsive promoters address this question by linking the presence of auxin in a cell to a quantifiable response.

Indirect visualization of auxin based on the use of auxin responsive elements/promoters presents several advantages over

the inference of auxin levels through localization of transport proteins. The largest of these advantages is that the promoter-based indirect auxin visualization systems respond specifically to the presence of IAA, and as a result give a relatively direct measurement of the amount of auxin present in cells or tissues [12, 13]. Additionally, the promoter-based systems provide the opportunity to not only indirectly visualize auxin accumulations, but also to quantify them, since the intensity of the observed response (commonly increases in glucuronidase (GUS) [12] activity or VENUS [13] yellow fluorescent protein expression) is proportional (directly proportional in the case of GUS and inversely proportional in the case of VENUS) to the amount of auxin present in the cell/tissue. Quantifications can be performed either visually, spectrophotometrically (i.e., through the use of enzymatic activity assays for GUS reporter constructs), or fluorimetrically (i.e., by comparing extractable fluorescence to a VENUS protein standard using a fluorimeter, for VENUS constructs). DR5::GUS is currently the most widely used auxin responsive element, and consists of five tandem repeats of an auxin-responsive element (TGTCTC) fused to the glucuronidase enzyme [12]. This construct has been successfully used to visualize auxin in both herbaceous (*Arabidopsis* [14], tomato [15], rice [16]) and woody (poplar [17]) species. In plants containing the DR5::GUS reporter construct, accumulation of auxin in cells will result in the expression of glucuronidase (GUS) in these cells. When stained with X-gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid), the GUS enzyme cleaves this molecule to release glucuronic acid (colorless), and 4-chloro-bromo-indigo, a blue precipitate [18]. The end result of this process is that cells and tissues where auxin levels were sufficient to trigger expression of the DR5:GUS reporter construct are stained a blue color following X-gluc treatment [12, 18]. Here we present a protocol detailing the use of the DR5::GUS reporter construct to visualize auxin in *Arabidopsis* [14]. Protocols for visualizing DII::VENUS are similar to those presented above for visualizing DR5::GUS, with the exception of the final visualization step. Imaging of DII::VENUS will require the use of confocal, rather than light, microscopy, in a manner similar to that described for the immune-localization of ABCB and PIN proteins, presented in chapter 6 of this volume.

1.2 Visualization of Auxins Using Fluorescent Auxin Analogs

While use of the DR5::GUSsynthetic auxin reporter construct provides several advantages (specifically fast turn-around time and the use of simple light microscopy to visualize stained tissues), the method does have a few drawbacks. As noted in the DR5::GUS protocol provided, it is very easy to over-stain tissues, resulting in an overestimation of auxin distribution in the cells or tissues under examination. Additionally, cell-to-cell movement of GUS stain over time can, if not carefully controlled for, provide a false picture

of auxin distribution in the target tissues. Finally, this method only indirectly detects auxin, meaning that it visualizes the effect of auxin on a given cell, i.e., the effect of the auxin present but not the auxin itself. In other words, the amount of GUS staining present in the cells of a DR5::GUS plant is indicative of the total amount of auxin present in the cell, as a result of both transport and synthesis, and is not a direct visualization of auxin itself. Initial attempts to visualize auxin directly focused on the use of antibodies generated to auxin molecules conjugated to bovine serum albumin through the indolic nitrogen [19], the carboxylic acid group [20], or one of the carbons of the benzyl ring [21]. While these antibodies have shown usefulness in radioimmunoassay [19] and enzyme-linked immunosorbent assay [22, 23] designed to quantify auxin levels in cellular or tissue extracts, these antibodies do not allow visualization of auxin or auxin metabolites in intact tissues. Recently, however, the development of fluorescent auxin analogs has progressed to the point where use of these molecules, in conjunction with current state-of-the-art auxin microdeposition methodologies, allows direct visualization of auxin, and limited visualization of auxin transport, at the cellular level [24]. The development of fluorescent auxin analogs has been complicated by the fact that endogenous auxins "auto-regulate" IAA transport and metabolism by altering the localization of PIN auxin efflux transporters and GH3, an auxin–amino acid conjugating enzyme [24]. Application of fluorescent auxin analogs with auxinic activity would therefore result in an almost immediate shift in both auxin transport profiles and auxin metabolism, and the profile of auxin transport gained by imaging these analogs would not be indicative of the native auxin gradient present in the cell, tissue, or organ being studied.

Recently developed 7-nitro-2,1,3-benzoxadiazole (NBD) conjugated indole-3-acetic acid (NBD-IAA) and naphthalene-1-acetic acid (NBD-NAA) provide a new and highly precise way to view auxin accumulations directly at the cellular and subcellular levels. NBD-IAA and NBD-NAA fluorescent auxins are recognized and transported (at least to some degree) by several auxin transport proteins, including both the uptake AUX/LAX permease AUX1 and the PIN1 major efflux facilitator protein auxin transporter [24]. The kinetics and rate of this transport, however, are currently unclear, and it appears as though NBD-IAA and NBD-NAA are not transported (or transported only minimally) by PIN3 and the ATP-binding cassette transporter/p-glycoprotein auxin transporters ABCB1 and ABCB19 [24]. While NBD-auxin analogs may not be transported by all auxin transporters, the cellular and subcellular accumulation patterns of these molecules appear to mimic those of endogenous auxins, and the NBD-analogs have an additional advantage in that they do not interact with the TIR1-Aux/IAA auxin receptor complex or GH3 and

therefore are significantly less active in triggering auxin responses than other auxin analogs (for example, 1-*N*-naphthylphthalamic acid), making them ideal substrates with which to directly and non-disruptively visualize auxin gradients at the cellular and subcellular levels as well as, to a lesser extent, auxin transport at the cellular level [24]. Additionally, since NBD-auxins are themselves fluorescent, these small molecules can be used to visualize auxin distribution in any plant line chosen, without the need to transform plants with a reporter construct and select for homozygosity. Finally, the rapid uptake of these compounds (15–20 min, on average) into tissues allows for real-time visualization of auxin transport in plant responses such as gravitropic bending of roots [24]. Here we detail protocols to utilize NBD-IAA or NBD-NAA to visualize auxin accumulations at the cellular and subcellular level in intact *Arabidopsis* seedlings and excised *Arabidopsis* hypocotyls.

1.3 Visualization of Auxins Using Liquid Chromatography Tandem Mass Spectroscopy

Although the methods above allow the detection of auxin through microscopy-based imaging, they do not allow precise determination of the amount of auxin present in a given tissue or organ. In order to generate a map which will allow visualization of the levels of auxin (or auxin analogs) present in a given plant tissue or organ, it is necessary to extract IAA from the target tissues, concentrate it, and quantify it. Initial methods used to detect auxins relied on thin-layer chromatography [25–27] or high-pressure liquid chromatography (HPLC) [28, 29]. While these methods are still used to quantify auxins in some high-throughput scenarios (e.g., when screening auxin levels in soil samples or microbial extracts [30]), in most cases they have been replaced by either enzyme-linked immunosorbent assays employing antibodies generated against auxins [23] or gas chromatography-mass spectroscopy (GC-MS). To date, most GC-MS methods used to detect auxin and auxin metabolites have required the derivatization of auxin (often with diazomethane [31]) prior to analysis, a process that results in sample loss and a consequent decrease in the sensitivity of this technique [32]. Modern GC-MS techniques, however, have begun to address issues of sample loss, and have considerably enhanced the limit of detection achievable via GC-MS [33]. More recently, the advent of highly sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have allowed the detection of underivatized auxins from plant tissues [34]. In addition to eliminating sample loss during the derivatization process, LC-MS/MS methodologies have the ability to utilize capacity of the tandem mass spectrometers to detect mass transitions (i.e., discrete pairs of precursor and product ions) specific to auxins, auxin metabolites, and auxin analogs, while filtering out molecular species generated by "non-auxin" molecules. Because of this, LC-MS/MS techniques significantly reduce the background commonly associated with GC-MS-based quantitation, resulting in increased sensitivity to

auxins, and decreased limits of detection. Performed carefully, LC-MS/MS-based methods allow detections of auxin in the femtogram range (loaded onto the high-pressure liquid chromatography column), and from as little as 10–20 mg of tissue.

2 Materials

2.1 Plant Material

Plant material of almost any age or developmental type, as well as protoplasts or suspension cells can be employed in the following protocols, with the exception of visualization of IAA using NDB-auxins (*see* **Note 1**). For example, we have successfully performed DR5::GUS staining on *Arabidopsis* mature leaves, roots, and shoots; seedling roots, hypocotyls, and cotyledons; flowers; and siliques. Additionally, we have employed LC-MS/MS to quantify auxin and auxin metabolites from *Arabidopsis* shoots, roots, and hypocotyls; rice shoots and roots; tomato flowers and ovules; soybean roots; pine wood, and both poplar and rhododendron roots.

2.2 DR5::GUS Visualization of Auxin

1. Forceps (Dumont #4 or Dumont #5).
2. Incubator set from 21 to 37 °C.
3. 50 mL conical tubes or 6-well cell culture plates.
4. Vacuum chamber.
5. Orbital shaker.
6. Staining solution (*see* **Note 2**): 100 mM Sodium phosphate, pH 7.0; 10 mM EDTA, free acid; 0.5 mM Potassium ferricyanide; 0.5 mM Potassium ferrocyanide; 0.1 % (m/m) Triton X-100; 1 mM X-glucuronide.
7. Clearing solution: 90 % (v/v) ethanol in deionized water.

2.3 NBD-IAA/ NBD-NAA Visualization of Auxins

1. Forceps (Dumont #4 or Dumont #5).
2. Petri plates.
3. 5 μL Hamilton syringe.
4. Dark box/chamber at 21–22 °C.
5. Microscope slides.
6. Coverslips.
7. Clear nail polish.
8. Ultrathin double-edged razor blades.
9. Germination medium, pH 5.8 (GM): 0.5× Murashige-Skoog salts, 1 % (m/m) sucrose, 1× B5-Vitamins, 0.5 g/L 2-(*N-morpholino*)ethanesulfonic acid, *for soft plates:* 0.8 g/L gellan gum, f*or vertical plates:* 12 g/L agarose.

10. NBD-IAA or NBD-NAA for intact seedling assays (*see* **Note 3**): 80 μM NBD-IAA or NBD-IAA, 0.1 % (m/v) ultrapure agarose, *optional:* 20–250 μM auxin transport inhibitors (i.e., tri-iodobenzoic acid, 1-*N*-naphthylthalamic acid).
11. NBD-IAA or NBD-NAA for excised/decapitated hypocotyl assays (*see* **Note 4**): 100 μM NBD-IAA or NBD-NAA, 0.1 % (m/v) ultrapure agarose, *optional:* 20–250 μM auxin transport inhibitors (i.e., tri-iodobenzoic acid, 1-N-naphthylphthalamic acid).

2.4 LC-MS/MS Quantification of Auxins

1. Plastic pestles.
2. Liquid nitrogen.
3. Analytical balance.
4. Benchtop centrifuge, refrigerated.
5. Nutator shaker.
6. 10 mL and 25 mL volumetric flasks.
7. Oasis HLB columns (Waters Corp, Milford, MA, www.waters.com) 30 mg 1 cc.
8. Nitrogen evaporation system.
9. Target vial and caps.
10. Small spatula.
11. Isotopically labeled standards (*see* **Note 5**).
12. Nylon syringe filters, 0.2 μm, 13 mm diameter.
13. Extraction solution: 50 mM Sodium phosphate, pH 7.0; 1 % diethyldithiocarbamate, sodium salt (DETC).
14. 1 N HCl solution (deionized, 18.0 MΩ water).
15. Oasis HLB solid-phase extraction buffers (*see* **Note 6**): water; methanol; 5 % methanol in water; 80 % methanol in water; 50 mM sodium phosphate, pH 2.7.
16. LC-MS/MS buffers (*see* **Note 6**): Buffer A: 0.1 % Acetic acid in water; Buffer B: 99.9 % Methanol, 0.1 % Acetic acid; 100 % Methanol.

3 Methods

3.1 Visualization of Auxin Using the DR5::GUS Reporter Construct

3.1.1 Plant Growth Conditions

Growth conditions will vary according to the species being stained and tissues being examined. For *Arabidopsis*, seedlings can be grown on 0.25× Murashige–Skoog media with Gamborg's vitamins, pH 5.8, with 0.5 % sucrose, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid, and 12 g/L agarose. Seedlings should generally be grown at a constant temperature of 21–22 °C, with a light intensity of 100 μM m^{-2} s^{-1}; but the precise temperature and light

requirements and settings for each individual experiment will vary depending upon the experimental hypothesis being investigated.

3.1.2 DR5::GUS Staining

1. Grow plants containing the DR5::GUS auxin reporter construct under the appropriate conditions until the desired developmental stage is reached.
2. Prepare staining solution and clearing solution (*see* **Note 2**).
3. Fill either a 50 mL conical tube or one well of a six-well cell culture plate with enough staining solution to submerge all seedlings for the experimental replicate being stained.
4. *Gently* transfer seedlings from the Petri plate or vertical mesh transfer system into the conical tube or six-well cell culture plate, allowing seedlings to initially float on top of the staining solution.
5. Repeat **steps 1–4** for all experimental replicates and treatments. Gently submerge all seedlings in the staining solution by slowly swirling the plate or conical tube.
6. Place the un-capped conical tube or un-lidded six-well cell culture plate in the vacuum chamber.
7. Slowly vacuum infiltrate to allow the staining solution to penetrate the tissue. A vacuum treatment of about 30–40 s is usually sufficient. Cease vacuum treatment when bubbles appear on the plant material and/or the sides of the tube or well.
8. *Slowly* re-pressurize the system by opening the air intake on the vacuum chamber.
9. Repeat **steps 7** and **8** twice, for a total of three vacuum treatments.
10. Remove conical tubes or six-well cell culture plates from the vacuum chamber. If conical tubes are being used, cap the tubes and seal them with Parafilm. For six-well culture plates, place the lid on the plate and seal with Parafilm.
11. Place conical tubes or six-well cell culture plates in the incubator for 3–12 h. The duration and temperature of the incubation will depend on the tissue being stained. Slightly higher temperatures (i.e., 32–37 °C) will accelerate the reaction. More lignified or thicker tissues, as well as organs with more waxy cuticles will require longer staining periods. *Arabidopsis* tissues are usually adequately stained when incubated between 22 and 32 °C for a period of between 6 and 12 h (*see* **Note 7**). *Arabidopsis* roots are generally stained to a degree sufficient to distinguish altered auxin accumulations in root tips after about 12 h (Fig. 1).
12. Remove conical tubes or six-well cell culture plates from the incubator and visually examine the plant materials to determine

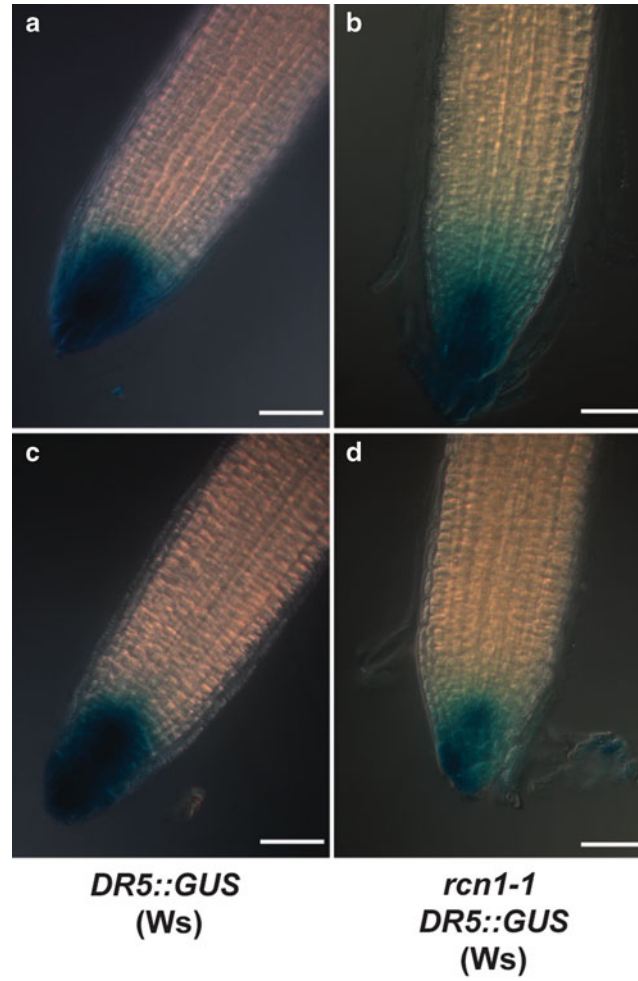

Fig. 1 DR5::GUS visualization of auxin maxima in *Arabidopsis* roots. Roots of *Arabidopsis DR5::GUS* wild-type (Ws; **a**, **c**) and *roots curl in naphthylphthalamic acid1-1 DR5::GUS(rcn1-1 DR5::GUS*; **b**, **d**) seedlings were stained for 12 h as described previously [14] and in Subheading 3.1 above. *rcn1 DR5::GUS* lines were backcrossed twice into the Ws genetic background as described in [14]. *rcn1-1* mutants have been demonstrated to exhibit a decreased auxin accumulations at the root apical meristem resulting from the loss of the A1 scaffolding/regulatory subunit of the protein phosphatase 2A (PP2A) heterotrimeric enzyme complex and a concomitant 60 % decrease in PP2A activity [14]. The loss of the auxin maximum at the root apical meristem (shown here by decreased DR5::GUS staining of the root tip compared to Ws wild-type) leads to a gradual loss of root tip organization which can be moderate (**b**) or more severe (**d**) [14]. Size bar = 50 μm

the degree of staining. Staining will result in the accumulation of blue 4-chloro-bromo-indigo precipitates in cells and tissues where auxin has accumulated. This staining is difficult to visualize against the background of chlorophyll or flavonoids in many plant tissues, however, necessitating destaining of the tissues prior to microscopic examination.

13. Unseal and open the conical tubes or six-well cell culture plates. With a Pasteur pipette, gently remove the staining solution from the tubes or wells.
14. Replace the staining solution with clearing solution. Re-cap conical tubes or six-well culture plates. Allow the tissues to destain for 6–24 h (until almost all chlorophyll has been removed from the tissues) at room temperature on an orbital shaker set to approximately 5–10 rpm. Replace the clearing solution as needed (*see* **Notes 8** and **9**).
15. Proceed to imaging using a light microscope equipped with an appropriate camera.

3.2 Visualization of Auxins Using NBD-IAA/NBD-NAA Fluorescent Auxin Analogs

3.2.1 Plant Growth Conditions

Growth conditions will vary according to the species being stained and tissues being examined. For *Arabidopsis*, seedlings should be grown on germination media gellan gum (for soft plates) or agarose (for vertical plates) plates (0.8 g/L gellan gum or 12 g/L agarose). Seedlings should generally be grown at a constant temperature of 21–22 °C with a light intensity of 100 μM m^{-2} s^{-1}, but the precise temperature and light requirements and settings for each individual experiment will vary depending upon the experimental hypothesis being investigated.

3.2.2 NBD-IAA/NBD-NAA Visualization of Auxins in Intact Seedlings

1. Grow seedlings on GM-agarose or GM-gellan gum under the appropriate conditions until the desired developmental stage is reached.
2. Prepare the 80 μM NBD-IAA/NBD-NAA 0.1 % agarose solution and store it on ice. Keep this solution chilled for the duration of the assay.
3. Carefully remove the lid from the plate containing the seedlings.
4. Using a Hamilton 5 μL syringe, deposit a 0.7 μL droplet of NBD-IAA/NBD-NAA solution on the apical meristem of each seedling to be imaged (*see* **Note 10**).
5. Replace the lid on the plate containing the seedlings, being careful not to dislodge the microdroplets of auxin or shift the position of the seedlings.
6. Transfer the plates to the dark box/chamber and incubate for 4 h at 21–22 °C.
7. Repeat **steps 2–5** for all seedlings to be imaged.

8. After 4 h of incubation in the dark, remove the seedlings from the dark chamber.
9. Using Dumont #4 or #5 forceps, place seedlings on a microscope slides with water (wet mount). Place a coverslip on top of the seedlings, and seal the edges with the clear nail polish.
10. Proceed to imaging using a confocal microscope.

3.2.3 NBD-IAA/NBD-NAA Visualization of Auxins in Excised Arabidopsis Hypocotyls

1. Grow seedlings on GM-agarose or GM-gellan gum under the appropriate conditions until the desired developmental stage is reached.
2. Prepare the 100 μM NBD-IAA/NBD-NAA 0.1 % agarose solution and store it on ice. Keep this solution chilled for the duration of the assay.
3. Label one 1.5 mL centrifuge tube for each hypocotyl to be assayed.
4. To each 1.5 mL tube, add 5 μL of NBD-IAA/NBD-NAA solution. Ensure that the solution is deposited directly into the bottom of the tube and that the droplet of solution is positioned at the very base of the tube (*see* **Note 11**).
5. Remove the top from the plate containing the *Arabidopsis* seedlings.
6. Using an ultra-thin double-edged razor blade, excise the top (apical) 1 mm of the hypocotyl, including the shoot apical meristem, from the first seedling. During this process, use the Dumont #4/#5 forceps to very gently (avoid bruising or damaging the hypocotyl tissues) hold the seedling steady to ensure a clean, straight cut.
7. Using the ultrathin double-edged razor blade, immediately complete the hypocotyl excision by cutting through the base of the hypocotyl, immediately above the shoot–root transition zone. During this process, allow the hypocotyl to rest on the needle-tip forceps above the surface of the plate so that the hypocotyl *does not touch* the GM media as it is harvested.
8. Immediately invert the excised hypocotyl into the appropriate labeled tube containing 5 μL of NBD-IAA/NBD-NAA 0.1 % agarose solution. Ensure that the "top" of the hypocotyl (i.e., where the apical meristem was formerly attached) is placed in the center of the NBD-IAA/NBD-NAA solution, the base of the excised hypocotyl rests on the lip or wall of the tube, and that the side of the excised hypocotyl does not rest against the wall of the tube (*see* **Note 12**).

9. Carefully place the tube containing the inverted hypocotyl in the dark box/chamber and incubate at 21–22 °C for 3 h.
10. Repeat **steps 6–9** for each hypocotyl to be imaged.
11. After 3 h of incubation in the dark, remove the hypocotyls from the dark chamber.
12. Using forceps, place hypocotyls on a microscope slides with water (wet mount). Place a coverslip on top of the hypocotyls, and seal the edges with the clear nail polish.
13. Proceed to imaging using a confocal microscope.

3.3 Quantification of Auxins Using LC-MS/MS

3.3.1 Plant Growth Conditions

Growth conditions will vary depending on the species being investigated and the tissue from which auxins are being extracted. For *Arabidopsis*, seedlings can be grown on 0.25× Murashige-Skoog media with Gamborg's vitamins, pH 5.8, with 0.5 % sucrose, 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid, and 12 g/L agarose. Seedlings should generally be grown at a constant temperature of 21–22 °C with a light intensity of 100 μM m^{-2} s^{-1}, but the precise temperature and light requirements and settings for each individual experiment will vary depending upon the experimental hypothesis being investigated.

3.3.2 Extraction of Auxins

1. Grow plant material under appropriate conditions until the desired developmental stage is reached. Harvest the target tissue into a pre-weighed plastic, sealable tube. Seal the tube and weigh tube and tissue together. Immediately freeze tissue in liquid nitrogen and store at −80 °C until extraction. Calculate the weight of the sample. For most samples, ideal sample masses are between 10 and 50 mg (larger sample amounts may be needed for lignified tissues).
2. Prepare extraction buffer immediately prior to extraction of auxins from tissue. Prechill the buffer on ice, and keep it at 4 °C throughout all extractions.
3. Remove the sample to be extracted from −80 °C storage. Grind the sample to a fine powder in the tube in liquid nitrogen, using the plastic pestle.
4. Add 1 mL of extraction buffer per 20 mg of tissue to the tube containing the ground sample (*see* **Note 13**).
5. Add isotopically labeled internal standards (i.e., auxins and auxin metabolites). The concentration of the internal standards will vary, but should be close to the levels of endogenous auxins present in the tissue.
6. Extract samples for 20 min at 4 °C on the nutator/shaker. Spin down samples at 12,000 × *g* for 15 min at 4 °C.

7. Collect supernatants and adjust the pH to 2.7 using the 1 N HCl solution.
8. Repeat **steps 1–7** for each sample being extracted.

3.3.3 Sample Concentration (Solid-Phase Extraction)

1. Store the supernatants generated in the previous section on ice. Each sample will be run through an Oasis HLB solid phase extraction column (1 cc, 30 mg of resin).
2. Condition the HLB columns by running 1 mL of 100 % methanol through each column, followed by 1 mL of water.
3. Complete the column conditioning by running 0.5 mL of 50 mM sodium phosphate buffer (pH 2.7) through each column.
4. Load the supernatants generated in the previous section onto the Oasis HLB columns (one sample per column). Allow the supernatants to flow through the column.
5. Wash columns with 2 mL 5 % methanol per column. Collect washes for any necessary future analyses.
6. Elute auxins by adding 2 mL 80 % methanol to each column. Collect each eluate in a new 1.7 mL tube.
7. Dry eluates under nitrogen at 21–22 °C.
8. Redissolve samples in 100 % methanol. The total volume used in reconstituting samples will be dependent upon the amount of auxin present. For *Arabidopsis*, between 20 μL and 1 mL should be sufficient. Samples should be filtered, transferred to target vials, sealed, and stored at 4 °C prior to LC-MS/MS analysis.

3.3.4 LC-MS/MS Analyses

1. Samples should be stored at 4 °C prior to LC-MS/MS analysis.
2. Exact LC-MS/MS conditions will vary depending upon the configuration of the mass spectrometer used, the ionization source employed, and the column used to separate auxins. Auxin and auxin metabolites can be separated efficiently (baseline resolution) within 15–20 min using a C-18 column, with 0.1 % acetic acid in water used as buffer A and 99.9 % methanol, 0.1 % acetic acid used as buffer B (*see* Fig. 2).
3. Prior to LC-MS/MS analysis, it will be necessary to generate a system-specific spectral library for both auxin and the isotopically labeled internal standard. This library can be used to monitor auxin- and standard-specific mass transitions. Ideally, at least two mass transitions will be monitored for both the auxin or auxin metabolite and its respective isotopically labeled standard.

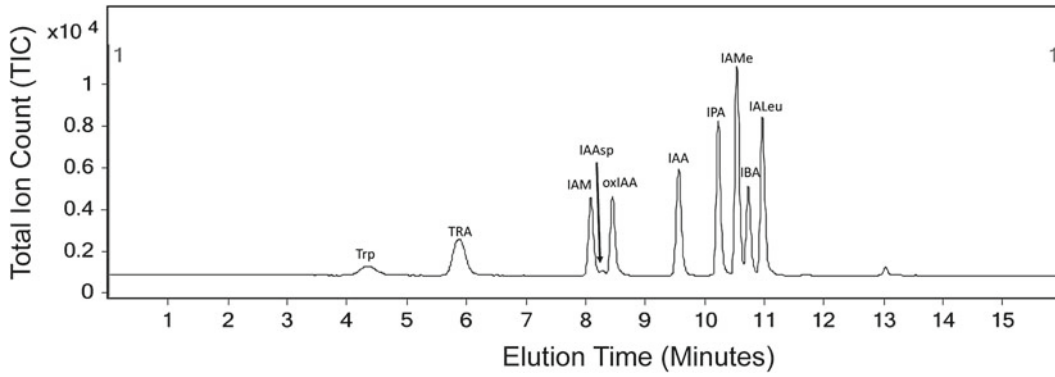

Fig. 2 LC-MS/MS separation of auxin and auxin metabolites. Authentic standards of auxin and auxin metabolites were separated using high-pressure liquid chromatography tandem mass spectroscopy. Standards were injected into an Agilent 1260 Infinity HPLC system and separated using an Agilent Poroshell 120 EC-C18 column and an acidified water (Buffer A) : acidified methanol (Buffer B) buffer system with a flow rate of 0.3 mL per min, as described previously [34] and in Subheading 3.3 above. Gradient conditions were as described previously [34], with the following modifications: 2 min 0–10 % B, 4 min 10–60 % B, 5 min 60–100 % B, hold at 100 % B for 3 min and then return to 0 % B in 2 min. Eluted standards were quantified using an Agilent 6460 Triple Quadrupole (QQQ) tandem mass spectrometer using electrospray ionization source. Compounds were quantified in positive ion mode, as described previously [34]. Trp = tryptophan; TRA = tryptamine; IAM = indole-3-acetamide; IAAsp = IAA–aspartic acid conjugate; oxIAA = 2-oxindole-3-acetic acid; IAA = indole-3-acetic acid; IPA = indole-3-propionic acid; IAMe = indole-3-acetic acid methyl ester (methyl indole-3-acetate); IBA = indole-3-butyric acid; IALeu = IAA–leucine conjugate

4 Notes

1. Since NBD-auxins appear not to be efficiently transported by auxin transporters located in the central cylinder of *Arabidopsis* shoots, shoot tissues are not optimal for this assay. NBD-auxins were able to precisely visualize auxin gradients at the cellular and subcellular levels in roots, root primordia, embryos, leaves, and protoplasts, however, and are ideal for visualizing auxin levels in these cells/tissues [24].
2. Staining solution for DR5::GUS assays should be prepared using deionized, 18 MΩ water. This solution should be prepared the day of use, stored at 4 °C, and allowed to warm to room temperature immediately prior to use.
3. NBD-IAA/NBD-NAA solutions for intact seedling assays made using deionized, 18 MΩ water. This solution should be prepared immediately prior use and maintained at 4 °C.
4. NBD-IAA or NBD-NAA solutions for excised/decapitated hypocotyl assays should be prepared using germination medium (GM). These solutions should be prepared immediately prior to use and maintained at 4 °C.

5. Auxin or auxin metabolites incorporating deuterium or ^{13}C atoms should be used as isotopically labeled standards for mass spectroscopy work. These molecules will behave identically to the target metabolite, elute from the HPLC column at the same time, and are easily distinguished from their target endogenous compound in the tandem mass spectrometer due to the peak shift of the parent ion. OlchemIm Ltd. (Czech Republic, www.olchemim.cz), Santa Cruz Biotechnology, Inc. (Dallas, TX, www.scbt.com), and Sigma-Aldrich (St. Louis, MO, www.sigmaldrich.com) provide a range of deuterated and/or ^{13}C labeled auxins and auxin metabolites. In our hands, isotopically labeled auxins with the deuterium or ^{13}C atoms on the benzyl or indole rings provide the most stable signal and serve as ideal internal standards, as these labels are not easily lost during sample preparation (compared to labels incorporated into the carboxylic acid group of IAA).
6. Oasis HLB and LC-MS/MS buffers should be prepared using LC-MS/MS grade water.
7. In this protocol, establishing and maintaining stable staining times are critical. Over-staining of tissues can occur quite easily, and will result in an overestimation of the amount of auxin present in specific cells, the distribution of auxin in a given tissue, or both. If this is the first time a particular tissue is being stained, it is best to run a time-course study to determine the optimal staining time.
8. In addition to the over-staining issues described in *see* **Note 7** above, it should also be noted that certain tissues, such as the hydathodes of cotyledons and cells in the root apical meristem, will almost always exhibit GUS staining if incubated in staining solution for a long enough period of time. In addition to the use of appropriate incubation times during staining, care should be taken to use appropriate controls, such as non-DR5::GUS containing plants, to differentiate a genuine signal from "background staining."
9. Once tissues are destained, they should be imaged as quickly as possible. It is possible for GUS stain (the 4-chloro-bromo-indigo precipitate) to move from cell-to-cell over time, particularly if cells are poorly or improperly destained. Delaying imaging for a prolonged period following destaining may result in an overestimation of the area of auxin distribution.
10. As the droplet of NBD-IAA/NBD-NAA being placed on each seedling is quite small, changes in ambient humidity can dramatically affect both drop placement and droplet integrity. For example, high ambient humidity can cause the droplet to lose integrity and run down the hypocotyl, while very low ambient humidity can result in the droplet wholly or partially evaporat-

ing prior to proper placement on the apical meristem. For this reason, it is recommended that assays are performed at 20–40 % ambient humidity.

11. Do *not* allow the solution or pipette tip to touch the side of the tube, as this may result in "off-target" depositions of auxin along the sides of the hypocotyls which will be placed in the tube.
12. It is important that the hypocotyl does not touch the side of the tube except at the very top of the tube (i.e., where the hypocotyl rests against the upper inside rim of the tube lip). Permitting the hypocotyl to touch the side of the tube below this point will allow solution containing NBD-IAA/NBD-NAA to move up the side of the hypocotyl via capillary action, yielding a false positive and giving an inaccurate visualization of both auxin movement and localization.
13. This amount of extraction buffer has been optimized for *Arabidopsis*; the ratio of extraction buffer to tissue will vary depending on plant species, tissue type, and degree of lignification of the tissue being extracted.

References

1. Soeno K, Goda H, Ishii T, Ogura T, Tachikawa T, Sasaki E, Yoshida S, Fujioka S, Asami T, Shimada Y (2010) Auxin biosynthesis inhibitors, identified by a genomics-based approach, provide insights into auxin biosynthesis. Plant Cell Physiol 51(4):524–536
2. Mano Y, Nemoto K (2012) The pathway of auxin biosynthesis in plants. J Exp Bot 63(8):2853–2872
3. Blakeslee JJ, Peer WA, Murphy AS (2005) Auxin transport. Curr Opin Plant Biol 8(5):494–500
4. Peer WA, Blakeslee JJ, Yang HB, Murphy AS (2011) Seven things we think we know about auxin transport. Mol Plant 4(3):487–504
5. Multani DS, Briggs SP, Chamberlin MA, Blakeslee JJ, Murphy AS, Johal GS (2003) Loss of an MDR transporter in compact stalks of maize br2 and sorghum dw3 mutants. Science 302(5642):81–84
6. Blilou I, Xu J, Wildwater M, Willemsen V, Paponov I, Friml J, Heidstra R, Aida M, Palme K, Scheres B (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature 433(7021): 39–44
7. Grunewald W, Friml J (2010) The march of the PINs: developmental plasticity by dynamic polar targeting in plant cells. EMBO J 29(16):2700–2714
8. Krecek P, Skupa P, Libus J, Naramoto S, Tejos R, Friml J, Zazimalova E (2009) The PIN-FORMED (PIN) protein family of auxin transporters. Genome Biol 10(12):249
9. Friml J (2010) Subcellular trafficking of PIN auxin efflux carriers in auxin transport. Eur J Cell Biol 89(2-3):231–235
10. Friml J, Wisniewska J, Benkova E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. Nature 415(6873):806–809
11. Mravec J, Kubes M, Bielach A, Gaykova V, Petrasek J, Skupa P, Chand S, Benkova E, Zazimalova E, Friml J (2008) Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. Development 135(20):3345–3354
12. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9(11):1963–1971
13. Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ et al (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature 482(7383):103–U132
14. Blakeslee JJ, Zhou HW, Heath JT, Skottke KR, Barrios JAR, Liu SY, DeLong A (2008)

Specificity of RCN1-mediated protein phosphatase 2A regulation in meristem organization and stress response in roots. Plant Physiol 146(2):539–553
15. Pattison RJ, Catala C (2012) Evaluating auxin distribution in tomato (*Solanum lycopersicum*) through an analysis of the PIN and AUX/LAX gene families. Plant J 70(4):585–598
16. Zhou J, Yu FB, Wang XM, Yang Y, Yu CL, Liu HJ, Cheng Y, Yan CQ, Chen JP (2014) Specific expression of DR5 promoter in rice roots using a tCUP derived promoter-reporter system. PLoS One 9(1):e87008
17. Chen YR, Yordanov YS, Ma C, Strauss S, Busov VB (2013) DR5 as a reporter system to study auxin response in Populus. Plant Cell Rep 32(3):453–463
18. Jefferson RA, Burgess SM, Hirsh D (1986) Beta-glucuronidase from Escherichia-coli as a gene-fusion marker. Proc Natl Acad Sci U S A 83(22):8447–8451
19. Pengelly W, Meins F (1977) Specific radioimmunoassay for nanogram quantities of auxin, indole-3-acetic-acid. Planta 136(2):173–180
20. Takahashi N (1986) Chemistry of plant hormones. CRC Press, Boca Raton, FL
21. Marcussen J, Ulvskov P, Olsen CE, Rajagopal R (1989) Preparation and properties of antibodies against indoleacetic-acid (Iaa)-C5-Bsa, a novel ring-coupled Iaa antigen, as compared to 2 other types of Iaa-specific antibodies. Plant Physiol 89(4):1071–1078
22. Fernandez B, Centeno ML, Feito I, Sancheztames R, Rodriguez A (1995) Simultaneous analysis of cytokinins, auxins and abscisic-acid by combined immunoaffinity chromatography, high-performance liquid-chromatography and immunoassay. Phytochem Anal 6(1):49–54
23. Jiraskova D, Poulickova A, Novak O, Sedlakova K, Hradecka V, Strnad M (2009) High-throughput screening technology for monitoring phytohormone production in microalgae. J Phycol 45(1):108–118
24. Hayashi KI, Nakamura S, Fukunaga S, Nishimura T, Jenness MK, Murphy AS, Motose H, Nozaki H, Furutani M, Aoyama T (2014) Auxin transport sites are visualized in planta using fluorescent auxin analogs. Proc Natl Acad Sci U S A 111(31):11557–11562
25. Ohwaki Y (1966) Thin-layer chromatography of diffusible auxin of corn coleoptiles. Bot Mag Tokyo 79(934):200
26. Bayer MH, Ahuja MR (1968) Tumor formation in Nicotiana—auxin levels and auxin inhibitors in normal and tumor-prone genotypes. Planta 79(4):292–8
27. Raj RK (1970) Indoles and Auxins.8. partition chromatography of naturally occurring indoles on cellulose thin layers and sephadex columns. Anal Biochem 33(2):471–4
28. Akiyoshi DE, Morris RO, Hinz R, Mischke BS, Kosuge T, Garfinkel DJ, Gordon MP, Nester EW (1983) Cytokinin auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. P Natl Acad Sci-Biol 80(2):407–411
29. Morgan PW, Durham JI (1983) Strategies for extracting, purifying, and assaying auxins from plant-tissues. Bot Gaz 144(1):20–31
30. Goswami D, Thakker JN, Dhandhukia PC (2015) Simultaneous detection and quantification of indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) produced by rhizobacteria from L-tryptophan (Trp) using HPTLC. J Microbiol Methods 110:7–14
31. Cohen JD, Lilly N (1984) Changes in (45)calcium concentration following auxin treatment of protoplasts isolated from etiolated soybean hypocotyls. Plant Physiol 75:109–109
32. Rivier L (1986) GC-MS of auxins. In: Linskens HF, Jackson JF (eds) Modern methods of plant analysis, volume 3: gas chromatography/mass spectrometry. Springer-Verlag, Berlin Heidelberg, pp 146–188
33. Barkawi LS, Tam YY, Tillman JA, Normanly J, Cohen JD (2010) A high-throughput method for the quantitative analysis of auxins. Nat Protoc 5(10):1609–1618
34. Novak O, Henykova E, Sairanen I, Kowalczyk M, Pospisil T, Ljung K (2012) Tissue-specific profiling of the *Arabidopsis thaliana* auxin metabolome. Plant J 72(3):523–536

Chapter 6

Immunolocalization of PIN and ABCB Transporters in Plants

Nicola Carraro and Wendy Ann Peer

Abstract

PIN auxin efflux carriers and ABCB auxin transporters are important for polar auxin transport, organogenesis and long distance auxin transport. Along with the auxin influx symporter AUX1, they are essential for tropic responses such as gravitropism and phototropism where lateral redistribution of auxin is required for the tropic response to occur. Immunolocalization of plant membrane transporters is an effective technique to determine the transporters' subcellular localization patterns in the tissues of interest, especially when fluorescent protein fusions of the protein of interest are not available. Immunolocalization is also a valuable tool for validation of the localization of fluorescent protein fusions when the fusions are available. Here we describe the procedures to prepare plant tissue samples and fix them for whole mount or embedding and sectioning. We focus on immunolocalizations of PINs and ABCBs in *Arabidopsis* and maize tissues. In addition, we describe treatments of roots with inhibitors of cellular trafficking: brefeldin A (BFA), a fungal compound that blocks exocytosis; wortmannin, a fungal compound that inhibits phosphatidylinositol 3-kinase and induces fusion of pre-vacuolar compartments and multi-vascular bodies; and oryzalin, a fungal compound that depolymerizes microtubules. Inhibitor treatments are performed prior to fixation and affect the localization patterns of PINs and ABCBs, giving insight into cell type -specific trafficking mechanisms. The procedures described for *Arabidopsis* and maize can be easily adapted for other herbaceous plants.

Key words ABCB, Antibody, Antisera, *Arabidopsis*, Fixation, Maize, Membrane proteins, PIN, Trafficking inhibitor, Whole mount

1 Introduction

Immunolocalization of proteins is a very effective technique that helps visualize in vivo localization patterns of proteins in plant tissues. The localization of the protein of interest can be "frozen in time" by fixation following a treatment of interest, such as a variety of time points after a tropic stimulus or chemical treatment. Immunolocalization has been extensively used for the model species *Arabidopsis*, and its importance is even greater in plant species were stable transformation and plant regeneration are long and less efficient processes. This is the case of maize, as transgenic lines

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carrying fluorescently-labeled reporter proteins are still scarce. In addition, fluorescently-labeled protein fusions (FPF) may exhibit subcellular localization patterns that differ from immunolocalization of the native protein. For example, if FPFs are overexpressed through the use of a 35S promoter or under the native promoter but in the wild type background, aggregations of the protein in subcellular compartments may be observed that are not observed with the native protein as shown by immunolocalization. In addition, the insertion site of the fluorescent tag in the FPF may also affect the function of the protein, and therefore its localization. Therefore, immunolocalization is one of the tools that can be used to validate FPFs localization as well as elucidate protein subcellular localization in species that are recalcitrant to transformation.

The quality of the immunolocalizations depends on two key factors: primary antibody specificity and false positive signals during imaging. A primary antibody needs to be developed against an epitope of the protein of interest and must be validated for specificity before using it in plant cells or sections (for example in western blot analyses). Afterwards, the antibody can be tested on the tissues of interest and adequate negative controls should be conducted with pre-immune sera. This will reduce the possibility of false positive results due to nonspecific binding of the primary (or secondary) antibody. Commercial antibodies are available for PINs, but nevertheless we advise verification of their specificity in the tissue of interest, as it might vary according to the species, organ and embedding technique. In the case of PINs and ABCBs, immunolocalization has allowed the study of their expression patterns at the tissue and subcellular levels both in *Arabidopsis* and maize. The localization of PINs and ABCBs at specific sites in the plasma membrane (PM) and the endoplasmic reticulum (ER) have helped establish the directionality of auxin fluxes in plant tissues and, coupled with imaging of auxin response reporters such as DR5 and DII-VENUS, provide high-resolution spatiotemporal information about auxin distribution and response during plant development [1, 2]. While the methods presented here have been used successfully in *Arabidopsis* and maize, these protocols may be applied directly, or with modifications, to other plant species.

2 Materials

All solutions should be prepared with ultrapure water (18 MΩ) and analytical grade reagents. Stock and working solutions are stored at room temperature unless otherwise indicated or recommended by the manufacturer. Waste should be disposed in accordance with your local regulations.

2.1 Plant Material

Any plant material at the appropriate age and the tissue of interest that is required to test your hypothesis can be used for immunolocalization. For example, *Arabidopsis* embryos, seedlings, and mature plants or maize seedlings and mature plants are commonly used.

2.2 Equipment

1. Microtome.
2. Vibratome.
3. Humid chambers for slide incubation with the antibodies.
4. Charged slides, such as Polysine® slides (Menzel-Gläser) or Superfrost+® slides (Fisher).
5. Magenta boxes.
6. Hot plate.
7. Whatman paper.

2.3 Buffers and Solutions

1. 10× Microtubules Stabilization Buffer (MTSB): 50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$. Adjust pH to 7.0 with KOH and bring volume to 1 L with simplicity H_2O.
2. 10× Phosphate Buffered Saline (PBS) Buffer: 1.37 M NaCl, 27 mM KCl, 65 mM $Na_2HPO_4 \times 2H_2O$, 15 mM KH_2PO_4. Adjust pH to 7.3 with HCl and bring volume to 1 L with simplicity water.
3. Wax (PEG–1-Hexadecanol, 9:1).
4. Agarose (4–6 % Electrophoresis grade agarose in 1× PBS).
5. Brefeldin A (BFA) stock solution: 2.5 mg/mL in DMSO. Working solution is 50 μM BFA in water. Store both stock and working solutions at −20 °C.
6. Wortmannin stock solution: 200 mM in DMSO. Working solution is 33 μM wortmannin in water. Store both stock and working solutions at −20 °C.
7. Oryzalin stock solution: 100 mM in DMSO. Working solution is 1.5–30 μM oryzalin in water or directly dissolved in solid growing media. Store both stock and working solutions at −20 °C.
8. Mounting Media: Samples can be preserved using mounting media such as a 5 % aqueous solution of glycerol or commercially available solutions such as VECTASHIELD® anti-fade mounting medium. Once mounted and sealed with nail polish, samples can be safely preserved in the dark at 4 °C for several months.
9. Blocking sloution: 1 % BSA in 1× PBS.

2.4 Primary and Secondary Antibodies

Primary antibodies may be monoclonal (recognizing one epitope) or polyclonal (recognizing multiple epitopes) and bind to the protein of interest. Secondary antibodies bind to an epitope on the primary antibody and amplify the signal, usually via a fluorescent molecule (e.g., FTIC) or fluorescent protein (e.g., GFP).

2.4.1 Primary Antibodies or Antisera

Primary antibodies or antisera may be obtained commercially, from researchers who developed them, or synthesized by the researcher. Antibodies may be synthesized against the whole protein or against an antigenic peptide region of the protein. Software and online tools exist to help predict the location of antigenic regions in the proteins of interest. Antigenic peptides may be obtained commercially from peptide and antibody synthesis companies.

2.4.2 Secondary Antibodies

Secondary antibodies may be obtained commercially. The secondary antibody needs to be compatible with an antigen on the primary antibody. For instance, if the primary antibody was synthesized in a rabbit, then the secondary antibody could be goat anti-rabbit. The choice of secondary antibody, especially if a particular fluorescent molecule or protein is selected, should take into account the type of microscope and camera available for imaging, i.e., optics, filters set, and excitation sources, such as laser lines in the case of confocal microscopy.

2.5 Counterstains

Counterstains may be used for contrast to highlight cell walls or other organelles to provide a subcellular context for the immunolocalization of the proteins of interest. In some cases, cell wall autofluorescence may be used instead of a counterstain.

1. Schiff reagent with propidium iodide [3, 4] (cell walls, nuclei/DNA): 100 mM sodium metabisulfite, 0.15 N HCl, propidium iodide to a final concentration of 100 μg/mL (freshly added). Stain until you see the tissue turns pink, and then rinse. Using mounting media of your choice, add a coverslip, and image immediately. Excitation 488 nm, Emission 600–650 nm.
2. Berberine-Aniline [5] (callose, lignin, suberin): 0.1 % (w/v) berberine hemi-sulfate, 0.5 % (w/v) aniline blue, 0.1 % (w/v) $FeC1_3$ in 50 % (v/v) glycerin. Stain with berberine ~30–60 min and rinse. Stain with aniline ~30 min and rinse. Stain with $FeC1_3$ in glycerin ~5 min. Using mounting media of your choice, add a coverslip, and image. Excitation 365 nm (or 405 nm), Emission > 420 nm.
3. DAPI (nuclei/DNA) stock solution: 10 mg/mL in water; Working solution is 1 μg/mL in water. Stain for 10 min, then rinse. Excitation 405 nm, emission 420–480 nm.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. When preparing fixatives, work under a fume hood and let fixative solution cool down to room temperature before use.

3.1 Plants Growth Conditions

Plant material, seedlings and mature tissue, should be grown according to the experimental conditions appropriate to test your hypothesis. *Arabidopsis* seedlings are typically grown on Phytagar or Phytogel plates so that the plants have a reduced cuticle to aid histochemical studies. Similarly, maize seedlings grown in magenta jars with agar will have a reduced cuticle. In addition, seedling roots may be damaged when removed from soil. Therefore, if seedlings are grown vertically on plates, instead of horizontally, or on the vertical mesh transfer system [6], then the seedlings can be readily harvested for fixation with minimal disturbance.

Arabidopsis seedlings (wild type and *abcb19*) are grown on 1 % Phytagar plates, ¼ Murashige and Skoog basal salts, pH 5.5, 22 °C and 14 h, 100 μmol m^{-2} s^{-1} light. Five-day-old seedlings are used for immunolocalizations. Maize seedlings are grown in sterile magenta boxes with two layers of Whatman filter paper soaked in sterile water, for 3–6 days after radicle emergence.

3.2 Inhibitor Studies

3.2.1 BFA Treatment

Seedlings are treated with 50 μM brefeldin A in eppendorf tubes (wrapped with aluminum foil) for 30 min and then fixed for immunolocalization. For brefeldin A washout experiments, treated seedlings are washed prior to fixation for three times in water followed by three washes in MTSB buffer.

3.2.2 Wortmannin Treatment

Seedlings are treated in 33 μM wortmannin for 1 h in an Eppendorf tube (wrapped with aluminum foil) and then fixed for immunolocalization.

3.2.3 Oryzalin Treatment

Arabidopsis seedlings are treated in 1.5–30 μM oryzalin in liquid MS media for 2–24 h (*see* **Note 1**). Maize pre-germinated seedlings are grown in magenta boxes so that the primary root inserts into the solid growth media containing 10–30 μM oryzalin for 48 h (*see* **Note 1**).

3.3 Tissue Fixation

Fixation of plant cells is adjusted according to the tissue type (root, leaf, shoot, meristem) and the thickness and composition of the cell walls. As a general rule, 4 % paraformaldehyde in MTSB or PBS is used. Dissolve paraformaldehyde in hot (60 °C) MTSB and adjust to pH 10 with 1 N KOH. After it has completely dissolved, adjust pH back to ~7.0 with H_2SO_4 (*see* **Note 2**). Fixation is performed in glass vials at room temperature, and a vacuum chamber can be used to help fixative infiltration into larger samples. Alternatively, fixation may be performed at 4 °C overnight, and tissues can be stored in fixative for several weeks until embedding.

The two protocols described below for whole mount fixation were developed over several years and adapted for PINs and ABCBs immunolocalizations in *Arabidopsis* seedlings (*see* **Notes 3** and **4**).

3.3.1 Method I (Triton X-100 and NP-40)

1. Fix for ~1 h with freshly prepared 4 % paraformaldehyde in MSTB + 5 % DMSO (*see* **Note 5**).
2. Wash three times with MTSB for 10 min each time.
3. Wash with MTSB + 0.1 % NP-40 for 10 min each time.
4. Digest cell wall with MTSB + 0.5 % Pectolyase for 30 min to 1 h (*see* **Note 6**).
5. Wash with MTSB + 0.1 % Triton X-100 for 10 min each for three times.
6. Wash with MTSB for 10 min each for three times.
7. Add permeabilization step: MTSB + 10 % DMSO + 0.8 % NP-40 incubate at room temperature for 1 h.
8. Wash with MTSB for 10 min each for three times.
9. Block with 3 % BSA in MTSB (or PBS) for 1 h.
10. Incubate immediately with the primary antibody (*see* Subheading 3.6.1).

3.3.2 Method II (NP-40)

1. Incubate whole seedlings in MSTB + 10 % DMSO for 15 min at room temperature in a small Petri dish.
2. Remove MTSB and DMSO and fix seedlings in fresh 4 % paraformaldehyde in MTSB and 5 % DMSO solution for 1 h.
3. Wash seedlings three times with MTSB for 10 min each.
4. Wash seedlings with MSTB+ 0.1 % NP-40 for 10 min.
5. Digest cell walls with 0.5 % Pectolyase (Seishin Corp, Tokyo) for 30 min at 37 °C, and for another 30 min at room temperature with gentle shaking.
6. Wash seedlings three times with 0.1 % Triton X-100 in MSTB for 10 min each.
7. Wash seedlings MSTB for another three times, 10 min each.
8. Permeabilize seedlings with 10 % DMSO and 1 % NP-40 for 1 h at room temperature.
9. Wash seedlings six times in MSTB for 10 min each.
10. Block with 3 % BSA for 1 h.
11. Incubation with the primary antibody should proceed immediately (*see* Subheading 3.6.1).

3.4 Embedding

Embedding is used to maintain the cellular structure of the tissue for sectioning. A cross section or longitudinal section is needed, when the tissue is not translucent or when the sample is thick and the internal cellular structure needs to be visualized. Whole seedlings and tissues may be embedded in wax and or agarose, as described below (*see* **Note 7**).

3.4.1 Wax (PEG–1-Hexadecanol, 9:1)

1. Melt PEG 400 distearate at 60 °C in a water bath and add 1-hexadecanol: 1 part hexadecanol + 9 parts PEG 400 (w/w). Stir the solution for 4 h, pour into plastic Petri dishes or Falcon tubes, and cool down to room temperature. Petri dishes containing the wax are stored at room temperature or at 4 °C in the dark and can be conserved for several months. While embedding, maintain wax liquid by keeping the tube at 40–45 °C in a water bath or incubator.
2. Perform sample dehydration at room temperature (ethanol solutions are freshly prepared in PBS buffer): 30 min in 30 % ethanol, 30 min in 50 % ethanol, 30 min in 70 % ethanol, 30 min in 90 % ethanol, and 30 min in 100 % ethanol.
3. Replace the 100 % ethanol with 4 mL 100 % ethanol and transfer the samples to 42 °C for 5 min.
4. Slowly add liquid wax to the samples and leave them at 42 °C overnight. The wax is added multiple times with a pre-warmed plastic Pasteur pipette, until samples are completely submerged by the wax and not floating in the ethanol upper phase.
5. The wax-infiltrated samples will lean against the bottom of the vial the next morning, while the ethanol phase will sit on top. Pour off the upper ethanol phase and replace wax three times at 2-h intervals. Carefully avoid letting the samples out of the wax by leaving a sufficient amount to cover them. After the final substitution, there should be only pure wax.
6. Properly orient the samples in the molds, let the wax solidify at room temperature, and store embedded samples at 4 °C.

3.4.2 Agarose

1. Fix (maize roots): 3 % paraformaldehyde in MTSB for 2 h under vacuum.
2. Rinse twice in MTSB.
3. Embed in 4–6 % agarose using a mold or plastic tube (*see* **Note 8**).

3.5 Sectioning and Dewaxing of Samples

While sectioning wax-embedded tissues, keep the slides at room temperature and do not heat the slides above 35 °C.

3.5.1 Sectioning of Wax-Embedded Tissues

1. Apply a few drops of sterile water on Polysine® slides (see **Note 9**).
2. Cut a small block from the wax embedded sample and place on mounting block in the desired orientation (cross or longitudinal sectioning).
3. Place the mounted sample at 4 °C or at −20 °C for 5–10 min, as cold blocks are easier to section and less prone to deformation.
4. Cut wax sections of 10–12 μm and do not form long ribbons during sectioning. Collect the single sections with a paint

brush, place them on the water over the slides, and let the slides dry at room temperature overnight.

5. Store the slides in sealed boxes at room temperature or proceed immediately with immunolocalization (*see* Subheading 3.6.2).

3.5.2 Sectioning of Agarose-Embedded Tissues

In order to section agarose-embedded tissues, mount the blocks in the desired orientation and place on a vibratome.

1. Cut 80–100 μm sections. The sections are single and do not form ribbons. Typically, only one of every two sections will be recovered, so plan the number of samples to be embedded accordingly.
2. Collect sections from the water-filled lower compartment of the vibratome and place into 96-well Elisa plates, depression well slides or Eppendorf tubes filled with PBS. Usually the agarose matrix detaches easily from the sample sections, but gentle pipetting or shaking might help.
3. Perform all subsequent washes changing the solutions with a fine point plastic Pasteur pipette.
4. Incubate immediately with the primary antibody (*see* Subheading 3.6.3).

3.6 Immunolocalization

It is crucial to perform appropriate negative controls in an immunolocalization experiment, as tissue autofluorescence and nonspecific binding of the primary and secondary antibodies are not uncommon. A negative control consists in the incubation without primary antibody (only the secondary antibody is used) to verify that the secondary antibody does not bind to membranes and cell walls nonspecifically, thus generating a false positive signal. Positive controls can be generated using an antibody which binds to proteins such as actin or tubulin (both proteins are generally abundant in most cell types), to verify that the immunolocalization assay works correctly. If available, pre-immune serum can be used on some sections instead of primary antibody to verify the presence of a nonspecific signal.

3.6.1 Whole Mount I

1. Incubate with the primary antibody at 4 °C overnight.
2. Wash six times in MSTB, for 10 min each time.
3. Incubate with the secondary antibody in 3 % BSA/MTSB at 37 °C for 4 h.
4. Wash six times in MSTB, for 10 min each time.
5. Place samples on slide with MTSB, place a coverslip on top of the samples, and seal with nail polish.
6. Image the samples with a microscope (Fig. 1).

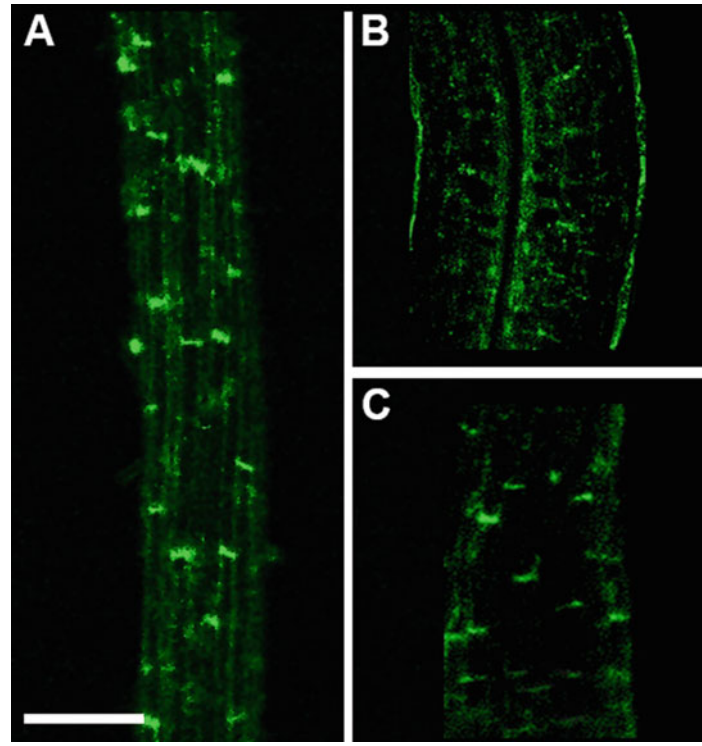

Fig. 1 Immunolocalization of ABCB1 in *Arabidopsis* roots and PIN1 in hypocotyl and hypocotyl–root junction. ABCB1 shows polar localization in the root tip. Immunolocalization of ABCB1-cmyc fusion with anti-cmyc antibody in shown (**a**). PIN1 shows polar localization in the vascular tissue of the hypocotyl using anti-PIN1 anti-sera from Prof. Klaus Palme (**b**). PIN1 shows polar localization in the root transition zone (**c**). Images were collected with a Bio-Rad 2100 confocal microscope. An Alexa Fluor® 488 secondary antibody was used. Scale bars = 100 μm

3.6.2 Whole Mount II

1. Incubate seedlings overnight at 4 °C with the primary antibody.
2. Wash six times in MSTB, for 10 min each time.
3. Incubate seedlings with a secondary antibody in 3 % BSA/MTSB for 3 h at 37 °C.
4. Wash six times in MTSB for 10 min each.
5. Place samples on a slide with MTSB, place a coverslip on top of the samples, and seal with nail polish.
6. Image the samples with a microscope.

3.6.3 Embedded in Wax

1. Incubate slides for 1 h at room temperature in blocking solution.
2. Add the desired amount of primary antibody (typical dilutions range from 1:200 to 1:500, v/v) and place the slides in the humid chamber.

3. The incubation in the primary antibody solution is performed overnight at 4 °C or for 2–5 h at room temperature. However, the incubation time must be determined empirically by the user, as it depends on the antibody and tissue types.
7. Wash the slides twice for 5 min in PBS.
8. Incubate slides for 1 h at room temperature with blocking solution containing the secondary antibody diluted according to the manufacturer's instructions (*see* **Note 10**).
9. Wash the slides twice for 5 min with PBS.
10. Briefly dry the slides keeping them in a vertical position on a paper towel.
11. Apply two drops of VECTASHIELD® anti-fade mounting medium containing DAPI, if staining of the nucleus is desired, or a solution of 5 % glycerol in water (*see* **Note 11**).
12. Cover the slides with coverslips, seal them with nail polish, and store at 4 °C until imaging the samples. Mounted slides can be stored safely at 4 °C for several months if kept in the dark. In the case of PIN and ABCB proteins the fluorescent signal will be retained for several months and can be imaged repeatedly (Fig. 2a, b).

3.6.4 Embedded in Agarose

1. Add primary antibody and leave overnight at 4 °C slowly shaking.
2. Rinse twice in PBS.
3. Add secondary antibody solution (dilution 1:400 for Alexa Fluor® antibodies 2 mg/mL) and incubate 1 h at room temperature.
4. Rinse twice in PBS.
5. Mount tissues on slides with a coverslip or leave into depression well slides with PBS.
6. Immediately image the samples with a microscope. If using depression wells slides, the samples can be conserved only for a few days in PBS at 4 °C, as in the case of maize root sections of Fig. 2d, e.

4 Notes

1. The treatment with oryzalin is effective at a range of different concentrations and can be performed for a few hours up to 2 days. When treating *Arabidopsis* roots, lower concentrations and treatments of 2–4 h can be sufficient to depolymerize microtubules and effectively cause mislocalization of PIN1. In maize, longer treatments are necessary until root swelling is observed (Fig. 2c–e). Longer treatments (for example 48 h) are effective even with lower oryzalin concentrations (1.5–10 μM) [7, 8].

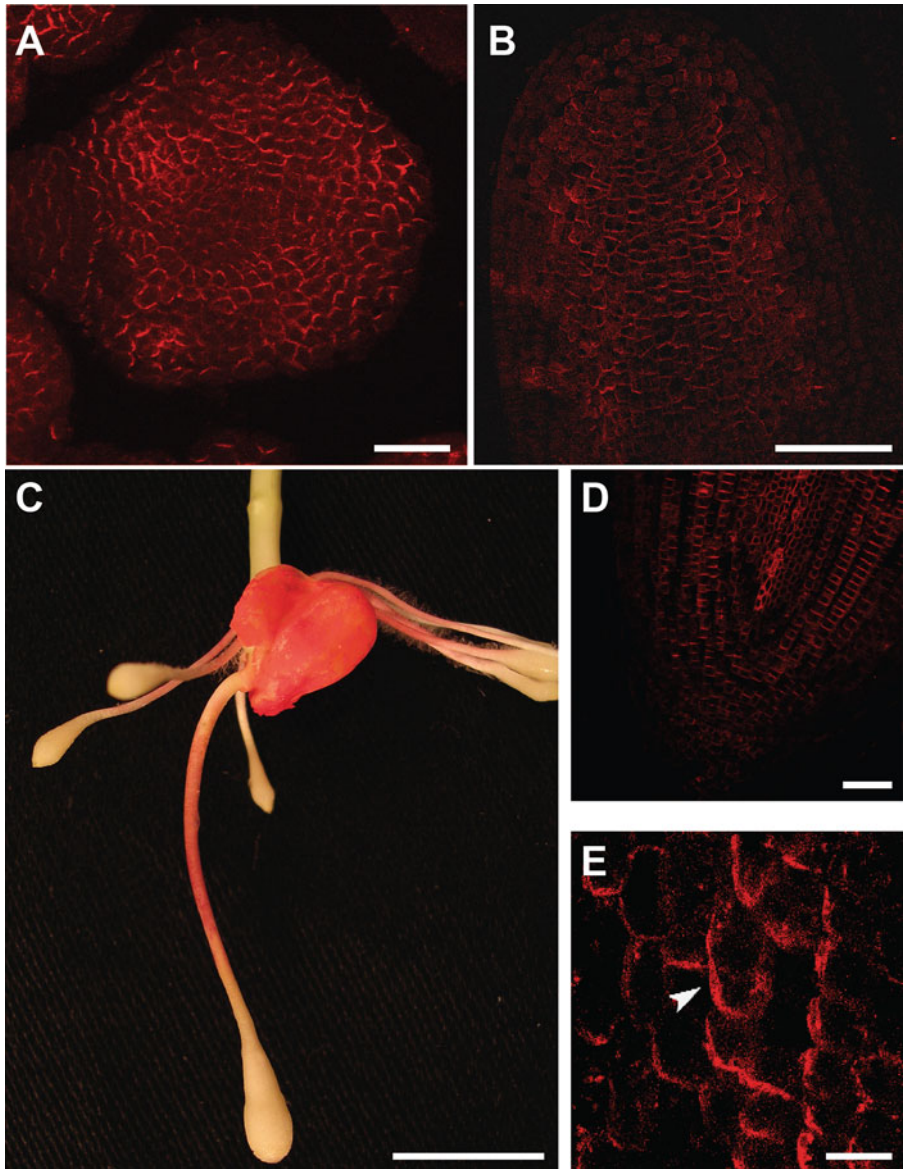

Fig. 2 Immunolocalization of PIN1 in *Arabidopsis* and maize inflorescence and roots. PIN1 shows polar localization in the shoot, inflorescences and primary root in maize and *Arabidopsis*. PIN1 is polarized at the plasma membrane in the shoot apical meristem and developing primordia of *Arabidopsis* (**a**). PIN1 shows a polar pattern in the developing tassel in maize (**b**). (**c**, **d**). PIN1 is also present in the primary root of maize, with a ladder-like pattern indicating acropetal auxin movement towards the root tip and after treatment with oryzalin, the root tip swells. Also PIN1 relocates to the lateral plasma membranes resulting in altered polar auxin transport (**e**). Images were collected with a Leica TCS SP2 confocal microscope after incubation with an anti-PIN1 monoclonal antibody and Alexa Fluor® 568 secondary antibody, with the exception of the picture in panel **d**. *Arrowhead* indicates laterally relocalized maize PIN1 after treatment of the root with oryzalin. Scale bars = 100 μm in A, B, D, 1 cm in **c** and 20 μm in **e**

2. The fixative can be stored in aliquots at –20 °C for up to 2 months, although it is recommended to prepare it fresh.
3. Triton X-100 vs. NP-40 solubility of ABCBs and PINs [9]: Although the subcellular localizations of ABCB19 and PIN1 in the reciprocal mutant backgrounds were like those in wild type, PIN1 plasma membrane localization in *abcb19* roots was more easily perturbed by the detergent Triton X-100, but not other non-ionic detergents such as NP-40. ABCB19 is stably associated with sterol/sphingolipid-enriched membrane fractions containing BIG/TIR3 and partitions into Triton X-100 detergent-resistant membrane (DRM) fractions. In the wild type, PIN1 was also present in DRMs, but was less abundant in *abcb19* DRMs.
4. Robots, such as the Intavis Insitu Pro Vsi (Intavis Bioanalytical Instruments), can be used for the serial fixation steps and primary and secondary antibody incubations for whole mounts (in baskets) and slides. Antibodies can be recovered from whole mount incubations, but not from immunolocalizations on slides. This is extremely useful for high throughput of a large number of samples and, of course, robots can work while we sleep.
5. DMSO introduces water molecules into the membranes and can destabilize some transporters, such as ABCB4 in *Arabidopsis*, from the plasma membrane [10]. Therefore, solvent controls are important to correctly interpret results.
6. Dissolve 0.05 g Pectolyase in 10 mL of MTSB and incubate at 37 °C for 30 min followed by room temperature incubation for another 30 min.
7. Other embedding materials include LR-white and other resins for structural and histochemical studies. We have only performed immunolocalizations on plant material embedded in wax or agarose and have not tried other media.
8. Solutions with agarose percentage (w/v) higher than 3 % solidify very quickly so embedding should be performed as quickly as possible. One solution is to use agarose with a lower melting point, although the final firmness of the embedding might be lower. Moreover, molds and tubes can be kept on a hot plate or in the water bath so that the agarose solution is warm while the sample position is adjusted. In addition, whole mounts may also be embedded in agarose.
9. Poly-L-lysine slides can be prepared inexpensively by dipping clean, washed and dried slides into a slide mailer, Copland jar or 50-mL tube with 0.1 % w/v poly-L-lysine. Let the slides dry, and then you have charged slides for your whole mounts and sections.

10. The choice of the secondary antibody depends on the user's preference and is often dictated by conditions: the available set of filters and lasers for the fluorescence or confocal microscopes and the wavelength at which the specific tissue autofluorescence is detected.
11. As mounting media, we use fluorescence anti-fade media such as CFM1 (Citifluor), VECTASHIELD® or VECTASHIELD® + DAPI, the latter will also counterstain the nuclei. Slides may be stored at 4 °C in the dark and fluorescence may be stable for several weeks to months, depending on the fluorophores used.

Acknowledgements

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References

1. Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. Plant Cell 9:1963–1971
2. Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ, Vernoux T (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. Nature 482:103–106
3. Truernit E, Bauby H, Dubreucq B, Grandjean O, Runions J, Barthélémy J, Palauqui J-C (2008) High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of phloem development and structure in *Arabidopsis*. Plant Cell 20:1494–1503
4. Peer WA, Hosein FN, Bandyopadhyay A, Makam SN, Otegui MS, Lee GJ, Blakeslee JJ, Cheng Y, Titapiwatanakun B, Yakubov B, Bangari B, Murphy AS (2009) Mutation of the membrane-associated M1 protease APM1 results in distinct embryonic and seedling developmental defects in *Arabidopsis*. Plant Cell 21:1693–1721
5. Brundrett MC, Enstone DE, Peterson CA (1988) A berberine-aniline blue fluorescent staining procedure for suberin, lignin, and callose in plant tissue. Protoplasma 146:133–142
6. Murphy A, Taiz L (1997) Correlation between potassium efflux and copper sensitivity in ten *Arabidopsis* ecotypes. New Phytol 136: 211–222
7. Boutté Y, Crosnier M-T, Carraro N, Traas J, Satiat-Jeunemaitre B (2006) The plasma membrane recycling pathway and cell polarity in plants: studies on PIN proteins. J Cell Sci 119:1255–1265
8. Carraro N (2002) Auxin transport and organs formation in *Arabidopsis* and maize. Dissertation, Università Degli Studi di Padova—Université Paris XI
9. Titapiwatanakun B, Blakeslee JJ, Bandyopadhyay A, Yang H, Mravec J, Sauer M, Cheng Y, Adamec J, Nagashima A, Geisler M, Sakai T, Friml J, Peer WA, Murphy AS (2009) ABCB19/PGP19 stabilises PIN1 in membrane microdomains in *Arabidopsis*. Plant J 57:27–44
10. Yang H, Richter GL, Wang X, Młodzińska E, Carraro N, Ma G, Jenness M, Chao D, Peer WA, Murphy AS (2013) Sterols and sphingolipids differentially function in trafficking of the *Arabidopsis* ABCB19 auxin transporter. Plant J 74:37–47

Part II

Photoperiodism and Circadian Rhythms

Chapter 7

Analysis of Circadian Leaf Movements

Niels A. Müller and José M. Jiménez-Gómez

Abstract

The circadian clock is a molecular timekeeper that controls a wide variety of biological processes. In plants, clock outputs range from the molecular level, with rhythmic gene expression and metabolite content, to physiological processes such as stomatal conductance or leaf movements. Any of these outputs can be used as markers to monitor the state of the circadian clock. In the model plant *Arabidopsis thaliana*, much of the current knowledge about the clock has been gained from time course experiments profiling expression of endogenous genes or reporter constructs regulated by the circadian clock. Since these methods require labor-intensive sample preparation or transformation, monitoring leaf movements is an interesting alternative, especially in non-model species and for natural variation studies. Technological improvements both in digital photography and image analysis allow cheap and easy monitoring of circadian leaf movements. In this chapter we present a protocol that uses an autonomous point and shoot camera and free software to monitor circadian leaf movements in tomato.

Key words Circadian rhythms, Leaf movements, Tomato, *Solanum lycopersicum*, Time-lapse imaging

1 Introduction

The circadian clock is an internal oscillator that allows organisms to anticipate daily changes. In plants, the clock regulates the expression of about one-third of all genes [1], which in turn control multiple physiological processes. A direct and accurate method for studying the circadian clock is to monitor gene expression, either with qRT-PCR or blots, or by using a reporter gene driven by a clock-controlled promoter [2]. The disadvantages of these methods are that they require costly setups, transformation or that they are time-consuming. Monitoring leaf movements is a fast and convenient alternative.

In fact, the existence of an endogenous timekeeper was proposed for the first time in the eighteenth century by de Mairan, based on the observation that the leaves of a heliotropic plant, most likely *Mimosa pudica*, fold up during the night and unfold during the day, and that this movement continues under constant

Paula Duque (ed.), *Environmental Responses in Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1398,
DOI 10.1007/978-1-4939-3356-3_7,

darkness [3]. Today it is widely accepted that rhythmic leaf movements are indeed controlled by the circadian clock, as they show all the hallmarks of circadian rhythms and are affected by mutation of circadian clock genes [4].

In legumes, leaf movements are caused by expansion and contraction of a specialized motor organ at the base of the petiole called pulvinus [5]. Plants without pulvini, however, also exhibit circadian leaf movements. The abaxial and adaxial sides of the leaf blades and petioles exhibit rhythmic growth in antiphase leading to oscillations in the leaf position [5]. Such leaf movements have been used to monitor circadian rhythms for several plant species, e.g., *Arabidopsis*, tobacco, potato, *Brassica oleracea*, and *Brassica rapa* [6–10]. Because of its simplicity, recording leaf movements is the main method used to study natural variation in circadian rhythms in plants. At the beginning of the last century, Bünning reported that different pea genotypes exhibit circadian leaf movement rhythms with different periods [11]. Later, variation in circadian leaf movements was employed to identify genetic loci responsible for differences in circadian period in *Arabidopsis* [12, 13] and *Brassica* species [9, 10].

The methods for measuring circadian leaf movements changed tremendously over time. In the early twentieth century a complicated setup consisting of a thread fixed to a leaf of the plant was used. This thread was connected to a lever equipped with a pen that would convey the information on the leaf position onto a paper roll [14]. In the 1990s, digital cameras coupled to computers made it possible to record the leaf movements of several plants without complicated preparations [6]. These image acquisition systems, however, still required specialized hardware and software, which made them expensive. Cameras needed to be connected to a camera switcher, which in turn had to be connected to a computer via a flash bus and a parallel port controller unit [15]. The enormous progress in digital photography over the last decade has made available relatively cheap point-and-shoot cameras that can acquire image sequences over several days with a defined interval without the need of a computer or any additional software. In addition, efforts have been made to develop open source image analysis software that can be easily customized for scientific use.

Here we present a method that uses autonomous compact cameras and open source image analysis software to monitor circadian leaf movements in virtually any growth cabinet that can be set to constant light and temperature conditions. Four cameras can monitor more than 100 tomato seedlings, making the method suitable for high-throughput phenotyping of circadian rhythms in basically every dicotyledonous plant species.

2 Materials

1. Two climate chambers (one with diurnal and one with constant conditions).
2. Clamp tripods and bar to fix the tripods (*see* **Note 1**).
3. Digital camera (Pentax Optio WG-1) (*see* **Note 2**).
4. External power supply (AC Adapter Kit K-AC117E for Pentax Optio WG cameras) (*see* **Note 3**).
5. SD card.
6. Polystyrene balls (diameter approximately 2 mm).
7. Petroleum jelly (Vaseline or similar).

3 Methods

3.1 Imaging System

1. Fix cameras in the climate chamber at the appropriate positions to enable a maximum number of plants to be imaged at the same time. We use two cameras per 120×60×50 cm shelf (Fig. 1).
2. (Optional) Build pot stands in order to fill the complete field of view of the cameras. For this we use the same pots in which seedlings are grown (Fig. 1).
3. Fix a black cardboard (or similar) to the back of the climate chamber to ensure that the background of the pictures is dark. This will facilitate the isolation of the white polystyrene balls used to follow the movements of the leaves during image analysis.
4. Set the climate chamber to continuous light and constant temperature (*see* **Note 4**).

3.2 Seed Preparation and Growth Conditions

Seeds have to be prepared according to the species requirements for growth on soil. The following steps describe the seed preparation appropriate for tomato.

1. Place tomato seeds in a petri dish and add saturated tri-sodium phosphate (Na_3PO_4). Keep seeds in this solution for 15 min.
2. Rinse seeds with tap water three times. Fill the petri dish with the rinsed seeds halfway with water and close with the lid. Keep petri dishes in the dark at room temperature for 3 days.
3. Fill single 7×7 cm pots with appropriate soil (*see* **Note 5**) and place single seeds in the middle of each pot, approximately 1 cm deep into the soil (*see* **Note 6**).
4. Place the pots into the entrainment chamber set to diurnal conditions (*see* **Notes 7** and **8**).

Fig. 1 Overview of the imaging system. Pentax Optio WG-1 cameras are fixed to shower curtain rods with clamp tripods. Twenty-seven 7 × 7 cm pots can be placed on the pot stands in front of each camera allowing imaging of 108 tomato seedlings using four cameras

3.3 Seedling Preparation and Imaging

It usually takes 3 days for the seedlings to emerge from the soil and to unfold their cotyledons (here defined as germination). Tomato seedlings are in an ideal state to start the time-lapse imaging on the third day after germination (*see* **Note 9**).

1. Fix a polystyrene ball to the tip of one cotyledon using a little bit of petroleum jelly on the day before the experiment starts (Fig. 2) (*see* **Note 10**).
2. Transfer the plants to the imaging chamber immediately after the lights of the entrainment chamber turn on (defined as Zeitgeber (ZT) = 0).
3. Start the cameras (*see* **Note 11**).
4. Image plants for as many days as needed. We have obtained good results by monitoring plants for 5 days.

3.4 Image Analysis

To extract the vertical positions of the cotyledon tips over time, i.e., the leaf movements, we use the open source software ImageJ (available at http://imagej.nih.gov/ij/). This software can be installed on every operating system.

1. Transfer the pictures from the camera to your computer and import them as an image sequence into ImageJ (*see* **Note 12**).

Fig. 2 Tomato seedling with a polystyrene ball fixed to one cotyledon tip on the day before being transferred to the imaging chamber

2. Split the colors of the stack with the Image > Color > Split Channels command to get 8-bit images. Since the polystyrene ball is most easily isolated from the blue channel, continue the analysis using only this channel.
3. Set the threshold with the Image > Adjust > Threshold command in such a way that only the polystyrene balls remain visible as black points (Fig. 3).
4. Check Centroid in the Analyze > Set Measurements menu to obtain horizontal and vertical positions of the polystyrene ball, i.e., leaf tip.
5. Choose the rectangle selection tool, normally located in the first position of the toolbar. Select the area in which the leaf tip of one seedling is located over the course of the experiment.
6. Process all images of the stack with the Analyze > Analyze Particles command. Column Y of the results represents the horizontal position of the centroid of the polystyrene ball and is copied to an excel spreadsheet.
7. Repeat **steps 5** and **6** for every seedling.
8. Analyze data online using BioDare to obtain circadian variables as period, phase, amplitude and relative amplitude error. A detailed step by step protocol describing how to do this was recently published [16]. Alternatively, the Biological Rhythm Analysis Software Suite (BRASS), available on http://millar.bio.ed.ac.uk/ can be used following the instructions on the website. Figure 4 shows representative results from a leaf movement experiment using tomato.

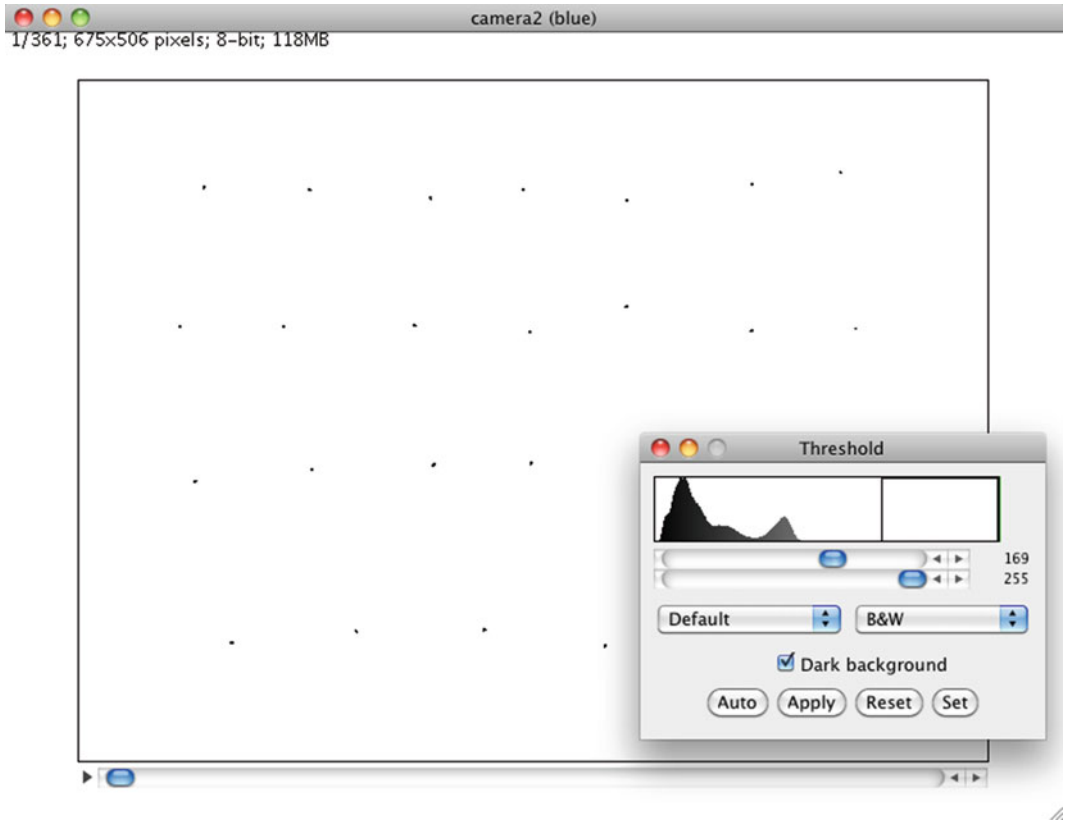

Fig. 3 Screenshot of ImageJ after the threshold adjustment. The first picture of the stack is shown. Every *black speck* represents one polystyrene ball attached to the cotyledon tip of one seedling

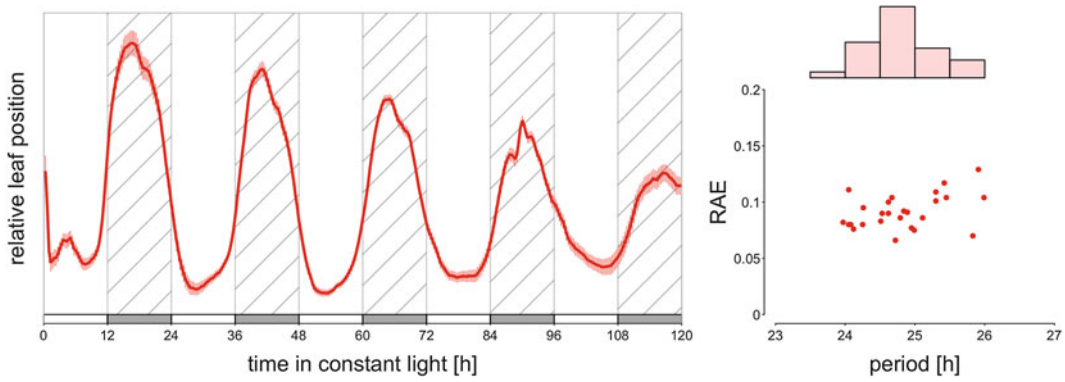

Fig. 4 Leaf movements of the wild tomato species *Solanum pimpinellifolium*. *Left panel*: Mean relative position of cotyledon tip ± standard error of the mean (SEM) from two independent experiments ($n = 27$). *Hatched areas* indicate subjective nights. *Right panel*: Circadian period and relative amplitude error (RAE) estimates obtained from BRASS. A histogram of period estimates is shown on above the scatterplot

4 Notes

1. We find that using clamp tripods fixed to a bar allows risk-free manipulation of plants (Fig. 1), although freestanding tripods can also be used. We use extensible shower curtain rods that can be easily clamped into various climate chambers to provide support for the clamp tripods (Fig. 1).
2. All the models from the Pentax Optio WG series (WG-1, WG-2, WG-10, WG-3, WG-4, WG-20 and all corresponding GPS models) feature an internal intervalometer, which can be programmed to take up to 1000 pictures at a defined interval. Another option is to use Canon cameras for which a firmware enhancement is available on the CHDK website: http://chdk.wikia.com/wiki/CHDK. This enhancement makes it possible to install custom written scripts such as the countdown intervalometer available on: http://chdk.wikia.com/wiki/Countdown_Intervalometer. It should be noted that the combination of this script with a hacked Canon PowerShot A495 was not 100 % stable in our hands. On average the script in one out of six cameras crashed during each experiment.
3. The Pentax Optio WG-1 battery lasts for at least 504 pictures (7 days with 20 min intervals) without being charged. Longer experiments may require an AC adapter.
4. For tomato we use constant white light provided by fluorescent tubes with an intensity of approximately 100 μmol m^{-2} s^{-1} at 24 °C. However, for other species lower light intensities may be beneficial. For *Arabidopsis*, for example, standard conditions provide 25 μmol m^{-2} s^{-1} of light [15].
5. Bright spots in the soil such as perlite or course sand can complicate image analysis. Adding a layer of finely sieved soil produces a dark surface and greatly facilitates image analysis.
6. If seeds already sprouted in the petri dish, care has to be taken not to break the radicle during sowing.
7. In principle, any diurnal conditions can be used to entrain the plants prior to monitoring leaf movements. It should be noted, however, that entrainment conditions do influence circadian rhythms. The standard method is to entrain plants in 12 h light/12 h dark cycles.
8. We place the lines in each experiment using a completely randomized design. Depending on the number of genotypes, plants can also be arranged according to a randomized block design with every camera representing one block.
9. We observe that starting the imaging 2 or even 1 day after germination does not cause any differences in period or phase of the circadian leaf movements in tomato. For this reason, we

start imaging all plants on the same day regardless of their day of germination, as long as they have been entrained between 2 and 4 days.

10. We thoroughly water the plants on the day before their transfer to constant conditions. The soil will store enough water to sustain the tomato seedlings for at least 5 days in constant conditions without any sign of droughtstress. If experiments last longer than 5 days, one option is to cover the soil surface with foil to avoid desiccation (also see Ref. 9).
11. We use an interval of 20 min. We take the first picture at ZT = 0.33 (20 min after the lights turn on) and a total of 361 pictures to obtain 120 h of data. We set the camera to "program" mode to be able to manually optimize settings such as white balance, exposure compensation, and sensitivity according to the light conditions and the background.
12. Make sure to set the maximum amount of memory available to ImageJ to an appropriate value with the Edit > Options > Memory & Threads command. You may need to scale the images to less than 100 % when importing the image sequence to not exceed this maximum memory.

References

1. Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 9(8):R130
2. Millar AJ, Short SR, Chua NH, Kay SA (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell 4(9):1075–1087
3. de Mairan JJO (1729) Observation botanique. Histoire de l'Academie Royale des Sciences, Paris: 35
4. McClung CR (2001) Circadian rhythms in plants. Annu Rev Plant Physiol Plant Mol Biol 52:139–162
5. Uehlein N, Kaldenhoff R (2008) Aquaporins and plant leaf movements. Ann Bot 101(1):1–4
6. Engelmann W, Simon K, Phen CJ (1992) Leaf movement rhythm in *Arabidopsis thaliana*. Z Naturforsch C 47(11-12):925–928
7. Siefritz F, Otto B, Bienert GP, van der Krol A, Kaldenhoff R (2004) The plasma membrane aquaporin NtAQP1 is a key component of the leaf unfolding mechanism in tobacco. Plant J 37(2):147–155
8. Yanovsky MJ, Izaguirre M, Wagmaister JA, Gatz C, Jackson SD, Thomas B, Casal JJ (2000) Phytochrome A resets the circadian clock and delays tuber formation under long days in potato. Plant J 23(2):223–232
9. Salathia N, Lynn JR, Millar AJ, King GJ (2007) Detection and resolution of genetic loci affecting circadian period in Brassica oleracea. Theor Appl Genet 114(4):683–692
10. Lou P, Xie Q, Xu X, Edwards CE, Brock MT, Weinig C, McClung CR (2011) Genetic architecture of the circadian clock and flowering time in Brassica rapa. Theor Appl Genet 123(3):397–409
11. Bünning E (1932) Über die Erblichkeit der Tagesperiodizität bei den Phaseolus-Blättern. Jahrbücher für Wissenschaftliche Botanik 77:283–320
12. Swarup K, Alonso-Blanco C, Lynn JR, Michaels SD, Amasino RM, Koornneef M, Millar AJ (1999) Natural allelic variation identifies new genes in the *Arabidopsis* circadian system. Plant J 20(1):67–77
13. Michael TP, Salome PA, Yu HJ, Spencer TR, Sharp EL, McPeek MA, Alonso JM, Ecker JR, McClung CR (2003) Enhanced fitness con-

ferred by naturally occurring variation in the circadian clock. Science 302(5647): 1049–1053

14. Bünning E (1931) Untersuchungen über die autonomen tagesperiodischen Bewegungen der Primärblätter von *Phaseolus multiflorus*. Jahrbücher für Wissenschaftliche Botanik 75:439–480
15. Edwards KD, Millar AJ (2007) Analysis of circadian leaf movement rhythms in *Arabidopsis thaliana*. Methods Mol Biol 362:103–113
16. Moore A, Zielinski T, Millar AJ (2014) Online period estimation and determination of rhythmicity in circadian data, using the BioDare data infrastructure. Methods Mol Biol 1158:13–44

Chapter 8

Sample Preparation of *Arabidopsis thaliana* Shoot Apices for Expression Studies of Photoperiod-Induced Genes

Fernando Andrés, Stefano Torti, Coral Vincent, and George Coupland

Abstract

Plants produce new organs from a population of pluripotent cells which are located in specific tissues called meristems. One of these meristems, the shoot apical meristem (SAM), gives rise to leaves during the vegetative phase and flowers during the reproductive phase. The transition from vegetative SAM to an inflorescence meristem (IM) is a dramatic developmental switch, which has been particularly well studied in the model species *Arabidopsis thaliana*. This developmental switch is controlled by multiple environmental signals such as day length (or photoperiod), and it is accompanied by changes in expression of hundreds of genes. A major interest in plant biology is to identify and characterize those genes which are regulated in the stem cells of the SAM in response to the photoperiodic signals. In this sense, techniques such as RNA in situ hybridization (RNA ISH) have been very successfully employed to detect the temporal and spatial expression patterns of genes in the SAM. This method can be specifically optimized for photoperiodic-flowering studies. In this chapter, we describe improved methods to generate plant material and histological samples to be combined with RNA ISH in flowering-related studies.

Key words Flowering, Photoperiod, Shoot apical meristem, Gene expression studies, In situ hybridization, *Arabidopsis thaliana*

1 Introduction

In plants, the transition from vegetative growth to flowering is regulated by several environmental stimuli and by the age of the individual. One of these environmental stimuli is the day length or photoperiod. The model plant species *Arabidopsis thaliana* has been widely used to identify genes involved in the photoperiodic induction of flowering [1]. *Arabidopsis* plants exposed to long days undergo a series of genetic and morphological changes in the shoot apical meristem (SAM) that culminate in the formation of flowers [2]. Hundreds of genes are deregulated in the SAM during this developmental switch and many of them are specifically transcribed in response to photoperiod [3–6]. These genes might play important roles in photoperiodic-flowering and therefore defining their

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specific spatial and temporal patterns of expression is a major goal in molecular studies on mechanisms controlling flowering time. However, this is a challenge because this tissue is embedded within the shoot apex and is therefore difficult to access. For this reason, specific technologies have been developed to study the expression of genes in the SAM, such as RNA in situ hybridization (RNA ISH) [7, 8]. RNA ISH is a technique that allows the detection of a specific RNA within a histological section. This is possible because nucleic acids can be preserved adequately within a histologic specimen and are detected with a probe consisting in a complementary strand of nucleic acid attached to a reporter molecule [9]. Many reports have already described excellent protocols of RNA ISH to detect gene expression in the SAM [8, 10, 11]. However, this technique requires specific optimization in order to be employed in studying the pattern of expression of photoperiodic-flowering genes. For instance, flowering of *Arabidopsis* plants must be properly induced in order to ensure a synchronized developmental progression [3, 5, 6]. This is tremendously important as the expression of genes induced by photoperiod is differentially regulated at different developmental stages. Therefore, comparing the expression of a gene between plants which are not perfectly synchronized might lead to misinterpretations. In addition, the expression pattern of a particular gene might vary among different layers of the SAM cells. Therefore the histological sections must represent the longitudinal center of the SAM to ensure a reliable comparison between two samples. In this chapter, we describe methods (1) to induce a synchronized photoperiod flowering-response in *Arabidopsis* plants, permitting the developmental progression of the SAM to be followed during the switch to the reproductive phase, and (ii) a straightforward technique to collect shoot apices and process the tissue (i.e., fixation, embedding, and sectioning) in order to obtain histological samples suitable for RNA ISH experiments. These methods are particularly relevant in the study of photoperiodic-flowering in *Arabidopsis*. A complete account of procedures for performing RNA ISH is beyond the scope of this chapter, but interested readers should refer to other important reports [7, 10, 11].

2 Materials

Prepare all the buffers with distilled and autoclaved water.

2.1 Plant Materials and Growing Conditions

1. Plant material: *Arabidopsis thaliana* (L.) Heynh (Ecotype Col-0).
2. Square 9 × 9 cm pots.
3. Soil.

4. Grow chambers: short days (SD, 8 h light–16 h dark); long days (LD, 16 h light–8 h dark) photoperiod. Light intensity: 150–180 μmol m^{-2} s^{-1}. Temperature: 21 °C.

2.2 Sampling and Fixation

1. Glass vials with screw caps (~20 ml).
2. Microdissection forceps.
3. Vacuum pump and desiccator.
4. 10× PBS: 1.5 M NaCl, 0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4 (pH 7). Prepare at least 1 L and store it at RT.
5. 4 % PFA: 40 ml 10× PBS, 360 ml H_2O, and 16 g of paraformaldehyde, 0.1 % Tween 20, 0.1 % Triton X-100 (*see* **Notes 1** and **2**). To prepare 400 ml of 4 % PFA fill a glass bottle with 360 ml of distilled water and 40 ml of 10× PBS to get 1× PBS. Warm the 1× PBS in a microwave for 1 min and 30 s. Weigh 16 g of PFA in the fume hood and transfer it into a glass bottle (500 ml), add the warm 1× PBS (~60 ° C), 500 μl of NaOH 4 M and stir until the PFA is dissolved. PFA vapors are toxic, so close the bottle with the cap while stirring. Adjust the pH to 7 with 550 μl of a 1:10 dilution of H_2SO_4. Do not use HCl, as it can react violently with PFA to produce a carcinogenic compound. Check the pH at room temperature with a pH indicator.
6. Graded ethanol series: 30, 40, 50, 60, 70, 85, 95, and 100 % in water. Store them at 4 °C (*see* **Note 3**).
7. Staining buffer: 0.1 % eosin in 100 % EtOH. Store it at RT.

2.3 Embedding

1. Embedding cassettes of 1 mm square mesh.
2. 100 % Ethanol.
3. Histological clearing agent.
4. Paraffin.
5. An automated tissue processing machine (Optional, *see* Subheading 3.3).
6. Paraffin dispenser.
7. Petri dishes (Ø 9 cm).
8. Bunsen burner.

2.4 Histological Sectioning

1. Retractile blade.
2. Single edge blades.
3. Artistic brushes.
4. Spatula.
5. Bunsen burner.
6. Wooden/plastic cubes (~2 × 2 × 2 cm).
7. Rotary microtome.
8. Electrostatically charged slides.
9. Stereomicroscope.

10. Slide dryer bench.
11. Pasteur pipette.
12. Cloth or absorbent paper.

3 Methods

3.1 Synchronized Induction of Photoperiodic-Flowering and Sampling

1. Sow around 30 *Arabidopsis* seeds per sample group (*see* **Note 4**) equally distributed in 9×9 pots (*see* **Note 5**) with soil (*see* **Note 6**).
2. Pre-germinate the seeds at 4 °C in the dark for 3 days and then transfer to a short day growth chamber.
3. Prepare fresh 4 % PFA (in the fume hood), distribute around 20 ml in glass vials (one per sample group) and keep the vials on ice.
4. Grow all the sample groups under SDs for 14 days (*see* **Note 7**). Collect the sample group "14SD" at 8 h after dawn (Zeitgeber time 8, ZT8), just before lights turn off (*see* **Note 8**). Grab individually each seedling from the soil with the microdissection forceps and pull it up. Hold the seedling with two fingers and remove the leaves and part of the petioles with the forceps (Fig. 1) (*see* **Note 9**). Then remove the root and put the sample (hypocotyls and shoot apex) into a glass vial containing 4 % PFA (on ice). Repeat for around 15–20 seedlings per sample group.
5. Transfer the remaining sample groups to a long day growth chamber to induce flowering (s*ee* **Note 10**). Samples will be collected as described above after 3 (sample group "3LD"), 5 (sample group "5LD"), and 7 days (sample group "7LD").

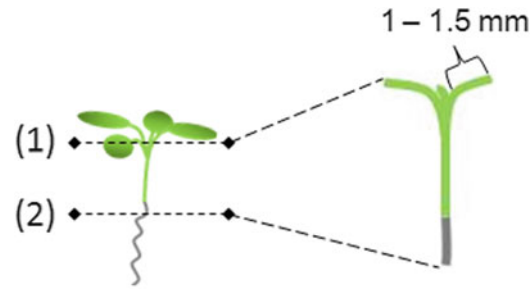

Fig. 1 Sampling and dissection of *Arabidopsis* shoot apex for ISH. (**a**) Grab a seedling with microdissection forceps from the petioles (be sure to grab all the petioles, *dashed line* (*1*)) and pull it up. Holding the hypocotyl firmly with two fingers, press strongly the forceps and remove petioles, leaves, and cotyledons (a twist should be enough). Try to keep 1–1.5 mm of the first true leaf petioles. Then, remove the root (*dashed line* (*2*)) with the forceps and place the sample in the cold 4 % PFA

6. Keep the vials on ice until the next step.

3.2 Fixation by Vacuum and Sample Dehydration

1. Put the vials (with the caps unscrewed) and some ice inside of the desiccator. Connect the desiccator to a vacuum pump and turn it on (*see* **Note 11**).
2. Keep the vacuum for 10 min and release gradually the pressure. Repeat (*see* **Note 12**).
3. Replace the 4 % PFA with fresh 4 % PFA solution and keep at 4 °C overnight on ice.
4. Dehydrate the samples through an ethanol series (30, 40, 50, and 60 %). Incubate for 1 h in each solution on ice.
5. Replace the 60 % ethanol with 70 % ethanol. Incubate for 2 h at 4 °C (*see* **Note 13**).
6. Replace the 70 % ethanol with 85 % ethanol and keep at 4 °C overnight.
7. Replace the above step with 95 and 100 % for 2–4 h each at 4 ° C (*see* **Note 14**).

3.3 Tissue Staining and Embedding

1. Transfer the samples from the vials to the embedding cassettes (*see* **Note 15**).
2. Immerse the cassettes in 0.1 % Eosin (staining solution) and keep for 1–2 h at RT (*see* **Note 16**).
3. Wash the samples twice with fresh 100 % ethanol.
4. Incubate the tissues with 50 % ethanol/50 % Histo-Clear for 1 h at RT (*see* **Note 17**).
5. Then, incubate three times for 1 h each with 100 % Histo-Clear at RT.
6. 50 % Histo-Clear/50 % paraffin overnight at 60 °C.
7. Replace with 100 % paraffin (60 °C) twice daily during 3 days.
8. Preheat the heating plate of the paraffin dispenser at 60 °C 30 min in advance.
9. Place a petri dish on the hot plate for 2–3 min.
10. Pour melted paraffin into a petri dish (use one petri dish per sample group) to fill up 80 % of its capacity.
11. Remove the embedding cassettes from the hot 100 % paraffin (or from the tissue processor, *see* **Note 18**) with preheated tweezers.
12. Open the cassettes' cap and use preheated tweezers to drop off the samples onto the hot paraffin (*see* **Note 19**).
13. Distribute the shoot apices with the preheated tweezers until all the shoot apices are laying horizontally on the bottom of the petri dish (Fig. 2a).
14. Very carefully, move the petri dish onto a cold surface (for example a precooled square piece of aluminum) (*see* **Note 20**).

15. Wait until the paraffin has solidified and then place the petri dish on ice for a 1–2 h.
16. Repeat these steps with all the sample groups and keep at 4 °C for at least 24 h.

3.4 Histological Sectioning

1. Using a preheated retractile knife make rectangular blocks (~10 × 5 mm) of paraffin containing the samples in the middle (*see* **Note 21**).
2. Glue with melted paraffin the sample blocks on the side of a wooden (or plastic) cube and keep them at 4–8 °C for 10 min (*see* **Note 22**). The plant sample must be oriented as indicated in Fig. 2b (*see* **Note 23**).
3. Section the tissue at 7 μm (*see* **Note 24**).
4. Produce a continuous ribbon of sections including all the tissue (*see* **Note 25**).
5. Visualize the sections in a stereomicroscope and find those sections showing the center of the SAM (*see* **Note 26**).
6. Separate with a blade the sections containing the center of the SAM.
7. Add water on the surface of a microscope slide and warm it up on the slide dryer bench (set-up at 35–40 °C).

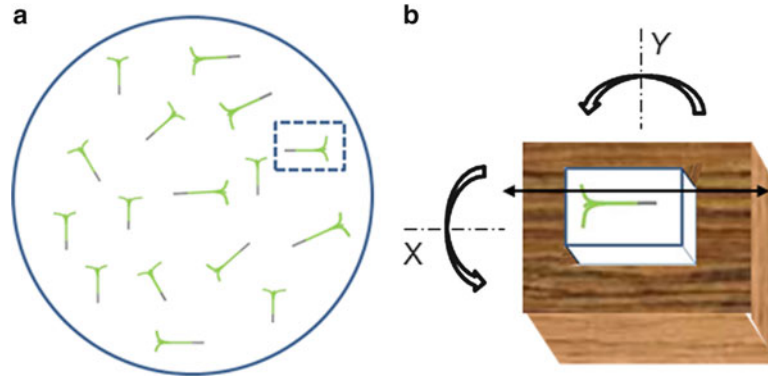

Fig. 2 Preparation of shoot apices embedded in paraffin for microtome dissection. (**a**) Transfer the shoot apices from the embedding cassettes to a petri dish containing melted paraffin (Subheading 3.3). Distribute the samples evenly spaced and laying horizontally on the bottom of the petri dish. Once the paraffin has completely solidified (at least 24 h after at 4 ° C) cut a piece of paraffin with a preheated knife including a shoot apex (*dashed line*). (**b**) Glue the paraffin piece with melted paraffin on a rectangular wooden or plastic block, put it at 4 ° C for a few minutes and fit it in the microtome head. Adjust properly the position of the block: Orientate the block to get the two petioles perpendicular to the blade (*x*-axis). Cut the upper part of the paraffin block (*arrow*), visualize the sample from above and orientate the block in order to get the hypocotyl in parallel with the blade (*y*-axis)

8. Put the ribbon of sections on the slide (matt side upwards), leave it floating on the water and place the slide on the slide dryer bench.
9. Wait a few minutes until the ribbon gets completely smooth.
10. Remove most of the water with a Pasteur pipette and finish drying the slide by using a cloth or absorbent paper. Be sure that the ribbon of sections sticks in the middle of the slide.
11. Put the slide on the slide dryer bench and leave it there until the next day (*see* **Note 27**).

4 Notes

1. Paraformaldehyde powder (PFA) and solutions containing PFA are very toxic and should be handled wearing gloves and in the fume hood. PFA residues and materials directly exposed to PFA should be disposed of in an appropriate waste container.
2. Due to instability, solutions should be freshly prepared from PFA just before use or frozen/thawed only once.
3. After preparing the ethanol series keep the bottles at 4 °C for 24 h to cool down and remove all the bubbles.
4. A sample group is a set of plants that will be exposed to a particular photoperiod for a number of days. By changing the photoperiod conditions, it is possible to synchronize the developmental stage of the plants and obtain a very uniform floral induction in all of the SAMs within a sample group. In this protocol all the plants are grown for 14 short days. The sample group collected at that time will represent plants staying at the vegetative stage (sample group "0 LD"). The other groups of plants are then shifted to long days and collected after 3, 5 and 7 long days (samples group 3 LD, 5 LD and 7 LD). These three sample groups define plants at the reproductive phase (from the initial floral induction to the floral development stages (Fig. 3)). In ISH experiments it is common to use marker genes to verify the developmental stage of the SAM. For instance, *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) should not be expressed in the SAM of a non-induced plant (14 short days, Fig. 3a). However, after 3 long days (Fig. 3b), *SOC1* expression is induced in the center of the SAM, indicating the beginning of the floral induction [12]. A good marker gene for the end of floral induction and the start of floral primordium development (after 5 long days, Fig. 3c) is *APETALA1* (*AP1*), as it is expressed specifically in the floral primordium [12].

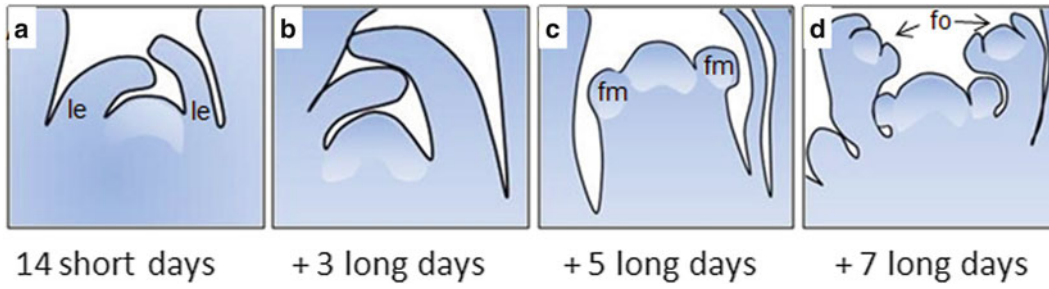

Fig. 3 Developmental progression of a SAM during photoperiodic induction. A good section should allow the visualization of all plant micro-structures shown in these representations. (**a**) A SAM of a plant grown for 14 short days stays vegetative and produces leaves (le) at the flanks. Flowering is induced by exposing the plants to long days (**b**–**d**). (**b**) After 3 long days the SAM grows and becomes domed. At this stage, hundreds of genes are already differentially expressed in the SAM (**c**) Five long days triggers the formation of floral meristems (fm) at the flanks of the SAM. Specific meristem identity genes are expressed at this stage. (**d**) After 7 long days several genes involved in floral development are expressed and floral organs (fo, approximately stage 3) are already visible

5. Plants should be evenly spaced in order to avoid shade effects which might affect the flowering induction. One week after germination, thin out the excess of seedlings and remove those showing a non-synchronized growth compared to the majority.
6. Seedlings grown on soil perform better than those grown on medium. This is an advantage for sectioning the tissue.
7. In *Arabidopsis*, flowering time tremendously differs within ecotypes and mutants. In this protocol we describe how to induce a synchronized photoperiodic-flowering response in *Arabidopsis* Col-0 plants. Col-0 plants grown for 14 days under short day conditions still persist at vegetative stage. However, in early flowering ecotypes or mutants flowering might be already induced after 14 short days. Therefore, for some genotypes the growing period under short day needs to be reduced in order to ensure that all the plants of the experiment stay at the vegetative stage before the photoperiodic floral induction.
8. The floral induction not only depends on the genotype of the plant, but also on the light quality of the lamps installed in the growth chambers. The strongest floral induction is usually achieved by combining white fluorescence lamps with a source of far red light (incandescent bulbs or LEDs [Light-emitting diode]).
9. Leave approximately 1–1.5 mm of the first true leaf petioles. The remaining petioles will contribute to keep the shoot apex

horizontal inside of the paraffin and will help to define the sample orientation during the sectioning (Figs. 1 and 2).

10. In order to avoid differences in gene expression mediated by the circadian clock rhythms, the samples should be collected at the same time of the day independently of the photoperiod. Collecting samples after ZT8 (8 h after dawn) implies that plants will be exposed to the dark under short days and to the light under long days. To avoid this situation is recommended to collect the samples before ZT8.
11. The samples should float and stay on the top level of the liquid because of the vacuum action.
12. When the pressure is completely released the samples should sink and lay on the bottom of the vial. If after the first vacuum step all the samples already lay on the bottom, it is not necessary to repeat this step again.
13. Samples can be stored in 70 % ethanol for several months at 4 °C.
14. Samples in 100 % ethanol can be stored for several months at 4 °C.
15. Proceed with the next step quickly; the ethanol evaporates very fast, do not allow samples to dry out.
16. Staining with Eosin is relevant for visualizing the tissue during the sectioning.
17. Embedding can be performed with an automatic tissue processor by running the following program: 100 % ethanol (three times of 1 h), Histo-Clear (three times of 1 h), Paraffin (three times of 1 h) at 60 °C.
18. Check the tank of the paraffin dispenser the day before using it and refill it if necessary. Paraffin might take a few hours to completely melt.
19. Petri dishes should be always on the heater plate until all the samples have been transferred to avoid the premature solidification of the paraffin and a bad arrangement of the samples.
20. After moving the petri dish onto a cool surface the shoot apex samples embedded in the paraffin can still be reorientated for a few seconds. Use this time to place the misplaced samples at the right position (lying horizontally on the bottom).
21. Solid paraffin breaks very easily. Using a preheated knife helps a lot to cut the paraffin without damaging the samples. Leave approximately 2–3 mm of paraffin around the sample.
22. Samples embedded in paraffin can be kept for at least 6 month at 4 °C.
23. Once the plant sample is approximately 1 mm close to the microtome blade, use a single edge blade to cut a horizontal section of the block approximately 1 mm above the plant sam-

ple. In this way, the shoot apex should be visible from the upper side of the block. Looking at the plant sample from above orientate the block to get the plant hypocotyl parallel to the microtome blade. Cut the sides of the block to get a smaller rectangle surrounding the plant sample. Once the hypocotyl is completely in parallel with the blade, cut ribbons of sections until the plant sample is reached. Readjust again the block orientation if necessary and continue sectioning.

24. The size of the section can be reduced to 4–5 μm, but it is tedious and the quality of the sections might decrease as well.
25. Cut the block until the tissue is over.
26. The expression pattern of genes in the SAM might vary depending on the layer of cells which is being observed. For this reason it is crucial to obtain perpendicular sections of the middle of the SAM for all the analyzed samples (Fig. 2).
27. At this point, the histological samples are ready for being used in RNA ISH experiments. Specific protocols for probe design and synthesis, and in situ hybridization could be found within the specific literature [7, 10, 11].

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References

1. Koornneef M, Hanhart CJ, van der Veen JH (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol Gen Genet 229(1):57–66
2. Andres F, Coupland G (2012) The genetic basis of flowering responses to seasonal cues. Nat Rev Genet 13(9):627–639
3. Blazquez MA, Soowal LN, Lee I, Weigel D (1997) LEAFY expression and flower initiation in *Arabidopsis*. Development 124(19):3835–3844
4. Mantegazza O, Gregis V, Chiara M, Selva C, Leo G, Horner DS, Kater MM (2014) Gene coexpression patterns during early development of the native *Arabidopsis* reproductive meristem: novel candidate developmental regulators and patterns of functional redundancy. Plant J 79(5):861–877
5. Schmid M, Uhlenhaut NH, Godard F, Demar M, Bressan R, Weigel D, Lohmann JU (2003) Dissection of floral induction pathways using global expression analysis. Development 130(24):6001–6012
6. Torti S, Fornara F, Vincent C, Andres F, Nordstrom K, Gobel U, Knoll D, Schoof H, Coupland G (2012) Analysis of the *Arabidopsis* shoot meristem transcriptome during floral transition identifies distinct regulatory patterns and a leucine-rich repeat protein that promotes flowering. Plant Cell 24(2):444–462
7. Jackson D (ed) (1992) In sit hybridization in plants. Molecular plant pathology. A practical approach. Oxford University Press, Oxford
8. Carles CC, Ha CM, Jun JH, Fiume E, Fletcher JC (2010) Analyzing shoot apical meristem development. Plant Dev Biol 655:105–129
9. Gall JG, Pardue ML (1969) Formation and detection of RNA-DNA hybrid molecules in cytological preparations. Proc Natl Acad Sci U S A 63(2):378–383

10. Hejatko J, Blilou I, Brewer PB, Friml J, Scheres B, Benkova E (2006) In situ hybridization technique for mRNA detection in whole mount *Arabidopsis* samples. Nat Protoc 1(4): 1939–1946
11. Drea S, Derbyshire P, Koumproglou R, Dolan L, Doonan JH, Shaw P (2009) In situ analysis of gene expression in plants. Methods Mol Biol 513:229–242
12. Turck F, Fornara F, Coupland G (2008) Regulation and identity of florigen: FLOWERING LOCUS T moves center stage. Annu Rev Plant Biol 59:573–594

Chapter 9

A Luciferase-Based Assay to Test Whether Gene Expression Responses to Environmental Inputs Are Temporally Restricted by the Circadian Clock

Amaury de Montaigu, Markus Christian Berns, and George Coupland

Abstract

Gating is the mechanism by which the influence of an environmental signal on a particular output is temporally restricted by the circadian clock, so that the maximum response of the output to the signal occurs at a specific time. Gated regulation mechanisms have been described for several genes whose expression is strongly induced by light or temperature at certain times but repressed by the circadian clock at others. To reveal a gated pattern of expression in response to light, light pulses are applied in the dark at different times of the 24 h cycle and the transcriptional response of the gene of interest is then monitored with an appropriate technique. Luciferase (LUC) reporters have been the method of choice to study circadian rhythms in the past decades, but this methodology also provides an ideal platform for performing a gating assay. In this chapter, we describe a LUC imaging based protocol designed to test whether the influence of light on the expression of a gene of interest is gated by the circadian clock.

Key words Circadian clock, Gating, Gene expression, Light, Luciferase

1 Introduction

Plants are sessile organisms that experience and adjust to important daily fluctuations of external environmental conditions. In order to adapt to their cycling environment, plants have evolved temporal regulation mechanisms that promote the activity of biological processes at the most favorable time of the day or night. A wide range of processes cycle on a 24 h basis and, consequently, many aspects of plant biology ranging from development to stress resistance are temporally controlled [1, 2]. The molecular mechanisms that generate temporal patterns of biological activities often involve daily oscillations in the transcription of single genes or entire gene networks. Peaks and troughs of gene transcription are detectable in at least 30% of the *Arabidopsis thaliana* transcriptome [3], which

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partly explains the wealth and diversity of temporally regulated processes reported to date. Genes whose transcription oscillates during day/night cycles control traits of agricultural importance, and alterations of these daily expression patterns contribute to adaptive behaviors of certain crop varieties [4–6]. For these reasons, appropriate tools are required to decipher the complex mechanisms that generate temporal expression patterns in cycling environmental conditions.

Daily changes in gene expression are mainly caused by fluctuations of external cues or by the circadian clock, an endogenous mechanism that generates internal rhythms of approximately 24 h. A fundamental property of circadian rhythms is that, although they persist in the absence of environmental transitions at dawn and dusk, they are strongly influenced by environmental inputs. Conversely, the circadian clock can influence environmental signals during the day by controlling the time at which these signals regulate their outputs. In this regulatory process called "gating," the circadian clock represses environmental responses at certain times but leaves an open "gate" during the day for these responses to take place (Fig. 1). As a result, the intensity of an environmental response will vary depending on the time of day and reach a maximum during a specific time window. There are several known examples of genes whose light or cold inducible expression is gated by the circadian clock [7–9], suggesting that this regulatory mechanism might not be uncommon. By focusing on a gated transcriptional response to light signals, the current chapter provides an example for how the gating of environmental inputs contributes to defining complex daily expression patterns.

To reveal that light induction of a gene is gated by the circadian clock, plants are transferred to constant darkness (DD) and are subjected to light pulses applied at regular time intervals during a 24 h cycle. The rapid and transient activation of gene expression in response to the light pulses, also known as the acute response to light, is then monitored during several hours with an appropriate technique. Measurements at high temporal frequency are necessary to study the dynamics of the acute response and to precisely define the peak of expression after each light pulse. Thus, one of the difficulties of a gating experiment is that it requires measuring numerous time points at high temporal resolution, which implies that the number of samples to harvest rapidly becomes unattainable if the technique of choice is destructive. The Luciferase (LUC) gene reporter technology, however, provides a noninvasive and automated platform suitable for the study of gene expression at high temporal resolution. In addition to increasing the frequency of time points, this technology also monitors rhythms of expression in individual seedlings. LUC measurements therefore capture

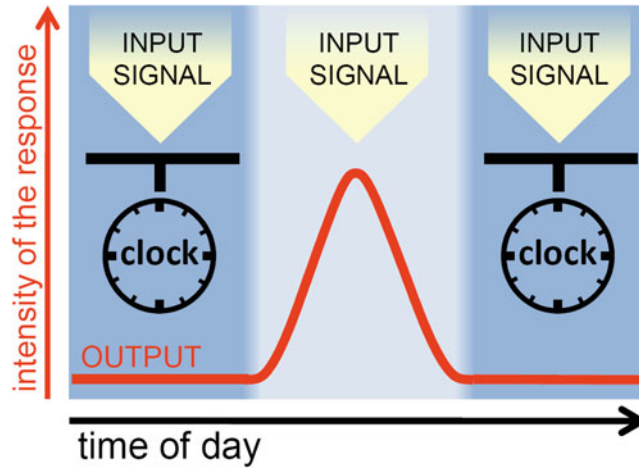

Fig. 1 Scheme illustrating the principle of a gated response. An input signal from the environment influences an output process during the day, but the circadian clock antagonizes the response of the output to the signal at certain times. As a consequence, the response of the output to the signal only occurs during a specific time window or gate (*light blue*)

the true variability in gene expression dynamics that exists between individuals [10], and also allows introducing high numbers of replicates in the experiments. In summary, LUC assays are suitable for performing a gating experiment because they enhance the temporal resolution and the throughput of gene expression measurements.

Luciferases are enzymes that oxidize a substrate and produce a photon. The resulting luminescence is proportional to the amount of active enzyme present in a sample and can be monitored relatively easily with different systems. Luciferases are found in many species, and luciferases from different species use different substrates [11, 12]. The *LUC* gene from the firefly (*Photinus pyralis*) uses D-luciferin as a substrate [11] and was successfully developed in the 1990s as a reporter for the study of rhythms of expression in plants [13]. Fusions of the *LUC* cDNA to promoters of interest were demonstrated to faithfully track the transcription patterns of endogenous genes [13–16] and have been used by an increasing number of laboratories in the past two decades. One of the main applications of the LUC system has been to study circadian rhythms in constant conditions of light and temperature, but this technology can also be applied in more complex experimental settings. Here, we describe the design and execution of a LUC based gating experiment that aims to reveal how light induction of gene expression can be gated by the circadian clock.

2 Materials

2.1 Seeds and Plants

1. Murashige and Skoog (MS) medium supplemented with 1 % sucrose and adjusted to pH 5.7.
2. Transgenic lines that express the *gene promoter*:*LUC* fusion of interest (*see* **Note 1**).

2.2 LUC Measurements

1. D-luciferin. Prepare a 50 mM stock solution by resuspending 1 g of D-luciferin in 71.3 mL of 0.1 mM triphosphate buffer (pH 8). Store at −80 °C.
2. Filter for sterilizing D-luciferin. We use a Syringe-driven Filter Unit 0.45 μm.
3. Sterile injection needle.
4. Black 96-well OptiPlate (Perkin Elmer). White plates are also available and used by other laboratories. The plates must be opaque to avoid luminescence contamination between wells.
5. TopSeals for 96-well microplates (Perkin Elmer).
6. Bar codes used by the TopCount as an identifier for each plate.
7. Stackers (*see* **Note 2**).
8. A box to keep the TopCount plates in darkness when carrying the plates from the chambers to the TopCount.
9. A TopCount Microplate Scintillation Counter (Perkin Elmer) to measure LUC activity. Other systems such as the CCD camera are also widely used for LUC measurements, but the TopCount is more suitable for high throughput assays (*see* **Note 3**).

3 Methods

3.1 Growth of the Seedlings

1. Seeds are surface-sterilized with ethanol. The amount of seeds required for the experiment is placed in a 1.5 mL vial. Add 1 mL of ethanol 70 % and leave 5–10 min. Resuspend the seeds by regularly inverting the vial during this time. Decant seeds (avoid centrifuging and vortexing the seeds in order to minimize damage). Remove the ethanol 70 % and apply 1 mL ethanol 100 % during 5–10 min. Resuspend the seeds by inverting the vial and decant. Remove as much ethanol 100 % as possible and wash seeds with 1 mL sterile water, resuspending the seeds by inverting the vial as before. Repeat the washing step twice and leave the seeds in water.
2. Stratification of the seeds can be performed at this point by placing the vials (containing seeds in 1 mL sterile water) at 4 °C during 3 days. In this case, seeds are stratified before plat-

ing. Alternatively, seeds can be plated and stratification performed on plates. Both methods are equally efficient in synchronizing germination.

3. Seeds are plated on square petri dishes (12 × 12 cm) containing MS medium with 1 % sucrose (*see* **Note 4**). Take particular care in evenly and sufficiently spacing the seeds on the medium to avoid seedlings growing in clumps (*see* **Note 5**). We transfer the seeds from the vial to the medium with a pipette and corresponding fine tips whose aperture dimension is appropriate for individual seeds to come out of the tip in single droplets. Cut the edge of the tip in order to make the aperture wider, otherwise seeds might be too large to pass through the aperture of the tip.
4. Seedlings are left to grow 7–10 days in the growth chamber set to the appropriate entraining condition, i.e., the external condition to which the circadian clock will adjust so that biological activities will be adequately timed relative to dawn and dusk. Typical entraining conditions are day/night cycles of fixed photoperiods, but the circadian clock can also be entrained by temperature cycles. The choice of the entraining condition will influence the result of a gating experiment (*see* **Note 6**).

3.2 Preparing the TopCount Plates

In a gating experiment, the design and the number of plates essentially depends on the temporal resolution of the light pulses. If a light pulse is applied every 2 h during a 24 h cycle, the experiment requires 14 plates including the control. An inexperienced experimenter must consider that transferring the seedlings from the petri dishes to the TopCount plates is time consuming (**step 1**), and preparing 14 full plates might not be doable in 1 working day. As an indicator, preparing a full plate (96 seedlings) will take approximately 30–60 min if performing this task for the first time. The number of plates and/or seedlings per plate must be planned accordingly. We recommend using 12–24 replicates per genotype in each plate to obtain robust results and suitable statistical power. Importantly, seedlings of the same genotype should preferably be organized in rows rather than in columns. The six TopCount detectors each measure luminescence in two adjacent columns, so organizing genotypes in rows will reduce a possible detector bias.

1. To prepare the TopCount plates, the 96 wells must be filled with 200 µL of solid MS medium supplemented with 1 % sucrose. Boil the MS medium and use a multichannel pipette to load the wells. We pour a volume of medium in a square a petri dish tilted to one side in order to facilitate the pipetting of the medium with the multichannel device. Once the MS medium has solidified inside the plates, wrap the plates in a plastic foil and keep them at 4 °C until the following day.

2. The seedlings are transferred (picked) from the petri dishes to the TopCount plates on the last day of the entrainment period. Picking the seedlings is the most time consuming step of a TopCount experiment, and this is particularly true for gating experiments in which the number of plates is high. All plates must be prepared and placed back in the growth chamber before the lights go off at dusk (*see* **Note 7**). Work in sterile conditions. Grab the seedling with the forceps at the hypocotyl–root junction and gently pull the seedling out of the medium. This is a delicate step, as applying too much pressure with the forceps will harm the seedling, and pulling the seedling out of the medium too abruptly will break the root. When transferring the seedling in the corresponding well, place the base of the hypocotyl in the medium so that the seedling stands up right with cotyledons facing upwards, well exposed to the TopCount detectors. Introduce the entire root in the medium and make sure that parts of the root do not get stuck on the side of the wells. Discard any damaged seedling. Although picking the seedlings is a tedious procedure, do not try to avoid it by sowing the seeds and growing the seedlings directly on the TopCount plates (*see* **Note 8**).
3. Add 20 µL of D-Luciferin 2.5 mM to each well. Sterile D-Luciferin 2.5 mM is prepared by diluting 20-fold the 50 mM stock solution in sterile water and by filtering the solution. To gain time, we use a multichannel pipette to load the D-Luciferin in the wells. The filtered solution is placed in a petri dish tilted to one side to facilitate the pipetting.
4. Seal the plates with the TopSeals and puncture a hole on top of each well with a sterile injection needle to facilitate air exchange not only for the seedlings but also because the Luciferase enzymatic reaction requires oxygen.
5. On the right side of each plate, stick a bar code that will be used by the TopCount machine as an identifier.
6. Put the plates in the same growth chamber that was used to grow the seedlings. It is important that all the seedlings experience the last environmental transition of the day/night cycle (dusk), meaning that the plates must be placed back into the growth chamber before lights off (*see* **Note 7**).
7. After dusk place the seedlings in controlled environmental conditions of constant darkness (DD) by, for example, changing the settings of the growth chamber so that the lights remain permanently switched off. Importantly, the seedlings will no longer be exposed to light until the light pulses are applied, and the plates must always be manipulated in the dark.

3.3 The Gating Experiment

The design and principle of the experiment are as follows. After the lights turn off at dusk on the last day of the entrainment period, the seedlings are released into DD conditions. From this point onwards the seedlings will not experience environmental transitions, and the beginning of the next cycle is now called "subjective dawn" because the lights will not come on at this time. At subjective dawn, 30 min light pulses are applied to the seedlings at 2 h intervals during 24 h (*see* **Note 9**). Each plate is subjected to one light pulse, and the response of the *promoter*:*LUC* reporter to each light pulse is monitored with the TopCount at high temporal resolution (*see* **Note 10**). If dawn was set at 8 a.m. in the growth chamber since the beginning of the experiment, then the first light pulse should be applied at 8 a.m. One plate that we call the "DD control" will not be exposed to any light pulse and will be measured continuously in DD. Note that, unlike circadian experiments performed in constant environmental conditions, a gating assay is only semi-automated and the experimenter must be present throughout the whole 24 h cycle (*see* **Note 11**).

1. The TopCount machine must be prepared before the experiment starts, ideally on the previous day. Choose the adequate settings (*see* **Note 10**) and run a dummy plate to make sure that the data are being stored in the correct file. Avoid any delay between plate measurements in the settings to maximize the temporal resolution of the assay. Also make sure that the parts of the TopCount in which the plates are stacked and automatically loaded in the machine (the stackers) are working properly, as this is a typical TopCount failure (*see* **Note 2**).
2. LUC activity measurements start at 7.30 a.m., before the application of the first light pulse. Two plates are carried from the growth chamber to the TopCount and placed in the stacker. Any exposure of the seedlings to light must be avoided while opening the growth chamber or transporting the plates (*see* **Note 12**). One of the two plates will be exposed to the first light pulse of 30 min at 8 a.m. (Plate 1), but prior to the pulse it is necessary to perform at least one LUC activity measurement that will be used as a reference. The other plate is the DD control. Make sure that an empty plate with a double bar code is placed on top of the pile after Plate 1 and the DD control, otherwise the plates will be measured only once (*see* **Note 2**).
3. At 8 a.m. and once the reference measurements are finished, transport Plate 1 to a chamber with the desired light quality and quantity settings, expose Plate 1 to the light source for 30 min and transport Plate 1 back to the TopCount. The plate must be left at least 2 min in the dark before the first measurement in order to reduce the background due to seedling-delayed fluorescence. LUC activity is then measured

continuously and automatically during 4 h every 10 min approximately (*see* **Note 10**).

4. The second light pulse is applied 2 h after the first, i.e., at 10 a.m. if dawn was set at 8 a.m. since the beginning of the experiment. As for Plate 1, the next plate (Plate 2) is removed from the growth chamber at 9.30 a.m. and transported to the TopCount in order to perform the reference LUC activity measurements before the pulse. At 10 a.m. Plate 2 is removed from the stacker and transported from the TopCount to the chamber in which the light pulse will be applied. After the pulse, Plate 2 is transported back to the TopCount and again placed into the stacker with Plate 1 and the DD control. LUC activity is then monitored every 10 min during 4 h.
5. The third light pulse is applied to Plate 3 6 h after subjective dawn (12 a.m.). Follow the same procedure than described in **steps 3** and **4**. Importantly, by the time of the third light pulse Plate 1 will have been measured during 4 h. Therefore, substitute Plate 1 by Plate 3 in the pile when starting the reference LUC measurements for Plate 3. At this step of the protocol, Plate 2, Plate 3 and the DD control are present in the stacker and Plate 1 is no longer required for the experiment. Do not keep more than three plates in the stacker as this will lower the temporal resolution of the measurements.
6. Repeat the previous steps with Plate 4 and the fourth light pulse applied at 2 p.m. Replace Plate 2 by Plate 4 in the stacker when starting the measurements.
7. Continue with the same procedure until the last light pulse is applied to Plate 13 at 8 a.m. the following subjective morning. A scheme of the whole procedure is provided in Fig. 2.
8. The data are downloaded from the TopCount and analyzed with the Microsoft Excel workbook TopTemp (www.amillar.org) that imports the raw data in an Excel format, organizes the plate measurements chronologically and by genotype, and produces graphs (*see* **Note 13**). LUC measurements in constant darkness (DD control) should oscillate and confirm that the gene is under circadian control (Fig. 3). As the baseline expression level of the gene is not expected to be constant in darkness, the raw data obtained after each light pulse will not be directly comparable. It is therefore necessary to normalize the luminescence data measured after each light pulse to the luminescence measured before the pulses so that the magnitude of the response can be compared at different times of the day. If the acute response of the gene to the light pulses is gated by the circadian clock, the data will reveal variation in the magnitude of the response with a maximum clearly occurring during a specific time window (Fig. 3).

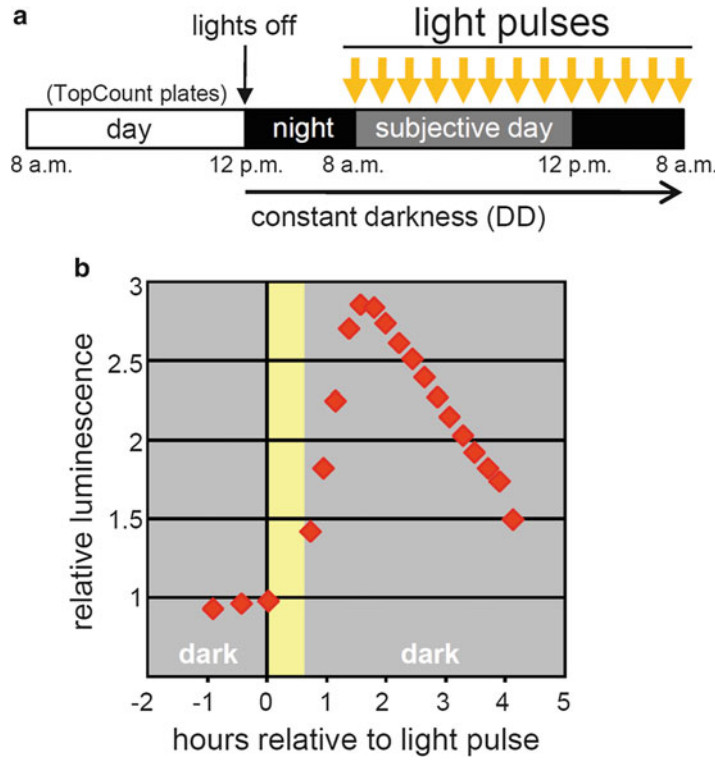

Fig. 2 Experimental design and logic of a gating assay. In this example light is the input signal and gene expression is the output. Gene expression is monitored with LUC activity measurements. (**a**) TopCount plates are prepared on the last day of the entrainment period. The plates must be put back in the growth chamber before lights off and experience the last environmental transition of the experiment (dusk). From this point onwards the seedlings will be released into constant darkness (DD) and will only be exposed to light at the time of the light pulses. The first light pulse is applied at the beginning of the next cycle, e.g., 8 a.m. if lights on was set at this time since the beginning of the experiment. Light pulses are applied to different batches of plants (one plate per light pulse) throughout the whole 24 h cycle. (**b**) The acute response of gene expression to each light pulse is monitored with high resolution LUC activity measurements during 4 h. At least one reference measurement is required in DD before the 30 min pulse

4 Notes

1. As the number of lines that can be included in a gating experiment is limited, we recommend the use of homozygous lines that have been established as reference lines beforehand. To generate the gene promoter:*LUC* constructs, we use a vector in which the *LUC* cDNA was previously fused to a GATEWAY® cassette. Plasmid details can be found in GeneBank (reference

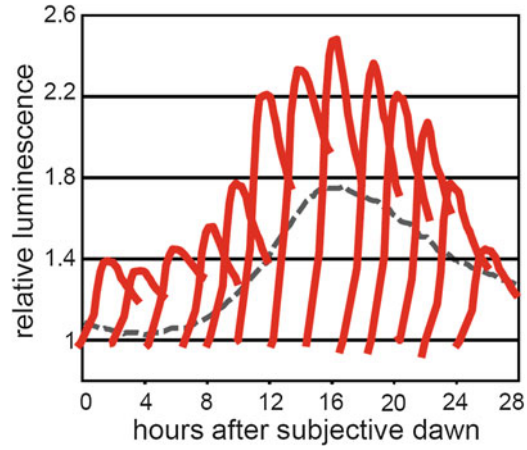

Fig. 3 Outcome of a gating experiment. The intensity of the acute response of gene expression (*red lines*) to the light pulses varies depending on the time of day. The maximum response of gene transcription to the light pulses is observed 16 h after the subjective morning, which also corresponds to the time when the gene reaches its peak of expression in constant darkness (DD control: *dashed line*). To compare the intensity of the response to each light pulse, LUC measurements are normalized to the luminescence measured before the pulse. Therefore, the scale of the acute response does not apply to the DD control whose presence on the graph only aims to show when the gene reaches its peak of expression in the control DD condition

number: AM295157). Promoters of interest are PCR-amplified and inserted in the vector with the GATEWAY® system following the recommendations of the manufacturer. The constructs are introduced into *Arabidopsis thaliana* via Agrobacterium mediated transformation using the floral dip method [17].

2. The stackers are hollow and vertical tower-like structures that are fixed to the TopCount machine and that are used to stack the TopCount plates in a pile. The use of stackers allows the automatic feeding of the plates to the TopCount machine during the experiment. Two stackers are necessary for the assay to be automated: the first stacker feeds the plates to the TopCount, and the second stacker stores the plates after the measurements. At the start of the experiment, all the plates must be placed in the first stacker. The plates are read one by one and progressively stored in the second stacker until the measurement of the last plate has been completed. A "stop plate" must be placed after the last "experimental plate" in the first stacker, at the top of the original pile, so that the TopCount knows that all the plates of the pile have been read. The "stop plate" is distinguishable by the presence of a double bar code instead of the single bar code used for the other plates. Once the double bar code has been read, all the plates are moved back from the

second stacker to the first stacker and the plate reading procedure starts again from the beginning. Always make sure that the stackers work before the beginning of an experiment as stacker defects are a typical TopCount failure.

3. The current protocol describes how to perform a gating experiment with a TopCount, but other alternatives such as the coupled charged device (CCD) camera are also suitable for quantifying LUC activity. Circadian biologists will normally use the TopCount as the system of choice if the throughput of an experiment needs to be high. However, there are advantages in the use of the CCD camera over the TopCount. CCD camera images provide spatial information on where the *promoter*:*LUC* construct is expressed in the seedling. LUC activity can also be measured in groups of plants, which generally tends to increase the quality of the curves and the precision of rhythmic data. Note that extracting data from the CCD camera is more complex, as it requires the use of additional software (Metamorph) to process the camera images and to convert the information from the images to luminescence data.
4. By default, we always add 1 % sucrose to the MS medium in our experiments. The presence of sucrose in the medium contributes to maintaining robust circadian rhythms in DD conditions which is particularly relevant for the protocol described in this chapter. Although studying circadian rhythms is not the goal of a gating assay, altered circadian rhythms due to lack of sucrose is expected to affect the outcome of the experiment. The sucrose concentration also affects circadian rhythms in continuous light [18].
5. Seedling development is influenced by the presence of neighboring plants, and isolated seedlings will likely respond differently to a light treatment than seedlings that have grown in dense clusters. Moreover, spacing the seedlings on the medium allows working with evenly developed individuals and will greatly facilitate the picking procedure.
6. Endogenous circadian rhythms vary depending on whether the plants were entrained in short days versus long days or in different temperature regimes. Changes in circadian rhythms between conditions are expected to shift the time window during which a gene strongly responds to a light pulse. Thus, the entraining condition will influence the outcome of a gating experiment and it is for the researcher to decide which condition is most relevant to his biological question.
7. On the last day of the entrainment period, the transfer of the seedlings from the petri dishes to the TopCount plates must be completed before lights off (dusk). Dusk is the last environmental transition that the seedlings will experience before the

light pulses, as the lights will not come on in the growth chamber in the following 24 h DD cycle. A fundamental property of the circadian clock is that it is reset by environmental transitions at dawn and dusk, meaning that endogenous circadian rhythms of the seedlings will be synchronized by the last resetting signal (dusk). If all the plates do not experience lights off at the same time, circadian rhythms will not be synchronized and the results of the gating assay will not be interpretable. This is usually not a problem if the plants were entrained in long day photoperiods as there will be plenty of time to complete the picking procedure. In short day photoperiods, less time will be available and the picking step must be planned accordingly.

8. We do not recommend placing the seeds directly in the TopCount plates after sterilization to try and skip the picking step. Germinating and growing the seedlings in a petri dish beforehand allows excluding from the experiment those seedlings that have not germinated or developed properly. Moreover, if the seeds germinate in the well of a TopCount plate the seedling will develop in an unfavorable environment with very little space for the root and aerial part of the plant to grow. Reduced light availability will also lead to etiolation of the seedlings which could have important consequences on the response to an environmental input.
9. The sensitivity to the light pulse will depend on the gene, and the response will also vary with light quantity, quality and with the duration of the pulse. We have proposed 30 min pulses in our protocol as an example but shorter pulses can also be applied, although reducing the duration of the pulse beyond a certain threshold will be expected to affect the magnitude of the response. Light pulses can be applied with fluorescent tubes that emit white light containing the whole spectrum of wavelengths, and also with LEDs that emit red, blue or far red monochromatic light. Note that fluorescent tubes can emit white light of more than 100 μmol m^{-2} s^{-1}, which is usually considered as high light intensity in standard growth chamber settings. Light intensities emitted by LEDs are usually lower than the ones obtained with fluorescent tubes.
10. The TopCounts used in our laboratory have six detectors, and when the reading time for each well is set to 2 s it takes the TopCount less than 2 min to a measure a full plate. To these 2 min have to be added the time that the TopCount needs (1) to move the plates from the first stacker to the detectors and from the detectors to the second stacker, (2) to move and measure the "stop plate," and (3) to reload the first stacker with all the plates (*see* **Note 2**). In total, the whole procedure with a pile of three plates takes 10–15 min. Two seconds of reading

time might not be sufficient for reporter constructs that yield low LUC activity. The reading time can be modified in the settings of the TopCount before the experiment and increased to, for example, 5 s. Longer reading times will of course reduce the temporal resolution of the assay. Importantly, do not introduce in the settings of the TopCount a temporal delay before the plate measurements as this is not useful in a gating experiment. Finally, the temporal resolution of an assay can also be enhanced with TopCounts that are equipped with twelve detectors instead of six. Note that in TopCount assays designed to study circadian rhythms in constant conditions, 1–2 h temporal resolution is usually sufficient.

11. In circadian studies, the most common application of LUC reporters is to measure circadian rhythms during several days in constant conditions of light and temperature where the circadian clock is not influenced by environmental transitions. Measuring circadian rhythms is fully automated because the experimental condition does not change throughout the experiment, and light can be constantly applied to the plants with a set of LED panels that are fixed to the stackers. Unfortunately, the experimental design of a gating assay cannot take advantage of fully automated measurements and of a light source fixed to the stackers. First, the number of plates in the stackers must be kept low to maintain the resolution of measurements high after the light pulses. Second, the LED panels fixed to the stackers would not allow selectively applying the light pulse to a specific plate. The gating protocol therefore requires a person to move the plates back and forth from the growth chambers to the TopCount during the entire 24 h cycle, including late at night and early in the morning.
12. We use a box to transport the plates from the chambers to the TopCount. Use a source of green light when manipulating plates in the dark.
13. Rhythmic data extracted from several days of measurements in constant conditions are analyzed with BRASS (www.amillar.org), an Excel workbook that has been extensively used by the plant circadian community. The aim of a gating experiment is not to measure rhythms, however, and BRASS is not strictly required to analyze the results of a gating assay. Nevertheless, the use of BRASS can be useful if changes in circadian rhythms are anticipated between two genotypes, as altered circadian rhythms will affect the outcome of the gating experiment. Circadian rhythms can be determined by leaving the DD control four extra days in DD conditions. Note that new tools have recently been developed to analyze circadian rhythms [19, 20].

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References

1. de Montaigu A, Toth R, Coupland G (2010) Plant development goes like clockwork. Trends Genet 26(7):296–306
2. Wang W, Barnaby JY, Tada Y, Li H, Tor M, Caldelari D, Lee DU, Fu XD, Dong X (2011) Timing of plant immune responses by a central circadian regulator. Nature 470(7332):110–114
3. Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL (2008) Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. Genome Biol 9(8):R130
4. Turner A, Beales J, Faure S, Dunford RP, Laurie DA (2005) The pseudo-response regulator Ppd-H1 provides adaptation to photoperiod in barley. Science 310(5750):1031–1034
5. Bohlenius H, Huang T, Charbonnel-Campaa L, Brunner AM, Jansson S, Strauss SH, Nilsson O (2006) CO/FT regulatory module controls timing of flowering and seasonal growth cessation in trees. Science 312(5776):1040–1043
6. Kloosterman B, Abelenda JA, Gomez Mdel M, Oortwijn M, de Boer JM, Kowitwanich K, Horvath BM, van Eck HJ, Smaczniak C, Prat S, Visser RG, Bachem CW (2013) Naturally occurring allele diversity allows potato cultivation in northern latitudes. Nature 495(7440):246–250
7. Millar AJ, Short SR, Chua NH, Kay SA (1992) A novel circadian phenotype based on firefly luciferase expression in transgenic plants. Plant Cell 4(9):1075–1087
8. Li G, Siddiqui H, Teng Y, Lin R, Wan XY, Li J, Lau OS, Ouyang X, Dai M, Wan J, Devlin PF, Deng XW, Wang H (2011) Coordinated transcriptional regulation underlying the circadian clock in *Arabidopsis*. Nat Cell Biol 13(5):616–622
9. Fowler SG, Cook D, Thomashow MF (2005) Low temperature induction of *Arabidopsis* CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol 137(3):961–968
10. Edwards KD, Akman OE, Knox K, Lumsden PJ, Thomson AW, Brown PE, Pokhilko A, Kozma-Bognar L, Nagy F, Rand DA, Millar AJ (2010) Quantitative analysis of regulatory flexibility under changing environmental conditions. Mol Syst Biol 6:424
11. Vieira J, Pinto da Silva L, Esteves da Silva JC (2012) Advances in the knowledge of light emission by firefly luciferin and oxyluciferin. J Photochem Photobiol B 117:33–39
12. Close D, Xu T, Smartt A, Rogers A, Crossley R, Price S, Ripp S, Sayler G (2012) The evolution of the bacterial luciferase gene cassette (lux) as a real-time bioreporter. Sensors (Basel) 12(1):732–752
13. Millar AJ, Kay SA (1996) Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in *Arabidopsis*. Proc Natl Acad Sci U S A 93(26):15491–15496
14. Strayer C, Oyama T, Schultz TF, Raman R, Somers DE, Mas P, Panda S, Kreps JA, Kay SA (2000) Cloning of the *Arabidopsis* clock gene TOC1, an autoregulatory response regulator homolog. Science 289(5480):768–771
15. Alabadi D, Oyama T, Yanovsky MJ, Harmon FG, Mas P, Kay SA (2001) Reciprocal regulation between TOC1 and LHY/CCA1 within the *Arabidopsis* circadian clock. Science 293(5531):880–883
16. Covington MF, Panda S, Liu XL, Strayer CA, Wagner DR, Kay SA (2001) ELF3 modulates resetting of the circadian clock in *Arabidopsis*. Plant Cell 13(6):1305–1315
17. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16(6):735–743
18. Haydon MJ, Mielczarek O, Robertson FC, Hubbard KE, Webb AA (2013) Photosynthetic entrainment of the *Arabidopsis thaliana* circadian clock. Nature 502(7473):689–692
19. Moore A, Zielinski T, Millar AJ (2014) Online period estimation and determination of rhythmicity in circadian data, using the BioDare data infrastructure. Methods Mol Biol 1158:13–44
20. Zielinski T, Moore AM, Troup E, Halliday KJ, Millar AJ (2014) Strengths and limitations of period estimation methods for circadian data. PLoS One 9(5):e96462

Chapter 10

Identification of *Arabidopsis* Transcriptional Regulators by Yeast One-Hybrid Screens Using a Transcription Factor ORFeome

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Abstract

Genetic and molecular approaches revealed that the circadian clock network structure is comprised of several interlocked positive and negative transcriptional feedback loops. The network evolved to sense and integrate inputs from environmental cues to adjust daily rhythms in physiological processes. Compiling evidence indicates that part of this regulation happens at the transcriptional level through subtle adjustments in the expression of core clock genes. Thus, to better understand the network and identify the molecular mechanisms of clock input pathways, it is imperative to determine how core clock genes are regulated. For this purpose we developed reagents for an unbiased approach to identify transcription factors (TFs) interacting with the promoters of core clock genes. At the center of this approach lies the yeast one-hybrid (Y1H) assay in which a pool of proteins fused to the GAL4 transcriptional activation domain are tested for their ability to interact with a selected promoter fragment in yeast cells. Taking advantage of the fact that *Arabidopsis* TF genes are well annotated, we generated a comprehensive TF clone collection (TF ORFeome) and used it to replace the standard cDNA pool strategy traditionally used in Y1H screens. The use of this TF clone collection substantially accelerates the comprehensive discovery of promoter-specific DNA binding activities among all *Arabidopsis* TFs. Considering that this strategy can be extended to the study of the promoter interactome of any *Arabidopsis* gene, we developed a low throughput protocol that can be universally implemented to screen the ~2000 TF clone library.

Key words Yeast one-hybrid, Transcription factor ORFeome, Circadian clock, *Cis*-regulatory network, Protein–DNA interaction, β-galactosidase reporter, Plant genomics

1 Introduction

Albeit developed more than 20 years ago, the yeast split GAL4 assay remains one of the most popular techniques to investigate protein–protein and protein–DNA interactions in vivo [1]. Some important features behind its popularity are its elegant simplicity and reliable results. Two fusion proteins, one containing the transcription activation domain of the GAL4 transcription factor (GAL4-AD) and the other its DNA binding domain, will be

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reconstituted into an active GAL4 only if their fusion partners have the capacity to interact with each other. Reconstitution of a functional GAL4 will then lead to binding and activation of a synthetic promoter containing a gal4 *cis*-element that regulates the expression of the β-galactosidase reporter gene (*lacZ*). Yeast one-hybrid (Y1H) is a derivative of the two-hybrid approach in which only proteins fused to the GAL4-AD are used and the promoter containing the gal4 *cis*-element is replaced by a promoter fragment of interest in the reporter construct. With this setup it is possible to determine if the protein in fusion with the GAL4-AD activates the reporter gene expression (i.e., *lacZ*), which is indicative of its interaction with the promoter region of interest.

In its original design, a cDNA library built from a tissue or cell extract of interest provided yeast one-hybrid preys (GAL4-AD protein fusions). The search was then limited by the presence of TFs in the prey library, which ultimately depends on the expression of TFs in the tissue and conditions used for isolation of the template RNA [2, 3]. Several technological advances allowed the Y1H assay to become an approach of choice for investigating transcriptional networks at a genomic scale. With the advent of genome sequencing and recombination-based cloning strategies, it is now possible to determine all TF encoding genes for a particular organism and to assemble genome-wide TF clone collections (TF ORFeome). For example, by combining several different prediction approaches, we recently estimated that the plant model *Arabidopsis thaliana* (*Arabidopsis*) contains at least 2492 TFs [4]. Taking advantage of the Gateway cloning technology, we assembled a collection of TF-GAL4-AD constructs for 1956 of these TFs [4]. Other Gateway compatible reagents for Y1H assays were previously developed [5]. By generating these reagents, it is now possible to use TF-specific libraries of Y1H prey clones, instead of cDNA libraries, to perform unbiased high-throughput Y1H screens.

Pioneer work in *C. elegans* laid the foundation for high throughput methods to perform TF-centered Y1H screens [6, 7]. Similar approaches were recently developed for other organisms such as plants (*A. thaliana*), flies (*D. melanogaster*), mice (*M. musculus*) and humans [8–11]. While Y1H is not the only alternative to gather information on TF–DNA interactions, it remains one of the few with the potential to be performed in a high-throughput mode. For example, it would be significantly challenging to use chromatin immunoprecipitation (ChIP) to determine TF–DNA interactions for each of the 2492 *Arabidopsis* TFs in all possible experimental conditions and tissues. Thus, Y1H constitutes a powerful alternative to start defining the cohort of TFs that interact with a specific gene promoter region. In fact, by using this approach we discovered a number of TFs that were proven to play an important regulatory role in the control of their associated promoters [8, 12–16].

We recently reported the construction of a comprehensive *Arabidopsis* TF clone collection suitable for Y1H screens [4]. Considering the universal availability of this clone collection, the current protocol was developed to provide a low cost and fast procedure that could be performed in any laboratory. Our hope is that it will provide the community with additional tools to expand the exploration of *Arabidopsis* transcriptional networks.

2 Materials

2.1 Yeast Media

1. Dextrose solution: 20 % dextrose solution filter-sterilized.
2. Adenine solution: 0.2 % adenine hemisulfate filter-sterilized.
3. YPDA medium: Dissolve 10 g Bacto peptone and 20 g yeast extract in 800 mL of water and adjust the pH to 5.8 with hydrochloric acid. Adjust the final volume to 885 mL with water and autoclave for 20 min (for petri dishes add 20 g/L of agar before autoclaving). Let the solution cool down and add 100 mL of 20 % dextrose and 15 mL of 0.2 % adenine solutions.

   4 L of liquid medium will be required to generate your TF library glycerol stock. Subsequently, 1.5 L of liquid medium and six petri dishes will be required for each library screen.
4. SD medium: Dissolve 6.7 g of yeast nitrogen base and the appropriate dropout (-Trp, -Ura/-Trp, or -Ura) in 800 mL of water, and adjust the pH to 5.8 with sodium hydroxide. Adjust the final volume to 900 mL with water and autoclave for 20 min (for petri dishes add 20 g/L of agar before autoclaving). Let the solution cool down and add 100 mL of 20 % dextrose solution.

   1.5 L of liquid medium and 84 petri dishes of SD-Trp medium will be required to generate your TF library glycerol stock. Subsequently, 1.2 L of SD-Trp and 1 L of SD-Trp/Ura liquid media and nine petri dishes of SD-Ura medium will be required for each library screen.

2.2 Yeast Transformation Solutions

1. Tris stock solution (1 M Tris–HCl pH 8.0). Sterilize before use.
2. EDTA stock solution (0.5 M EDTA pH 8.0). Sterilize before use.
3. LiAc stock solution (1 M LiAc). Filter-sterilize before use.
4. PEG stock solution (50 % polyethylene glycol 3350). For 500 mL, add water to 250 g of PEG 3350 and mix slowly with a stir bar (add enough water to solubilize while keeping the volume below 500 mL). After full dissolution (it takes several hours), stop stirring and let settle until all bubbles are gone. Complete volume to 500 mL and filter-sterilize.

5. 10× TE stock solution (100 mM Tris–HCl pH 8.0, 10 mM EDTA). For 1 L, mix 100 mL of 1 M Tris stock solution and 20 mL of 0.5 M EDTA stock solution. Adjust the volume to 1 L with water and sterilize in autoclave or by filtration.
6. 1× TE solution. For 1 L, mix 100 mL of 10× TE stock solution and 900 mL of sterile water.
7. TE–LiAc solution (1× TE and 0.1 M LiAc). For 10 mL, mix 1 mL of 10× TE, 1 mL of 1 M LiAc stock solution, and 8 mL of sterile water.
8. TE–LiAc–PEG solution (1× TE, 0.1 M LiAc, 40 % PEG). For 50 mL, mix 5 mL of 10× TE stock solution, 5 mL of 1 M LiAc, and 40 mL of 50 % PEG stock solution.
9. Salmon sperm DNA (10 mg/mL). For 100 mL, dissolve 1 g of salmon sperm DNA in 100 mL of water. Autoclave for 20 min and aliquot. Store at −20 °C.
10. PLATE solution (1× TE, 0.1 M LiAc, 40 % PEG). For 1 mL, mix 100 μL of 10× TE stock solution, 100 μL of 1 M LiAc stock solution, and 800 μL of PEG stock solution [17].

2.3 β-Galactosidase Assay Solutions

1. Z buffer (60 mM Na_2HPO_4, 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$). Adjust pH to 7. Around 1 L will be required per screen.
2. Z buffer/β-mercaptoethanol/ONPG solution. Prepare fresh. For 400 mL, dissolve 280 mg of ONPG (o-nitrophenyl β–d-galactopyranoside) in 400 mL of Z buffer (final concentration of 0.7 mg/mL). It may take several minutes to dissolve. When ready to use, add 688 μL of β-mercaptoethanol.

2.4 Strains, Plasmids, Primers, and 96-Well Plasticware

1. Yeast strain for promoter integration, YM4271 promoter strain (Clontech).
2. Yeast strain for TF library transformation, YU strain (Ura3-52 mutation restored in the Y187 strain—Clontech).
3. pEXPAD502 control plasmid (Life Technologies). To be transformed into the YU strain.
4. Gateway cloning genotyping primers:
   GATE-Fw: CCGCCCCCTTCACC;
   GATE-Rv: GTCGGCGCGCCCACCCTT.
5. Yeast genotyping primers:
   YGENO-Fw: TCTTCCTTCTGTTCGGAGATT;
   YGENO-Rv: GGACCTAATGTATAAGGAAAGAATA.
6. 96-well deep well plates (Plate One cat 1896-1000). 21 plates are required to generate the TF library glycerol stock. Afterward, 42 plates are required per screen.
7. 96-well PP plates (Greiner cat 781281). 84 plates are required to generate the TF library glycerol stock. Afterward, 21 plates are required per screen.
8. 96-well reading plates (Greiner cat 655101). 42 plates are required per screen.

3 Methods

3.1 Construction Reporter Plasmid

1. Select promoter fragment to be analyzed (*see* **Note 1**).
2. Design PCR primers for cloning into pENTR/D-TOPO (Life Technologies) (*see* **Note 2**).
3. Perform PCR reaction and purify PCR fragment from agarose gel.
4. Perform TOPO cloning reaction and transform into *E. coli* (plate cells on LB-kanamycin).
5. Perform colony PCR using GATE-Fw/-Rv primers on a few colonies.
6. Select one positive clone and start overnight culture.
7. Purify plasmid DNA and sequence using the universal M13 forward primer.
8. Perform LR clonase reaction to transfer the cloned promoter into pGLacZi [18] (Gateway-compatible version of the pLacZi plasmid—Clontech), and transform into *E. coli* (plate cells on LB-Ampicillin).
9. Perform colony PCR using GATE-Fw/-Rv primers on a few colonies.
10. Select one positive clone and start overnight culture.
11. Purify plasmid DNA and linearize the plasmid using the restriction enzymes *ApaI*, *EcoRV* or *NcoI*. Choose an enzyme that does not cut into your promoter fragment (*see* **Note 3**).

3.2 Construction of Yeast Reporter Strains

1. Streak YM4271 yeast strain on YPDA medium. Incubate 2–3 days at 30 °C. (Section adapted from Ref. 5).
2. Using a sterile toothpick, transfer a fresh 2–3 mm size colony into a 1.5 mL sterile microtube.
3. Add 10 μL carrier salmon sperm DNA (10 mg/mL).
4. Add 1 μg of the linearized pGlacZi [17] reporter plasmid DNA. Vortex well.
5. Add 500 μL of PLATE solution. Vortex briefly.
6. Add 57 μL of DMSO. Vortex briefly.
7. Incubate for 15 min at room temperature.
8. Heat shock cells for 15 min at 42 °C in water bath.
9. Pellet cells by centrifugation for a few seconds.
10. Remove supernatant without disturbing the pellet.
11. Resuspend pellet in 200 μL of sterile 1× TE.
12. Plate cells on SD-Ura medium and incubate at 30 °C (growth may take 3–5 days).
13. Pick four colonies and re-streak on SD-Ura medium. Incubate at 30 °C for 2 days.
14. Pick single colonies and re-streak on SD-Ura medium. Incubate at 30 °C for 2 days.
15. Pick single colonies and re-streak on YPDA medium. Incubate at 30 °C for 2 days.

16. Isolate genomic DNA from each reporter strain and assess reporter plasmid genomic integration by PCR using primers YGENO-Fw or promoter specific primer and YGENO-Rv (*see* **Note 4**).
17. Prepare glycerol stocks and store confirmed promoter strain at −80 °C.

3.3 Preparation of TF Library in Yeast (Glycerol Stocks)

1. Start a culture for each of the ~2000 plasmids from the pDEST22-TF clone collection and purify plasmid DNA (only ~100 ng is required from each clone in order to generate the yeast library stock) (Section adapted from ref *7*) (*see* **Notes 5** and **6**).
2. Start a 5 mL culture of the YU strain in YPDA medium.
3. Inoculate 500 mL of YPDA medium with 1 mL of the overnight yeast culture.
4. The next morning inoculate 3 L of YPDA with 500 mL of the yeast culture.
5. Grow the culture until OD_{600} reaches 0.4–0.8 (3–5 h).
6. Centrifuge culture at 1000 × *g* for 5 min (room temperature).
7. Discard supernatant and resuspend pellet in 500 mL of sterile water.
8. Centrifuge at 1000 × *g* for 5 min (room temperature).
9. Discard supernatant and resuspend in 100 mL of TE–LiAc.
10. Centrifuge at 1000 × *g* for 5 min (room temperature).
11. Discard supernatant and resuspend with 40 mL of TE–LiAc and 4 mL of salmon sperm DNA (10 mg/mL).
12. Distribute 20 μL of cells per well of 21 × 96-well PP plates (*see* **Note 7**).
13. Transfer 100 ng of the pDEST22-TF clone collection plasmid DNA into the 96-well PP plates (*see* **Note 8**).
14. Add 100 μL of TE–LiAc–PEG solution per well (*see* **Note 9**).
15. Incubate plates 20–30 min at 30 °C.
16. Heat shock yeast cells for 20 min at 42 °C (*see* **Note 10**).
17. Centrifuge the cells for 5 min at 1000 × *g* (room temperature).
18. Discard supernatant using a multichannel pipette without disturbing the cell pellet.
19. Add 110 μL of 1× TE into each well.
20. Centrifuge 5 min at 1000 × *g* (room temperature).
21. Remove 100 μL of supernatant using a multichannel pipette. This should leave just enough TE for plating the cells.
22. Resuspend the pellet by pipetting using a multichannel pipette or using a 48-well cell replicator (frogger) (*see* **Note 11**).

23. Using a 48-well cell replicator or a multichannel pipette, transfer an array of 48 wells onto SD-Trp plates (*see* **Note 12**).
24. Incubate at 30 °C for 2–3 days.
25. Fill sterile 96-well PP plates with 50 μL of sterile 1× TE buffer (*see* **Note 13**).
26. Using a 48-well cell replicator or multichannel pipette, transfer a fraction of the transformed yeast cells into the 96-well PP plates.
27. Carefully mix and using a 48-well cell replicator or multichannel pipette transfer an aliquot of the resuspended cells onto a new SD-Trp plate.
28. Incubate at 30 °C for 2–3 days.
29. Fill sterile 96-deep well plates with 500 μL of SD-Trp medium per well.
30. Fill sterile 96-well PP plates with 50 μL of sterile 1× TE buffer.
31. Using a 48-well cell replicator or multichannel pipette transfer a fraction of the transformed yeast cells into the 96-well PP plates.
32. Mix well and using a multichannel pipette, transfer 5 μL of the resuspended cells into the 96-deep well plates.
33. Incubate at 30 °C for 36–48 h with agitation (800 rpm) using a microplate shaker.
34. Using a multichannel pipette add 500 μL of 50 % glycerol into each well in order to obtain a final concentration of 25 % glycerol in each well.
35. Mix well and aliquot into 96-well PP plates to generate multiple glycerol stock copies of each yeast TF library plate.
36. Seal 96-well PP yeast TF library plates with aluminum plate seal and store at −80 °C.

3.4 Perform Yeast One-Hybrid Screens

1. Streak the YM4271 promoter strain on YPDA medium. Incubate 2–3 days at 30 °C.
2. Thaw glycerol stocks for one copy of the yeast TF library.
3. Fill sterile 96-deep well plates with 600 μL of SD-Trp medium per well.
4. Using a multichannel pipette transfer 5 μL of the yeast TF library into the 96-deep well plates. Seal plates with breathable seal and incubate at 30 °C for 24–36 h with agitation (600 rpm) using a microplate shaker.
5. Add 50 mL of YPDA into a 250 mL flask and inoculate with the YM4271 promoter strain. Incubate overnight at 30 °C with agitation (180 rpm) (*see* **Note 14**).

6. Mix 25 mL of the overnight promoter strain culture with 75 mL of fresh YPDA medium.
7. Fill sterile 96-well PP plates with 90 μL of YPDA medium per well.
8. Using a multichannel pipette add 10 μL of the library strain into each well.
9. Pour the diluted promoter strain culture into a sterile pipetting reservoir. Using a multichannel pipette add 40 μL of the cell suspension into each well.
10. Seal 96-well PP plates with a breathable seal and incubate for 12–48 h at 30 °C (no shaking needed).
11. Centrifuge 96-well PP plates for 3 min at 1000 × *g* (room temperature).
12. Remove 140 μL of the supernatant.
13. Add 120 μL of SD-Trp/Ura into each well and carefully vortex or mix by pipetting.
14. Centrifuge 96-well PP plates for 3 min at 1000 × *g* (room temperature).
15. Remove 120 μL of the supernatant.
16. Add 180 μL of SD-Trp/Ura into each well.
17. Fill a new 96-deep well plate with 100 μL of SD-Trp/Ura medium per well.
18. Using a multichannel pipette transfer 3 μL of the diploid cell suspension in the 96-well PP plates into the 96-deep well plates.
19. Shake plates in a microplate shaker (600–800 rpm) at 30 °C for 24–36 h.

3.5 Quantification of β-Galactosidase Activity

1. Add 400 μL of YPDA into each well of the 96-deep well plates.
2. Continue shaking in a microplate shaker (600–800 rpm) at 30 °C for 5–6 h.
3. Mix well. Using a multichannel pipette transfer a 120 μL aliquot of each well into a 96-well reading plate (*see* **Note 15**).
4. Determine the optical density (OD) at 600 nm using a 96-well plate absorbance reader.
5. Centrifuge 96-deep well plates for 3 min at 1000 × *g* (room temperature).
6. Remove supernatant (~450 μL).
7. Add 150 μL of Z buffer into each well.
8. Centrifuge 96-deep well plates for 3 min at 1000 × *g* (room temperature).
9. Remove supernatant (~150 μL).

10. Add 25 μL of Z buffer into each well (*see* **Note 16**).
11. Perform four freeze–thaw cycles. Each cycle consists of incubations for 3 min in liquid nitrogen and 5–10 min at 30–37 °C (*see* **Notes 17** and **18**).
12. Add 170 μL of Z buffer/BME/ONPG solution.
13. Incubate until yellow color is developed (*see* **Note 19**).
14. Add 80 μL of 1 M Na_2CO_3 to stop the enzymatic reaction.
15. Centrifuge deep well plates for 8 min at 1000 × *g* (room temperature).
16. Using a multichannel pipette, take a 120 μL aliquot from the supernatant without disturbing the pellet, and transfer into a 96-well reading plate.
17. Determine the OD at 420 nm using a 96-well plate absorbance reader.
18. Calculate the β-galactosidase enzymatic activity using the following equation:
    $[OD_{420} \times 1000] / [OD_{600} \times \text{time(s)} \times \text{volume(mL)}]$ (*see* **Note 20**).
19. Normalize the β-galactosidase activity obtained in each well to the β-galactosidase activity obtained in control wells (YU yeast strain transformed with the control plasmid).

4 Notes

1. We typically select 250–400 bp fragments. Overlapping fragments should be selected when performing promoter scanning (hiking). To date, no exhaustive analysis has been performed to determine the optimal promoter size for Y1H screens. However, we found that a TCP transcription factor induces the expression of the β-galactosidase reporter when the promoter bait contains a TCP binding site located at 100 nucleotides but not when the same site is located at 500 nucleotides upstream of the transcription start site [4].
2. For small DNA fragments, such as promoter fragments in the 250 nucleotides range, we find that TOPO cloning (Life Technologies) is a simple and efficient approach. pENTR/D--TOPO allows directional cloning if the CACC sequence is added to the 5′ end of the forward primer used to amplify the promoter fragment. As an alternative, the promoter fragment can be directly cloned into the pLacZi plasmid (Clontech) using restriction endonuclease digestion.
3. While plasmid linearization improves the efficiency of the reporter plasmid genomic integration, it is also possible to generate the reporter strains using an intact reporter plasmid.

4. The promoter fragment carried by the YM4271 reporter strain can be further confirmed by sequencing this PCR product
5. We typically used the PureLink 96 plasmid purification system (Life Technologies); however, due to the minimal DNA amount required for yeast transformation other low cost options could also be used [19–21].
6. We grow cultures in 96-deep well plates using microplate shakers (VWR) with a 3 mm orbit, housed in a bacterial incubator.
7. Yeasts cells have the tendency to settle quickly. Be careful and agitate the suspension as often as possible.
8. An example of the screen setup was provided in Pruneda-Paz, Breton et al. [4], which includes empty wells and control plasmid wells.
9. This solution is very viscous. Pay great care when dispensing the solution. It is imperative to properly mix the plasmid and yeast at this point. We carefully mix the content of every well by pipetting up/down at least three times (this is the longest step of the whole procedure).
10. When transforming the entire pDEST22-TF clone collection into yeast (21×96-well plates) we use a bacterial incubator at 42 °C in order to have enough space to process all plates simultaneously.
11. Cell replicators (froggers) are available from several companies (i.e., V&P scientific, Sigma-Aldrich). In our experience the ones that use metal screws instead of plain metal pins give the best performance as the cupped end of screws hold around 5–10 μL of liquid.
12. It is important to dry the plate surface and lid well (we usually leave open plates next to a flame for several minutes prior to cell transfer). Moisture in the plate surface or plate lid will usually lead to cross contamination of yeast cells in adjacent wells.
13. We found that replating the cells after resuspending them in TE provides the best way to generate a TF library master stock with a homogeneous and comparable cell density in each well.
14. The two cultures in points 4 and 5 have to be coordinated so that both incubations finish at the same time.
15. Avoid dispensing the last drop when transferring into the reading plate in order to avoid the presence of bubbles that will affect the readings.
16. At this point, plates can be sealed (aluminum foil) and stored at −80 °C.
17. After the last cycle let plates warm up to room temperature (this step can be speed up if the last 30–37 °C incubation step

after the last incubation in liquid nitrogen is extended to 10–15 min).

18. Plates will float in liquid nitrogen or water. This step can be performed in sets of 2–3 plates using a small Styrofoam box filled with liquid nitrogen and a regular size water bath. To increment the throughput of this step a large steel wire grid basket can be used to accommodate between 9 and 12 × 96-well plates. The basket containing 96-well plates is transferred in/out of a larger sized liquid nitrogen container and 30–37 °C water bath.
19. We usually do it at 30 °C but this step could be faster if performed at 37 °C.
20. Volume: volume of cells used to perform the β-galactosidase assay; Time: incubation time until the β-galactosidase reaction is stopped.

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References

1. Fields S, Song O (1989) A novel genetic system to detect protein-protein interactions. Nature 340:245–246
2. Wang MM, Reed RR (1993) Molecular cloning of the olfactory neuronal transcription factor Olf-1 by genetic selection in yeast. Nature 364:121–126
3. Li JJ, Herskowitz I (1993) Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science 262:1870–1874
4. Pruneda-Paz JL, Breton G, Nagel D et al (2014) A genome-scale resource for the functional characterization of *Arabidopsis* transcription factors. Cell Rep 8(2):622–632
5. Deplancke B, Dupuy D, Vidal M et al (2004) A gateway-compatible yeast one-hybrid system. Genome Res 14:2093–2101
6. Deplancke B, Vermeirssen V, Arda HE et al (2006) Gateway-compatible yeast one-hybrid screens. CSH Protoc. doi:10.1101/pdb.prot4590
7. Reece-Hoyes JS, Walhout AJ (2012) Gene-centered yeast one-hybrid assays. Methods Mol Biol 812:189–208
8. Pruneda-Paz JL, Breton G, Para A, Kay SA (2009) A functional genomics approach reveals CHE as a component of the *Arabidopsis* circadian clock. Science 323:1481–1485
9. Hens K, Feuz JD, Isakova A et al (2011) Automated protein-DNA interaction screening of Drosophila regulatory elements. Nat Methods 8:1065–1070
10. Gubelmann C, Waszak SM, Isakova A et al (2013) A yeast one-hybrid and microfluidics-based pipeline to map mammalian gene regulatory networks. Mol Syst Biol 9:682
11. Reece-Hoyes JS, Barutcu AR, McCord RP et al (2011) Yeast one-hybrid assays for gene-centered human gene regulatory network mapping. Nat Methods 8:1050–1052
12. Walhout AJ (2011) What does biologically meaningful mean? A perspective on gene regulatory network validation. Genome Biol 12:109

13. Li L, Ljung K, Breton G et al (2012) Linking photoreceptor excitation to changes in plant architecture. Genes Dev 26:785–790
14. Niwa M, Daimon Y, Kurotani K et al (2013) BRANCHED1 interacts with FLOWERING LOCUS T to repress the floral transition of the axillary meristems in *Arabidopsis*. Plant Cell 25:1228–1242
15. Ito S, Song YH, Josephson-Day AR et al (2012) FLOWERING BHLH transcriptional activators control expression of the photoperiodic flowering regulator CONSTANS in *Arabidopsis*. Proc Natl Acad Sci U S A 109: 3582–3587
16. Chow BY, Sanchez SE, Breton G et al (2014) Transcriptional regulation of LUX by CBF1 mediates cold input to the circadian clock in *Arabidopsis*. Curr Biol 24(13):1518–1524
17. Burke D, Dawson D Steams T (2000) Methods in yeast genetics: a Cold Spring Harbor Laboratory Course Manual. pp 205
18. Helfer A, Nusinow DA, Chow BY et al (2011) LUX ARRHYTHMO encodes a nighttime repressor of circadian gene expression in the *Arabidopsis* core clock. Curr Biol 21:126–133
19. Li JF, Sheen J (2012) DNA purification from multiple sources in plant research with home-made silica resins. Methods Mol Biol 862:53–59
20. Lezin G, Kosaka Y, Yost HJ (2011) A one-step miniprep for the isolation of plasmid DNA and lambda phage particles. PLoS One 6:e23457
21. Marra MA, Kucaba TA, Hillier LW (1999) High-throughput plasmid DNA purification for 3 cents per sample. Nucleic Acids Res 27:e37

Chapter 11

Monitoring Alternative Splicing Changes in *Arabidopsis* Circadian Clock Genes

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Abstract

Posttranscriptional control makes an important contribution to circadian regulation of gene expression. In higher plants, alternative splicing is particularly prevalent upon abiotic and biotic stress and in the circadian system. Here we describe in detail a high-resolution reverse transcription-PCR based panel (HR RT-PCR) to monitor alternative splicing events. The use of the panel allows the quantification of changes in the proportion of splice isoforms between different samples, e.g., different time points, different tissues, genotypes, ecotypes, or treatments.

Key words Alternative splicing, Circadian rhythm, RNA-binding proteins, Splicing factors

1 Introduction

Alternative splicing is a regulatory mechanism in the cell that can generate a plethora of transcript variants from one and the same pre-mRNA [1]. It deviates in several ways from constitutive splicing, which removes all introns and joins the flanking exons. During alternative splicing, exons can be removed together with flanking introns, designated exon skipping, or introns can remain in the mRNA, generally designated intron retention. The intron removed can also vary due to the use of alternative 5′ or 3′ splice sites. The usage of the splice sites is dictated by *cis*-acting motifs located in the mRNAs that serve as recognition sites for RNA-binding proteins and accessory factors involved in the splicing process.

Alternative splicing has major consequences for the resulting mRNA variants: The encoded proteins can be composed of distinct domains, producing more than one protein isoform from the same gene. These protein isoforms can thus vary in their function, the interaction with other proteins or their subcellular localization.

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Furthermore, alternative splice isoforms can be recognized as "aberrant" and degraded, thus ultimately changing transcript abundance [2].

Conventional microarrays like the Affymetrix AG or ATH1 GeneChip rely on hybridization of labeled probes to exonic sequences and do not discriminate between alternative splice isoforms [3, 4]. The use of TILING arrays monitors expression of both coding and noncoding regions. A study using TILING arrays detected introns that showed a circadian rhythm in efficiency of splicing [5]. In many cases, they were in phase with adjacent rhythmic exons. Thus, the transcript with the retained intron likely would give rise to a truncated protein variant. Some genes displayed rhythmic introns where exon expression was arrhythmic, suggesting that the corresponding alternative splicing events are controlled by the circadian clock.

At the same time, we developed a medium-throughput alternative splicing panel based on Reverse Transcription-PCR and separation of fluorescently labeled amplicons by capillary sequencing [6]. This panel measures the proportion of alternatively spliced products with great precision. The procedure can detect statistically significant alternative splicing changes of a few percent between different samples. In its initial version, the panel comprised 90 primer pairs to detect alternative splice events in 89 genes. Subsequently, it has been expanded to around 350 primer combinations, detecting alternative splicing events in nearly 300 genes.

The panel has been widely used in the plant community to demonstrate alternative splicing in different plant organs or in plants grown under different light regimes [6]. Moreover, the analysis of mutants defective in candidate splicing regulators, e.g., serine–arginine rich proteins or the subunits of the CAP binding complex, as well as transgenic plants overexpressing hnRNP (heterogeneous nuclear ribonucleoproteins)—like proteins has demonstrated the global impact of these regulators on plant alternative splicing [6–8]. Furthermore, natural variation in alternative splicing and changes in alternative splicing patterns upon changes in ambient temperature of a few degrees were found [8, 9].

More recently, Next Generation Sequencing (NGS) pointed to 61 % of all *Arabidopsis* genes being alternatively spliced [10, 11]. NGS clearly has the advantage of allowing global *de novo* detection of transcript variants compared to strategies based on RT-PCR. Nevertheless, the HR RT-PCR panel proved superior in detecting small differences that are statistically different. In addition, NGS-based assays require significant specialized bioinformatics support and are more expensive.

Plants, like most organisms, employ an endogenous timekeeping device, the circadian clock, to coordinate physiological, biochemical, and developmental processes with the day–night cycle [12, 13]. Clock proteins build an auto-regulatory feedback loop

and generate their own 24-h rhythm through inhibiting transcription of their own genes.

Recently, a suite of genes encoding core components of the circadian clock have been shown to undergo alternative splicing [10, 14, 15]. Moreover, the *prmt5* mutant defective in PROTEIN ARGININE N-METHYLTRANSFERASE 5 that posttranslationally modifies splicing factors has been found to have a long period phenotype [16]. TILING arrays had uncovered 471 introns out of a total of 67,791 that were retained in the *prmt5* mutant. Subsequently, the use of the HR RT-PCR panel detected a widespread effect on alternative splicing in this mutant [16]. Another mutation within the putative RNA binding protein SPLICEOSOMAL TIMEKEEPER LOCUS1 shows a long period phenotype [17]. Again, the HR RT-PCR panel showed that many introns were spliced less efficiently in the mutant [17]. In a reverse genetic approach, the hnRNP-like RNA-binding protein *At*GRP7 has been shown to be part of a negative feedback loop controlled by the circadian clock [18, 19]. This negative autoregulation occurs by alternative splicing. *At*GRP7 also affects alternative splicing of numerous downstream targets some of which undergo circadian oscillations themselves [8].

2 Materials

2.1 Plant Growth

1. A list of *Arabidopsis* seed stock centers is available at The *Arabidopsis* Information Resource (TAIR). http://www.arabidopsis.org/portals/mutants/stockcenters.jsp.
2. Soil consists of four parts potting compost, three parts vermiculite, and two parts perlite mix.
3. 0.5× MS 20 agar medium: Mix 2.2 g/l Murashige and Skoog medium, 20 g/l sucrose, and 8 g/l agar pH to 5.8 with 1 M NaOH in distilled water. Sterilize by autoclave and pour medium into 9 cm petri plates.
4. Gamborg's medium: Mix 3.2 g/l Gamborg's B5 salts and minimal organics, 1 ml/l 1000× Gamborg's vitamins, 0.5 g/l MES and 3 % (w/v) sucrose. Bring to pH 5.9 with 1 M KOH.

2.2 RNA Isolation and cDNA Synthesis

Qiagen RNeasy Mini Kit (50) (Cat No. 74104).
Sigma Aldrich RNAzol® RT (Cat No. R4533).

1. Solution D: Mix 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5 % (w/v) sarkosyl, and 0.1 M 2-mercaptoethanol.
2. Water-saturated phenol: Dissolve 100 g of solid phenol at 40 °C for 1 h. Add an equal volume of distilled water and mix. An aqueous layer should be present above the saturated phenol. If not, add more water. Store at 4 °C in a dark bottle.

3. Chloroform–isoamyl alcohol: Mix chloroform and isoamyl alcohol at a ratio of 24:1.
4. TE Buffer: 10 mM Tris and 1 mM EDTA pH 8 HCl.
5. Promega RQ1 RNase-Free DNase (Cat No. M6101).
6. Promega RNasin (Cat No. N2611).
7. Phenol–chloroform: Dissolve 250 g phenol with 100 ml Tris–HCl pH 8 at 40 °C for 1 h. Add an equal volume of chloroform mixed and store at 4 °C in a dark bottle.
8. Ethanol–sodium acetate pH 4.8 mix: Mix 19 vol. 100 % ethanol with 1 vol. 3 M sodium acetate pH 4.8.
9. Thermo Scientific, NanoDrop 2000 UV–Vis spectrophotometer.
10. Clontech RNA to cDNA EcoDry double primed premix (Cat No. 639549).

2.3 PCR Reagents

1. Roche Taq DNA polymerase and 10× Buffer (Cat No. 1 146 173).
2. Promega dNTPs (Cat No. U1240)—Dilute 100 mM stock of each deoxynucleotide (dATP, dCTP, dGTP, and dTTP) to 20 mM. Mix each nucleotide together with sterile distilled water to produce a 1.25 mM working dNTP stock.
3. Primers to detect alternative splicing events (MWG—Eurofin)—Dilute 100 μM stock to 20 μM.
4. PCR plates (Thermo-Fast 96, Semi-skirted. Millipore Cat No. AB-0900).

2.4 Product Separation

1. 500 or 1200 LIZ Size standard (ABI Cat No. 4322682) for reproducible sizing of RT-PCR fragments. The 500 marker contains 16 single-stranded labeled fragments of different lengths and the 1200 marker contains 68 single-stranded labeled fragments of different lengths.
2. Hi Di Formamide (ABI Cat No. 4311320).

2.5 Software

1. Applied Biosystems. GeneMapper v3.7 or above.
2. Microsoft Excel.
3. Genstat or R statistical analysis software.

3 Methods

3.1 Plant Growth

The plant growth protocols in this chapter describe the growth and use of *Arabidopsis*. Other plant species will have different growth requirements. Below are three methods of growing *Arabidopsis* plants that depend on plant testing conditions (*see* **Note 1**).

3.1.1 Growth in Soil

Sow selected *Arabidopsis* seed in 17 × 20 cm trays containing water-saturated soil (*see* **Note 2**). Place trays in black bags and vernalize seeds in the dark at 4 °C for 3 days. Grow seed in the glasshouse or under controlled conditions at a standard temperature of 22 °C and 16 h light–8 h dark cycle (*see* **Note 3**).

3.1.2 Growth on Agar

Sterilize around 15 mg of selected *Arabidopsis* seed with 0.6 % sodium hypochlorite for 5 min followed by multiple washes with distilled sterile water. Sow seed onto 0.5 × MS agar medium plates and vernalize the seed in the dark at 4 °C for 3 days. Grow seedlings under controlled conditions at 22 °C in a 16 h light–8 h dark cycle.

3.1.3 Growth in Liquid

Sterilize around 15 mg of selected *Arabidopsis* seeds with 0.6 % sodium hypochlorite for 5 min, followed by multiple washes with distilled sterile water. Grow seedlings in a 250-ml flask containing 25 ml of Gamborg's medium. Germinate seeds in the liquid medium with shaking. After germination (around 3–4 days) the seedlings remain on the surface of the medium and form rafts of plantlets where the roots are immersed but shoots are above the surface of the medium. Grow seedlings at 22 °C in a 16 h light–8 h dark cycle and with vigorous shaking (around 120 rpm) for 19 days (from seed germination). Exchange Gamborg's medium every 2 days after the seeds have germinated.

3.2 Gene Selection

Any gene that shows alternative splicing may be tested for changes in splicing. The alternative splicing panel continues to add more primer pairs covering many alternative splicing events as further genes are tested. For our studies, genes selected focused on RNA binding and processing factors, transcription factors, genes involved in phosphorylation/dephosphorylation and genes that have roles in temperature stress, pathogen response and circadian rhythm. Rubisco activase (At2g39730) was the first plant gene identified to show alternative splicing [20]. This gene is highly expressed and produces two transcripts through alternative splicing that are often found in an approximately 1:1 ratio. This alternative splicing event is included as an alternative splicing control. At5g25760 (Ubiquitin-conjugating enzyme 21) and At1g13320 (Protein phosphatase 2A subunit A3) are commonly used in our analyses as two transcriptional controls to normalize transcription to steady state levels.

Alternative splicing analysis is done in a 96-well format and genes are selected on the basis of multiples of this number and the biological question under study. For example, circadian clock genes and their alternative splicing events are included in a splicing analysis of circadian rhythm along with other genes not directly involved to produce two plates of 96 alternative splicing events.

3.3 Primer Design

Primers are designed by selection of sequences within exon sequences upstream and downstream of the alternative splicing event(s). To study intron retention events, primers are designed

across a constitutively spliced intron and the alternatively retained intron (*see* **Note 4).** This was done to avoid false positive intron retention results that may occur due to contaminating DNA. Select primers that are 19-25 nt long, about 50 % GC and produce spliced PCR products that are less than either 750 or 1200 bp in length, depending on the 500 or 1200 bp size marker used in the sequencer run. Design both 5′ and 3′ primers with one or two G or C nucleotides at their 5′ and 3′ ends to avoid primer dimerization. Primers are screened against the *Arabidopsis* genome to avoid primers that have a perfect match with the target sequence. The 5′ forward primer is labeled at its 5′ end with a 6-FAM (6-carboxyfluorescein) fluorescent dye (*see* **Note 5**).

3.4 RNA Extraction

1. Extract up to 100 mg of any selected plant tissue but, for circadian rhythm analysis, collect samples over a time course between light and dark.
2. Extract RNA using available RNA extraction kits. We use the RNeasy Plant Mini Kit (Qiagen Cat No. 74904) or RNAzol® RT following the manufacturer's instructions (*see* Quiagen RNeasy manual; Sigma Aldrich protocol). Alternatively, extract high quality total RNA using the single step RNA isolation method using acid guanidinium thiocyanate–phenol–chloroform [21]. Briefly, suspend 100 mg of ground plant material in 2 ml Solution D. Shake samples vigorously for 5 min at room temperature. The solution is acidified by the addition of 3 M sodium acetate pH 4 to 200 mM. Add an equal volume of water-saturated phenol along with 0.2 vol. of chloroform–isoamyl alcohol and mix vigorously for 1 min. Leave samples to stand on ice for 15 min. After centrifugation at 3,200 × *g* in a Eppendorf 5804 centrifuge for 15 min at room temperature, remove the aqueous phase to a fresh tub, precipitate RNA in an equal volume of isopropyl alcohol and leave at –20 °C for 1 h. Pellet RNA at maximum centrifuge rotor speed for 15 min and the wash the pellet with 70 % ethanol at –20 °C. Air-dry the RNA pellet and resuspend in sterile distilled water at a concentration of 1 μg/μl.
3. Determine RNA concentrations by NanoDrop (Thermo Scientific).
4. RNA extraction kits leave very little contaminating DNA but nevertheless can be picked up by sensitive PCR. Our alternative splicing analysis does not usually take into consideration any unspliced RNA products that will produce the same PCR product size as contaminating DNA. If the alternative splicing analysis needs to consider unspliced products, RNA preparations are further treated with RQ1 DNase to remove remaining DNA. A maximum of 50 μg of RNA (50 μl) is added to TE Buffer, 10 mM $MgCl_2$, 100 U RNasin, and 10 U of RQ1 DNase in a final volume of 100 μl. Incubate the reaction at 37 °C for 20 min

and terminate the reaction by adding 1/50th volume of 0.5 M EDTA pH 8 and 1/50th volume 10 % (w/v) SDS. Extract RNA with an equal volume of phenol–chloroform pH 8 mix and precipitate with 2.5 vol. of ethanol–sodium acetate pH 4.8 held at −20 °C. After pelleting by centrifugation the RNA pellets are washed with 70 % ethanol at −20 °C, air-dried, and resuspended at a concentration of 1 μg/μl (*see* **Note 6**).

3.5 First Strand cDNA Synthesis

1. Add 5 μg of total RNA (enough for 100 PCR reactions, i.e., one 96 well-plate) to sterile distilled water to a volume of 20 μl.
2. Transfer the sample to one microfuge tube supplied which contains the "RNA to cDNA EcoDry double primed" first strand cDNA synthesis bead and leave at room temperature for 1 min (*see* **Note 7**).
3. Gently mix the sample with the end of a pipette, spin briefly in a microfuge to collect the sample, and incubate at 37 °C for 1 h at 70 °C for 10 min. Add sterile distilled water to give a final volume of 100 μl.

3.6 PCR

1. Prepare a 100× PCR reaction mix as follows:

	×1 (μl)	×100 (μl)
10× buffer	2.5	250
1.25 mM dNTPs (200 μM[a])	4	400
Taq DNA polymerase	0.125	12.5
SDW	16.375	1637.5

[a]200 μM is the final dNTP concentration

2. For a 96-well plate reaction, add the complete first strand reaction mix to the 100× PCR reaction mix. Add 24 μl of this mix to each well of a 96-well plate containing 1 μl of the 96 different forward and reverse gene-specific primers (400 nM per primer per reaction). This gives a total PCR reaction volume of 25 μl in each well.
3. Mix the samples by vortex, spin briefly to collect the samples on the bottom of the well, and place on a PCR machine (Perkin Elmer 9700) using the following cycle program:

1 cycle	94 °C—2 min
24 cycles (*see* **Note 8**)	94 °C—15 s
	50 °C—30 s
	70 °C—1 min
1 cycle	70 °C—10 min

4. Store at 4 °C until ready to use.

3.7 Separation and Analysis of the Spliced Products

1. Mix the labeled RT-PCR products from the RT–PCR reactions with Hi Di Formamide (Applied Biosystems) and the selected LIZ labeled size marker (Applied Biosystems). For the 96 reactions in the 96-well plate, prepare the following mix depending on the size marker selected:

	×1 (μl)	×100 (μl)
500 LIZ Size standard	0.05	5
Hi Di formamide	8.95	895
1200 LIZ size standard	0.5	50
Hi Di formamide	8.5	850

2. Aliquot 9 μl of the mix into each of the 96 wells of a 96 well-plate and add 1 μl of each purified RT-PCR reaction. Store the remaining sample at −20 °C for downstream cloning and sequencing if required.
3. Inject samples, separate by capillary electrophoresis, and detect on the ABI3730 DNA Analyzer (Life Technologies). Set up the platform for fragment analysis using a 36-cm capillary array, POP7 polymer, and dye filter set G5. Run samples containing the LIZ 500 marker using the manufacturer's "GeneMapper36_POP7" Run Module (run time 1200 s, run voltage 15 KV). Samples containing the LIZ 1200 marker are run using the "3730_36cm_POP7_GS1200Lizv2_1" Run Module (run time 6000 s, run voltage 6.1 KV). Subsequently, the peak (RT-PCR product) sizes and areas are calculated and analyzed with Life Technologies GeneMapper v3.7 or the freely available PeakScanner software v1 (Life Technologies) (*see* **Note 9**).
4. RT-PCR products are accurately identified with ±1–2 bp resolution. Extract the relative fluorescent peak areas for RT-PCR products with expected sizes for the alternatively spliced products and tabulate in Microsoft Excel (Table 1) (*see* **Note 10**).

3.8 Basic Statistics

1. Calculate the proportion of the different alternative splicing events by dividing the value for each alternatively spliced product by the sum of the values for all the alternatively spliced products.
2. For an accurate statistical measurement of alternative splicing proportions, three biological repeats are routinely performed for all experiments. Mean alternative splicing proportions with standard deviations and standard errors are calculated for the three separate biological repetitions (*see* **Note 11**).
3. For each alternatively spliced transcript, analysis of variance (ANOVA) is used in turn to compare all the treatments after an angular transformation of the individual alternative splicing

Table 1
Extracted RT-PCR product length and peak areas for a single primer pair across two temperatures

Sample file name	Length found	Peak area	Proportion	Mean of three repeats	SD	SE	p
	(bp)	(RFU)					
20 °C							
292_wt_zt0_20_Rep1	165.91	635027	0.995	0.996	0.0007	0.0004	
292_wt_zt0_20_Rep1	247.42	2828	0.005	0.004	0.0007	0.0004	
292_wt_zt0_20_Rep2	166.08	439095	0.997				
292_wt_zt0_20_Rep2	247.42	1383	0.003				
292_wt_zt0_20_Rep3	166.09	489588	0.997				
292_wt_zt0_20_Rep3	247.36	1690	0.003				
4 °C							
292_wt_zt0_4_Rep1	165.11	681192	0.86	0.865	0.0057	0.003	0.0000007
292_wt_zt0_4_Rep1	247.4	111658	0.14	0.135	0.0057	0.003	
292_wt_zt0_4_Rep2	166.01	621365	0.87				
292_wt_zt0_4_Rep2	248.2	96697	0.13				
292_wt_zt0_4_Rep3	165.98	621493	0.87				
292_wt_zt0_4_Rep3	247.84	92383	0.13				

Sample file name indicates primer number, wild type plant, Zeitgeber time (*see* **Note 14**), temperature, and the repeat number
SD = Standard deviation = $\sqrt{\sum_{i=1}^{n}(x_i-\bar{x})^2/(n-1)}$ where n is the number of repeats, xi is the individual value and $\bar{x}$ is the mean of the repeats
SE = Standard error of mean. SE = SD/$\sqrt{}$n where n is the number of repeats
p = significance value from the analysis of variance F test
Analysis of variance compares 20 °C with 4 °C

proportions (*see* **Note 12**). Specifically, means are compared between wild type plants and the different treatments, stresses or mutant plant. The ANOVA assumes a completely randomized experimental design. The significance of the differences between treatments is determined using least significant differences. Alternative splicing events with significant variation ($p = <0.05$) are routinely selected (*see* **Note 13**).

3.9 Example

A HR RT-PCR analysis is shown for a single primer pair that covers the alternative splicing event found in the key circadian clock gene *LATE ELONGATED HYPOCOTYL* (*LHY*) (At1g01060). Alternative exon inclusion occurs at the long intron, which adds a premature termination codon (PTC) (Fig. 1a). The difference in length between the two transcripts is 82 nt and HR RT-PCR

identified the two HR RT-PCR products as 166 and 248 bp in length (Fig. 1b). An experiment was done on *Arabidopsis* plants which were grown at 20 °C and subsequently transferred to 4 °C for 4 days in three biological replicates [13]. RNA was extracted from plants at ZT 0, the time of maximal LHY expression (*see* **Note 14**), followed by HR RT-PCR and capillary electrophoresis to identify the different transcripts. Collected data on RT-PCR product length and peak area were extracted and tabulated for the three repeats and the proportions determined (Table 1). Means of the proportions were determined over the three repeats, followed by standard deviations, standard errors and ANOVA (Table 1). A graph of the data shows the significant proportionate increase in alternative exon inclusion and the truncated form of the protein at the lower temperature (Fig 1c).

4 Notes

1. Alternative splicing is affected at different developmental stages and tissues. It is also affected by environmental abiotic and biotic stresses and circadian times. High-resolution RT-PCR alternative splicing analysis is highly sensitive to these changes. It is therefore important that biological repeat plants including control plants are grown at the same time and in the same conditions with the exception of the condition tested.
2. The rate of seed sown is dependent on the mass or tissue that is required. Eight individual plants may be grown in the 17×20 cm seed tray. The rate of seed sown may be increased substantially to produce high leaf mass. Individual plants may be grown in 10-cm diameter pots with the same soil mix as described or in water-saturated Jiffy-7®-peat pellets (Jiffy International AS, Kristiansand, Norway).
3. Be aware of any local or country control measure requirements for the growth of transgenic plants.
4. PCR is very sensitive and despite careful removal of contaminating DNA, enough DNA may remain to produce products that are indistinguishable from unspliced pre-mRNA. To overcome this, retained intron event primers are designed across a constitutively spliced intron upstream or downstream of the retained intron. Retained intron spliced products are therefore easily distinguished from the unspliced products, which would result from retention of both introns.
5. Different fluorescent standard dye sets are available for genotyping applications. We have successfully used 6-FAM labeled primers in association with size markers labeled with ROX (6-Carboxyl-X-Rhodamine) or the proprietary LIZ label. As

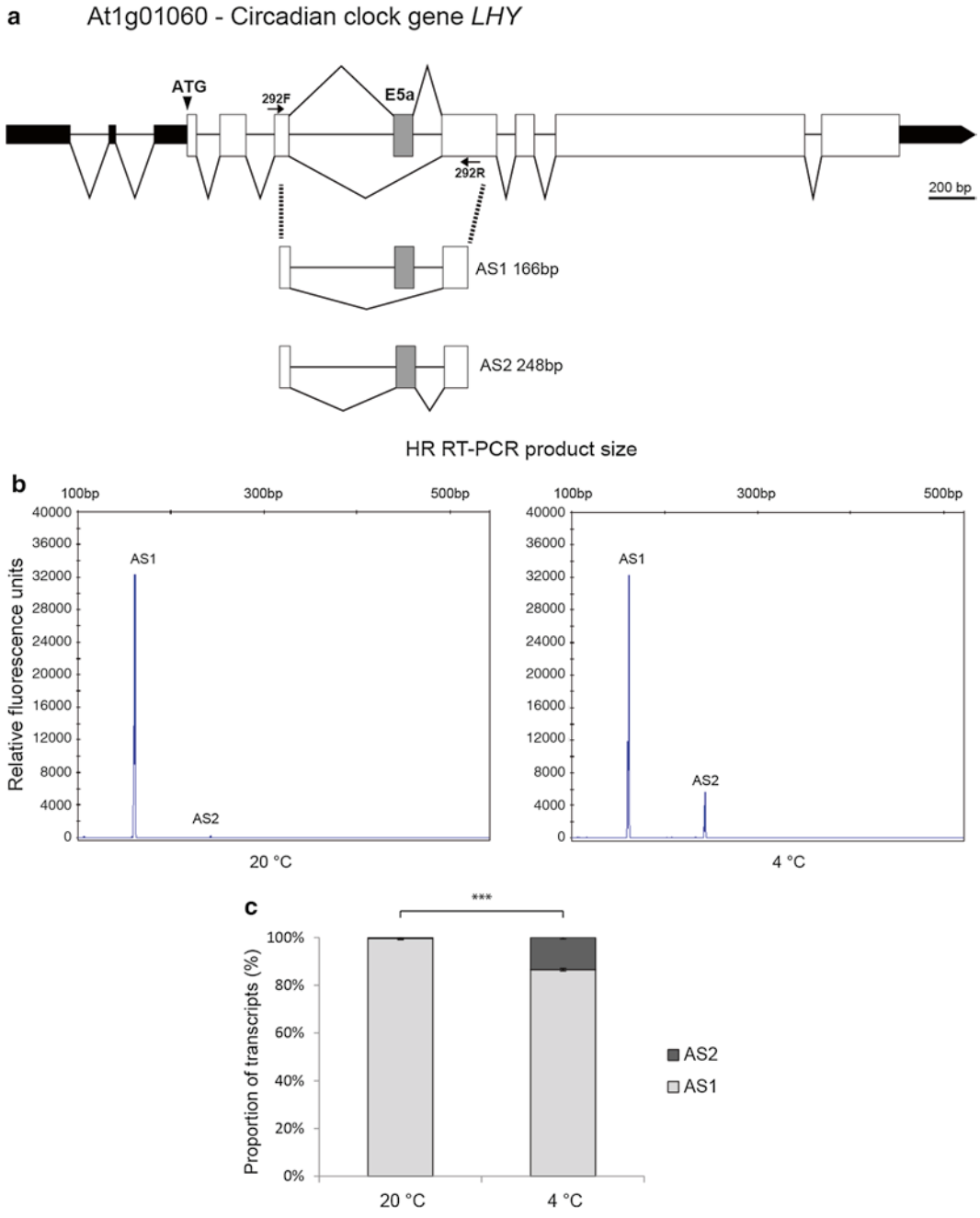

Fig. 1 High resolution RT-PCR alternative splicing analysis of a single alternative splicing event. (**a**) Schematic representation of the circadian clock gene *LHY* (At1g01060). Coding regions are shown as an *open box*, untranslated regions are shown as a *black box* and the introns are shown as a *line*. The alternative exon inclusion event is indicated and the products of HR RT-PCR with primer pair 292 (*arrowed*) are shown. (**b**) Electropherograms showing the output from the sequencer. The HR RT-PCR products are identified as peaks AS1 and AS2 (see (**a**)). The *X*-axis indicates length of HR RT-PCR product in base pairs (bp) and the *Y*-axis indicates the relative fluorescence units. Results are shown for the splicing analysis of plants exposed to different temperatures (°C). (**c**) Graph indicates the proportion of spliced products (AS1—*light grey bar* and AS2—*dark grey bar*) expressed as a percentage (%) across the two temperatures (°C). Error bars represent SD. *** statistical significance $p = <0.001$

these are used as size markers we avoid labeling primers with these fluorescent labels. We have further attempted to multiplex alternative splicing analysis using HEX (6-carboxy-1,4-dichloro-2′,4′,5′,7′-tetrachlorofluorescein) labeled primers. Depending on the amounts of transcripts made, we found overlap in the emission spectra between 6-FAM and HEX labeled primers, which led to the presence of unexpected peaks in the other labeled RT-PCR products and confused downstream analysis. We have returned to single fluorescent dye label analysis.

6. In some cases DNA may still remain after a single DNase treatment. A second DNase treatment will remove any remaining DNA.
7. The RNA to cDNA EcoDry Double primed cDNA synthesis beads contain both oligo dT and random hexamers. We have found an improvement in RT-PCR peaks using this double primed system.
8. 24 cycles was selected on the basis that PCR is in the logarithmic phase of amplification and allows us to screen both highly and more poorly transcribed genes. We previously tested the variation in alternative splicing results in technical replicates and the majority showed less than 1 % variability with a small number reaching up to 3 %. We, therefore, set 3 % variation as a cut off value for statistically significant changes in alternative splicing (*see* **Note 13**). Samples taken at >24 cycles showed that the more highly expressed genes were moving out of the exponential phase of PCR amplification [22].
9. GeneMapper software is constantly updated. Check Life Technologies website for updates (https://www.lifetechnologies.com/uk/en/home.html).
10. The nature of alternative splicing means that you may obtain expected peaks (RT-PCR products) that vary substantially in their peak areas. The GeneMapper software may be set to select a minimum peak size and reduce the number of small background peak areas that may be extracted and tabulated. However, we set the peak level low (200 relative fluorescent units) to capture as many of the alternatively spliced products as possible. This table, therefore, requires editing to remove background noise peaks.
11. The HR RT-PCR alternative splicing analysis measures changes in the proportions of the different alternative spliced transcripts found. Although, between the repeats, there may be apparent changes in the amounts of transcript produced, the proportion of the different alternatively spliced transcripts is maintained.
12. An angular transformation is often used with proportions to bring the data closer to a normal distribution. It is performed

on the raw proportions before any statistical analysis is done. If p is the proportion then the transformation is $x = \arcsin(\sqrt{p})$.

13. In a number of cases where there is very little variation between the repeats, the standard errors may be very low and even small changes in the proportion of splicing may be identified as statistically significant with $p = <0.05$. We select examples that are statistically significant and show >3 % splicing change (*see* **Note 8**).
14. Zeitgeber time (ZT) is a standardized scheme for a 24-h entrained circadian cycle in which ZT 0 indicates the beginning of day (light phase) and ZT 12 is the beginning of night (dark phase).

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References

1. Reddy ASN, Marquez Y, Kalyna M et al (2013) Complexity of the alternative splicing landscape in plants. Plant Cell 25:3657–3683
2. Staiger D, Brown JWS (2013) Alternative splicing at the intersection of biological timing, development, and stress responses. Plant Cell 25:3640–3656
3. Streitner C, Hennig L, Korneli C et al (2010) Global transcript profiling of transgenic plants constitutively overexpressing the RNA-binding protein AtGRP7. BMC Plant Biol 10:221
4. Hennig L, Menges M, Murray JA et al (2003) *Arabidopsis* transcript profiling on Affymetrix GeneChip arrays. Plant Mol Biol 53:457–465
5. Hazen SP, Naef F, Quisel T et al (2009) Exploring the transcriptional landscape of plant circadian rhythms using genome tiling arrays. Genome Biol. 10: R17.
6. Simpson CG, Fuller J, Maronova M et al (2008) Monitoring changes in alternative precursor messenger RNA splicing in multiple gene transcripts. Plant J 53:1035–1048
7. Raczynska KD, Simpson CG, Ciesiolka A et al (2010) Involvement of the nuclear cap-binding protein complex in alternative splicing in *Arabidopsis thaliana*. Nucleic Acids Res 38: 265–278
8. Streitner C, Köster T, Simpson CG et al (2012) An hnRNP-like RNA-binding protein affects alternative splicing by in vivo interaction with target transcripts in *Arabidopsis thaliana*. Nucleic Acids Res 40:11240–11255
9. Streitner C, Simpson CG, Shaw P et al (2013) Small changes in ambient temperature affect alternative splicing in *Arabidopsis thaliana*. Plant Signal Behav 8:e24638
10. Filichkin SA, Priest HD, Givan SA et al (2010) Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. Genome Res 20:45–58
11. Marquez Y, Brown JWS, Simpson CG et al (2012) Transcriptome survey reveals increased complexity of the alternative splicing landscape in *Arabidopsis*. Genome Res 22:1184–1195
12. Staiger D, Shin J, Johansson M et al (2013) The circadian clock goes genomic. Genome Biol 14:208
13. Hsu PY, Harmer SL (2014) Wheels within wheels: the plant circadian system. Trends Plant Sci 19(4):240–9

14. James AB, Syed NH, Bordage S et al (2012) Alternative splicing mediates responses of the *Arabidopsis* circadian clock to temperature changes. Plant Cell 24:961–981
15. James A, Syed N, Brown J et al (2012) Thermoplasticity in the plant circadian clock: How plants tell the time-perature. Plant Signal Behav 7:1219–1223
16. Sanchez SE, Petrillo E, Beckwith EJ et al (2010) A methyl transferase links the circadian clock to the regulation of alternative splicing. Nature 468:112–116
17. Jones MA, Williams BA, McNicol J et al (2012) Mutation of *Arabidopsis* SPLICEOSOMAL TIMEKEEPER LOCUS1 causes circadian clock defects. Plant Cell 24:4907–4916
18. Staiger D, Heintzen C (1999) The circadian system of *Arabidopsis thaliana*: forward and reverse genetic approaches. Chronobiol Int 16:1–16
19. Schmal C, Reimann P, Staiger D (2013) A circadian clock-regulated toggle switch explains AtGRP7 and AtGRP8 oscillations in *Arabidopsis thaliana*. PLoS Comput Biol 9:e1002986
20. Werneke JM, Chatfield JM, Ogren WL (1989) Alternative mRNA splicing generates the two ribulosebisphosphate carboxylase/oxygenase activase peptides in spinach and *Arabidopsis*. Plant Cell 1:815–825
21. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
22. Kim SH, Koroleva OA, Lewandowska D et al (2009) Aberrant mRNA transcripts and the nonsense-mediated decay proteins UPF2 and UPF3 are enriched in the nucleolus. Plant Cell 21:2045–2057

Chapter 12

Assessing the Impact of Photosynthetic Sugars on the *Arabidopsis* Circadian Clock

Michael J. Haydon and Alex A.R. Webb

Abstract

Circadian clocks drive 24 h biological rhythms to optimize physiology and development in response to the rotation of the planet. In plants, photosynthesis is modulated by the circadian clock and contributes to daily rhythms in cellular metabolism. In addition to light and temperature, sugar produced from photosynthesis acts as a *zeitgeber* to contribute to setting of the plant circadian clock. Here, we describe methods to manipulate photosynthetic output and sugar availability in *Arabidopsis* seedlings. These protocols have been applied to investigate the effects on the *Arabidopsis* circadian network, but are easily adaptable to other processes in plants.

Key words *Arabidopsis*, Circadian clock, Photosynthesis, Sugars, Luciferase, CO_2-free air, DCMU

1 Introduction

Circadian clocks are 24 h molecular timekeeping mechanisms that allow organisms to anticipate daily and seasonal rhythms in the environment associated with the rotation of the Earth. Circadian clocks drive rhythmic patterns in physiology, development, and metabolism to confer a fitness advantage [1, 2]. Circadian clocks comprise a molecular oscillator, consisting of multiple, interlocking transcription–translation regulatory feedback loops and associated posttranslational events including protein–protein interactions, targeted protein degradation and regulatory signaling events. In order to match physiology and behavior with the environment, the oscillator is set to daily cues, called *zeitgebers*, in the process of entrainment. Light is well-established as a key entraining signal in most organisms [3], but other cues can also act at *zeitgebers* [4]. Temperature cycles contribute to entrainment [5], as well as metabolic cues such as feeding regimes in animals [6, 7] or nutrient assimilation in plants [8, 9].

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As photoautotrophs, plants produce sugars in a light-dependent manner by photosynthesis. Photosynthesis is modulated by the circadian clock [2, 10, 11] and there are daily oscillations in the endogenous concentrations of soluble sugars in plants [12, 13]. Effects of sugars on the circadian network in the model plant *Arabidopsis thaliana* had been largely overlooked until relatively recently because the standard media composition used in the *Arabidopsis* circadian research community contained 3 % (w/v; 90 mM) sucrose [14]. When added to growth media, sugars can shorten circadian period in continuous light [9, 15] and sustain circadian rhythms in continuous dark [16]. In addition, endogenous rhythms of sugars produced from photosynthesis have been shown to act as a *zeitgeber* in *Arabidopsis* to entrain the clock to a "metabolic dawn" [9] and a mobile photosynthate, most likely sucrose, was proposed to drive a slave oscillator in *Arabidopsis* roots [17]. Thus, rhythmic sugar metabolism has a profound impact on the plant circadian network.

Here, we describe methods that manipulate sugar availability in *Arabidopsis*, which we used to define the effects of photosynthates on the circadian network [9]. We describe two methods to inhibit photosynthesis to abolish rhythmic production of sugars: chemical inhibition of Photosystem II with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU); and growth of plants in CO_2-free air. In addition, we describe a method to provide pulses of exogenous sugars to *Arabidopsis* seedlings. The methods are described in the context of luminescence imaging of transgenic luciferase reporter lines because this is a technically specialized technique requiring specific considerations. However, the methods described can be used for other quantitative methods. These protocols have been successfully applied for quantitative RT-PCR and metabolite measurements [9] and similar techniques have been used to describe a role for photosynthates in photomorphogenic root development [18]. We used these protocols to understand roles of photosynthetically derived sugars, but inhibiting photosynthesis also has other effects, such as on reactive oxygen species production and on the plastoquinone pool. With appropriate controls, these aspects of photosynthesis could also be investigated using these protocols.

2 Materials

2.1 Plant Growth

1. Transgenic *A. thaliana* seeds carrying a circadian promoter: luciferase reporter such as CCA1:LUC+, PRR9:LUC+, PRR7:LUC+, GI:LUC+, or TOC1:LUC+ [9]. These lines and others are available in various genetic backgrounds from seed stock centers or directly from the research community.
2. 100 × 100 mm square petri plates.

3. 25-well 100 × 100 mm square petri plates.
4. Imaging rings: clear PVC tubing (<7 mm external diameter), cut into <10 mm length and sterilized by autoclave.
5. Black PVC insulation tape, 19 mm diameter.
6. 0.2 mL PCR tube strips.
7. 1 μm nylon mesh.
8. 2-part epoxy resin.

2.2 Media and Chemicals

1. ½ MS media: 0.215 g MS salts, 0.8 g agar per 100 mL deionized H_2O. Autoclave to sterilize.
2. 20 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) stock solution: 47 mg DCMU in 10 mL ethanol.
3. 100 mM d-luciferin potassium salt stock solution: 100 mg in 3.14 mL sterile deionized H_2O. Prepare in dim light and store 100 μL aliquots wrapped in aluminum foil at −80 °C.
4. Self-indicating soda lime.

2.3 CO_2-Free Air Apparatus

1. Aquarium air pump: minimum flow 300 l/h, 0.19 mbar.
2. PVC tubing, various diameters.
3. Empty gas purifier column (*see* **Note 1**).
4. 500 mL plastic screw cap bottle.
5. 0.45 μm sterile filter.

2.4 Photon-Counting Camera

1. A plant luminescence imaging system, comprising a CCD camera mounted in a light-tight cabinet fitted with programmable LED arrays for both blue (660 nm) and red (470 nm) light. The cabinet must also permit light-tight input of a gas line. Two possible systems are the Berthold NightSHADE LB985 or Photek ICCD225. The imaging system should be housed in a temperature-controlled dark room, fitted with green light.

3 Methods

3.1 Inhibition of Photosynthesis with DCMU

1. Surface-sterilize and sow seeds in clusters of 5–10 seeds (*see* **Note 2**) on ½ MS media without added sucrose in square petri plates. Chill plates in the dark at 4 °C for 2 days before transfer to growth cabinets with light–dark cycles. Light should be supplied at 50–100 μmol m^{-2} s^{-1}.
2. Prepare an imaging plate by covering borders between wells of a 25-well square petri plate with black PVC tape and sterilize with 70 % (v/v) ethanol in a laminar flow bench. When dry, add 2 mL ½ MS media to each well, including control media, media containing 20 μM DCMU, and media containing

DCMU supplemented with sugars (*see* **Note 3**). When agar is set, place 4 imaging rings into each well using sterilized forceps.

3. Transfer clusters of seedlings (5–10-day old) to each ring using sterile toothpicks at around dusk (*see* **Note 4**).
4. In dim light, or in green light after dusk, add 50 µl of 1 mM d-luciferin to each ring. Repeat before dawn, 12 h later.
5. Commence luminescence imaging at dawn. Check light intensity of LED arrays using a Quantum (PAR) sensor. Program LED lights according to the desired light regime (*see* **Note 5**). Images are captured at 1 h intervals.

3.2 Inhibition of Photosynthesis in CO_2-Free Air

1. Surface-sterilize and sow seeds in clusters of 5–10 seeds (*see* **Note 2**) on ½ MS media without added sucrose in square petri plates. Chill plates at 4 °C for 2 days before transfer to growth cabinets with light–dark cycles. Light should be at 50–100 µmol m^{-2} s^{-1}.
2. Prepare two imaging plates by covering borders between wells of a 25-well square petri plate with black PVC tape and sterilize with 70 % (v/v) ethanol in a laminar flow bench. When dry, add 2 mL ½ MS media to each well including media supplemented with sugars (*see* **Note 3**). When agar is set, place 4 imaging rings into each well using sterilized forceps. Using a sharp, flame-hot metal instrument such as scissors or forceps, pierce two holes in the lid of one petri plate, ~5 mm diameter, to accommodate a gas inlet and outlet for the CO_2-free air treatment. The other plate will be used as an ambient air control plate.
3. To prepare the apparatus for CO_2-free air (Fig. 1), connect the aquarium pump to a column, filled with indicating soda lime, with PVC tubing. The CO_2-free air from the outlet of the column of soda lime is passed via a 0.45 µm syringe filter into an air-tight bottle of autoclaved H_2O. The humidified, CO_2-free air is collected from the top of the bottle by connecting an outlet line of sufficient length to pass into the light-tight imaging cabinet and connect to the imaging plate (*see* **Note 6**).
4. In dim light, or green light before dawn, add 50 µl of 1 mM d-luciferin to each seedling cluster.
5. Transfer clusters of seedlings (5–10 days old) to each ring using sterile toothpicks at around dusk, and repeat d-luciferin treatment in dim light or in green light after dusk.
6. At around dusk, turn on pump and connect humidified CO2-free air outlet to the inlet of the prepared imaging plate using a cut 200 µl tip. Ensure an air-tight seal at the inlet and between the lid and the plate using PVC insulation tape. Run the pump

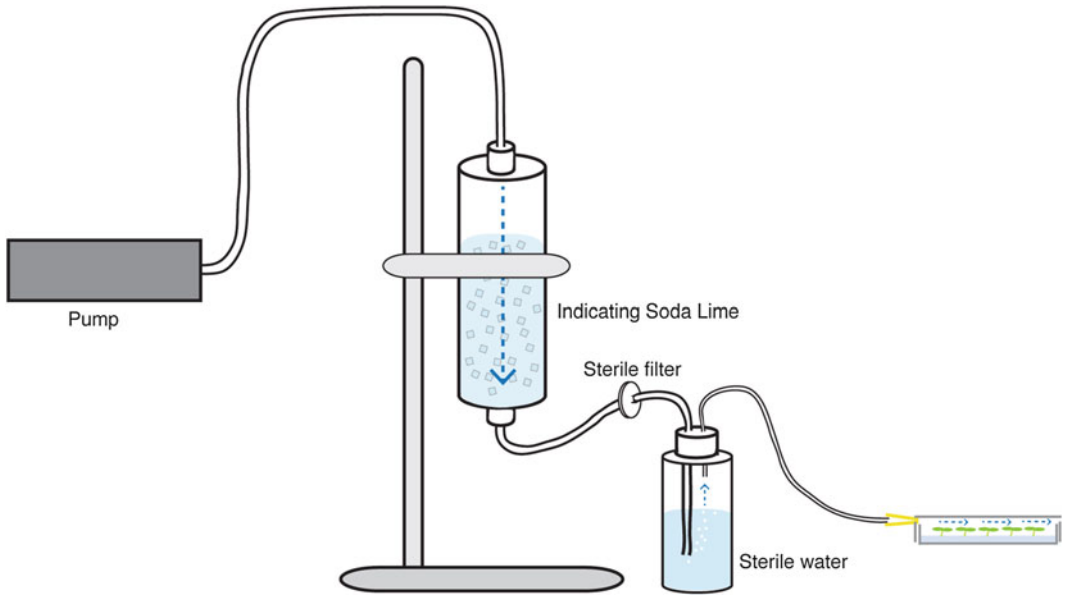

Fig. 1 Apparatus for generation of CO_2-free air. Ambient air is pumped through indicating soda lime to remove CO_2. Filtered, CO_2-free air is humidified by passing through sterile H_2O and into a sealed petri plate containing *Arabidopsis* seedlings on solid media

overnight to ensure removal of CO_2 from the sterile water and the imaging plate (*see* **Note 4**).

7. Commence luminescence imaging at dawn of the CO_2-free air treatment plate and the ambient air control plate. Check light intensity of LED arrays using a Quantum (PAR) sensor. Program LED lights according to the desired light regime (*see* **Note 5**). Images are captured at 1 h intervals.

3.3 Short-Term Addition of Exogenous Sugars

1. Prepare transfer rafts: Cut the bottom half from 0.2 mL PCR tube strips and then cut strips into pairs of tubes. Fix the inverted rims onto 1 μm nylon mesh with 2-part epoxy resin to form 4-well rafts (Fig. 2). When dry, trim excess mesh with scissors so that the rafts fit in wells of a 25-well square petri plate and sterilize with 70 % (v/v) ethanol in a laminar flow bench.
2. Prepare an imaging plate by covering borders between wells of a 25-well square petri plate with black PVC tape and sterilize with 70 % (v/v) ethanol in a laminar flow bench. When dry, add 2 mL of ½ MS media without added sucrose to each well.
3. Surface-sterilize and sow seeds in clusters of 5–10 seeds (*see* **Note 2**) in wells of transfer rafts placed on ½ MS media without added sucrose in imaging plates. Chill plates in the dark at 4 °C for 2 days before transfer to growth cabinets with light–dark cycles. Light should be at 50–100 μmol m^{-2} s^{-1}.

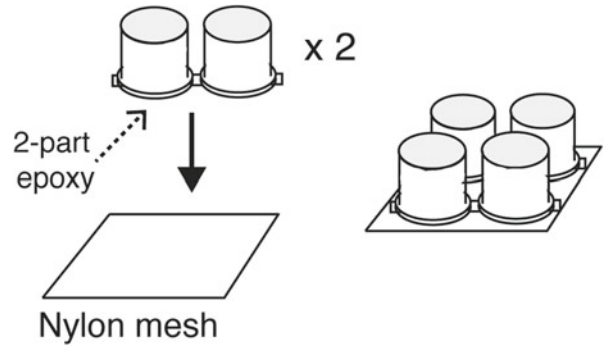

Fig. 2 Preparation of transfer rafts for short-term treatments. Two pairs of cut 0.2 ml PCR tubes are fixed, inverted, onto 1 μm nylon mesh using 2-part epoxy resin to create a 4-well transfer raft. The rafts are designed to fit into a compartment of square 25-well petri plates

4. Within 24 h of the start of imaging, dose plants twice with d-luciferin. In dim light or in green light, add 50 μl of 1 mM d-luciferin to each well and repeat around 12 h later.
5. Commence luminescence imaging at dawn. Check light intensity of LED arrays using a Quantum (PAR) sensor. Program LED lights according to the desired light regime (*see* **Note 5**). Images are captured at 1 h intervals.
6. Provide pulses of sugars at the desired concentration and duration by transferring rafts to sterilized liquid media, supplemented with treatments (e.g., sucrose) or osmotic controls (e.g., mannitol) immediately after an image capture. At the end of the pulse, wash briefly in liquid media without sugars and return to the imaging plate.

4 Notes

1. We use an empty In-Line 100 psig Gas Purifier column (Alltech). Alternatively a simple, low pressure column could be fashioned by cutting the base from two plastic bottles and joining with an air-tight seal.
2. For luminescence imaging, we routinely sow seeds in clusters. This ensures good luminescence signal intensity and robust data. The number of seeds in each cluster can be altered depending on the expected signal of the reporter in the experimental conditions.
3. The effects of DCMU or CO_2-free air on the circadian clock were suppressed by addition of sucrose to the media [9]. We added sucrose, glucose, fructose, mannitol, or 3-O-methyl glucose to media at 90 mM because this was appropriate context

for the circadian research community, but sucrose media concentrations as low as 5 mM suppressed the effects of DCMU on circadian rhythms in *Arabidopsis* [9].

4. For inhibition of photosynthesis, we transferred seedlings to treatment conditions at dusk, rather than at dawn, because we reasoned that inhibition of photosynthesis would have little effect in the night, but would ensure that there was minimal activation of photosynthesis at dawn. This is supported by detection of similar concentrations of soluble sugars in *Arabidopsis* seedlings at dawn after treatment overnight [9] and that DCMU has no effect on growth of seedlings germinated in the dark (unpublished data).
5. The standard light regime for entrainment in the Webb and Haydon labs is 12 h light–12 h dark, but short days (8 h light) or long days (16 h) could be used. For all circadian experiments testing the effects of inhibition of photosynthesis, entrainment continued for 2 days after treatment before release into continuous light [9]. In continuous light, circadian period lengthens with decreasing light quantity [19], so choice of light conditions is important in this context. We favored conducting free run experiments under low light quantity (~10 μmol m^{-2} s^{-1}) because the effects of exogenous sugar on circadian period are more pronounced than under higher light quantity (>50 μmol m^{-2} s^{-1}) but the effect of inhibition of photosynthesis on circadian period was similar in either condition [9]. Choice of light conditions is flexible, but Photosynthetically Active Radiation (PAR) should always be monitored with a Quantum sensor.
6. It is of utmost importance that the apparatus to generate CO2-free air (Fig. 2) is a closed, air-tight system to achieve good air flow. Ensure that all connections are secure, using PVC insulation tape, if necessary. Test air flow by bubbling the output tube in a beaker of water. To ensure positive pressure, sequentially reduce the diameter of tubing through the system. CO_2 concentration of the air coming from outlet should be close to zero and can be confirmed by measurement with an infra-red gas analyzer (e.g., ADC Gas Analysis Ltd.).

References

1. Woelfle MA, Ouyang Y, Phanvijhitsiri K, Johnson CH (2004) The adaptive value of circadian clocks : an experimental assessment in Cyanobacteria. Curr Biol 14:1481–1486
2. Dodd AN, Salathia N, Hall A et al (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309:630–633
3. Johnson CH (1992) Phase response curves: what can they tell us about circadian clocks? In: Hiroshige T, Honma K (eds) Circadian clocks from cell to Human. Hokkaido Univ. Press, pp 209–249
4. Haydon MJ, Hearn TJ, Bell LJ et al (2013) Metabolic regulation of circadian clocks. Semin Cell Dev Biol 24:414–421

5. Rensing L, Ruoff P (2002) Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. Chronobiol Int 19:807–864
6. Damiola F, Le Minh N, Preitner N et al (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14:2950–2961
7. Stokkan KA, Yamazaki S, Tei H et al (2001) Entrainment of the circadian clock in the liver by feeding. Science 291:490–493
8. Gutiérrez RA, Stokes TL, Thum K et al (2008) Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. Proc Natl Acad Sci U S A 105:4939–4944
9. Haydon MJ, Mielzcarek O, Robertson FC et al (2013) Photosynthetic entrainment of the *Arabidopsis* circadian clock. Nature 502:689–692
10. Harmer SL, Hogenesch JB, Straume M et al (2000) Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science 290:2110–2113
11. Noordally ZB, Ishii K, Atkins KA et al (2013) Circadian control of chloroplast transcription by a nuclear-encoded timing signal. Science 339:1316–1319
12. Lu Y, Gehan JP, Sharkey TD (2005) Daylength and circadian effects on starch degradation and maltose metabolism. Plant Physiol 138:2280–2291
13. Bläsing OE, Gibon Y, Günther M et al (2005) Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in *Arabidopsis*. Plant Cell 17:3257–3281
14. Millar AJ, Short SR, Hiratsuka K et al (1992) Firefly luciferase as a reporter of regulated gene expression in higher plants. Plant Mol Biol Report 10:324–337
15. Knight H, Thomson AJW, McWatters HG (2008) SENSITIVE TO FREEZING6 integrates cellular and environmental inputs to the plant circadian clock. Plant Physiol 148:293–303
16. Dalchau N, Baek SJ, Briggs HM et al (2011) The circadian oscillator gene GIGANTEA mediates a long-term response of the *Arabidopsis thaliana* circadian clock to sucrose. Proc Natl Acad Sci U S A 108:5104–5109
17. James AB, Monreal JA, Nimmo GA et al (2008) The circadian clock in *Arabidopsis* roots is a simplified slave version of the clock in shoots. Science 322:1832–1835
18. Kircher S, Schopfer P (2012) Photosynthetic sucrose acts as cotyledon-derived long-distance signal to control root growth during early seedling development in *Arabidopsis*. Proc Natl Acad Sci U S A 109:11217–11221
19. Aschoff J (1979) Circadian rhythms: influences of internal and external factors on the period measured in constant conditions. Z Tierpsychol 49:225–249

Chapter 13

Assessing Protein Stability Under Different Light and Circadian Conditions

Takatoshi Kiba and Rossana Henriques

Abstract

Plants use light as an indicator of time and space as well as the major energy source for photosynthesis. Due to the development of specific photoreceptors, plants can perceive a wide range of wavelengths and adjust their development accordingly to their surroundings. In addition to light, the circadian clock allows the anticipation of diurnal and seasonal changes thus providing organisms with the adequate physiological responses to ever changing surroundings, which are reflected in increased fitness and survival rate. Although initially described as a set of interconnected transcriptional loops, it is now accepted that posttranslational modifications are also important for proper clock function. In fact, not only the clock but also light signaling rely on posttranslational modifications, such as phosphorylation and ubiquitination, for proper signal transduction. We have designed a simple and yet reproducible method to determine protein stability and half-life under different light and circadian conditions. Our method only requires standard laboratory equipment, a relatively small amount of starting material and can be applied to young seedlings and mature plants. Besides our application to study light and circadian clock proteins, this protocol can be adapted to any other conditions that regulate protein stability.

Key words Protein, Degradation, 26S proteasome, Circadian clock, Light signaling

1 Introduction

Light is fundamental for plant life. Besides its role as an energy source for photosynthesis, plants use light quality, intensity, direction, and duration as an indicator of time and space. Therefore, since light perception is critical for survival, plants have developed several light sensors able to monitor different wavelengths from the UV-B range to the far-red [1]. Within those are the phytochromes (phyA, phyB, phyC, phyD, and phyE) that sense Red (R) and Far-Red (FR) light, whereas blue/UV-A photoreceptors include the cryptochromes (cry1 and cry2), phototropins (phot1, phot2), and the ZEITLUPE family [FKF1 (FLAVIN-BINDING, KELCH REPEAT, F-BOX 1), LKP2 (LOV KELCH PROTEIN 2), and ZTL (ZEITLUPE)] [2]. Although photoreceptors differ

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in their structure they share similar signaling mechanisms that culminate in light-dependent degradation/stabilization of transcription factors and/or protein–protein interactions. These signaling cascades regulate different environmental responses such as shade avoidance as well as developmental processes like flowering [1].

In addition to this role, light is also an input signal to the circadian clock, an internal timekeeping mechanism that includes several interconnected transcriptional loops able to control the rhythmic expression of output genes, able to regulate plant fitness and biomass [3]. At the core of the clock is the central oscillator that relies on the transcriptional feedback between morning and evening genes and their coordinated regulation contributes to the robustness of the rhythms generated. The heart of the central oscillator includes the MYB transcription factors CCA1 (CIRCADIAN CLOCK ASSOCIATED 1)/LHY (LATE ELONGATED HYPOCOTYL), and the Pseudo-Response Regulator TOC1/PRR1 (TIMING OF CAB EXPRESSION 1/PSEUDO RESPONSE REGULATOR 1) and their reciprocal inhibition is critical for clock function [4–6]. Besides *TOC1*, other PRRs (*PRR5*, *PRR7*, and *PRR9*) accumulate throughout the day at specific times, where *PRR9* accumulates at dawn, followed by *PRR7* and *PRR5* expression, and all three of them contribute to the repression of *CCA1* and *LHY* transcription during the day [7]. Recently, a new component of the oscillator, the Evening Complex (EC), was identified, including ELF3 (EARLY FLOWERING 3), ELF4 (EARLY FLOWERING 4), and LUX/ARRHYTHMO. Together these oscillator components function as a repressilator where LHY and CCA1 repress the EC that represses the *PRR*s (*TOC1*, *PRR9*) and in their turn PRRs repress *CCA1/LHY*, thus closing this triangle of repression events [6].

While initial studies focused mostly on transcriptional regulation of clock proteins, other layers of regulation are important for proper clock function. In fact, alternative splicing, chromatin remodeling and posttranslational modifications have been associated with circadian function [8–11]. Within posttranslational regulation, ubiquitination and phosphorylation have been the mostly studied in the regulation of circadian clock proteins, such as CCA1, LHY, PRR5, PRR7, and TOC1 [12–14]. These findings show that circadian proteins are tightly controlled and their activity depends on different factors, being their stability one of the most important. Interestingly, this is also the case for several light-dependent transcription factors (TFs). In fact, HFR1 (LONG HYPOCOTYL IN FAR-RED 1) and PIF1, PIF3–5 (PHYTOCHROME INTERACTING FACTOR) accumulate in the dark but are quickly degraded upon light exposure [15]. On the other hand, HY5 (HYPOCOTYL 5) protein is degraded under dark conditions but stabilized in the light to promote photomorphogenesis. However, not only light-dependent TFs are unstable but also pho-

toreceptors such as phyA, phyB, and cry2 [16]. Taken together these findings show that both light signaling and the clock rely on protein degradation mechanisms for adequate regulation.

Ubiquitination is the process of adding ubiquitin (Ub) molecules to specific lysine residues (K48) of target proteins. Proteins tagged with Ub side chains are then recognized by the 26S proteasome machinery and degraded. The transfer of Ub to specific target proteins is a multistep process that requires different protein–protein complexes: (1) the Ub activation by the E1 enzyme; (2) its conjugation by the E2 enzyme; and (3) the E3 ligases that recruit specific substrates for ubiquitination [16].

Arabidopsis E3 ligases are encoded by more than 1000 genes and are highly diverse in their domains. For instance, RING E3 ligases such as COP1 (CONSTITUTIVE PHOTOMORPHOGENESIS 1) and SINAT 5 (SEVEN IN ABSENTIA IN *Arabidopsis thaliana* 5) include both the substrate and E2-binding motifs. On the other hand, in multi-subunit E3s these two motifs are present in different proteins. This is the case of the SCF complex that includes SKP1 (named ASK for *Arabidopsis* SKP1), Cullin, F-box, and RBX1 (RING-BOX 1). The cullin subunit functions as a scaffolding protein binding both SKP1 and RBX1. In its turn, SKP1 binds to the F-box protein that confers the substrate specificity of the SCF. Since the *Arabidopsis* genome possesses more than 700 genes encoding F-Box proteins, the possible combinations for SCF complexes are extremely high [16].

One of the major regulators of light signaling pathways is COP1, a RING E3 ligase that acts as a major repressor of photomorphogenesis in the dark [17]. COP1 possesses autoubiquitination activity and directly targets for degradation LAF1 (LONG AFTER FAR-RED LIGHT 1), HY5, HFR1, and the clock protein GI (GIGANTEA) [17]. In addition, COP1 also ubiquitinates phyA and possibly cry2 to be degraded. Due to its wide range of substrates, COP1 is one of the major regulators of light signaling in *Arabidopsis*.

Interestingly, there are three circadian F-box proteins belonging to the ZEITLUPE family that control the stability of circadian proteins. These F-box proteins, FKF1, ZTL, and LKP2 target for degradation several components of the central oscillator such as TOC1 and PRR5 [13, 14, 18]. Their coordinated activity allied to the transcriptional regulation is critical for proper clock function.

In order to characterize the posttranslational regulation of PRR5 we designed a protocol to evaluate protein stability and half-life under different circadian and light conditions. This protocol can be used with older or younger seedlings (e.g., few days old) since it does not require an enormous amount of starting material. Although we have optimized this protocol to be used under circadian conditions, it can be adapted to any other type of treatments.

2 Materials

2.1 Laboratory Equipment

1. Refrigerated centrifuge.
2. Syringe filters (pore size 0.2 μm).
3. Sterile 1.5 ml, 15 ml, and 50 ml plastic tubes.
4. Sterile 200 μl, 1 ml tips.
5. Sterile 5 ml, 10 ml pipettes.
6. Sterile plastic petri dishes.
7. Multi-well plate (12–24 well/plate).
8. Fume-hood.
9. Sterile bench.
10. Small forceps.
11. Table top vacuum pump.
12. For circadian experiments: light and temperature controlled incubator, or growth chamber programmable for different photoperiods [12 h light/dark (12 L/D); 8hL/16hD (SD); 16hL/8hD (LD); continuous light (LL); continuous dark (DD)].
13. For light signaling experiments: specific light quality (LED) incubators with controlled light intensity and temperature that can be adjusted to Red (R), Far-Red (FR), or Blue (B) light conditions.
14. Liquid nitrogen.
15. Spectrophotometer.
16. Parafilm.
17. Plastic cuvettes.

2.2 Reagents

2.2.1 Plant Growth

1. MS basal salt mixture.
2. Myo-inositol.
3. MES.
4. Sucrose.
5. Bacto-agar.
6. Bleach.
7. Triton X-100.

2.2.2 MG132 and/or CHX Treatment

1. MG132—proteasome inhibitor XI, reversible proteasome inhibitor.
2. Proteasome inhibitor set:

   ALLN—also known as MG101, calpain inhibitor 1, proteasome inhibitor V, is an inhibitor of the neutral cysteine proteases and the proteasome.

Epoxomicin (EPX)—also known as Proteasome inhibitor XIV is an irreversible inhibitor of chymotrypsin-like (CT-L), trypsin-like (T-L), and the peptidyl-glutamyl peptide hydrolyzing activities of the proteasome.

clasto-Lactacystin β-Lactone (c-LβL)—also known as Omuralide, proteasome inhibitor IX, is a 20S proteasome inhibitor.

3. Cycloheximide (CHX) is an inhibitor of protein synthesis in eukaryotes.
4. Protease inhibitor cocktail.
5. Ethanol.

2.2.3 Protein Extraction, Quantification, and Detection

1. Lithium dodecyl sulfate (LDS) sample buffer for protein sample denaturation.
2. β-mercaptoethanol (1.43 M).
3. Tris–HCl.
4. NaCl.
5. NP-40.
6. EDTA.
7. DTT.
8. Bradford reagent.
9. BSA.
10. Coomassie brilliant blue.
11. Methanol.
12. Glacial acetic acid.
13. Anti-tubulin antibody.

2.3 Preparation of Working Solutions

2.3.1 MS Solid Medium for Arabidopsis

1. Dissolve the appropriate amount of MS basal salt mixture, 100 mg/l of myo-inositol, and 1 % sucrose (w/v) (if necessary) in Milli-Q water.
2. Add 500 mg/l of MES, mix well and adjust pH at 5.7 with KOH. Finally add 8 g/l of Bacto-agar.
3. Autoclave at 120 °C for 15 min.
4. Pour in sterile plastic petri dishes while it is hot and let it cool to become solid.

2.3.2 MG132 and Mock Treatment

1. Mix the MS basal salt mixture with the appropriate amount of Milli-Q water and filter-sterilize with a syringe filter (MS liquid medium) (*see* **Note 1**).
2. Prepare stock solution of MG132 at 100 mM by dissolving the MG132 powder in DMSO. Keep the aliquots at –20 °C (*see* **Note 2**).

3. Add to the MS liquid medium in 50 ml sterile plastic tubes the necessary volume of MG132 stock solution to obtain a final concentration of 100 μM.
4. To prepare the mock solution, add the same volume of DMSO to another 50 ml tube with MS liquid medium.

2.3.3 CHX and Mock Treatment

1. Prepare MS liquid medium as described in Subheading 2.3.2.
2. Prepare 100 mM of CHX in ethanol. This solution is always prepared fresh and for immediate use.
3. Dilute the CHX for the working concentration of 100 μM in MS liquid medium.

2.3.4 PRR5 Extraction Buffer

Table 1

2.3.5 Coomassie Staining Solution

1. For 1 l of solution add 1 g of Coomassie brilliant blue, 50 % (vol/vol) of methanol, and 10 % (vol/vol) of glacial acetic acid in Milli-Q water. Mix by stirring for several hours and filter through Whatman filter paper. We routinely reuse this solution up to 3–4 times.
2. Incubate this solution with the Western membrane for at least 1 h, although it can be left for several hours.

2.3.6 Coomassie Destaining Solution

1. Mix 40 % (vol/vol) of methanol and 10 % (vol/vol) of acetic acid in Milli-Q water.
2. After discarding the Coomassie staining solution incubate the membrane with destaining solution for 30 min. Replace with new destaining solution and leave for at least 1 h before changing, this can be done until proper destaining is seen (*see* **Note 3**).

Table 1
Detailed formulation of PRR5 extraction buffer

Reagents (final concentration)	Stock solutions	50 ml	10 ml
Tris–HCl pH 7.5 (50 mM)	1 M (20×)	2.5 ml	0.5 ml
NaCl (150 mM)	5 M (33.3×)	1.5 ml	300 μl
NP-40 (0.5 %)	20 % (40×)	1.25 ml	250 μl
EDTA (1 mM)	0.5 M (500×)	100 μl	20 μl
DTT (3 mM)	1 M (33.3×)	Add fresh	Add fresh
Protease inhibitor cocktail (1x)	100×	Add fresh	Add fresh
ALLN (100 μM)	100 mM (1000×)	Add fresh	Add fresh
EPX (1 μM)	1 mM (1000×)	Add fresh	Add fresh
clasto-LβL (4 μM)	4 mM (1000×)	Add fresh	Add fresh

3 Methods

The protocols described below were used to determine protein stability under different light qualities and different circadian conditions in etiolated seedlings. For this reason the seedlings were grown in medium without sucrose. Nevertheless they can also be used for older seedlings grown under circadian conditions with sucrose.

We describe in detail the conditions for MG132 treatments to assess protein degradation by the 26S proteasome and CHX to determine protein half-life (Fig. 1). We also provide different experimental set ups combining the two types of treatments, that show how the proteasome regulates protein half-life (i.e., CHX combined with MG132 treatments).

3.1 Plant Growth Conditions

3.1.1 For Etiolated Seedlings Used for Light Signaling and Also Circadian Experiments

1. Prepare MS solid medium without sucrose.
2. (**Steps 2–4** have to be done in a sterile bench.)
3. Sterilize seeds in 0.05 % Triton X-100 for 5 min with agitation, followed by 10 min incubation in 50 % bleach in 0.05 % Triton X-100.

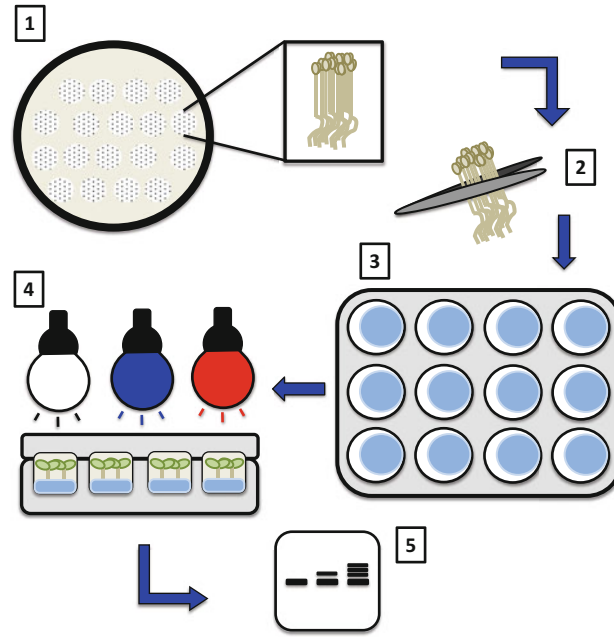

Fig. 1 Schematics of the procedure to assess stability of light-unstable proteins. We show in detail the more important steps of this protocol, namely (*1*) the plating of seeds in small circles; (*2*) their collection, and transfer to multi-well plates (*3*); followed by incubation under different light/circadian conditions (*4*) with the different treatments (MG132 and/or CHX). Finally protein amounts are determined by Western blot (*5*)

4. Wash several times (normally 6) with sterile water, after the last wash leave some water to resuspend seeds for sowing.
5. Sow seeds by pipetting them onto the MS solid medium (*see* **Note 4**).
6. Wrap plates with a double layer of aluminum foil and incubate them at 4 °C for 4 days.
7. Remove plates from the dark and expose to white light at 22 °C for 3 h to induce germination.
8. Put these plates in the dark by wrapping them with a double layer of aluminum foil and incubate at 22 °C for 4–5 days (*see* **Note 5**).

3.1.2 For Older Seedlings Grown under Different Circadian Conditions

1. Prepare MS solid medium with 1 % sucrose.
2. Repeat the previous **steps 2–5**.
3. Put plates under appropriate circadian conditions (12 L/D, SD, LD, LL, or DD).

3.2 MG132 Treatment (to Determine Protein Degradation by 26S Proteasome)

3.2.1 On the Day before the Experiment

1. Label multi-well plates with time points to be analyzed (1 well/time point).
2. The seedlings are then divided into two groups by transferring them to the multi-well plates where they are incubated with MS liquid medium either supplemented with 100 μM MG132 or with DMSO mock solution (*see* **Notes 6–8**).
3. Infiltrate in weak vacuum for 10 min.
4. Incubate the infiltrated seedlings overnight in the same growth conditions the seedlings were grown (*see* **Note 9**).

3.2.2 On the Day of the Experiment

1. For light regulated proteins: collect T0, transfer the plates to the appropriate light conditions and start collecting the time points.
2. For circadian regulated proteins: maintain the plates under the appropriate circadian conditions, and detect their stability at the time they should accumulate.
3. Freeze samples with liquid nitrogen and keep them at −80 °C until analysis.

3.3 CHX Treatment (to Detect Protein Half-Life)

Follow **steps 1–4** described in Subheading 3.2.1 (*see* **Notes 6–8**), and on the day of the experiment perform these steps:

1. Transfer seedlings to multi-well plates with MS liquid medium supplemented with 100 μM of CHX.
2. Infiltrate with weak vacuum for 10 min and leave the plates for 1 h at 22 °C (for CHX to fully enter the cells).
3. After 1 h collect the T0.

4. Transfer the other plates to the appropriate light conditions (RL, FR, BL, or white light) and collect the time points (e.g., dark, 10 min, 30 min, 1 h, 3 h).
5. Freeze samples with liquid nitrogen and store them at −80 °C until analysis.

3.4 MG132 and CHX Co-treatment

Perform the **steps 1–4** described in Subheading 3.2.1 so that seedlings are incubated with 3 ml of MS liquid medium + MG132 or MS liquid medium + DMSO (mock) solution, and on the day of the experiment follow these instructions:

1. Normally we set up the following conditions: CHX treatment of MG132 pre-incubated or mock pretreated seedlings with or without MG132 [(+CHX/+MG132) and (+CHX/-MG132)] (Fig. 2). The schematics of these treatments are shown below.

Pre-incubation (O/N)			
+MG132		−MG132 (mock)	
Treatments (day of experiment)			
+CHX		+CHX	
+MG132	−MG132	+MG132	−MG132

2. To remove MG132 in the pre-incubation medium, wash the seedlings with new MS liquid medium (normally 3 ml/well) 2–3 times. This is done by removing the medium with a vacuum pump and pipetting in fresh medium.
3. Prepare MS liquid medium supplemented with MG132 and CHX to the appropriate final concentrations, add to all the seedlings and do infiltration in weak vacuum.
4. Wait 1 h for CHX to penetrate the cells, and then collect the T0 (if necessary collect seedlings under dark conditions).
5. Transfer seedlings to the appropriate light conditions and collect the time points.
6. Freeze samples with liquid nitrogen and store them at −80 °C until analysis.

3.5 Protein Extraction, Quantification, and Detection

1. We initially tested the stability of our protein and tried different protein extraction methods. We could extract total protein either in 2x LDS-sample buffer supplemented with 10 % of β-mercaptoethanol or in the PRR5 extraction buffer supplemented with protease inhibitor cocktail and several proteasomal inhibitors (Table 1).
2. If the extraction is done in 2× LDS-sample buffer we confirm the equal loading of total protein either by staining the Western blot membrane with Coomassie staining solution or immune-

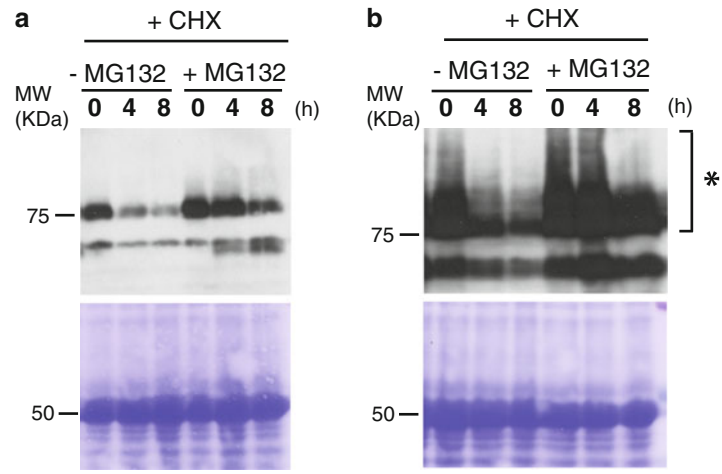

Fig. 2 PRR5 stability and half-life determined with combined MG132 and CHX treatments. Transgenic seedlings overexpressing PRR5 tagged with the FLAG epitope were grown under 12 L/D conditions and subjected to different treatments. The seedlings were pre-incubated with mock solution (**a**) or with MG132 (**b**), divided into two groups and incubated with CHX in the presence (+MG132) or absence (–MG132) of MG132. These experiments revealed that PRR5 half-life is extended with the inhibition of the 26S proteasome and PRR5 is poly-ubiquitinated before degradation (*). Upper panels show the result of Western blot probed with the anti-FLAG antibody and lower panels are the Coomassie staining of the membranes to show equal loading

detection with the anti-tubulin antibody. On the other hand, for PRR5 extraction buffer we determine protein concentration using the Bradford protein assay method.

3. In order to do so we prepare a 10 % (weight/vol) stock solution of BSA and use it to set up a dilution series (e.g., 10 μg/μl, 5 μg/μl, 2 μg/μl, 1 μg/μl). We then determine the Absorbance of each BSA dilution, in a 1 ml plastic cuvette by mixing 200 μl of Bradford reagent with 800 μl of Milli-Q water and adding 1 μl of each dilution series. This is covered with Parafilm and mixed well. The Absorbance at 595 nm is measured and we plot in a graph the BSA concentration (x axis) against the Absorbance value (y axis). We calculate the linear regression [Absorbance (y) = slope*protein concentration (x) + constant value (when $x = 0$)] and determine the protein concentration using the following equation: Protein concentration (x) = [Absorbance (y) – constant value]/slope].
4. The same protein levels were then loaded and determined by Western blot.

4 Notes

1. Normally the treatments with MG132 and/or CHX are performed in MS liquid medium, which is a simplified version of the autoclaved MS liquid medium since the seedlings will only stay in this solution for a few hours. We also do not add sucrose to the MS liquid medium as a way to avoid contamination since the treatments are performed on the bench.
2. We routinely used 100 μM of MG132 but lower concentrations (50 μM) can also be used, accordingly to each particular case.
3. We normally add some folded absorbent paper or a little piece of sponge to the destaining solution since it helps to adsorb Coomassie brilliant blue.
4. If samples are to be collected in the dark it is better to sow seeds for a sampling point all together as a small circle since they can be easily grabbed with forceps even when the light conditions are dim such as when working under safety light conditions (Fig. 1, step 1).
5. Check under safety light in a dark room after 2 days to confirm germination and that the seedlings are not contaminated.
6. It is important to maintain the darkness in every step of the pretreatments in case of light-unstable proteins, if this is not the case, then the treatments can be done in the light. In case of circadian regulated proteins it is necessary to check their accumulation pattern before setting up this experiment, otherwise they will not be detected.
7. When dealing with many samples with very short intervals, it saves time to organize the samples in the multi-well plates by time, especially if you have to do sampling in the dark (Fig. 1, steps 2 and 3).
8. Determining the light/circadian regulation of your protein of interest (e.g., determine its peak/trough periods) in advance is advised in order to set up the stability conditions at a time your protein accumulates.
9. For circadian proteins, if overnight incubation is not practical, then keep in mind that MG132 needs at least 9 h in order to work fully. Therefore it is advised to calculate the infiltration time in order to have MG132 working at the time the protein is expressed [e.g., for PRR5 that peaks in the afternoon, the seedlings were grown under 12 L/D, incubated with or without MG132 from early morning till afternoon, and then samples were divided and CHX added; after 1 h of pre-incubation different time points were collected: 0, 4, and 8].

References

1. Kami C, Lorrain S, Hornitschek P, Fankhauser C (2010) Chapter Two – Light-regulated plant growth and development. In: Marja CPT (ed) Current topics in developmental biology, vol 91. Academic Press, pp 29–66. doi: http://dx.doi.org/10.1016/S0070-2153(10)91002-8
2. Ito S, Song YH, Imaizumi T (2012) LOV domain-containing F-Box proteins: light-dependent protein degradation modules in *Arabidopsis*. Mol Plant 5(3):573–582. doi:10.1093/mp/sss013
3. Dodd A, Salathia N, Hall A, Kevei E, Toth R, Nagy F, Hibberd J, Millar A, Webb A (2005) Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309(5734):630–633
4. Gendron JM, Pruneda-Paz JL, Doherty CJ, Gross AM, Kang SE, Kay SA (2012) *Arabidopsis* circadian clock protein, TOC1, is a DNA-binding transcription factor. Proc Natl Acad Sci U S A 109(8):3167–3172. doi:10.1073/pnas.1200355109
5. Huang W, Pérez-García P, Pokhilko A, Millar AJ, Antoshechkin I, Riechmann JL, Mas P (2012) Mapping the core of the *Arabidopsis* circadian clock defines the network structure of the oscillator. Science 336(6077):75–79. doi:10.1126/science.1219075
6. Pokhilko A, Fernandez AP, Edwards KD, Southern MM, Halliday KJ, Millar AJ (2012) The clock gene circuit in *Arabidopsis* includes a repressilator with additional feedback loops. Mol Syst Biol 8. doi:http://www.nature.com/msb/journal/v8/n1/suppinfo/msb20126_S1.html
7. Nakamichi N, Kiba T, Henriques R, Mizuno T, Chua N, Sakakibara H (2010) PSEUDO-RESPONSE REGULATORS 9, 7, and 5 are transcriptional repressors in the *Arabidopsis* circadian clock. Plant Cell 22(3):594–605
8. Hemmes H, Henriques R, Jang I-C, Kim S, Chua N-H (2012) Circadian clock regulates dynamic chromatin modifications associated with *Arabidopsis CCA1/LHY* and *TOC1* transcriptional rhythms. Plant Cell Physiol 53(12):2016–2029
9. Henriques R, Mas P (2013) Chromatin remodeling and alternative splicing: Pre- and post-transcriptional regulation of the *Arabidopsis* circadian clock. Semin Cell Dev Biol 24(5):399–406, http://dx.doi.org/10.1016/j.semcdb.2013.02.009
10. Malapeira J, Khaitova LC, Mas P (2012) Ordered changes in histone modifications at the core of the *Arabidopsis* circadian clock. Proc Natl Acad Sci U S A 109(52):21540–21545. doi:10.1073/pnas.1217022110
11. Song YH, Ito S, Imaizumi T (2010) Similarities in the circadian clock and photoperiodism in plants. Curr Opin Plant Biol 13(5):594–603, doi: http://dx.doi.org/10.1016/j.pbi.2010.05.004
12. Kusakina J, Dodd AN (2012) Phosphorylation in the plant circadian system. Trends Plant Sci 17(10):575–583, doi: http://dx.doi.org/10.1016/j.tplants.2012.06.008
13. Kiba T, Henriques R, Sakakibara H, Chua N (2007) Targeted degradation of PSEUDO-RESPONSE REGULATOR5 by an SCFZTL complex regulates clock function and photomorphogenesis in *Arabidopsis thaliana*. Plant Cell 19(8):2516–2530
14. Mas P, Kim W-Y, Somers D, Kay S (2003) Targeted degradation of TOC1 by ZTL modulates circadian function in *Arabidopsis thaliana*. Nature 426(6966):567–570
15. Leivar P, Monte E (2014) PIFs: systems integrators in plant development. Plant Cell 26(1):56–78. doi:10.1105/tpc.113.120857
16. Henriques R, Jang I-C, Chua N-H (2009) Regulated proteolysis in light-related signaling pathways. Curr Opin Plant Biol 12(1):49–56
17. Lau OS, Deng XW (2012) The photomorphogenic repressors COP1 and DET1: 20 years later. Trends Plant Sci 17(10):584–593, doi: http://dx.doi.org/10.1016/j.tplants.2012.05.004
18. Baudry A, Ito S, Song YH, Strait AA, Kiba T, Lu S, Henriques R, Pruneda-Paz JL, Chua N-H, Tobin EM, Kay SA, Imaizumi T (2010) F-Box proteins FKF1 and LKP2 act in concert with ZEITLUPE to control *Arabidopsis* clock progression. Plant Cell 22(3):606–622

Part III

Abiotic Stress Responses

Chapter 14

Screening for Abiotic Stress Tolerance in Rice: Salt, Cold, and Drought

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Abstract

Rice (*Oryza sativa*) is the primary source of food for more than half of the world population. Most rice varieties are severely injured by abiotic stresses, with strong social and economic impact. Understanding rice responses to stress may help breeding for more tolerant varieties. However, papers dealing with stress experiments often describe very different experimental designs, thus making comparisons difficult. The use of identical setups is the only way to generate comparable data. This chapter is organized into three sections, describing the experimental conditions established at the Genomics of Plant Stress (GPlantS) unit of ITQB to assess the response of rice plants to three different abiotic stresses—high salinity, cold stress, and drought. All sections include a detailed description of the materials and methodology, as well as useful notes gathered from the GPlantS team's experience. We use rice seedlings as plants at this stage show high sensitivity to abiotic stresses. For the salt and cold stress assays we use hydroponic cultures, while for the drought assay plants are grown in soil and subjected to water withholding. All setups enable visual score determination and are suitable for sample collection along the imposition of stress. The proposed methodologies are simple and affordable to implement in most labs, allowing the discrimination of several rice genotypes at the molecular and phenotypic level.

Key words *Oryza sativa*, Rice, Abiotic stress, Seedling stage, Hydroponic culture, Cold stress, Drought, Salt stress

1 Introduction

Rice is the most important food crop, feeding almost 2/3 of the human population. However, rice sensitivity to abiotic stresses, including high salinity, cold, drought, or flooding, poses major problems for cultivation in numerous countries, including the top rice producers such as Japan, China, Korea, Brazil, India, the USA, the Philippines, or Thailand. In this chapter, we cover some

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methodologies to screen for rice tolerance/susceptibility to high salinity, cold, and drought.

High soil salinity is one of the most serious abiotic stresses affecting agriculture worldwide and one of the major obstacles to increase rice production in growing areas worldwide [1]. High salinity negatively impacts plant growth, development, and ultimately productivity. Globally, it is estimated that 19.5 % of irrigated land (230 million ha) and almost 2.1 % of dry land agriculture (45 million ha) is affected by salt [2]. Salt-affected soils have an excessive accumulation of a complex combination of soluble salts. However, NaCl is considered the primary cause of soil salinization, because of its abundance in many affected soils and its high solubility [3]. Soils are classified as salt affected when the electrical conductivity (EC) is 4 dS/m or higher, which is equivalent to approximately 40 mM NaCl [4]. Rice salt stress sensitivity varies along its life cycle. The higher sensitivity is observed at the seedling (2–3 leaves) and reproductive (pollen development and fertilization) stages [5]. Although the response mechanisms may be different depending on the developmental stages, screening seedlings may be more convenient, as they require less space and assays are more efficient in terms of time and costs. Despite rice being considered a salt stress-sensitive crop, some degree of salt tolerance can be found in rice germplasm [6]. There have been attempts to screen for rice salt stress tolerance under field conditions. However, this is often difficult due to the highly variable environmental conditions [7], such as soil natural structure variation, irrigation, management practices, and meteorological conditions (temperature, humidity, etc.) [8]. On the other hand, hydropony is highly suitable for salt stress tolerance screenings. This plant culture system creates a simple, efficient, and accurate system for screening for salinity tolerance. Moreover, salt tolerance at seedling stage under hydroponic conditions has been correlated with salt tolerance under field conditions [5].

Low temperature is another abiotic stress severely affecting rice yield [9, 10]. The level of cold stress injury in rice plants normally depends on the severity of the stress, the exposure duration, and the plant developmental stage [11]. Although rice is more sensitive to cold stress injury at the booting stage [12], low temperature (in the atmosphere or in the irrigation water) can have a negative impact on growth and development of rice plants at any developmental stage: germination, seedling, vegetative, reproductive, or grain maturity, leading to a reduction of grain yield [13, 14]. The development and selection of cold stress-tolerant varieties is the most effective way to prevent crop damage caused by low temperature. Several methodologies have been used to screen cold tolerance/susceptibility under low-temperature conditions at different stages of development [15, 16, 13, 17]. Adopting a common/uniformized strategy would be highly advantageous, as it

would allow an easier and more efficient result comparison among different labs. Kim et al. [18] have shown that visual assessment of cold stress tolerance in rice seedlings and the use of physiological indicators, in particular electrolyte leakage (EL), are highly correlated. The cold stress symptoms reported at seedling stage include leaf rolling, stunting, yellowing, and wilting. Electrolyte leakage measurements are used as an indirect diagnosis of membrane damage, induced by cold stress. This parameter allows detecting chilling injury before visible symptoms arise.

Drought is one of the most important abiotic stresses limiting the productivity of crop plants around the world. The occurrence of extreme climate events has been increasing in recent years and is expected to keep rising in the near future. The increase of the world population combined with diminishing freshwater resources for irrigation is posing a real threat for food security in the next decades. Rice is particularly susceptible to water deficit at specific stages of development, mainly at the seedling and reproductive stages. However, the identification of drought-tolerant genotypes (in rice as well as in other species) may be a complex task at the experimental design level and, in most cases, stress assays performed in the lab do not correlate with data from field conditions [19]. For instance, there are examples in the literature in which the same genotypes are described with opposite drought tolerance phenotypes due to different experimental designs [20, 21]. The establishment of a single drought assay protocol for rice may be difficult to transpose among labs, as numerous variables must be considered. Nevertheless, further research is needed at this level, and the identification of genotypes better adapted to water deficit is imperative to reduce the yield losses in regions of water scarcity.

The experiments described in this chapter are compatible with plant stress phenotyping and sample collection throughout the stress assays aiming at several analysis purposes. A combination of invasive and noninvasive methodologies may be used. As a noninvasive method to determine differences in stress, the chlorophyll fluorescence can be a good method to evaluate photosynthesis by assessing the efficiency of photosystem II (PSII) [22, 23] using a pulse amplitude modulated (PAM) fluorometer (e.g., PAM-2500, Walz, Germany). Using a dark adaptation step to measure the integrity of PSII and/or different light intensities to measure the efficiency of PSII, the researcher may have an indication on how stress is affecting photosynthesis. It is advisable that these measurements are performed before plants reach the permanent wilting point, as after that stage excessive manipulation is needed (unrolling the leaf). As an invasive (destructive) method, the analysis of the relative water content (RWC) [22, 24] is an effective method to determine the percentage of water lost by the leaf during stress (*see* **Note 1**). Other noninvasive (e.g., *I*nfra-*R*ed *G*as *A*nalyzer) and invasive techniques (e.g., shoot and root fresh and dry weight or pre-dawn water potential) may be used as well.

This chapter describes the experimental conditions adapted and optimized by the GPlantS unit team to study high salinity, cold stress, and drought responses in rice. For salt stress, two types of assays are proposed aiming distinct goals: screening for salt stress tolerance or collection of samples for molecular and biochemical analysis. For cold stress, two types of assays are proposed in order to evaluate rice chilling tolerance (cold shock assay, at 4 °C) and rice cold acclimation response (cold acclimation assay, with a pre-treatment at 10 °C before subjecting the seedlings to 4 °C treatment). For drought, a soil culture system is proposed and evaluation of drought symptoms after water withholding. Important and extensive details are provided in order to facilitate assay implementation and guide the researcher to tailor the experiment to suit his/her specific goals.

2 Materials

2.1 Materials and Instruments

1. Test and standard rice accessions—In order to help rating the visual symptoms of the imposed stress, in addition to the genotypes to be tested, it is desirable to include standards (rice genotypes with known stress tolerance) (*see* **Note 2**). At least two standards should be used: one stress tolerant and one stress susceptible (see Tables 1 and 2). In the case of hydroponics systems (salt and cold stress assays), include the standards in every seedling float to assess eventual differences between floats (*see* **Note 3**). Most of these materials can be obtained free of charge from IRRI (International Rice Research Institute, The Philippines) under a Standard Material Transfer Agreement.
2. Scale (1000 g capacity and 0.0001 g sensitivity)—salt/cold.
3. Reagents (analytical grade) for nutrient solutions (Table 3)—salt/cold.
4. Magnetic stirrer—salt/cold.
5. NaOH and HCl solutions (for pH correction)—salt/cold.
6. NaCl (analytical grade) (*see* **Note 4**)—salt.
7. Carbendazim (methyl-*1H*-benzimidazol-2-ylcarbamate)—salt/cold/drought.
8. Styrofoam sheets (1.5–2 cm thick for constructing of seedlings floats)—salt/cold.
9. Nylon net (insect proofing type)—salt/cold.
10. Mixing container (big enough to prepare nutrient solution)—salt/cold.
11. Black plastic trays for hydroponics (*see* **Note 5**)—salt/cold.

Table 1
Standard salt stress-tolerant/sensitive accessions available from IRRI, through MTA

Tolerance	Accession
Salt tolerant	Nona Bokra
Moderately salt tolerant	FL478
	Bicol
	STDV (induced mutant from IR 29)
Salt susceptible	IR 74
	Taipei 309

In the protocol, we propose to use only two standard accessions. However, three standards are advisable: salt stress tolerant, moderately tolerant, and susceptible. The moderately tolerant standard helps to rate test accessions with moderate tolerance. As alternative to Pokkali and IR 29, local varieties or breeding lines of known salt stress tolerance/sensitivity may be used as standards
Note: The comparison of test and standards should ideally be done within the same tray, as the conditions within trays do not evolve in the same way every time

12. Plastic pots and black plastic trays (*see* **Note 6**)—drought.
13. Portable electrical conductimeter (EC)—salt.
14. Portable pH meter—salt/cold.
15. Convection oven at 50 °C—salt/cold/drought.
16. Chamber at 28–30 °C—salt/cold/drought.
17. Radiometer—salt/cold/drought.
18. Conductimeter (measurement range: 0.2 μS/cm-30000 μS/cm)—cold.
19. Glass vials of 40–50 ml (*see* **Note 7**)—cold.
20. Plant growth chambers set at 28 °C with cool-white light (300–500 $\mu E/m^2/s$), relative humidity 70 %, and 12-h photoperiod (*see* **Note 8**)—salt/cold/drought.
21. Plant growth chamber set to 4 °C or 10 °C, with cool-white light (100 $\mu E/m^2/s$), relative humidity 50–70 %, under 12-h photoperiod—cold.
22. Scale (10 kg capacity and 0.01 g sensitivity)—drought.
23. Soil mixture for rice (2 vols. of sieved soil, 2 vols. of peat, 1 vol. of vermiculite) (*see* **Note 9**)—drought.
24. Osmocote Fertilizer (NPK 16-9-12)—drought.

Table 2
Cold stress tolerance of rice accessions at the seedling stage

Accession	Origin	Group	Cold tolerance[a]	Reference
Benjiemang	China	Japonica	TT	[13]
HSC 55	Hungary	Japonica	T	
Lijiangheigu	China	Japonica	TT	
B55	China	Japonica	T	
YRM 64	Australia	Japonica	S	
YRL 39	Australia	Japonica	S	
Somewake	Japan	Japonica	T	[18]
M-201	USA	Japonica	T	
Mutsukogane	Japan	Japonica	T	
IAC 25	–	Japonica	S	[31]
Jambu	–	Japonica	S	
Langrue	–	Japonica	S	
Guan-Yin-Tsa	–	Indica	M	
I-Geo-Tze	–	Indica	M	
29 Lu 1	China	Indica	M	[18]
Byakkoku	Australia	Indica	M	
IR64	Philippines	Indica	SS	
IR50	Philippines	Indica	SS	
Guichao #2	China	Indica	SS	
Pusa 33	India	Indica	SS	

[a]Cold stress tolerance: *SS* very susceptible, *S* susceptible, *M* moderately tolerant, *T* tolerant, *TT* very tolerant
Note: The comparison of test and standards should ideally be done within the same tray, as the conditions within trays do not evolve in the same way every time

2.2 Preparation of Yoshida's Nutrient Solution

In order to avoid nutrient deficiencies and mineral toxicities not associated to high salinity or cold stress, the preparation of stock solutions is critical. Stock solutions should not be stored for more than 2 months at room temperature. Details for preparing 1 L of stock solution are shown in Table 3. In total, five macronutrients and one micronutrient 1000× concentrated stock solutions will be prepared.

1. For each macronutrient stock solution 1000× concentrated, prepare 1 L weighing the required amount of each reagent (Table 3) and transfer it to a 2 L beaker with 750 ml distilled water. Stir the mixture until complete solubilization (at least 15 min) using a magnetic stirrer, transfer to a volumetric flask, and make the volume up to 1 L. After stirring the solution for another 10 min, transfer it to a stock solution bottle and sterilize it by autoclaving (120 °C, 1 atm, 20 min for 1 L bottles with a maximum of 700 ml of solution).

Table 3
Composition of macro- and micronutrient 1000× stock solutions (adapted from [32])

Element	Reagent	Yoshida medium (g/L)
Macronutrient (five independent solutions)		
N	Ammonium nitrate (NH_4NO_3)	91.40
P	Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$)	35.60
K	Potassium sulfate (K_2SO_4)	71.40
Ca	Calcium chloride, dihydrate ($CaCl_2 \cdot 2H_2O$)	117.35
Mg	Magnesium sulfate, 7-hydrate ($MgSO_4 \cdot 7H_2O$)	324.00
Micronutrient (one solution)		
Mn	Manganese chloride, 4-hydrate ($MnCl_3 \cdot 4H_2O$)	1.500
Mo	Ammonium molybdate, 4-hydrate [$(NH_4)6Mo_{24} \cdot 4H_2O$)]	0.074
Zn	Zinc sulfate, 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	0.035
B	Boric acid (H_3BO_3)	0.934
Cu	Copper sulfate, 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.031
Fe	Ferric chloride, 6-hydrate ($FeCl_3 \cdot 6H_2O$)	7.700
	Citric acid, monohydrate ($C_6H_8O_7 \cdot H_2O$)	11.900

Notes: For easy handling and storage, hydrated reagents are preferred. Five independent macronutrient stock solutions and a single micronutrient stock solution are prepared 1000× concentrated. If a large amount of distilled water is not available, demineralized water may be used to prepare the nutrient solutions. Local tap water should not be used, as it may lead to mineral precipitation and alteration of mineral concentration

2. For the micronutrient 1000× concentrated stock solution, each micronutrient is initially dissolved independently and then combined as described below to form a single stock solution. Due to the small amount of some required reagents, precise weighing is critical. Use 25 ml distilled water to dissolve each reagent separately, except for ferric chloride that must be dissolved in 50 ml distilled water. For a final volume of 1 L, mix all micronutrient solutions together in a 2 L beaker with 700 ml distilled water, adding one solution at a time, following the

order presented in Table 3. Add ferric chloride solution to the mixture just before adding citric acid. Stir the mixture for 15 min using a magnetic stirrer. Add 50 ml of concentrated sulfuric acid to the mixture and stir for another 15 min. Then, transfer the solution to a volumetric flask and make up volume to 1 L. The final solution should have a yellowish brown color. Store in a dark glass bottle (translucid bottles must be covered with aluminum foil) and sterilize by autoclaving (120 °C, 1 atm, 20 min for 1 L bottles with a maximum of 700 ml of solution).

3. Prepare the required volume of 1× Yoshida nutrient solution by adding 1/1000 volume of each of the five macronutrients and the micronutrient stock solution (1000× concentrated) to 3/4 final volume of distilled water. Adjust final volume and pH to 5.1.

3 Methods

3.1 Evaluation of Salt Stress Tolerance in Rice at the Seedling Stage

In this section, we describe an adaptation of the protocol originally developed by Gregorio et al. [5] to screen rice accessions for different levels of salt stress tolerance. The protocol is also suitable for analysis/collection of sample(s) along the stress period.

The screening method developed by the IRRI for identification of salt stress tolerance at seedling stage is based on the ability of seedlings to grow in salinized nutrient solutions. Rice is naturally adapted to waterlogged environment with minimal oxygen supply, making this technique suitable to screen for rice salt stress tolerance [5].

High salinity tolerance is determined by visual analysis of salt stress symptoms, such as reduced leaf area, leaves becoming whitish, leaf tip death, and leaf rolling.

3.1.1 Construction of the Seedlings Floats

Seedling floats are made of a rectangular piece of styrofoam with a nylon net on the bottom. Their size will depend on plastic tray size.

1. Cut a rectangular piece of 1.5–2.0 cm thick styrofoam. The float must completely fit the plastic tray (Fig. 1c).
2. Drill holes with about 1–2 cm diameter (Fig. 1a) (*see* **Note 10**).
3. Glue the nylon net to one side of the styrofoam (hot glue) (Fig. 1b) (*see* **Note 11**).
4. Insert the floats in the plastic trays and add water until the water level is about 1–2 mm above the seedlings floats (Fig. 1c) to define the volume needed per tray.

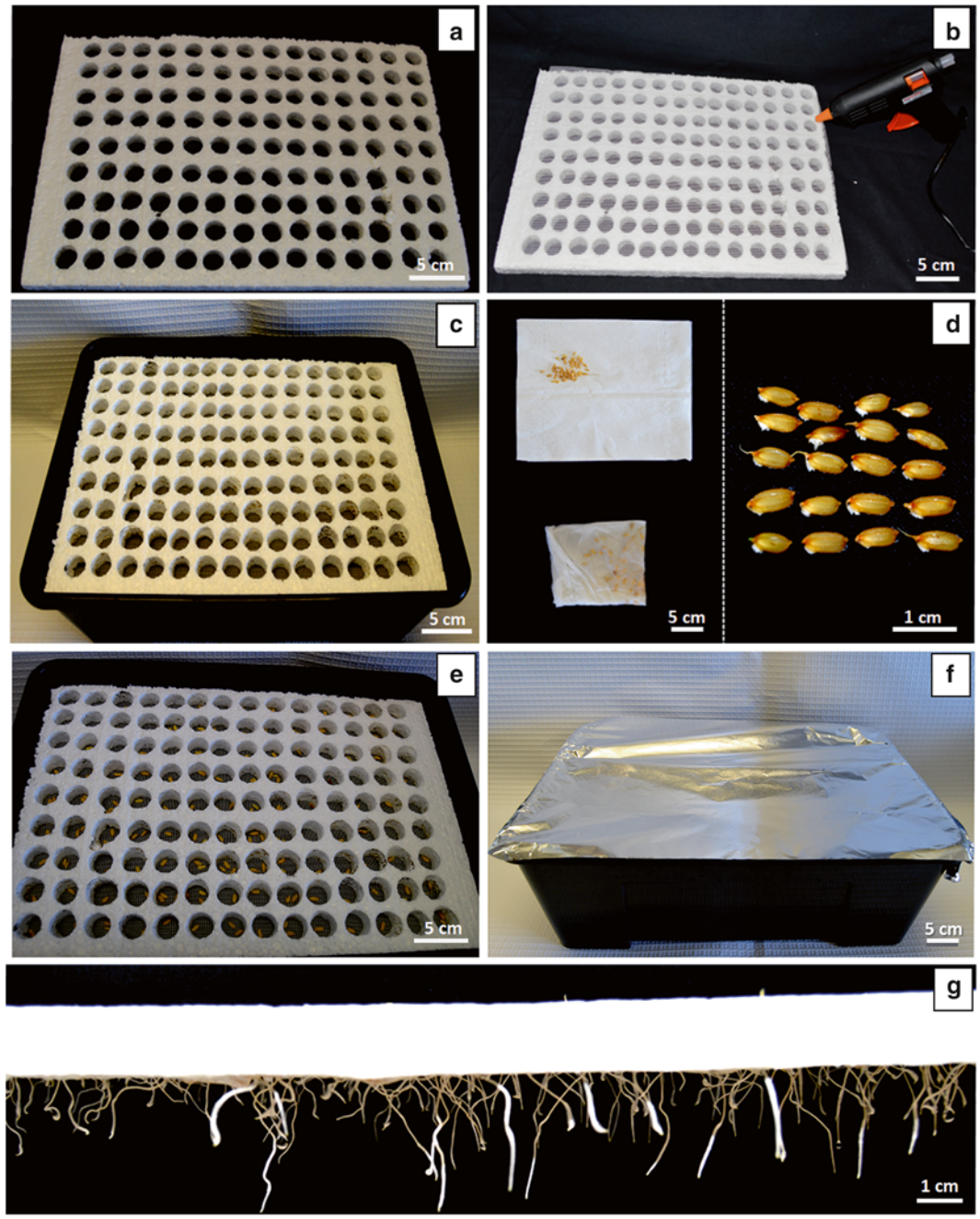

Fig. 1 Steps of seedling float construction, seed preparation, and germination. (**a**) The seedling float is prepared using a styrofoam sheet drilled to make holes of 1–2 cm diameter. (**b**) A nylon net is then attached to one side of the float using hot glue. (**c**) The hydroponics system is set up by tightly fitting the styrofoam sheet to the tray and adding water until a level of about 1–2 mm above the net. (**d**) For establishment in hydroponics, the seeds are first incubated at 28 °C in the dark, on moistened paper towel for 1–2 days until radical emergence. (**e**) For setting up the hydroponics solutions, the water/nutrient solution level is brought to about 1–2 mm above the seedling float, just touching the net. Two pre-germinated seeds are sown per hole on the styrofoam float. (**f**) The tray is covered with aluminum foil for 2–3 days and placed in a growth chamber for complete seed germination in darkness. (**g**) After 4 days of germination (1–2 days in paper + 2–3 days floating), the roots are approximately 3–4 cm long

3.1.2 Preparation of the Plant Material

1. Seed batch—All seeds of one genotype should be from the same batch, and ideally all rice genotypes should have been amplified under the same conditions and at the same time. Do not use seeds from different batches as these plants can behave differently under the same conditions.
2. Breaking dormancy—Heat-treat test and standard seeds, stored in paper bags, for 5 days in a convection oven set to 50 °C to break dormancy (*see* **Note 12**).

3.1.3 Seedling Establishment in Hydroponics

The establishment of rice seedlings in hydroponic culture, to analyze salt stress tolerance, must be as follows:

1. Surface-sterilize the heat-treated seeds with fungicide by soaking them in 1 g/L carbendazim (methyl-*1H*-benzimidazol-2-ylcarbamate) water solution for 30 min at 50 °C in a water bath, followed by four rinses in distilled water (*see* **Note 13**).
2. Germinate seeds on moistened paper towels. Place seeds on a moistened paper towel, folding it over to cover them, and incubate at 28–30 °C (in the dark) for 24–48 h, until they start germinating (Fig. 1d) (*see* **Note 14**).
3. Fill the tray(s) with distilled water (*see* **Note 15**). Place the seedling styrofoam float on the tray and sow two pre-germinated seeds per hole (Fig. 1e), using forceps. Be careful not to damage the embryo. Sow at least 15 seedlings per line (*see* **Note 16**). The water level must be 1–2 mm above the seedling floats´ net. Cover the tray with aluminum foil and transfer to the growth chamber for 2 days for complete seed germination (Fig. 1f). The growth chamber should be set to 28 °C with 70 % humidity and 12-h photoperiod.
4. Two days after germination, when roots are around 2–3 cm long (Fig. 1g), remove the aluminum foil to expose the seedlings to photoperiod.
5. Rotate the seedling floats daily and also swap the floats among the several trays used in the assay (*see* **Note 3**).

3.1.4 Salt Stress Treatment for Tolerance Screening

To screen for salt stress tolerance, 3 days after germination, rice seedlings are well developed and salinization can be performed by addition of NaCl to the nutrient solution:

1. Replace distilled water with salinized Yoshida nutrient solution (*see* **Note 17**). Prepare the required volume of Yoshida nutrient solution and add NaCl (analytical grade) (*see* **Note 4**) until it reaches the desired electric conductivity (EC), stirring to ensure total NaCl solubilization. Set initial salinity at EC = 6 dS/m. After 3 days, increase salinity to EC = 12 dS/m (*see* **Note 18**).

2. Renew the solution every 8 days and adjust the pH daily to 5.1 (*see* **Note 19**). Nutrient solution level must be kept about 1–2 mm above the seedling floats by adding distilled water to maintain the original volume and the nutrient/salt concentration.
3. The genotypes may be scored at 10 and 16 days after initial salinization.

3.1.5 Salt Stress Treatment for Molecular and Biochemical Analysis

In order to collect samples during salt stress for molecular or biochemical analysis, the beginning of the salt treatment may be delayed. If salt is applied 3 days after germination, susceptible genotypes will be dead in about 14 days. In addition, salt stress imposed 3 days after germination leads to severe growth arrest on susceptible genotypes, thus limiting the amount of sample material available.

Therefore, one must proceed as follows:

1. Three days after germination, replace distilled water by Yoshida nutrient solution (no NaCl added).
2. Renew the solution every 8 days and adjust pH daily to 5.1 (*see* **Note 19**). Keep nutrient solution level at about 1–2 mm above the seedling floats by addition of distilled water.
3. Allow the seedlings to develop for 12 days (after germination) until all plants are at the three-leaf stage (with third leaf still developing). At that stage, subject the seedlings to salt stress by adding NaCl (analytical grade) (*see* **Note 4**) to nutrient solution, until it reaches the desired electric conductivity (EC), stirring to ensure total NaCl solubilization (*see* **Note 17**). Set salinity at EC = 12dS/m (*see* **Note 20**). Sample collection/analysis can be performed throughout the entire assay.

3.1.6 Phenotyping and Sample Collection for Analysis

The salt stress scoring method, calibrated with standards, allows discriminating susceptible from tolerant test genotypes. The method is based on visualization of salt stress symptoms: reduced leaf area, older leaves becoming whitish, leaf tip death, leaf rolling, and plant death. Scoring may start 10 days after salinization with a final scoring 16 days after salinization. Symptoms on test genotypes should be compared to standards (included in each test float) to estimate the degree of salt stress tolerance.

1. Score test genotypes by visualization of salt stress symptoms, applying the modified standard evaluating score (Table 4).
2. Ten days after salinization, standard genotypes like “Pokkali” (tolerant) scores 1 and “IR29” (susceptible) scores 7. At this point, susceptible genotypes can be distinguished from the tolerant ones.

Table 4
Modified Standard Evaluation System (SES), adapted from IRRI Screening Rice Salinity Tolerance [5]

Score	Observation	Tolerance
1	All leaves normal, no leaf symptoms	Highly tolerant
3	Nearly normal growth, but leaf tips or few leaves whitish and rolled	Tolerant
5	Growth severely retarded; most leaves rolled; only a few are elongating	Moderately tolerant
7	Complete growth cessation; most leaves dry, some plants dying	Susceptible
9	Almost all plants are dead or are dying	Highly susceptible

Visual salt stress injury is evaluated at seedlings stage, after 3 days imposition of 6 dS/m NaCl, followed by 13 days at 12 dS/m NaCl. Scoring can be performed over time (each day) if quantitative data is required

3. Sixteen days after salinization "Pokkali" scores 3 and "IR29" scores 9. At this point, it is possible to clearly distinguish susceptible from tolerant genotypes (Fig. 2).
4. Sample collection for analysis can be performed throughout the salt stress assay.

3.2 Evaluation of Cold Stress Tolerance in Rice at the Seedling Stage

In this section, we also use a hydroponic system to assess cold stress tolerance in rice seedlings.

3.2.1 Preparation of the Plant Material

1. Seed batch and dormancy break—As previously described (*see* Subheading 3.1.2, **steps 1** and **2**) proper care should be taken to ensure the use of homogeneous seed batches and to break seed dormancy, in order to synchronize seed germination.

3.2.2 Seedling Establishment in Hydroponics

1. Prepare styrofoam floats as described in Subheading 3.1.1 and perform seed surface-sterilization, germination, and sowing in the styrofoam floats as in Subheading 3.1.3 (**steps 1–3**).
2. Two days after germination, remove the aluminum foil and replace the water by Yoshida nutrient solution (Fig. 1g).
3. Rotate the seedling floats daily and also swap the floats among the several trays used in the assay (*see* **Note 3**).
4. Renew the solution every 8 days and adjust pH daily to 5.1 (*see* **Note 19**). Nutrient solution level must be kept about 1–2 mm above the seedling floats by adding distilled water to maintain the original volume and the nutrient concentration.
5. Allow the seedlings to develop for 12 days (after germination) at 28 °C with 12-h photoperiod until all plants are at the three-leaf stage (with third leaf still developing). The seedlings should be well synchronized, as they might be differently

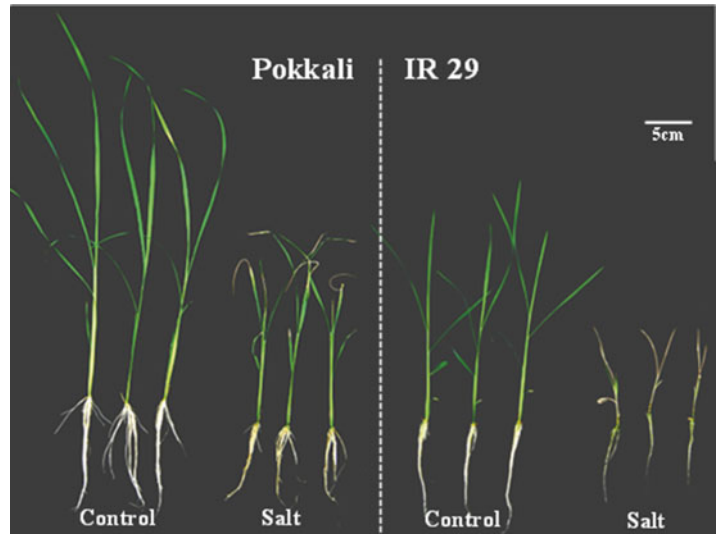

Fig. 2 Evaluation of salt stress visual symptoms. Visual symptoms of salt stress injuries after 16 days of salinization. *Left side*: Pokkali, a salt-tolerant variety, scores 3, showing little growth arrest and few leaves whitish and rolled. *Right side*: IR 29 a salt-susceptible variety scores 9, showing severe salt injury

affected by cold stress (*see* **Note 21**). Thus removal of all seedlings showing discrepancies in developmental stage is recommended.

6. On the day before cold stress imposition, prepare fresh nutrient solution and keep it at the incubation temperature required: 4 °C for cold shock or 28 °C for cold acclimation assay.

3.2.3 Cold Shock Assay (4 °C)

For the cold shock assay, three-leaf stage seedlings are directly transferred from normal growth conditions (28 °C, 70 % relative humidity, light intensity 300–500 μE/m²/s) to 4 °C chilling temperature (50–70 % relative humidity, light intensity 100 μE/m²/s), using a growth chamber with similar conditions.

1. Place new trays filled with precooled nutrient solution inside a growth chamber set to 4 °C, 12-h photoperiod. Reduce the light intensity to 100 μE/m²/s, in order to avoid photoinhibition stress of the seedlings.
2. When the chamber temperature stabilizes at 4 °C, start the cold shock assay by transferring the seedling floats kept at control conditions to the trays filled with solution cooled in the 4 °C chamber (*see* **Note 22**).
3. Allow seedlings to be subjected to these conditions for 5–11 days (*see* **Note 23**). An example of cold shock-treated seed-

Analyzed parameter	**a** Cold shock assay	**b** Cold acclimation assay
Electrolyte leakage	92.9 %	68.9 %
Cold injury visual symptoms at the end of the cold treatment		
Survival rate (assessed 1-2 weeks after the cold)	0 %	87 %

Fig. 3 Screening rice seedlings for cold stress tolerance in a hydroponic system. The percentage of electrolyte leakage was monitored to evaluate the degree of cold stress injury and determine the length of cold period of the assay. (**a**) Cold shock assay. Three-leaf stage seedlings were subjected to cold stress treatment at 4 °C for 9 days. These plants, after 9 days at low temperature, revealed 92.95 % ± 2.53 % of electrolyte leakage and showed 0 % survival rate after a 1–2-week recovery period.(**b**) Cold acclimation assay. Three-leaf stage seedlings were subjected to a pretreatment at 10 °C for 1 day, before being transferred to 4 °C for 9 days. After 9 days in cold stress conditions, these plants revealed 68.9 % ± 5.03 % of electrolyte leakage and showed 87 % survival rate in the subsequent recovery period

lings, at the end of the low-temperature period, is shown in Fig. 3a.

4. After cold stress treatment, transfer the seedling floats to the growth chamber, set to control conditions (28 °C, 70 % relative humidity) (*see* **Note 24**), and let the plants recover under these conditions for 1–2 weeks (*see* **Note 25**).

3.2.4 Cold Acclimation Assay (10 °C→ 4 °C)

For the cold acclimation assay, the process is similar to that of the cold shock assay with an additional initial step to mimic cold acclimation. Before being transferred to 4 °C, the three-leaf stage seedlings, grown in control growth conditions (28 °C, 70 % relative humidity, light intensity 300–500 μE/m^2/s), are subjected to a pretreatment period of 1 day at 10 °C (50–70 % relative humidity, light intensity 100 μE/m^2/s).

1. Set the growth chamber to 10 °C, 50–70 % relative humidity, and 12-h photoperiod. Reduce the light intensity to 100 μE/m^2/s, in order to avoid photoinhibition stress of the seedlings.
2. When the chamber temperature stabilizes at 10 °C, transfer the trays with the seedling floats to the 10 °C growth chamber (do

not precool the solution), for the acclimation step incubation (*see* **Note 22**).

3. After 1 day of incubation at 10 °C, move the trays with the seedling floats to a 4 °C growth chamber, 50–70 % relative humidity, and 12-h photoperiod, with light intensity of 100 μE/m²/s.
4. Allow the seedlings to stay in these conditions for 7–15 days (*see* **Note 23**). An example of cold acclimation-treated seedlings, at the end of the low-temperature period, is shown in Fig. 3b. Given that in the cold acclimation assay rice seedlings will tolerate 4 °C for longer periods, this assay may have to be extended in order to properly screen the different genotypes.
5. After cold stress treatment transfer the seedling floats to the growth chamber set to control growth conditions (28 °C, 70 % relative humidity, 12-h photoperiod) (*see* **Note 24**) and let the plants recover under these conditions for 1–2 weeks (*see* **Note 25**).

3.2.5 Phenotyping and Sample Collection for Analysis

1. Determining Electrolyte Leakage
   The percentage of electrolyte leakage can be used to evaluate the degree of cold stress injury in the genotypes throughout the assay [18]. Samples for electrolyte leakage measurements should be collected right before cold stress imposition and then every 2–3 days (or at shorter intervals in the case of indica rice genotypes) until the end of cold stress period incubation. Samples must always be collected at the same time point of the photoperiod.
   (a) At each time point, cut the second fully expanded leaf of 3–5 random plants into 1 cm segments (Fig. 4a, b), with a sharp blade (total of 15–25 middle segments per pool/replicate). Make at least six replicates per genotype (*see* **Note 26**). Replicates should be collected one at a time in order to reduce the time taken from leaf cutting to incubation.
   (b) Immediately wash the leaf sections thoroughly and rapidly with distilled water to remove the electrolytes released by tissue disruption (*see* **Note 27**).
   (c) Remove the excess water and transfer the pool of 15–25 leaf sections into a capped vial (*see* **Note 7**) with 15 ml of distilled water (Fig. 4c). Keep the vials for 20 h at room temperature with gentle shaking. Reserve two vials with 15 ml of distilled water to use as blanks in the measurements.
   (d) After this incubation period, measure the initial electrical conductivity of each sample (S1) and blank vial (B1) using a conductimeter with measurement range from 0.2 μS/cm

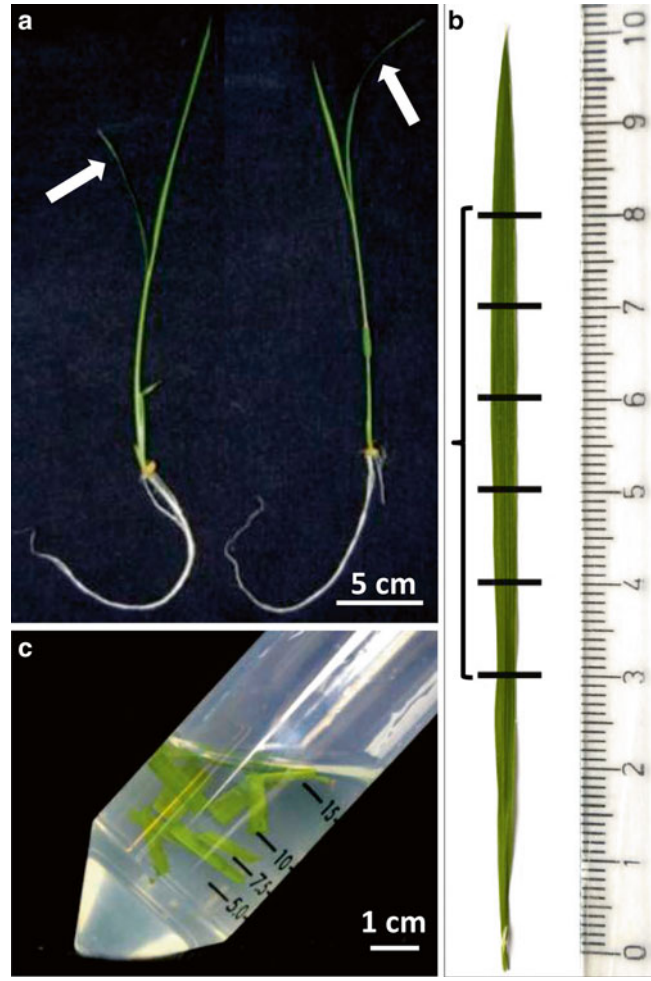

Fig. 4 Preparation of samples for electrolyte leakage measurements, from three-leaf stage seedlings subjected to cold stress. The second fully expanded leaf of 3–5 random seedlings (**a**: indicated by an *arrow*) is cut into 1 cm segments (**b**). 15–25 middle sections are incubated in capped vials with 15 ml of distilled water, for 20 h at room temperature, with gentle rocking

to 30,000 μS/cm (e.g.: Crison conductimeter GLP31) (*see* **Note 28**).

(e) Incubate the vials, tightly closed, at 80 °C for 2 h (*see* **Note 29**) in order to destroy the cells and release the remaining electrolytes. Allow them to cool down to room temperature and then measure again the electrical conductivity of each sample (S2, maximum conductivity of tissues) and blanks (B2) (*see* **Note 28**). The relative degree of electrolyte leakage is calculated as follows: EL (%) = [(S1-B1)/(S2-B2)] × 100.

Table 5
Modified Standard Evaluation System (SES) of visual cold stress injury at the seedlings stage, adapted from IRRI [25] and Andaya and Mackill [14]

Score	Observation	Tolerance
1	All leaves normal, no apparent visual injury	Highly tolerant
3	Some leaves rolled or wilted	Tolerant
5	About half of the leaves wilted	Moderately tolerant
7	Most leaves wilted	Susceptible
9	All leaves wilted, seedlings apparently dead	Highly susceptible

2. Visual Scoring of Cold Stress Injury
   Seedlings should be scored for cold stress injury at the end of the cold treatment period (*see* Subheadings 3.2.3 and 3.2.4). Symptoms on test genotypes may be compared to standards, included in each seedling float, to estimate the degree of tolerance. Use a scale of 1–9, as described in the Standard Evaluation Systems in rice [25] and adapted by Andaya and Mackill [14] (Table 5).
3. Assessing Survival and Viability Rate
   After the recovery period, evaluate the survival rate of the seedlings or otherwise the plant viability rate (*see* **Note 30**). The survival rate represents the percentage of seedlings that were alive and resumed growth after the recovery period (non-survivors correspond to dead seedlings). Plant viability rate can be determined by counting the number of seedlings that resumed growth and developed new leaves during the recovery period, as opposed to the ones that are wilted, failed to grow, or died.

3.3 Evaluation of Drought Tolerance in Rice at the Seedling Stage

Before starting a drought assay in rice, the researcher must take into account several aspects, depending on the goal of the study. Does it aim testing plant survival? Or detecting differences in plant growth after recovery? Is it a drought shock (fast water loss) or a progressive drought assay? If the genotype under study is thought to be primed to tolerate drought, it is likely that a drought shock could show differences. However, if the plant needs to activate a molecular and physiological response to drought, a progressive drought assay should be envisaged. Taking different purposes into account, the researcher may adapt the drought assay protocol that fits his/her specific needs.

To test drought tolerance, we use a strategy of plant growth in soil followed by water withholding to better reproduce natural

conditions. We monitor soil water content along the stress period and use visual observation combined with invasive and noninvasive phenotyping methods.

3.3.1 Designing the Drought Experimental Setup

1. Seed batch and dormancy break—As previously described (*see* Subheading 3.1.2) proper care should be taken to ensure the use of homogeneous seed batches and to break seed dormancy to synchronize seed germination.
2. Preliminary drought trial—Different rice accessions may uptake water from the soil at different rates. The different water uptake rate under drought may, by itself, reflect a tolerance mechanism. Therefore, a preliminary drought assay must be considered to evaluate the water uptake rate by the different target genotypes.
3. Developmental stage—As mentioned for the salt and cold stresses, the plant developmental stage will influence several aspects of the drought assay setup. Rice plants show higher drought sensitivity at the seedling/young plant stage (until approximately 25 days after germination) and then at the reproductive stage. Rice seedlings have a small root system and can be placed in small pots. However, if the drought assay is planned for older plants (e.g., 1-month-old plants or older), larger pots must be used to accommodate the larger root system.
4. Plant density—The spacing of the root system is important. A high density of plants or a small pot (0.5 L) for 1-month-old plants may cause root compression and influence the drought assay results. In addition, placing several genotypes in the same pot may not be the best approach to discriminate phenotypes as the root systems may interact, influencing the drought responses of one another.
5. Plant growth rates—Genotypes with different growth rates (height) will also have different rates of water uptake from the soil, thus complicating genotype comparisons. A big and fast-growing genotype will reach a lower soil water content faster, and exhibit drought symptoms sooner than a slow-growing and small genotype. These symptoms, however, may not correlate with the plants' ability to cope with drought.
6. Growth chambers—Different laboratories have different plant growth conditions (chambers, glasshouses, etc.) in which the drought assay may be conducted. The available conditions should be well known when designing the drought assay. In a controlled chamber, the optimal rice growth conditions are 28 °C, 70 % relative humidity, 300–500 μE/m^2/s, and 12-h photoperiod. In a drought assay, although the temperature and relative humidity may be maintained, the light intensity should not exceed 300 μE/m^2/s. Excessive light intensity in a

drought assay will generate over-production of reactive oxygen species (ROS), thus causing additional stress.

3.3.2 Preparation of the Potted Plants

1. Fill the pots with soil mixture for rice (soil, turf, and vermiculite; 2:2:1). All pots must have exactly the same amount of soil mixture and the soil dry weight must be determined (*see* **Note 31**).
2. Water the pots and place them in a tray filled with water. Allow the pots to equilibrate with the water-filled tray for 2 days.
3. Remove the pots from the water-filled tray and place them in a grid to allow water runoff by gravity. When no dripping is observed, weight the pot and consider this as the field capacity (100 % of soil water content, SWC). Place the pots back in the water-filled tray and check the water level in the trays regularly.
4. Prepare the seeds as described in Subheadings 3.1.2 and 3.1.3 (**steps 1** and **2**).
5. Transfer the germinated seedlings to the pots (*see* **Note 32**) and allow them to grow to the desired stage before starting the drought assay (*see* **Note 33**) (Fig. 5). After 5 days in soil, osmocote (NPK 16-9-12) must be added to the water in the tray (approx.: 0.5 g osmocote/L of soil). Set the chamber conditions to 28 °C, 70 % relative humidity, and 300–500 μE/m^2/s light intensity, with a 12-h photoperiod.
6. Randomize the location of the pots/trays frequently, both previously and during the drought assay, to minimize the chances of chamber zonation (*see* **Note 34**).

3.3.3 Drought Stress Imposition

1. At the start of the drought assay, remove the water from the trays (*see* **Note 35**).
2. Weigh the pots every day at the end of the light period and keep the record to evaluate if the different tested genotypes have a similar water uptake (*see* **Note 36**). To accurately determine water uptake, evaporation must be quantified. Therefore, several pots without plants must be included in the experiment and, after water withholding, weighed every day to assess water evaporation from soil.

3.3.4 Phenotyping and Sample Collection for Analysis

1. Check the plant phenotypes every day. Depending on the objective of the drought assay, it may have a variable duration. When scoring for survival after recovery, pots should reach approximately 10 % of SWC (*see* **Notes 36** and **37**) to accentuate differences in survival (Fig. 6). Another approach may be to determine SWC at which 50 % of the plants start to display leaf rolling (permanent wilting point) (*see* **Note 38**) and maintain that SWC in each pot for 10–14 days [26].
2. For molecular and biochemical analysis, leaf samples (root samples are difficult to collect without disturbing the experi-

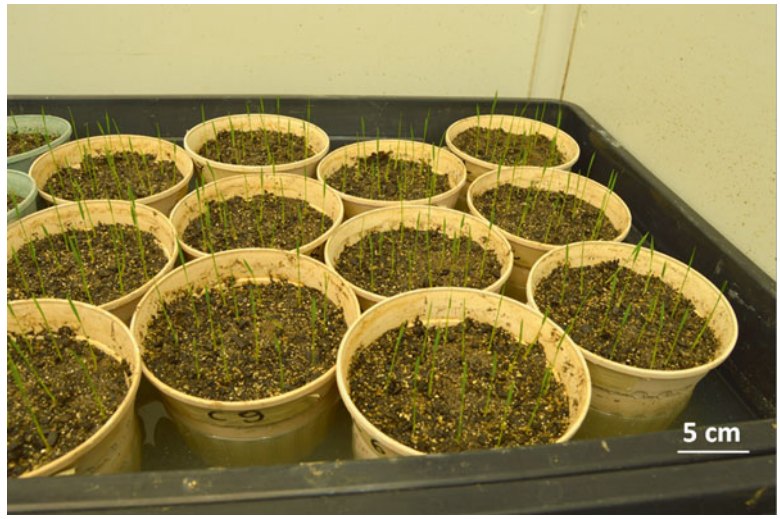

Fig. 5 Rice seedlings grown in pots. Rice germinated seeds were sown in fully irrigated soil mixture and let to grow for 1 week under control conditions

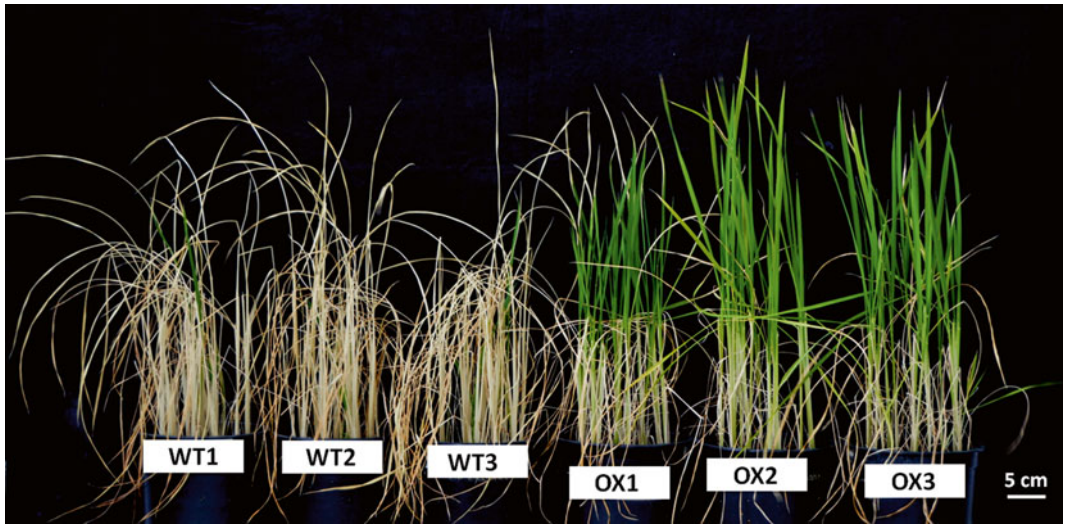

Fig. 6 Rice plants showing different levels of drought tolerance after drought treatment followed by recovery. Drought treatment performed to evaluate drought tolerance of a transgenic rice line, overexpressing (OX) a bHLH transcription factor, as compared with its wild-type (WT) Nipponbare. One-month-old rice plants grown under control conditions were subjected to water withholding until they reached 10 % SWC (approximately 12 days after water withholding). Plants were then re-watered and stayed fully irrigated for 1 week. OX1-3 and WT1-3 correspond to three replicates for each line

mental setup) can be collected. Collect control samples (fully irrigated pots) (*see* **Note 35**) and drought samples at different drought time periods (e.g., at 50, 25, and 15 % SWC and 24 and 48 h after re-watering) (*see* **Note 39**).

4 Notes

1. This technique is applied using mid-blade sections of the leaf (discarding the leaf tip and basal section) and measuring its fresh weight, turgid weight, and dry weight. RWC = (fresh weight—dry weight)/(turgid weight-dry weight) × 100.
2. Local varieties or breeding lines, with known salt/cold stress tolerance, may also be used. When screening for cold stress tolerance, have in mind that indica rice subspecies have typically a lower degree of cold stress tolerance as compared to japonicas [27] and therefore appropriate standards must be chosen accordingly. Thus, in case of screening japonicas, one must not use indica genotypes as standards, as they will not endure the whole cold stress period required to screen japonicas.
3. Ideally, all genotypes to be screened should be grown side by side in the same seedling float. Sometimes, because a large number of seeds are required for the assay, this is not possible and more than one seedling float is used. Growth chamber conditions are not totally homogeneous and the tray positions or even the position of the seedlings inside the same seedling float will be different, in terms of either light intensity, ventilation, or temperature. Also, the nutrient availability inside each tray may differ. Swapping seedling float positions will randomize, and thus minimize, the effect of eventual differences in the conditions to which the seedlings are exposed.
4. Commercial table salt must not be used for salinization of nutrient solution, as it may contain more salts than NaCl. Such "contaminants" may affect mineral concentration of nutrient solution and affect the salt stress screening.
5. Light promotes algal growth in nutrient solution. Therefore, black trays are preferred and the floats must be tightly fitted to the trays (Fig. 1c). Algae are not toxic but they tend to increase pH, especially during midday and early afternoon. If considerable algal growth is detected, it is recommended to adjust the pH twice a day, at the beginning and the end of the light cycle. The plastic tray size may vary depending on the amount of genotypes to be tested. A tray with at least 13 cm depth is recommended. Shallower trays may affect plant root growth.
6. Pot size is selected to suit the volume of soil mixture to the age and size of the plants to use in the drought assay. Pots with capacity for 500 mL of soil mixture may allow one plant per

pot, while larger pots (e.g., 7 L) may allow several plants (10–15 plants). The combination of the number of plants, plant size, and pot capacity will also influence the rate of water loss and the duration of the drought assay. The pots should be placed in plastic trays (preferentially black, in order to avoid algae overgrowth) filled with water.

7. Glass vials are preferable but Falcon tubes (50 ml) can also be used. Vials/tubes must be carefully washed with distilled water, in order to guarantee removal of all detergent contaminants. Distribute 15 ml of distilled water (the use of deionized water is not recommended, due to its extremely low osmolarity that can force electrolytes to leave non-damaged tissues as well) and close the vials. Use clean gloves while handling this material.
8. Screening can be performed in an ordinary greenhouse. However, in those conditions, temperature, solar radiation, and relative humidity will vary during the day. Salt toxicity decreases with low temperature and high humidity (low transpiration rates), and increase with high solar radiation incidence (high transpiration rates). If screening is performed under variable environmental conditions, the EC level must be adjusted along the assay. EC is adjusted according to the salt stress response of the standards. Preferably, assays should be performed in growth chambers with controlled conditions.
9. The type of soil mixture greatly affects the amount of water loss and, thus, the rate at which the plants will suffer from drought. Soil mixtures with a large volume of sand will dry quicker, but will also prevent the slower drought adaptation mechanisms.
10. The styrofoam sheet can be drilled with a sharp 1–2 cm diameter metal tube. Metal tube edge can be sharpened with sandpaper. Alternatively, holes can be made by heating a 1–2 cm diameter metal tube and pressing it against the styrofoam sheet. Do not heat the tube excessively, or it will burn/melt the styrofoam. Melting styrofoam will release hazardous by-products and thus requires working in a ventilated area (preferably in a fume hood). Avoid enclosed, non-ventilated areas.
11. Some hot glue guns melt the plastic glue at high temperatures, thus also melting styrofoam. Ensure that melted glue will not damage the styrofoam. Alternatively, the nylon net can be stitched to the styrofoam with nylon thread.
12. Proper breaking of seed dormancy is essential for the visual scoring as it helps to synchronize seed germination. Genotypes with delayed germination will likely behave as stress sensitive.
13. Carbendazim (methyl-*1H*-benzimidazol-2-ylcarbamate) is a widely used, broad-spectrum, fungicide. It is used on several crops including rice for the control of a wide range of fungal

diseases. Carbendazim is classified by the World Health Organisation (WHO) as unlikely to present hazard in normal use, but nevertheless care must be taken. Wear protective gloves; do not discard carbendazim residues on open water sources. One gram of carbendazim cannot be completely solubilized in 1 L of water. Always mix carbendazim solution vigorously before use.

14. Alternatively, seeds can be germinated in Petri dishes with moistened filter paper and incubated at 28–30 °C (dark) for 24–48 h. Some genotypes may require a longer incubation period on moistened paper for germination. In this event, delay germination of faster genotypes for 1 or 2 days. All genotypes must be transferred to seed floating at the same developmental stage and time. If seeds are incubated for germination longer than 48 h, radicle should be inserted through the nylon net hole. This is a laborious process and may damage the radicle affecting rice salt stress tolerance. Thus, it is recommended to transfer seedlings with underdeveloped radicle to floats (Fig. 1d).
15. There is no need to use nutrient solution during rice seed germination, as the seed endosperm contains adequate nutrients to ensure normal seedling growth for 3–4 days.
16. In case of simply scoring for visual symptoms we suggest screening (at least) 15 seedlings per genotype. Calculate carefully the amount of seeds required for the assay, depending on the type of analysis to be performed. Pre-germinate a larger amount of seeds, up to double the amount required, in order to guarantee selection of highly synchronized seeds.
17. Keep trays with Yoshida nutrient solution to use as controls. Leaf material collected at this stage (just before salinization of the nutrient solution) for molecular or biochemical analysis is referred to as a control. Nevertheless, and depending on the extent of the salt stress assay and the age of the plants, the ideal control samples should be collected from unsalinized Yoshida-filled trays at the same time points that samples are collected from salt stress-treated plants.
18. Initial seedling salinization should not be adjusted to EC = 12 dS/m, otherwise salt-sensitive genotypes may be dead in a few days after salinization.
19. The pH affects availability and absorption of several elements needed for plant growth. For rice nutrient solution (Yoshida), optimum absorption of these elements is found at pH values around 5.0–5.5. When pH varies ±1 from pH 5.0, some nutrients may become toxic and others inaccessible. A reliable/calibrated pH meter is thus essential.
20. It might be necessary to salinize with an EC higher than 12 dS/m (up until EC = 18 dS/m), for instance when assessing molecular response. Take into consideration that the effects of

these higher salt stress impositions will reflect a salt stress shock. Shorter incubation periods should be used.

21. It is advisable to previously assess the development time of the different rice genotypes, as all plants should be in the same developmental stage (three-leaf). If necessary, adjustments should be made in the germination dates so that all seedlings are at the three-leaf stage at the start of the stress.

22. Make sure that this transfer occurs always at the same time of the day, since several cold stress-responsive genes are regulated by the circadian clock [28–30]. We usually perform the transition 4 h after light period initiation, as it has been shown that *OsDREB1A-C*, key regulators of the cold stress response, show their highest level of cold-induced transcript accumulation at this time point [30]. Keep some seedling floats at control growth conditions (28 °C, 300–500 μE/m^2/s, 70 % relative humidity), to use as controls. Leaf material collected at this stage (just before transferring to cold stress conditions) for molecular or biochemical analysis is referred to as a control. Nevertheless, and depending on the extent of the cold stress assay, the ideal control samples should be collected from seedlings grown in control growth conditions at the same time points that samples are collected from cold stress-treated seedlings.

23. If the duration of the cold stress treatment exceeds 8 days, the nutrient solution should be replaced by a precooled fresh one. A pre-assay to determine optimum stress duration is strongly recommended. A very short period will result in the total survival of the seedlings whereas a too long incubation in cold stress conditions will lead to overall death. None of these situations will enable discrimination between cold stress-tolerant/susceptible phenotypes. The ideal duration of the cold incubation will depend on many factors, such as particular growth chamber conditions, like ventilation, humidity, and light quality/intensity but also the genotypes studied or even the conditions in which the seed batches were produced. In our experiments, we have observed significantly different cold stress behaviors using different batches of seeds. During the cold treatment period plants' growth will be nearly fully arrested and seedlings will gradually show increased wilting and some yellowing. The selection of the correct time to stop the cold stress treatment and start the recovery period is not easily determined by visual parameters. We suggest using electrolyte leakage measurements to monitor the best timing to do it. Using the described methodology in Subheading 3.2.5 (**step 1**), seedlings revealing electrolyte leakage above 80–90 % are not likely to recover from the cold stress injuries. On the other hand, seedlings showing electrolyte leakage below 70 % are likely to nearly fully recover, although they may exhibit dif-

ferent rates of recovery, proportional to their electrolyte leakage values. Having this in mind, we recommend stopping the cold stress treatment when the test genotypes reveal (differential) electrolyte leakage values between 50 and 80 %, and the most susceptible genotypes (possibly the susceptible standard used) already reach 80–90 % of electrolyte leakage.

24. Moving plants back to control conditions during the dark period is strongly advised, to avoid photoinhibition, which could have severe consequences to the plant and may mask the cold stress effects to be screened. If a specific procedure requires transfer during the light period, then it is recommended to considerably reduce the light intensity (e.g., to 10 % of the light intensity used for control growth conditions) during the first light period and only returning to full light intensity (300–500 $\mu E/m^2/s$) on the following day.
25. In our experience, 1 week of recovery is enough to assess if seedlings have survived the cold stress treatment or not, although there might be the need to extend this period to photo-document the results.
26. The number of replicates or the number of plants per pool will depend on the genetic heterogeneity of the genotypes under study.
27. We find it useful to transfer the leaf pieces (using forceps) into a strainer to be rinsed with distilled water.
28. Measure electrical conductivity of each sample three times and calculate the mean.
29. To disrupt plant tissues, it is possible to substitute the incubation at 80 °C by a freezing (at –20 °C)/thawing cycle.
30. In some cases all the seedlings will survive the treatment, but different genotypes may show distinct recovery rates. In these cases, it would be important to assess the recovery evolution process by registering new-leaf emergence rate in the seedlings.
31. To determine the weight of dry soil in each pot, the soil mixture from four or five extra pots must be completely dried (0 % of soil water content) in a tray kept in an oven (e.g., 80 °C) until the weight does not change. This will allow calculating the weight of the dried soil mixture per pot.
32. Make a hole in the soil, approximately 5 cm deep and 1 cm wide, and carefully transfer the seedling while avoiding damage of the root system. Accommodate the seedling without too much pressure. Transfer the same number of seedlings to each pot to allow an even water uptake among pots. Do not place different genotypes in the same pot to avoid adding an extra variable (interaction between root systems).

33. Take into consideration that the transferred seedling will need to adapt to the soil mixture for at least 5–7 days (Fig. 5).
34. Some glasshouses or growth chambers may lead to different evaporation rates in different locations. It will therefore be necessary to make sure that differences in water loss are not due to different chamber locations. Thus, one must randomize the location of the pots with the different genotypes and change their position regularly, to minimize the effects of different evaporation zones.
35. Keep some trays with water to use as controls. Leaf material collected at this stage (the beginning of water withholding) for molecular or biochemical analysis is referred to as a control. Nevertheless, and depending on the extent of the drought assay and the age of the plants, the ideal control material is collected from water-filled trays at the same time points that samples are collected from drought-stressed plants.
36. Calculating SWC is a good way of assessing the degree of stress imposed to the plants. SWC can be assessed by measuring the pot weight. After determining the soil dry weight and field capacity (100 % of water) of the soil mixture, it is possible to determine the percentage of water lost in each day after water withholding. This allows evaluating how different genotypes are losing water along time and at which percentage of SWC the drought symptoms become visible. It is necessary to evaluate the rice genotype water uptake profile. Some genotypes may use more water than others and the reduction of SWC may differ. The percentage of SWC was calculated as

    $$SWC\ \% = [(PW - PW_{min})/(PW_{max} - PW_{min})] \times 100$$

    (PW: Pot Weight after water withholding; PW_{min}: Pot weight with dry soil; PW_{max}: Pot Weight with maximum soil water content)
37. Plants at this drought stage (approximately 10 % of SWC) are severely stressed. The re-watering must be performed at the beginning of the dark period to avoid the over-production of reactive oxygen species (ROS).
38. Leaf rolling is a characteristic response of rice plants under drought (also common to many other plants). When the SWC reaches values of 10–15 % (e.g., Nipponbare), rice leaves start rolling. Permanent wilting point (PWP) is known as the minimal SWC that the plant stands without wilting. For practical reasons, we can define PWP as the SWC when the first rice leaf in a pot starts wilting. This allows determining the PWP for different genotypes even if they lose water differentially. Maintaining the SWC at the permanent wilting point for 50 % of the plants during a long term (10–14 days) will allow

discriminating between drought-tolerant and drought-sensitive rice genotypes [26].

39. When collecting leaf samples for analysis, collect them from the same number of plants per pot in order to avoid a differential water loss across pots.

Acknowledgments

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References

1. Shannon MC, Rhoades JD, Draper JH, Scardaci SC, Spyres MD (1998) Assessment of salt tolerance in rice cultivars in response to salinity problems in California. Crop Sci 38(2):394–398
2. FAO (2008) FAO Land and plant nutrition management service. http://www.fao.org/ag/agl/agll/spush.
3. Shavrukov Y, Genc Y, Hayes J (2012). The Use of Hydroponics in Abiotic Stress Tolerance Research, Hydroponics - A Standard Methodology for Plant Biological Researches, Dr. Toshiki Asao (Ed.), ISBN: 978-953-51-0386-8, InTech, DOI: 10.5772/35206
4. Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681. doi:10.1146/annurev.arplant.59.032607.092911
5. Gregorio GB, Senadhira D, Mendoza RD (1997) Screening rice for salinity tolerance. IRRI Discussion Paper Series N 22 22
6. IRRI (2006) Rice Breeding Course. http://www.knowledgebank.irri.org/ricebreedingcourse/bodydefault.htm#Breeding_for_salt_tolerance.htm. 2014
7. Afza R, Zapata-Arias FJ, Zwuletitsch F, Berthold G, Gregorio G (1999) Modification of a rapid screnning method of rice mutant for NaCl tolerance using liquid culture. Mutat Breed Newsl 44:25–28
8. Tavakkoli E, Fatehi F, Rengasamy P, McDonald GK (2012) A comparison of hydroponic and soil-based screening methods to identify salt tolerance in the field in barley. J Exp Bot 63(10):3853–3868. doi:10.1093/jxb/ers085
9. Farrell TC, Fox KM, Williams RL, Fukai S, Lewin LG (2006) Minimising cold damage during reproductive development among temperate rice genotypes. II. Genotypic variation and flowering traits related to cold tolerance screening. Aust J Agric Res 57(1):89–100
10. Zhou L, Zeng YW, Hu GL, Pan YH, Yang SM, You AQ, Zhang HL, Li JJ, Li ZC (2012) Characterization and identification of cold tolerant near-isogenic lines in rice. Breed Sci 62(2):196–201
11. Li TG, Visperas RM, Vergara BS (1981) Correlation of cold tolerance at different growth stages in rice. Acta Bot Sin 23:203–207
12. Satake T, Hayase H (1970) Male sterility caused by cooling treatment at the young microspore stage in rice plants. V. Estimation of pollen developmental stage and the most sensitive stage to coolness. Proc Crop Sci Soc Japan 39:468–473
13. Ye C, Fukai S, Godwin I, Reinke R, Snell P, Schiller J, Basnayake J (2009) Cold tolerance in rice varieties at different growth stages. Crop Pasture Sci 60(4):328–338. doi:10.1071/Cp09006
14. Andaya VC, Mackill DJ (2003) Mapping of QTLs associated with cold tolerance during the vegetative stage in rice. J Exp Bot 54(392):2579–2585. doi:10.1093/Jxb/Erg243

15. Bertin P, Kinet JM, Bouharmont J (1996) Evaluation of chilling sensitivity in different rice varieties. Relationship between screening procedures applied during germination and vegetative growth. Euphytica 89(2):201–210. doi:10.1007/Bf00034606
16. Suh JP, Jeung JU, Lee JI, Choi YH, Yea JD, Virk PS, Mackill DJ, Jena KK (2010) Identification and analysis of QTLs controlling cold tolerance at the reproductive stage and validation of effective QTLs in cold-tolerant genotypes of rice (*Oryza sativa* L.). Theor Appl Genet 120(5):985–995. doi:10.1007/s00122-009-1226-8
17. Yu X, Peng YH, Zhang MH, Shao YJ, Su WA, Tang ZC (2006) Water relations and an expression analysis of plasma membrane intrinsic proteins in sensitive and tolerant rice during chilling and recovery. Cell Res 16(6):599–608. doi:10.1038/sj.cr.7310077
18. Kim SI, Tai TH (2011) Evaluation of seedling cold tolerance in rice cultivars: a comparison of visual ratings and quantitative indicators of physiological changes. Euphytica 178(3):437–447. doi:10.1007/s10681-010-0343-4
19. Comas LH, Becker SR, Cruz VM, Byrne PF, Dierig DA (2013) Root traits contributing to plant productivity under drought. Front Plant Sci 4:442
20. Catala R, Ouyang J, Abreu IA, Hu Y, Seo H, Zhang X, Chua NH (2007) The *Arabidopsis* E3 SUMO ligase SIZ1 regulates plant growth and drought responses. Plant Cell 19(9):2952–2966
21. Miura K, Okamoto H, Okuma E, Shiba H, Kamada H, Hasegawa PM, Murata Y (2013) SIZ1 deficiency causes reduced stomatal aperture and enhanced drought tolerance via controlling salicylic acid-induced accumulation of reactive oxygen species in *Arabidopsis*. Plant J 73(1):91–104
22. Lourenço T, Saibo N, Batista R, Ricardo CP, Oliveira MM (2011) Inducible and constitutive expression of HvCBF4 in rice leads to differential gene expression and drought tolerance. Biol Plant 55(4):653–663
23. Maxwell K, Johnson GN (2000) Chlorophyll fluorescence—a practical guide. J Exp Bot 51(345):659–668
24. Barr HD, Weatherley PE (1962) A re-examination of the relative turgidity technique for estimating water deficit in leaves. Aust J Biol Sci 15:413–428
25. IRRI (1988) Standard evaluation system for rice. International Rice Research Institute (IRRI): Los Baños (The Philippines)
26. Degenkolbe T, Do PT, Zuther E, Repsilber D, Walther D, Hincha DK, Kohl KI (2009) Expression profiling of rice cultivars differing in their tolerance to long-term drought stress. Plant Mol Biol 69(1-2):133–153
27. Mackill DJ, Lei XM (1997) Genetic variation for traits related to temperate adaptation of rice cultivars. Crop Sci 37(4):1340–1346
28. Duan M, Huang P, Yuan X, Chen H, Huang J, Zhang HS (2014) CMYB1 Encoding a MYB Transcriptional Activator Is Involved in Abiotic Stress and Circadian Rhythm in Rice. Scientific World Journal doi:Artn 178038
29. Dong MA, Farre EM, Thomashow MF (2011) CIRCADIAN CLOCK-ASSOCIATED 1 and LATE ELONGATED HYPOCOTYL regulate expression of the C-REPEAT BINDING FACTOR (CBF) pathway in *Arabidopsis*. Proc Natl Acad Sci U S A 108(17):7241–7246. doi:10.1073/pnas.1103741108
30. Fowler SG, Cook D, Thomashow ME (2005) Low temperature induction of *Arabidopsis* CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol 137(3):961–968. doi:10.1104/pp.104.058354
31. Kim S-I, Kim D-M, Tai TH (2012) Evaluation of Rice Seedling Tolerance to Constant and Intermittent Low Temperature Stress. Rice Sci 19(4):295–308. doi:10.1016/S1672-6308(12)60054-7, http://dx.doi.org
32. Yoshida S, Forno DA, Cock JH, Gomez KA (1976) Laboratory manual for physiological studies of rice. International Rice Research Institute (IRRI), Manila (The Philippines)

Chapter 15

Basic Techniques to Assess Seed Germination Responses to Abiotic Stress in *Arabidopsis thaliana*

Urszula Piskurewicz and Luis Lopez-Molina

Abstract

The model organism *Arabidopsis thaliana* has been extensively used to unmask the molecular genetic signaling pathways controlling seed germination in plants. In *Arabidopsis*, the normal seed to seedling developmental transition involves testa rupture soon followed by endosperm rupture, radicle elongation, root hair formation, cotyledon expansion, and greening. Here we detail a number of basic procedures to assess *Arabidopsis* seed germination in response to different light (red and far-red pulses), temperature (seed thermoinhibition), and water potential (osmotic stress) environmental conditions. We also discuss the role of the endosperm and how its germination-repressive activity can be monitored genetically by means of a seed coat bedding assay. Finally we detail how to evaluate germination responses to changes in gibberellin (GA) and abscisic acid (ABA) levels by manipulating pharmacologically the germination medium.

Key words Seed germination, Testa rupture, Endosperm rupture, Seed coat bedding assay, Osmotic stress, Thermoinhibition, Gibberellins, Abscisic acid

1 Introduction

The appearance of seeds represents a major chapter in the history of embryophytes. Seeds have notably favored the spread of gymnosperms and angiosperms. Indeed, the seed is a capsule where the plant embryo lies in a metabolic inert and highly protected state allowing the plant to travel in time and in space. Eventually, the plant must resume its life cycle and this is accomplished through the process of seed germination, a crucial developmental transition absolutely necessitating seed imbibition with water.

Abandoning the highly protected state of the seed is hazardous since the newly formed seedling is highly fragile. It is therefore unsurprising that mechanisms controlling seed germination have appeared during evolution.

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DOI 10.1007/978-1-4939-3356-3_15,

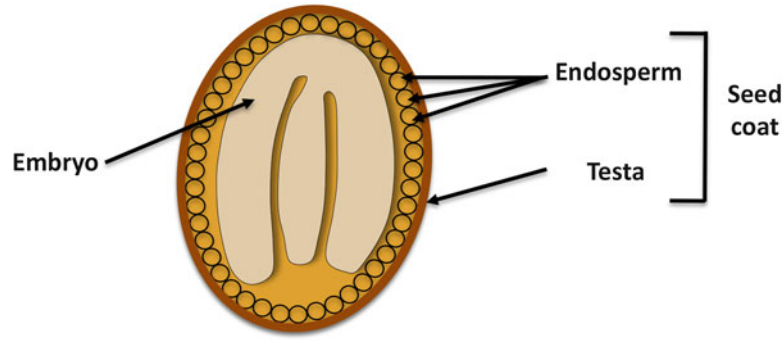

Fig. 1 A schematic drawing of an *Arabidopsis* dry seed

The model plant *Arabidopsis thaliana* has been widely used to study seed germination in angiosperms and particularly the molecular genetics of the signaling pathways controlling seed germination.

The mature *Arabidopsis* seed is the endpoint of embryogenesis and is composed of an external layer of maternal dead tissue, the testa, arising from ovular integuments. Underneath the testa lies a single cell layer of endosperm surrounding the mature embryo (Fig. 1). The *Arabidopsis* endosperm is a triploid tissue that is the result of double fertilization, which is typical of angiosperms, containing two copies of the maternal genome and one copy of the paternal genome. The micropylar part of the seed is where the first events of seed germination can be observed under standard germination conditions (SGC): testa rupture soon followed by micropylar endosperm rupture, where endosperm cells detach from each other while the radicle tip protrudes out of the seed [1] (Fig. 2). The occurrence of this latter event is usually chosen as the criteria defining "seed germination" [2]. However, there are numerous developmental steps taking a mature seed towards a young seedling. Each of those steps is expected to be under tight genetic and environmental control. This should be kept in mind when assessing "seed germination" since the order of execution of these steps might be altered depending on the genetic background and environmental conditions faced by the seed. For example, endosperm rupture can take place without prior visible testa rupture, as in the case of *abi5* mutants under low GA conditions (the so-called "explosive" germination, *see* Fig. 2) [3].

In *Arabidopsis* several seed germination control mechanisms can be studied. They include seed dormancy, light-dependent seed germination, osmotic stress-dependent seed germination, and high temperature-dependent seed germination. Control of seed germination is an active process involving the biosynthesis of two terpenoid plant hormones: abscisic acid (ABA) and gibberellins (GA). ABA inefficiently prevents testa rupture but efficiently prevents

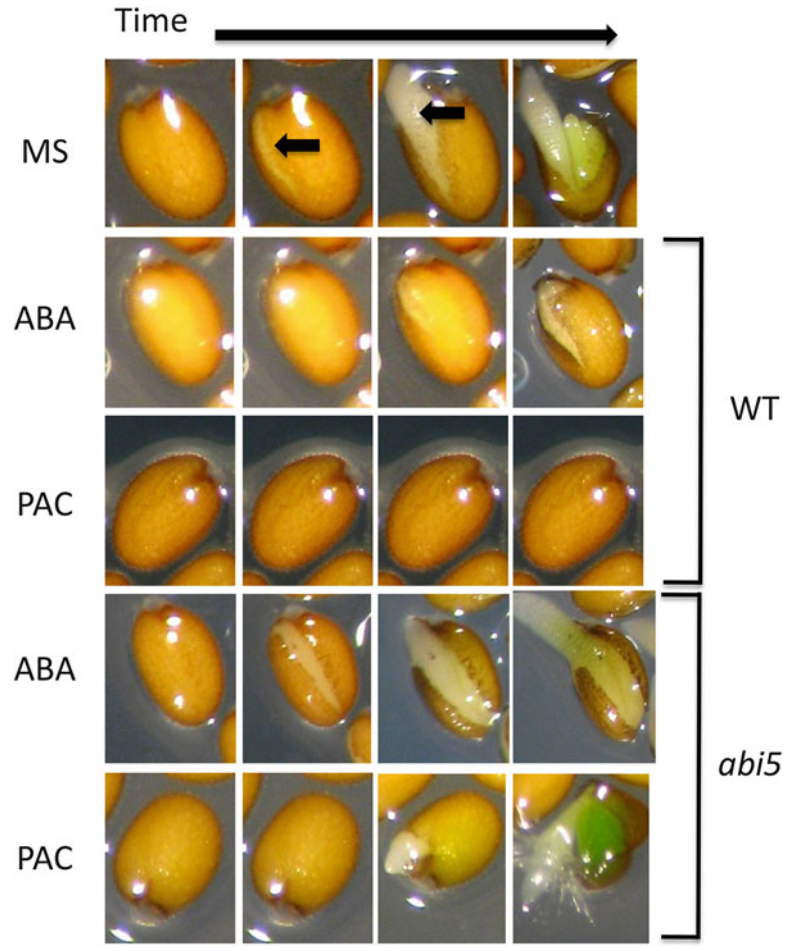

Fig. 2 Pictures represent Col-0 wild-type seeds (WT) plated on MS medium in the absence (MS) or presence of 5 μM ABA or 5 μM PAC. An *arrow* indicates testa and endosperm rupture under MS conditions. ABA in germination medium delays testa rupture and prevents endosperm rupture. PAC in germination medium blocks both testa and endosperm rupture. In the presence of ABA*abi5* mutant seeds rupture testa earlier than wild type and are also able to rupture endosperm. In the presence of PAC, *abi5* seeds rupture endosperm prior to testa rupture (the so-called explosive germination)

endosperm rupture [2, 3] (Fig. 2). It was proposed that ABA protects the plant by maintaining its embryonic state in a growth-arrested form [4, 5]. In contrast, GA efficiently promotes testa and endosperm rupture, cellular growth, and greening. Environmental conditions unfavorable for seed germination are associated with high ABA levels and low GA levels in seeds whereas the inverse situation is observed under favorable conditions [6, 7].

Seed dormancy is a default property of freshly produced seeds where seed germination is prevented even under favorable

germination conditions [8, 9]. Seeds lose their dormancy after a period of after-ripening or after seed stratification (a cold treatment under moist conditions) [10]. Different *Arabidopsis* accessions display variable levels of seed dormancy. This trait ensures that seeds do not germinate out of season.

When seeds become post-dormant, i.e. non-dormant, additional control mechanisms can be observed. Canopy light, unfavorable for photosynthesis, is enriched in far-red (FR) light and the ratio of red (R) to FR light in the environment has a strong influence on seed germination, a process involving the phytochromes A and B (phyA and phyB) [11–13]. Water potential is also perceived by the seed and high salinity conditions will block seed germination (osmotic stress). Similarly, high temperatures are also perceived by the plant and this blocks germination, a process called seed thermoinhibition. Here it is important to underline that newly post-dormant seeds, i.e., seeds newly able to germinate under normal conditions, will tend to be highly reactive upon exposure to even mildly unfavorable seed germination conditions. This must be taken into account when studying the germination responses of seeds in search for germination responses phenotypes. For example, newly post-dormant seeds may poorly germinate in darkness and even after an early R pulse followed by darkness. In certain cases, the high responsiveness of newly post-dormant seeds can prevent revealing even the most striking phenotypes. For example, the *PIF1* gene encodes a basic helix-loop-helix (bHLH) transcription factor (TF) necessary to repress seed germination after an early FR pulse followed by darkness [14]. However, newly post-dormant *pif1* seeds may not only fail to germinate after an early FR pulse but also after an early R pulse, which strongly stimulates germination of wild-type (WT) seeds provided that they are sufficiently after-ripened (UP and LLM, unpublished). Thus, the researcher must ensure that the conditions of the germination assay are carefully calibrated to maximize the chances of detecting alterations in seed germination responses of a given *Arabidopsis* mutant seed. This can be accomplished by systematically including in the seed germination response assay WT and known mutant seeds to ensure that they behave with the expected germination responses (*see* below).

Recent work has provided strong support to the notion that the endosperm plays a central role to control seed germination. Previous studies had shown that the endosperm, rather than the testa, exerted a germination-repressive activity essential to prevent the germination of dormant seeds upon their imbibition [15, 16]. Lee et al. developed a “seed coat bedding assay” where dissected *Arabidopsis* embryos are cultured on a bed of dissected seed coats allowing the monitoring of their growth [16, 17]. This assay allows to genetically dissect in vitro the signaling pathways operating in the endosperm that regulate the growth of the embryo. In particular, it

could be shown that the endosperm of dormant seeds or that of non-dormant seeds irradiated with an FR pulse synthesizes and releases ABA towards the embryo to block its germination [11, 16]. In certain cases "explosive" germination can be observed where the embryo exits the enclosure of the seed coat without prior visible rupture of the testa [3, 11]. This further illustrates the notion that the endosperm exerts a germination-repressive activity that may be overcome by the embryo under particular genetic or environmental germination conditions.

Here we detail a number of basic procedures to assess germination responses of *Arabidopsis thaliana* seeds.

2 Materials

1. Murashige and Skoog basal medium (MS medium): 4.3 g/L MS basal salt mixture (Sigma), 0.5 g/L 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma), 0.8 % (w/v) agar, pH 5.7.
2. Seeds sterilization solution: 30 % commercial bleach (bleach: 2.5 % sodium hypochlorite), 0.05 % Tween.

3 Methods

3.1 Assessing Seed Germination under Abiotic Stress

Under standard growth conditions (22–24 °C, 100 μmol/m^2/s, 16-h/8-h day/night photoperiod, 70 % relative humidity (RH)), dry siliques are obtained ~8 weeks after planting. Depending on the ecotype, freshly harvested seeds will exhibit different levels of dormancy. Here we describe how to assess the germination of wild-type (WT) seeds of the Col-0 ecotype 2 weeks after seed harvesting since they display weak dormancy. Seed responses to abiotic stress after stratification are described in Subheading 4. Seed storage at 4 °C markedly optimizes seed viability over time.

3.1.1 Surface Sterilization of Seeds with Bleach

1. Prepare fresh seed sterilization solution.
2. Incubate ~20–50 μL of seeds in microcentrifuge tube with seed sterilization solution for 10 min at room temperature with occasional shaking (*see* **Note 1**).
3. Shortly spin down sterilized seeds or wait for a few seconds until they settle down at the bottom of the tube.
4. Thoroughly remove supernatant.
5. Rinse seeds three times with sterile water under laminar flow hood. The volume of water should exceed the volume of seeds at least fivefold to ensure appropriate removal of all traces of sterilization solution.

6. After third rinse leave 200 μl of sterile water in microcentrifuge tube.
7. Use a 1 ml micropipette tip to resuspend seeds and immediately transfer seeds onto the surface of the germination medium.

3.1.2 Surface Sterilization of Seeds with Ethanol

1. Add 70 % ethanol to ~20–50 μL of dry seeds, resuspend them well so that each seed is in direct contact with ethanol, and incubate them for 30 s.
2. Shortly spin down sterilized seeds or wait for a few seconds until they settle down at the bottom of the tube.
3. Remove supernatant and add 100 % ethanol.
4. Use a 1 ml micropipette tip to resuspend seeds and immediately plate seeds onto the surface of a sterile filter paper and wait until ethanol evaporates.
5. Transfer seeds one by one on germination medium (*see* **Note 2**) by touching them with the tip of a sterile toothpick. Wetting the tip with sterile water facilitates this procedure.
6. Alternatively, after removing 70 % ethanol, add 1 ml of sterile water to the seeds and incubate them for 10 min. Spin down the seeds and remove the supernatant. Rinse seeds three times with sterile water. After the third rinse resuspend seeds in 200 μl of sterile water and transfer them onto the surface of the germination medium using a 1 ml micropipette tip.

3.1.3 Assessing Testa and Endosperm Rupture under Standard Germination Condition

1. Prepare MS medium plate.
2. Surface-sterilize seeds (*see* Subheading 3.1.1 or 3.1.2).
3. Plate between 100 and 300 seeds onto MS medium.
4. If necessary aspirate the excess water surrounding the seeds. Further leave the plate containing the seeds uncovered in the laminar flow cabinet until all water surrounding the seeds is dried out.
5. Place plates in growth chambers under standard growth conditions (22–24 °C, 100 μmol/m^2/s, 16-h/8-h day/light photoperiod).
6. 20 h after seed plating, start taking pictures every 6–8 h using a stereomicroscope coupled to a digital camera.
7. Enlarge electronically photographs and count testa and endosperm rupture events at different times after seed plating.

For statistical accuracy a minimum of three independently grown seed batches are necessary to assess differences in seed germination (*see* **Note 3**).

An example of testa and endosperm rupture frequency over time of Col-0 wild-type seeds is presented in Fig. 3.

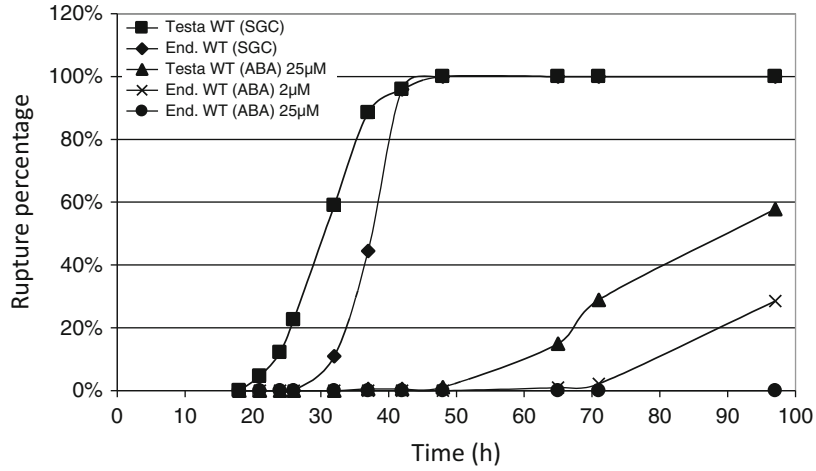

Fig. 3 Curves represent frequency of testa and endosperm (End.) rupture events of Col-0 wild-type (WT) seeds plated onto MS medium in the absence (SGC) or presence of ABA (ABA). For testa rupture inhibition high concentration of ABA was used (25 μM) whereas for endosperm rupture inhibition low (2 μM) and high (25 μM) concentrations of ABA were used

3.1.4 Assessing Testa and Endosperm Rupture under Osmotic Stress Conditions

Assess testa and endosperm rupture on MS medium supplemented with NaCl at concentrations ranging from 100 to 300 mM or with mannitol at concentrations ranging from 300 to 600 mM. Under such conditions, testa rupture is delayed when compared to standard germination conditions and endosperm rupture is either drastically delayed (by concentrations of NaCl and mannitol ranging from 100 mM to 150 mM and from 300 mM to 400 mM mannitol, respectively) or completely blocked (NaCl and mannitol concentrations greater than 200 mM and 500 mM, respectively).

Osmotic stress inhibits seed germination by triggering endogenous synthesis of ABA. As expected, supplementing germination medium with ABA prevents germination in a similar manner as osmotic stress; that is, testa rupture is delayed while endosperm rupture is blocked. This treatment is thus considered to mimic an osmotic stress upon seed imbibition. To mimic osmotic stress, ABA concentrations in the medium should be between 2 and 5 μM to prevent endosperm rupture and between 20 and 200 μM to delay testa rupture (*see* Figs. 2 and 3).

Several *Arabidopsis* mutants display testa and endosperm rupture responses that are insensitive to osmotic stress upon seed imbibition. We use *abi5* as a reference for weaker germination responses to osmotic stress [4, 18, 19] (*abi5* ruptures testa faster than wild type under osmotic stress and ruptures endosperm while wild type does not) (*see* Fig. 2).

3.1.5 Assessing Testa and Endosperm Rupture under Canopy Light

1. Prepare two MS medium plates.
2. Surface sterilize seeds (*see* Subheading 3.1.1 or 3.1.2).
3. Plate between 100 and 300 seeds onto two MS medium plates.

4. If necessary aspirate the excess water surrounding the seeds. Further leave the plate containing the seeds uncovered in the laminar flow cabinet until all water surrounding the seeds is dried out.
5. One hour after plating seeds onto MS medium transfer the plates to a dark room. Illuminate one plate with far-red light (FR) of intensity of 3.2–5 μmol/m^2/s for 5 min (*see* **Note 4**). As a control for light-induced germination, illuminate the second plate first with FR pulse of 5 min (3.2 μmol/m^2/s) and then immediately illuminate the same plate with red (R) light (20 μmol/m^2/s) for 5 min.
6. Wrap MS medium plates in aluminum foil ensuring that day light is not penetrating into the plates and incubate plates in a growth chamber under normal germination conditions.
7. Unwrap the plates after 3–5 days to assess germination events.

Wild-type Col-0 seeds treated with an FR pulse do not germinate even after 5 days after the treatment. In contrast, an FR pulse immediately followed by an R pulse leads to high germination rates. It is important to remind here that fresh seeds will tend to poorly germinate under these unfavorable germination conditions, even in response to an R light pulse. *pif1* mutant seeds can be used as a positive control for germination after an FR pulse [14, 20] (*pif1* ruptures testa 24 h after FR illumination and ruptures endosperm 3 days after FR illumination) (*see* **Notes 4** and **5**).

FR inhibits seed germination by inhibiting GA synthesis. Therefore, supplementing the germination media with paclobutrazol (PAC), an inhibitor of GA biosynthesis, causes a germination arrest similar to that observed after an FR pulse (i.e., testa and endosperm rupture blockade). PAC concentrations in the medium should be between 5 and 20 μM to ensure complete seed germination arrest (*see* Fig. 2). A more detailed discussion about the use of PAC can be found in the supplementary Fig. 10 of Piskurewicz et al. 2009 [20]. Indeed, at high concentrations, PAC may interfere with ABA biosynthesis [21].

3.1.6 Assessing Testa and Endosperm Rupture under High Temperatures

Same as Subheading 3.1.3 but MS medium plates incubated at temperatures ranging from 26 to 36 °C (*see* **Note 6**).

3.2 Molecular Genetic Assessment of Seed Germination under Abiotic Stress

3.2.1 Molecular Markers to Assess Germination under Standard Germination Conditions

1. Plate seeds under SGC (*see* Subheading 3.1.3).
2. Collect ~200 μL of imbibed seeds every 8–12 h after seed plating. Alternatively, at each time point seeds can be dissected so as to separate embryos and seed coats (*see* Subheading 3.3). Freeze seeds or dissected seed material in liquid nitrogen and store at −80 °C until use.
3. Isolate RNA from collected seed material [22, 23].
4. Synthesize cDNA using 0.5–2 μg of total RNA.

5. Analyze gene expression by qPCR (*see* **Note 7**). Genes positively correlated with seed germination include *GA20ox1* (AT4G25420), *GA20ox2* (AT5G51810), *GA20ox3* (AT5G07200), *GA3ox1* (AT1G15550), *GA3ox2* (AT1G80340), *CYP707A1* (AT4G19230), and *CYP707A2* (AT2G29090), while the expression of the following genes is negatively correlated with seed germination: *GA2ox1* (AT1G78440), *GA2ox2* (AT1G30040), *RGL2* (AT3G03450), *NCED6* (AT3G24220), *NCED9* (AT1G78390), *ABI5* (AT2G36270), *ABI3* (AT3G24650), and *ABI4* (AT2G40220).

3.2.2 Molecular Markers to Assess Germination under Osmotic Stress

Same as Subheading 3.2.1 but seeds are plated on plates that are supplemented with NaCl, mannitol, or ABA. For a precise characterization of seed responses to osmotic stress we recommend to isolate RNA separately from embryo and from the seed coat. A minimum of 200 seeds, embryos, or seed coats are necessary to isolate enough RNA for expression analysis (*see* **Notes 7** and **8**).

3.2.3 Molecular Markers to Assess Germination under Canopy Light

Same as Subheading 3.2.1 but seeds are illuminated with FR and R light as described in Subheading 3.1.5.

Additionally, the expression of *PIF1* (AT2G20180) and *RGL2* (AT3G03450) in the seed coat and that of *SOM* (AT1G03790), *GAI* (AT1G14920), and *RGA* (AT2G01570) in the embryo positively correlate with germination arrest (*see* **Note 7**) [11].

3.2.4 Molecular Markers to Assess Germination under High Temperature

Same as Subheading 3.2.1 but seeds plated onto MS medium are placed at high temperature.

Additionally, the expression of *NCED5* (AT1G30100) and *NCED9* (AT1G78390) in both the seed coat and embryo positively correlates with germination arrest under high temperatures (*see* **Note 7**) [24].

3.3 Seed Coat Bedding Assay

The seed coat bedding assay (SCBA) is an in vitro procedure where dissected embryos are cultured on a layer of dissected seed coats [11, 16, 17]. The SCBA allows following the growth of the embryos under the influence of the underlying layer of endosperm. Seed coat and embryonic material of different ages or genetic background can be used to dissect the genetic pathways operating in the endosperm and embryo controlling seed germination. This procedure was used successfully in the context of seed dormancy and control of seed germination by R and FR pulses [11, 16]. We previously described in detail how to assemble an SCBA [17]. However for the sake of completeness of this chapter we provide here the essential steps of the protocol.

1. Prepare three MS medium plates. Place a sterile 0.45 μm pore size nylon mesh on the plate on which seeds will be plated.

2. Surface sterilize seeds using seed sterilization solution or using 70 % ethanol followed by immediate water imbibition (*see* Subheading 3.1.1 or 3.1.2).
3. Plate between 100 and 300 sterile seeds on the surface of the nylon mesh prepared as described above.
4. Aspirate the excess water surrounding the seeds. Leave the plate and seeds uncovered in the laminar flow cabinet until all water surrounding the seeds is dried out.
5. Close the plate and incubate seeds for 4 h under standard germination conditions (22–24 °C, 100 μmol/m^2/s, 16-h/8-h day/light photoperiod). It is possible to leave the closed plates for 4 h in the laminar flow hood.
6. Place a stereomicroscope inside the hood.
7. Take a new MS medium plate. Place two juxtaposed, rectangular, autoclaved Whatman 3 MM papers onto the surface of the medium.
8. Using a pair of sterile blunt tip Dumont #5 forceps transfer the nylon mesh with the seeds onto the Whatman 3MM papers.
9. Using a 29 G insulin syringe needle tip, cut the seed coat along the longest semi-principal axis as close as possible to the location where the cotyledons are joined to the radicle (*see* Fig. 4a).
10. Using the forceps push the seed against the paper, applying gentle pressure on the side of the radicle tip to release the embryo from the seed coat (*see* Fig. 4a).
11. Carefully isolate at least 100–150 embryos from their seed coats. Make sure that embryos do not get damaged during the procedure.
12. To assemble the SCBA place a 3.5 × 5 cm piece of sterile nylon mesh onto a fresh MS medium plate.
13. Transfer the dissected embryos and seed coats onto the nylon mesh.
14. Using forceps and syringe assemble a single layer of at least 100–150 seed coats, making sure that the coats are in as close proximity as possible and the openings in the seed coats are facing up.
15. Place 10–20 embryos in a single, circular layer on the center of the seed coat bed, ensuring that the embryos are in as close proximity as possible (Fig. 4b, c).
16. Incubate the seed coat bedding assays under standard germination conditions or any suitable environmental condition (see **Note 9**).

Following this procedure, other methods such as the analysis of gene expression from seed coats and embryos cultured in the context of the SCBA can be performed (*see* **Note 7**).

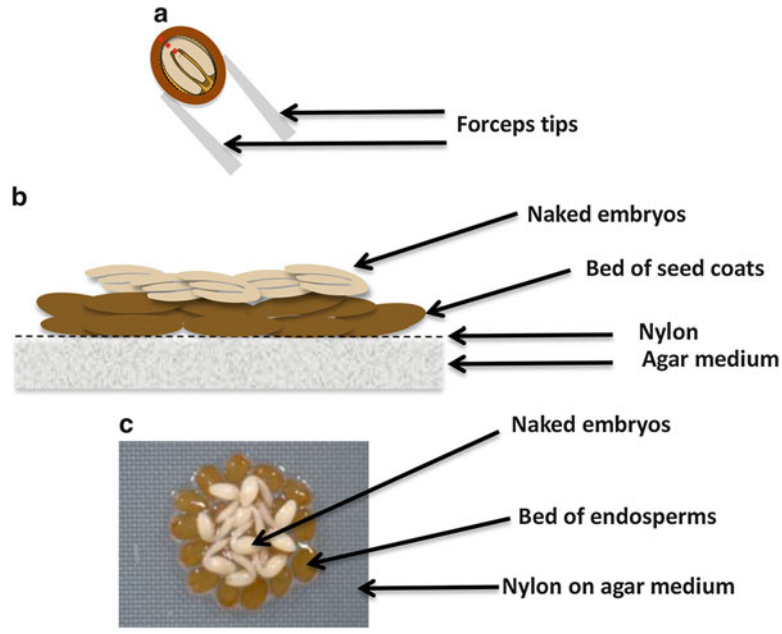

Fig. 4 (**a**) Procedure for *Arabidopsis* seed dissection. *Dashed red line* indicates the location where the seed coat is cut with a needle. The embryo is gently squeezed out of the seed coat enclosure by gently pressing with forceps as depicted. (**b**) A drawing showing an assembled seed coat bedding assay. Coat-less dissected embryos are cultured on a bed of seed coats resting on a nylon filter that is lying on a germination (agar) medium. (**c**) A picture of an assembled seed coat bedding assay

4 Notes

1. After adding seed sterilization solution to dry seeds, the seed mucilage is released, which increases the volume containing the seeds. The sterilization solution should exceed the volume of dry seeds by at least tenfold to ensure good contact of seeds with sterilization buffer.
2. Germination frequency curves presented in this chapter (Fig. 3) show germination dynamics of seeds that were surface sterilized using bleach solution. Seed sterilization with ethanol results in slight delay of germination when compared to seeds sterilized with bleach solution.
3. All seeds whose germination is assessed and compared have to be harvested on the same day from plants grown side by side (i.e., in identical environmental conditions) and stored in the same conditions for the same period of time.
4. Depending on the dormancy levels, seeds differ in responses to R and FR light. For example freshly harvested (i.e., dormant)

seeds of a *pif1* mutant do not germinate after a 5-min pulse of FR light at intensity of 3.2 μmol/m^2/s. Those seeds are able to germinate only when either the length or the intensity of the FR light pulse is markedly decreased (for example FR pulse of intensity 3.2 μmol/m^2/s can be decreased to 30 s and still repress germination of fresh wild-type seeds). However, after few weeks of after-ripening, non-dormant *pif1* seeds germinate after a 5-min pulse of FR light at intensity of 3.2 μmol/m^2/s. Similarly, after a stratification (i.e., dormancy breaking by imbibing seeds in 4 °C for 3 days) wild-type seeds become insensitive to FR illumination and germinate after a pulse of 5 min of FR light at intensity of 3.2 μmol/m^2/s. Similarly, fresh seeds of Col-0 ecotype fail to germinate after a 5-min pulse of R light (20 μmol/m^2/s) unless the intensity or the duration of the R pulse is increased. Similar trends in seed germination responses can be observed when using PAC treatments (i.e., hypersensitive seed germination arrest in fresh seeds versus older seeds in response to PAC).

5. Dormancy of seeds is affected by the environmental conditions faced by the mother plant at the time of seed maturation. Seeds that are produced by plants grown at low temperatures (10–15 °C) are very dormant in all ecotypes tested in our laboratory, including Col-0 and Ler ecotypes [25].
6. Dormancy levels of seeds determine the strength of their seed thermoinhibition responses. Prolonged after-ripening renders seeds less responsive to high temperature-dependent seed germination inhibition. Thus, higher temperatures are needed to arrest the germination of after-ripened wild-type seeds.
7. When studying endosperm- or embryo-specific gene expression, it is advisable to ensure that no cross contamination has taken place during seed dissection. This can be done by monitoring the expression of *EPR1* (AT2G27380), a gene specifically expressed in the seed coat, and that of ABI4 (AT2G40220), a gene specifically expressed in the embryo [11, 26–28].
8. Under osmotic stress wild-type seeds display high *ABI3* expression in both the embryo and the seed coat, high *ABI5* expression levels in the embryo and in the micropylar endosperm, and high *ABI4* expression specifically in the embryo [27].
9. *ga1* and *ga1/rgl2* mutant seeds can be used as controls to assess whether the SCBA has been properly assembled [17]. *Arabidopsis ga1* mutant seeds (unable to synthesize gibberellins) cannot germinate even 120 h after seed imbibition under standard germination conditions. However, when seed coats are removed from *ga1* seeds, the embryonic growth and greening can be observed as early as 24 h after removal of the seed coat. When the dissected embryos of *ga1* mutant seeds are

cultured on a bed of *ga1* seed coats, the greening and growth of *ga1* embryos are prevented. However, when *ga1* embryos are cultured on a bed of *ga1/rgl2* double-mutant seed coats, the growth and greening of *ga1* embryos are not inhibited. This experiment shows that the activity of endosperm to repress the growth of *ga1* embryo necessitates functional RGL2 in the seed coat.

References

1. Belin C, Lopez-Molina L (2008) *Arabidopsis* seed germination responses to osmotic stress involve the chromatin modifier PICKLE. Plant Signal Behav 3(7):478–479
2. Muller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of Lepidium sativum (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. Plant Cell Physiol 47(7):864–877
3. Piskurewicz U, Jikumaru Y, Kinoshita N, Nambara E, Kamiya Y, Lopez-Molina L (2008) The gibberellic acid signaling repressor RGL2 inhibits *Arabidopsis* seed germination by stimulating abscisic acid synthesis and ABI5 activity. Plant Cell 20(10):2729–2745. doi:10.1105/tpc.108.061515
4. Lopez-Molina L, Mongrand S, Chua NH (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. Proc Natl Acad Sci USA 98(8):4782–4787
5. Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua NH (2002) ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J 32(3):317–328
6. Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu Rev Plant Biol 56:165–185
7. Olszewski N, Sun TP, Gubler F (2002) Gibberellin signaling: biosynthesis, catabolism, and response pathways. Plant Cell 14(Suppl):S61–S80
8. Bentsink L, Koornneef M (2009) Seed dormancy and germination. In: The *Arabidopsis* book. The American Society of Plant Biologists, pp 1–18. doi:10.1199/tab.0119
9. Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. Annu Rev Plant Biol 59:387–415. doi:10.1146/annurev.arplant.59.032607.092740
10. Holdsworth MJ, Bentsink L, Soppe WJ (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. New Phytol 179(1):33–54, doi:NPH2437 [pii] 10.1111/j.1469-8137.2008.02437.x
11. Lee KP, Piskurewicz U, Tureckova V, Carat S, Chappuis R, Strnad M, Fankhauser C, Lopez-Molina L (2012) Spatially and genetically distinct control of seed germination by phytochromes A and B. Genes Dev 26(17):1984–1996. doi:10.1101/gad.194266.112
12. Shinomura T, Nagatani A, Chory J, Furuya M (1994) The induction of seed germination in *Arabidopsis thaliana* Is regulated principally by phytochrome B and secondarily by phytochrome A. Plant Physiol 104(2):363–371, doi:104/2/363 [pii]
13. Shinomura T, Nagatani A, Hanzawa H, Kubota M, Watanabe M, Furuya M (1996) Action spectra for phytochrome A- and B-specific photoinduction of seed germination in *Arabidopsis thaliana*. Proc Natl Acad Sci USA 93(15):8129–8133
14. Oh E, Kim J, Park E, Kim J-I, Kang C, Choi G (2004) PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key negative regulator of seed germination in *Arabidopsis thaliana*. Plant Cell 16(11):3045–3058. doi:10.1105/tpc.104.025163
15. Bethke PC, Libourel IG, Aoyama N, Chung YY, Still DW, Jones RL (2007) The *Arabidopsis* aleurone layer responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy. Plant Physiol 143(3):1173–1188
16. Lee KP, Piskurewicz U, Tureckova V, Strnad M, Lopez-Molina L (2010) A seed coat bedding assay shows that RGL2-dependent release of abscisic acid by the endosperm controls embryo growth in *Arabidopsis* dormant seeds. Proc Natl Acad Sci USA 107(44):19108–19113. doi:10.1073/pnas.1012896107

17. Lee KP, Lopez-Molina L (2013) A seed coat bedding assay to genetically explore in vitro how the endosperm controls seed germination in *Arabidopsis thaliana*. J Vis Exp 9(81):e50732
18. Finkelstein RR, Lynch TJ (2000) Abscisic acid inhibition of radicle emergence but not seedling growth is suppressed by sugars. Plant Physiol 122(4):1179–1186
19. Lopez-Molina L, Chua NH (2000) A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*. Plant Cell Physiol 41(5):541–547
20. Piskurewicz U, Tureckova V, Lacombe E, Lopez-Molina L (2009) Far-red light inhibits germination through DELLA-dependent stimulation of ABA synthesis and ABI3 activity. EMBO J 28(15):2259–2271. doi:10.1038/emboj.2009.170
21. Saito S, Okamoto M, Shinoda S, Kushiro T, Koshiba T, Kamiya Y, Hirai N, Todoroki Y, Sakata K, Nambara E, Mizutani M (2006) A plant growth retardant, uniconazole, is a potent inhibitor of ABA catabolism in *Arabidopsis*. Biosci Biotechnol Biochem 70(7):1731–1739
22. Piskurewicz U, Lopez-Molina L (2011) Isolation of genetic material from *Arabidopsis* seeds. Methods Mol Biol 773:151–164. doi:10.1007/978-1-61779-231-1_10
23. Vicient CM, Delseny M (1999) Isolation of total RNA from *Arabidopsis thaliana* seeds. Anal Biochem 268(2):412–413
24. Toh S, Imamura A, Watanabe A, Nakabayashi K, Okamoto M, Jikumaru Y, Hanada A, Aso Y, Ishiyama K, Tamura N, Iuchi S, Kobayashi M, Yamaguchi S, Kamiya Y, Nambara E, Kawakami N (2008) High temperature-induced abscisic acid biosynthesis and its role in the inhibition of gibberellin action in *Arabidopsis* seeds. Plant Physiol 146(3):1368–1385. doi:10.1104/pp. 107.113738
25. Kendall SL, Hellwege A, Marriot P, Whalley C, Graham IA, Penfield S (2011) Induction of dormancy in *Arabidopsis* summer annuals requires parallel regulation of DOG1 and hormone metabolism by low temperature and CBF transcription factors. Plant Cell 23(7):2568–2580. doi:10.1105/tpc.111.087643
26. Endo A, Tatematsu K, Hanada K, Duermeyer L, Okamoto M, Yonekura-Sakakibara K, Saito K, Toyoda T, Kawakami N, Kamiya Y, Seki M, Nambara E (2012) Tissue-specific transcriptome analysis reveals cell wall metabolism, flavonol biosynthesis and defense responses are activated in the endosperm of germinating *Arabidopsis thaliana* seeds. Plant Cell Physiol 53(1):16–27, doi:pcr171 [pii] 10.1093/pcp/pcr171
27. Penfield S, Li Y, Gilday AD, Graham S, Graham IA (2006) *Arabidopsis* ABA INSENSITIVE4 regulates lipid mobilization in the embryo and reveals repression of seed germination by the endosperm. Plant Cell 18(8):1887–1899
28. Tatematsu K, Nakabayashi K, Kamiya Y, Nambara E (2008) Transcription factor AtTCP14 regulates embryonic growth potential during seed germination in *Arabidopsis thaliana*. Plant J 53(1):42–52, doi:TPJ3308 [pii] 10.1111/j.1365-313X.2007.03308.x

Chapter 16

Assessing Tolerance to Heavy-Metal Stress in *Arabidopsis thaliana* Seedlings

Estelle Remy and Paula Duque

Abstract

Heavy-metal soil contamination is one of the major abiotic stress factors that, by negatively affecting plant growth and development, severely limit agricultural productivity worldwide. Plants have evolved various tolerance and detoxification strategies in order to cope with heavy-metal toxicity while ensuring adequate supply of essential micronutrients at the whole-plant as well as cellular levels. Genetic studies in the model plant *Arabidopsis thaliana* have been instrumental in elucidating such mechanisms. The root assay constitutes a very powerful and simple method to assess heavy-metal stress tolerance in *Arabidopsis* seedlings. It allows the simultaneous determination of all the standard growth parameters affected by heavy-metal stress (primary root elongation, lateral root development, shoot biomass, and chlorophyll content) in a single experiment. Additionally, this protocol emphasizes the tips and tricks that become particularly useful when quantifying subtle alterations in tolerance to a given heavy-metal stress, when simultaneously pursuing a large number of plant lines, or when testing sensitivity to a wide range of heavy metals for a single line.

Key words *Arabidopsis thaliana*, Chlorophyll content, Heavy-metal stress tolerance, Lateral root development, Primary root elongation, Root assay, Seedlings, Shoot biomass

1 Introduction

As sessile organisms, terrestrial plants need to acquire their nutrients from the soil solution and therefore their growth and development largely rely on the soil mineral status. One of the most pervasive causes of loss of crop productivity worldwide is the contamination of arable land with heavy metals. Heavy-metal soil contamination as a result of anthropogenic activities occurs in many regions of the world and, depending on environmental as well as societal factors, may pose health risks to both humans and animals when accumulating in food crops. Given the modern agricultural context, the impact that this unfavorable soil condition exerts on crop yields will grow to paramount importance in the years to come. Thus, the elucidation of the physiological and molecular mechanisms underlying plant heavy-metal stress tolerance will be

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crucial for the use of biotechnology to reclaim farmlands lost to agriculture as well as in phytoremediation strategies—i.e., the use of plants to decontaminate polluted environments—and has been the subject of intense research in the plant biology field [1, 2].

Heavy metals—i.e., in a broad sense, potentially toxic metallic elements—such as zinc (Zn), copper (Cu), or iron (Fe) among others are essential for plant development as they serve as catalytic cofactors or structural motifs in numerous enzymes and other proteins assuming a key role in many basic metabolic processes. Nonetheless, these micronutrients become potentially toxic when present in excess. Conversely, nonessential heavy metals, such as cadmium (Cd), arsenate (As), or cesium (Cs) constitute toxic elements that can adversely affect plant growth even when present in trace amounts in the soil solution. Depending on the chemical and physical properties of the heavy metal in question, heavy-metal toxicity mainly occurs as a result of a propensity to inactivate crucial proteins through blocking of functional groups—the case of Cd and lead (Pb)—or through displacement of essential metal ions from their site of action—the case of Zn—as well as a result of the ability to disturb cellular homeostasis of other essential elements—the case of As and Cs—or to induce oxidative stress through the generation of reactive oxygen species (ROS)—the case of Cu and Fe [3].

To deal with these opposing effects and adjust to environmental fluctuations in their availability, plants have developed a sophisticated and tightly controlled homeostatic network aimed at ensuring an adequate supply of crucial oligo-elements while preventing the toxic buildup of both essential and nonessential heavy metals at the cellular and whole-plant levels [4]. Heavy metals are primarily acquired from the soil solution as ions, which once absorbed into the root epidermis move mostly symplastically through the adjacent cell layers to reach the central stele. After secretion into the stellar apoplast followed by active loading into the xylem vessels, heavy metals are translocated to the shoot via root pressure and the transpirational stream and subsequently transferred to the phloem sap before allocation to aerial organs. Plants adapt to essential heavy-metal shortage supply prevalently by activating cellular heavy-metal uptake systems particularly at the root-soil interface [5]. At the whole-plant level, tolerance to heavy-metal excess is achieved mainly through reduced uptake at the root soil interface and through the rearrangement of its tissue partitioning via enhanced sequestration in leaves, whereas within the root both immobilization in the outer cell layers and exclusion from the epidermis contribute to limit heavy-metal entry into the root symplasm. At the cellular level, such mechanisms are primarily intended to restrict the cytosolic accumulation of free heavy metal, mainly though extrusion in the apoplasm, chelation with specific ligands, and/or vacuolar compartmentalization [6–8].

Deciphering the steps in plant heavy-metal stress tolerance and identifying the genetic determinants mediating heavy-metal uptake, translocation, chelation, and detoxification largely benefit from molecular genetic studies in the plant model *Arabidopsis thaliana*. In the presence of excessive amounts of a given heavy metal, *Arabidopsis* seedlings develop pleiotropic toxicity symptoms, generally including shoot growth retardation, leaf chlorosis, and remodeling of the root system architecture, i.e., inhibition of primary root elongation and altered lateral root development. The easiest and most commonly employed method to reliably appraise the extent of damage caused by heavy-metal stress in *Arabidopsis* seedlings remains the root assay [9, 10], which was initially adapted to heavy-metal susceptibility assessment by Howden and Cobbett in 1992 [11]. Root growth can be rapidly scored, and the assay requires relatively little specific equipment, providing both qualitative and quantitative data. It typically involves vertically oriented growth of seedlings on solid media imposing or not a given rhizotoxic stress and scoring followed by comparison of primary root elongation in exposed versus non-exposed seedlings. Such an experiment can at first seem quite straightforward but often turns out to be rather challenging to interpret, in particular when a large number of lines of interest must be simultaneously compared, when a wide range of heavy metals has to be tested, or when subtle alterations between genotypes need to be reproducibly quantified. In addition, most of the reports using this assay focus merely on primary root elongation and disregard evaluation of other susceptibility indicators, such as shoot growth, photosynthesis performance, or lateral root development. This chapter does not aim solely at describing the root assay itself but rather to provide a precise practical application of the method, which is particularly helpful to accurately and fully evaluate *Arabidopsis* heavy-metal stress tolerance by measuring all standard phenotypic parameters in a single experiment. Using this combined approach, we were able to assign a role in ion rhizotoxicity tolerance to three *Arabidopsis* transporters from the Major Facilitator Superfamily. In particular, we reported that Pht1;9 function confers oversensitivity to As [12], while activity of the ZIF2 and ZIFL2 carriers promotes tolerance to Zn and Cs toxicity, respectively [13, 14].

2 Materials

2.1 Plant Material

1. Good-viability *Arabidopsis* seeds of the appropriate genotypes along with seeds of the corresponding wild type(s).
2. Seeds of previously reported tolerant and/or sensitive heavy-metal stress lines may also be used as controls (*see* **Note 1**).

2.2 Reagents and Solutions

1. Murashige and Skoog (MS) [15] medium (or equivalent) freshly prepared before use: 1× MS basal salt mix, supplemented with 0.1 g l^{-1} *myo*-inositol and 0.5 g l^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES), pH adjusted to 5.7 with KOH 1 M, solidified with 0.8 % ultrapure agar and autoclaved (*see* **Note 2**).
2. Sterilization solution freshly prepared before use: 50 % [v/v] sodium hypochlorite and 0.02 % [v/v] Triton X-100 in sterile distilled water.
3. Appropriate antibiotics for seedling selection when mutant or transgenic seed batches are not derived from homozygous plants.
4. Stock solutions of the heavy metals to be tested (Table 1).

2.3 Consumables

1. Eppendorf tubes (1.5 and 2 ml).
2. Fine forceps.
3. Square Petri dishes (11.5 cm).
4. Razor blades.
5. Micropore tape (3 M).

2.4 Equipment

1. A climate-controlled growth cabinet set to long-day (16-h light, 22 °C/8-h dark, 18 °C) or under continuous light (20-h light, 22 °C/4-h dark, 18 °C) conditions with 60 % relative humidity and cool-white light (~100–120 µmol m^{-2} s^{-1}).
2. Space at 4 °C (room or fridge).
3. Laminar flow chamber.
4. Fume hood.

3 Methods

The pipeline of the whole process is presented in Fig. 1.

1. Under sterile conditions, prepare square Petri dishes containing equivalent amounts of control MS medium or selective MS medium in case seed selection is required. Allow the plates to dry before closing them in order to avoid any condensation on the lid.
2. Surface-sterilize the seeds by incubating them for 10 min in sterilization solution (in Eppendorf tubes) under constant and vigorous shaking followed by four rinses with sterile distilled water.
3. Under sterile conditions, immediately spread evenly the seeds with the help of a tip onto the appropriate control plates (*see* **Note 3**) in 1–4 rows starting 1.5 cm from the top of the plate without spacing between the seeds (*see* **Note 4**). Allow the plates to dry until the water containing the seeds has totally

Table 1
Heavy-metal stock solutions and concentrations to test for the root assay in the Columbia (Col-0) ecotype of *Arabidopsis thaliana*

Heavy metal	Cation	Compound	Stock solution		Range
			Concentration	Preparation	
Aluminum	Al^{3+}	$AlCl_3$	0.1 M	0.4 g in 30 ml H_2O	0.5, 0.75, 1, 1.5, 2 mM
Arsenate	AsO_4^{3-}	NaH_2AsO_4	500 mM	0.82 g in 10 ml H_2O	100, 200, 300, 400, 500 µM
Cadmium	Cd^{2+}	$CdCl_2$	30 mM	55 mg in 10 ml H_2O	10, 25, 50, 75, 100 µM
Cobalt	Co^{2+}	$CoCl_2.6H_2O$	100 mM	0.238 g in 10 ml H_2O	25, 50, 75, 100, 150 µM
Copper	Cu^{2+}	$CuCl_2$	30 mM	51.1 mg in 10 ml H_2O	25, 50, 75, 100, 150 µM
Iron	Fe^{2+}	FeSO4	0.1 M	0.278 g in 10 ml H_2O	0.25 mM
Lithium	Li^{2+}	$LiCl_2$	5 M	in 10 ml H_2O	5, 10, 12.5, 15, 20 mM
Manganese	Mn^{2+}	$MnCl_2.4H_2O$	0.5 M	0.99 g in 10 ml H_2O	1, 1.5, 2, 2.5, 3 mM
Nickel	Ni^{2+}	Ni $Cl_2.6H_2O$	100 mM	0.238 g in 10 ml H_2O	50, 75, 100, 150, 200 µM
Lead	Pb^{2+}	N_2O_6Pb	0.5 M	1.66 g in 9 ml H_2O + 1 ml HNO_3	0.1, 0.25, 0.5, 0.75, 1 mM
Cesium	Cs^{+}	CsCl	5 M	8.42 g in 10 ml H_2O	1, 2, 3, 4, 5 mM
Thallium	Tl^{3+}	$TlCl_3$	0.33 M	3.1 g in 5 ml HCl 37 % + 25 ml H_2O	2.5, 5, 10, 15, 20 µM
Zinc	Zn^{2+}	$ZnSO_4.7H_2O$	100 mM	0.288 g in 10 ml H_2O	100, 250, 500, 750, 1000 µM

All heavy-metal stock solutions are prepared with sterile distilled water

evaporated. Seal the plates using Micropore tape and wrap them together in aluminum foil (*see* **Note 5**).

4. Incubate the plates vertically (*see* **Note 6**) at 4 °C for 3 days to break seed dormancy.
5. After stratification, remove the aluminum foil and incubate the plates vertically (*see* **Note 7**) in the controlled-growth cabinet. Let the seeds germinate and the seedlings grow until root lengths reach roughly 1.0–1.2 cm (maximum 1.5 cm). In our hands, this corresponds to about 4–6 days depending on the light conditions.

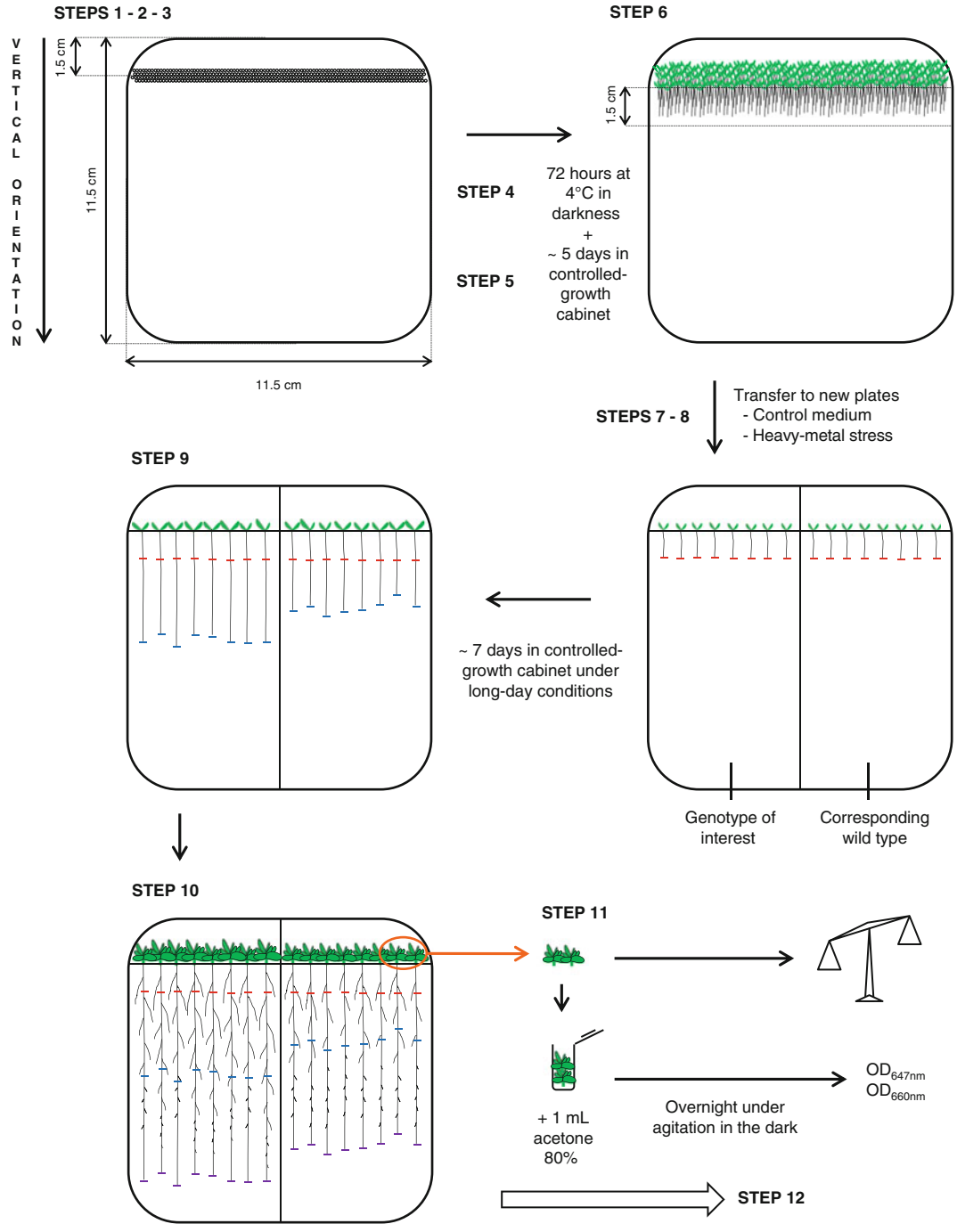

Fig. 1 Overview of the protocol. Steps are referred to according to Subheading 3. *Step 1*, preparation of the plates; *Step 2*, surface-sterilization of the seeds; *Step 3*, sowing of the seeds on control medium; *Step 4*, stratification of the seeds; *Step 5*, germination and synchronized growth of the seedlings; *Step 6*, visual inspection of the plates; *Step 7*, preparation of the heavy-metal stress and control plates; *Step 8*, transfer and growth of the seedlings; *Step 9*, scoring of primary root growth elongation; *Step 10*, scoring of lateral root development; *Step 11*, scoring of shoot biomass and chlorophyll content; *Step 12*, analysis of the data

6. At this point, careful visual inspection of all plates is essential to ensure that the predefined experiment design is still feasible. First, it is crucial to ensure that the number of seedlings capable of being transferred is sufficient: seedlings of all the genotypes to be concomitantly tested must be at the same developmental stage, in particular with roots of similar length (less than 0.2 cm variation), and the plates must be free of fungal or bacterial contaminations. We typically transfer 16 seedlings per genotype per condition onto two different plates, each accommodating two genotypes in parallel, i.e., two sets of eight seedlings. The appropriate controls should not be forgotten, i.e., transfer also of (1) each genotype to a control plate to ensure that the phenotypic parameters to be measured are not altered under control conditions (and later normalize heavy-metal stress effects), and (2) seedlings from the wild-type background to each of the analyzed conditions. For studies where a single mutant or transgenic line is being compared to the corresponding wild type, the easiest way to ensure a valid comparison is to grow the wild-type seedlings on the same plate as the genotype under evaluation in order to avoid any effects of plate-to-plate variability. Alternatively, when a relatively high number of distinct genotypes in the same background need to be tested, 2–3 repetitions of wild-type seedling transfer in between the genotypes of interest may be acceptable to avoid extensive measurements.
7. Prepare square Petri dishes containing equivalent amounts of control MS medium and MS medium supplemented with the heavy metal(s) to be tested. Allow the plates to dry completely before closing them to avoid any condensation on the lid. Heavy-metal-containing medium can be easily prepared by incorporating an appropriate amount of heavy-metal stock solution into previously autoclaved medium (*see* **Note 8**). The concentrations of the different heavy metals that we routinely test for the *Arabidopsis* ecotype Columbia (Col-0) are described in Table 1, but the appropriate concentrations should be empirically established depending on the accessions employed and the nature of the lines to be analyzed (tolerant or sensitive when compared to wild type). For a first screen, it is recommended to test a full range of heavy-metal concentrations.
8. Under sterile conditions, gently transfer seedlings using regularly disinfected forceps to the new plates by carefully allowing the root tip to touch the medium and, at an angle of approximately 30°, delicately sliding the root over the medium surface until the hypocotyl-root junction reaches a line drawn at 1.5 cm from the top of the plate. This way, the roots will be straight and contact with the medium surface will be maximized (*see* **Note 9**). Maintain a regular spacing between the

seedlings. When the transfer is finalized, seal the plates, mark the position of the root tips directly on the bottom of the plate, and incubate plates vertically in the controlled-growth cabinet with roots pointing downward (*see* **Note 10**).

9. After approximately 7 days (*see* **Note 11**) of growth on new media, primary root elongation can be scored by marking the new position of the primary root tips (*see* **Note 12**).
10. After a further incubation period, i.e., just before the longest root of one genotype reaches the bottom of the plate, lateral root development can be recorded by first marking the new position of the primary root tips and then scanning the plates from their bottom side (*see* **Note 13**).
11. Immediately following scanning or after a further incubation period in the control-growth cabinet (*see* **Note 11**), seedlings can be assessed concomitantly for shoot biomass and chlorophyll content. Shoot biomass is determined by measuring the fresh weight of two pooled plant shoots (*see* **Notes 13** and **14**). Immediately after weighing, place the two plant shoots together in the bottom of a 2 ml Eppendorf tube and add 1 ml of 80 % acetone. Once shoot biomass measurements are complete, incubate all the tubes overnight in the dark under gentle but continuous agitation. The following day, measure spectroscopically the absorbance of the acetone solution at 647 and 660 nm.
12. Even before the end of the experiment, the primary root elongation and lateral root development parameters can be quantified for each seedling on scanned images using an image analysis software, such as ImageJ (http://rsb.info.nih.gov/ij/index.html). Primary root elongation is evaluated by measuring the exact distance between the initial and the corresponding mark. Lateral root density is evaluated by counting the number of lateral roots (excluding adventitious roots) and normalizing to the total length of the corresponding primary root, determined by measuring the exact distance between the hypocotyl-root junction and the final mark. Total lateral root length is evaluated by adding up the length of each lateral root of a given seedling. Total chlorophyll content is determined according to the method and equation (total chlorophyll = $18.71A_{647\ \mathrm{nm}} + 7.15A_{660\ \mathrm{nm}}$) of MacKinney [16] and expressed on a fresh weight basis (*see* **Note 15**). Finally, the average value of each of the analyzed parameters under a given heavy-metal stress is normalized to the corresponding average value in the non-stress condition, typically using $n = 16$ for primary root elongation, $n = 8$ or 16 for lateral root development parameters, and $n = 8$ for shoot biomass and chlorophyll content. To ascertain that tolerance to a given heavy-metal stress is affected, similar results need to be obtained in at least three independent experiments (*see* **Note 16**).

4 Notes

1. Best results will be produced if seed batches have been obtained from plants cultured simultaneously.
2. Before use, it is imperative that all soap, detergent, and other cleaning fluids be completely removed from glassware, as even slight traces of such compounds in the medium will interfere with the assay. Glassware should be rinsed thoroughly 3–6 times with sterile distilled water before preparing the medium. Preparing the medium with sterile distilled water and avoiding storage of the plates before use significantly help preventing fungal and bacterial contaminations.
3. The root assay can be carried out by germinating the seeds directly onto heavy-metal-containing plates or by transferring seedlings grown beforehand on control medium plates to heavy-metal-containing plates. From our experience, the most informative is the transfer method described here, as it excludes a possible effect of heavy-metal stress on germination rate (which can be easily scored in an independent assay by measuring the germination rates of exposed versus non-exposed seedlings) while being relevant when seedling selection on medium supplemented with antibiotics is required prior to transfer.
4. Sowing the seeds at high density so that they touch each other on the plate rather than leaving space between them will greatly improve synchronization of seedling growth right after seed germination, in particular at the root level. In addition, this will favor straight growth of the roots and prevent their curling or curving. Another essential point when sowing the seeds is to avoid as much as possible scratching the medium surface with the tip to minimize root growth inside the medium rather than on its surface and thus prevent their subsequent transfer.
5. Spreading only one genotype per plate will prevent any cross-contamination between the lines to be tested. Alternatively, in the case of preliminary small-scale tests, two genotypes can be spread onto a single plate divided vertically. The number of seedlings amenable to transfer is often limiting, so be sure to plate enough seeds. The number of plates to be prepared depends on the germination rate of the seed batch and on the kind of screening to be performed (i.e., the type and range of heavy-metal concentrations to be tested). Nevertheless, we recommend preparing at least two plates per genotype in case fungal or bacterial contaminations appear.
6. We have observed that performing the stratification step with Petri dishes set up already in a vertical position promotes synchronization of seedling root growth.

7. Petri dishes must be incubated vertically but with a slight inclination—i.e., a forward shift of ~2 cm at the base of the dish—so that the seeds (and later the seedlings) face the light source. This will greatly favor the straight growth of the roots and is highly facilitated by the presence of gridded shelves (specific from the growth cabinet or, alternatively, from a fridge) holding the plates approximately at half their height. All the plates from a single experiment must be similarly inclined and if possible positioned on the same shelf so that they are exposed to the same amount of light.
8. Interpretation of the results can be erroneous if special care is not taken to ensure that the genotypes of interest are exposed to the exact same severity of heavy-metal stress as the wild-type control and thus uniform composition of the medium between plates is essential. We recommend preparing all the plates of a given concentration from the same heavy-metal stock solution.
9. Transfer of the seedlings is the most critical step of the protocol. Its success depends largely on intact seedlings and any damage needs to be strictly avoided. Initially, it may take some practice of the transfer procedure to achieve quick transfer and correct positioning without wounding the seedlings, particularly squashing at the hypocotyl region. We strongly advise to delicately lift the seedling shoot using the fine forceps as a lever rather than closing them. If correct positioning is not achieved at a first attempt, make the seedling root slide again but never touch the root in order to preserve its integrity. To minimize dehydration of the seedlings, keep the lid of the initial and receiver plates as closed as possible during the transfer procedure. Any clearly wounded or dry seedling should be discarded. We highly recommend checking root integrity, in particular root tip intactness, of each transferred seedling under a dissection microscope at the first transfer attempts while routinely ensuring that 24 h after transfer the roots have recovered and resumed steady-state growth even under heavy-metal stress (although at a slower rate than under control conditions).
10. Some studies indicate the inversion of the plates after transfer, so that the roots are pointed upward, to facilitate evaluation of primary root growth without having to mark root tip positions. However, we believe that this method is only amenable to qualitative assessment of root elongation upon exposure to heavy-metal stress and largely privilege continuous growth as it allows the full extent of primary root elongation and lateral root parameters to be accurately measured while eliminating possible effects of agravitropic behavior of the lines under evaluation.

11. One advantage of this method is that, as long as each specific trait is simultaneously quantified for all the genotypes under study, some slight variability in incubation times can be tolerated.
12. Susceptibility to heavy-metal stress will not necessarily follow a linear progression, particularly regarding primary root elongation. As scoring this parameter is a noninvasive method, we highly recommend marking the position of the root tips at 2-day intervals, at least in a first screen.
13. When recording phenotypical data, particular attention should be paid to the water frequently accumulating inside the plates. Water at the bottom of the plates can disturb seedling root position and it is crucial that they remain in place for later measurement from scanned images, while water condensed on the lid can easily wet seedling shoot and lead to highly erroneous conclusions. Keep the plates as vertical as possible before carefully opening the plate under sterile conditions, removing excess water by gently turning them over and drying the lid with paper. Seal back the plates in case further incubation is needed.
14. Still on the agar plates, cut two seedlings at the root-hypocotyl junction with a razor blade and immediately measure their combined weight using a precision weighing scale while avoiding seedling damage as much as possible. Note that it is essential to be in a calm environment without frequent movements or strong ventilation to avoid quick water loss from the seedling shoots. For the same reason, keep the plates closed between each measurement.
15. Even taking particular care during plate preparation and seedling manipulation, contaminations frequently occur. Any contaminated seedling should be eliminated from the data recording, as should those that do not recover quickly after transfer or that suddenly arrest growth for no apparent reason. It is therefore important to follow the plates daily, as fungal and bacterial contaminations usually appear during prolonged incubation times. It should also be noted that shoot biomass and chlorophyll content can be assessed earlier than initially planned, i.e., as soon as a first plant shows signs of contamination, in order to save the experiment.
16. Be aware that the root assay is instrumental to determine the level of susceptibility to a given heavy metal, but not when the observed differences are due to altered internal heavy-metal homeostasis or whole-plant heavy-metal accumulation. A similar assay to the one presented here, but set up on a larger scale, can be performed to prepare tissue samples for heavy-metal content quantification by methods such as atomic absorption spectroscopy.

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References

1. Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53(366):1–11
2. Rascio N, Navari-Izzo F (2011) Heavy metal hyperaccumulating plants: how and why do they do it? And what makes them so interesting? Plant Sci 180(2):169–181. doi:10.1016/j.plantsci.2010.08.016
3. Marschner P (ed) (2012) Mineral nutrition of higher plants, 3rd edn. Academic, London
4. Clemens S (2001) Molecular mechanisms of plant metal tolerance and homeostasis. Planta 212(4):475–486
5. Puig S, Penarrubia L (2009) Placing metal micronutrients in context: transport and distribution in plants. Curr Opin Plant Biol 12(3):299–306. doi:10.1016/j.pbi.2009.04.008
6. Clemens S (2006) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie 88(11):1707–1719. doi:10.1016/j.biochi.2006.07.003
7. Yadav SK (2010) Heavy metals toxicity in plants: An overview on the role of glutathione and phytochelatins in heavy metal stress tolerance of plants. S Afr J Bot 76(2):167–179. doi:10.1016/j.sajb.2009.10.007
8. Hossein MA, Piyatida P, Teixeira da Silva JA, Fujita M (2012) Molecular mechanism of heavy metal toxicity and tolerance in plants: central role of glutathione in detoxification of reactive oxygen species and methylglyoxal and in heavy metal chelation. J Bot: Article ID 872875. doi:10.1155/2012/872875
9. Müller AJ (1964) Keimwurzeltest zur Bewertung des somatischen Strahlenschadens bei *Arabidopsis*. Kulturpflanze 12(1):237–255
10. Contant RB (1966) *Arabidopsis* seedling growth for radiobiological studies. Arabid Inf Serv 3:36–37
11. Howden R, Cobbett CS (1992) Cadmium-sensitive mutants of *Arabidopsis thaliana*. Plant Physiol 100(1):100–107
12. Remy E, Cabrito TR, Batista RA, Teixeira MC, Sa-Correia I, Duque P (2012) The Pht1;9 and Pht1;8 transporters mediate inorganic phosphate acquisition by the *Arabidopsis thaliana* root during phosphorus starvation. New Phytol 195(2):356–371. doi:10.1111/j.1469-8137.2012.04167.x
13. Remy E, Cabrito TR, Batista RA, Hussein MA, Teixeira MC, Athanasiadis A, Sa-Correia I, Duque P (2014) Intron retention in the 5′ UTR of the novel ZIF2 transporter enhances translation to promote zinc tolerance in *Arabidopsis*. PLoS Genet 10(5):e1004375. doi:10.1371/journal.pgen.1004375
14. Remy E, Cabrito TR, Batista RA, Teixeira MC, Sa-Correia I, Duque P (2015) The major facilitator superfamily transporter ZIFL2 modulates cesium and potassium homeostasis in *Arabidopsis*. Plant Cell Physiol 56(1):148–162. doi:10.1093/pcp/pcu157
15. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497
16. MacKinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140:315–322

Chapter 17

Assessing Drought Responses Using Thermal Infrared Imaging

Ankush Prashar and Hamlyn G. Jones

Abstract

Canopy temperature, a surrogate for stomatal conductance, is shown to be a good indicator of plant water status and a potential tool for phenotyping and irrigation scheduling. Measurement of stomatal conductance and leaf temperature has traditionally been done by using porometers or gas exchange analyzers and fine-wire thermocouples attached to the leaves, which are labor intensive and point measurements. The advent of remote or proximal thermal sensing technologies has provided the potential for scaling up to leaves, plants, and canopies. Thermal cameras with a temperature resolution of <0.1 K now allow one to study the temperature variation within and between plants. This chapter discusses some applications of infrared thermography for assessing drought and other abiotic and biotic stress and outlines some of the main factors that need to be considered when applying this to the study of leaf or canopy temperature whether in controlled environments or in the field.

Key words Thermal imaging, Stomatal conductance, Plant stress, Drought, Water use efficiency

1 Introduction

Water deficit is one of the major constraints for agriculture and future climatic predictions suggest an increase in the frequency of extreme weather conditions. Thus, breeding of crops for drought avoidance, escape, and tolerance is likely to be needed for sustainable agriculture. Under water-deficit or stress conditions, root-sourced abscisic acid (ABA) is conveyed through xylem resulting in stomatal closure, which, especially in isohydric plants, often occurs before plant water status declines [1, 2]. Thus, stomatal closure can be used as an indication for response to water stress and in regulating crop irrigation [3]. Most of the traditional ways of measuring stomatal conductance use porometer or infrared gas analyzers which give point measurements and are time consuming and labor intensive. With recent technological advances, infrared thermography (IRT) has become viable as an alternative for the indirect estimation of stomatal conductance, because the temperature of

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leaves, plants, or canopies is an indicator of leaf transpiration rate (and hence of stomatal opening and closing). The fact that stomata tend to close with water-deficit stress means that stomatal closure, indicated by the use of IRT, has become increasingly used as a tool for irrigation scheduling and in phenotyping for drought tolerance [3–7]. IRT has also been used to understand the variation in leaf/canopy temperature measurements in response to other abiotic and biotic stresses [8–10], for energy balance and aerodynamic studies, and for studies of biochemical activity (especially relating to thermogenic respiration) in plants [11, 12].

1.1 Thermal Imaging Theory

Temperature sensing in the thermal infrared is based on the fact that all objects emit thermal radiation (R; W m^{-2}) as a function of surface temperature according to the Stefan–Boltzmann equation:

$$R = \epsilon \sigma T^4 \quad (1)$$

where ε is the emissivity, σ is the Stefan-Boltzmann constant (5.6697×10^{-8} W m^{-2} K^{-4}), and T is the temperature (K). The emissivity is introduced to take account of the fact that not all surfaces are perfect emitters of radiation (i.e., black bodies) and relates the actual radiance for a body to the amount that would be emitted by a perfect emitter, and varies between 0 and 1 (for a perfect emitter or "black body"). In order to allow measurements to be made in sunlight, thermal imagers for use outdoors need to be restricted to the long-wave infrared radiation window (c. 9.5 μm to 14 μm) and to exclude shorter wavelengths. Other cameras (so-called short-wave thermal cameras sensitive to radiation between 3 and 5 μm) are available that are optimized for engineering applications where surface temperatures may be of the order of 500–1000 K: these are unsuitable for vegetation studies as they can detect reflected solar radiation, thus giving incorrect results when used in the daytime outdoors. Long-wave cameras are little affected by solar radiation and are therefore useful in the field.

1.2 Plant Environment Interaction: Leaf Temperature Regulation

Plants interact with environment through interface "stomata," maintaining carbon-water and energy exchange balance, and adapt to ever-changing conditions. Thus stomata play an important role in plant adaptation and growth by balancing the need to minimize water loss while maintaining photosynthetic gains [13]. Evaporative cooling through transpiration is a major component of the leaf energy balance, and the leaf temperature (T_l) at any time is given by Eq. 2:

$$T_l - T_a = [r_{HR}(r_{aW} + r_s)\gamma R_{ni} - \rho c_p r_{HR} D] / [\rho c_p [\gamma (r_{aW} + r_s) + s r_{HR}]] \quad (2)$$

where T_a is the air temperature (K), R_{ni} is the net isothermal radiation absorbed by the leaf (W m^{-2}), D is the atmospheric air humidity deficit (Pa), ρ is the density of air (kg m^{-3}), c_p is the specific heat capacity of air (J kg^{-1} K^{-1}), s is the slope of the curve relating saturating water vapor pressure to temperature (Pa K^{-1}), γ is the psy-

chrometric constant (Pa K^{-1}), r_{HR} is the parallel resistance to heat and radiation transfer, r_{aW} is the boundary layer resistance to water vapor transfer, and r_s is the stomatal resistance to water vapor transfer (refer to Ref. 13 for details).

The use of IRT for remotely sensing stomatal closure and transpiration offers a great potential for irrigation scheduling and as a high-throughput phenotyping tool. Indeed IRT can be used as an effective tool not only in evaluating crop water status but also for other abiotic and biotic stresses in several agricultural crops.

In this chapter we highlight different steps involved in experimental setup, image acquisition, processing using different normalization techniques, and data analysis involved typically in a field trial, with examples on controlled conditions also addressed.

2 Materials

2.1 Equipment

Thermal cameras that are sensitive to radiation in the 8–14 μm band and having a thermal resolution of 100 mK or better are available from a range of companies. This high temperature sensitivity is essential for most plant stress-sensing applications (*see* **Note 1**) with their absolute accuracy being less important.

2.2 Plant Trials

In general a replicated case–control designed trial consisting of a control and corresponding water stress treatments either in glasshouse conditions (controlled) or field conditions (natural environment) is required. This can also include different genotypes if genotypic variability needs to be assessed for case–control studies and future breeding.

2.3 Reference Surfaces

Reference markers in the form of marked banners or labels are placed in the field as position indicators; these can also be used as artificial wet or dry references [4, 14]. Environment data is also often needed for data normalization. In addition to environment data, different referencing methods can be used (*see* **Note 2** and Ref. 14 for details).

2.4 Software

Thermal cameras come with their own proprietary software for extracting temperatures from images; in adddition statistical or other image analysis software for image processing (e.g., Excel, Genstat, R) are often required.

3 Methods

3.1 Thermal Imaging

A flow diagram indicating the various steps involved in thermal imaging is shown in Fig. 1. Software built into the camera transforms the detected radiation into temperature, taking account as

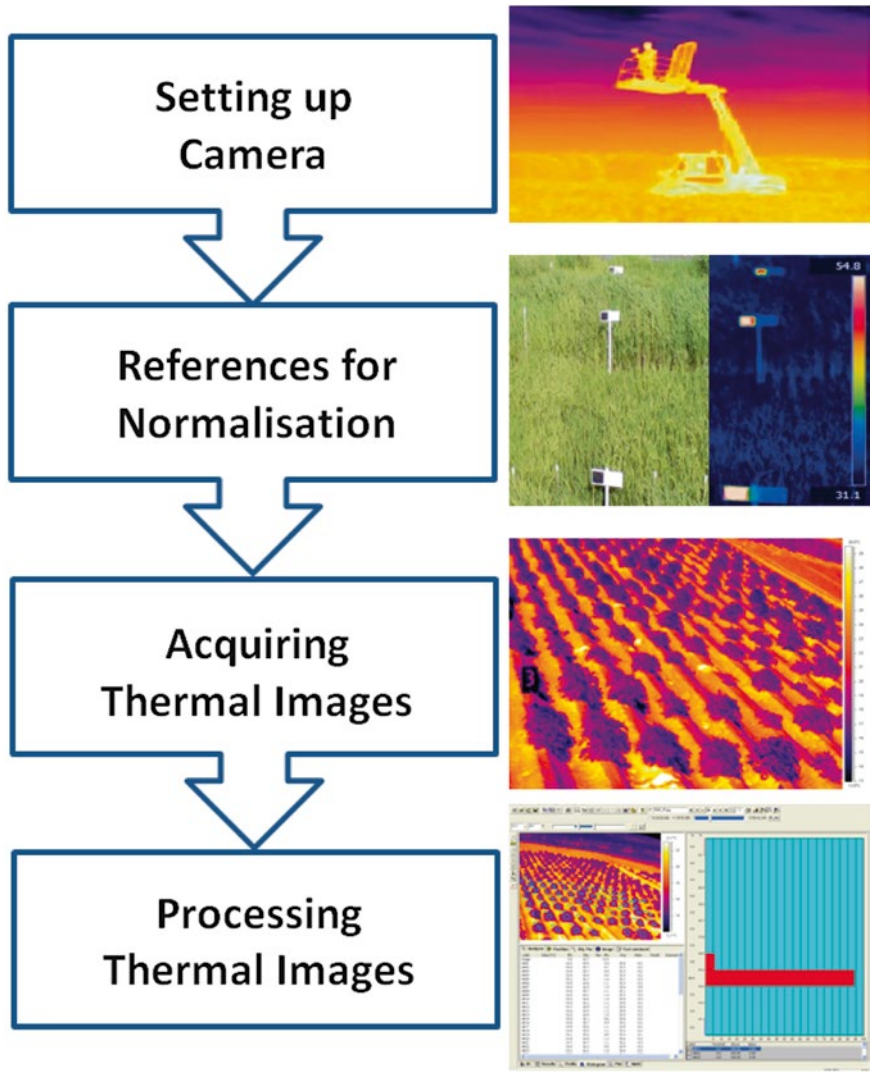

Fig. 1 Steps involved in using the imaging as a tool for phenotyping or crop monitoring

necessary of atmospheric humidity, object-to-camera distance, and surface emissivity. These temperatures are then commonly displayed as false color images or can be analyzed using raw digital numbers.

3.1.1 Setting Up Thermal Camera

1. Imaging can be done using either handheld or mounted (*see* **Note 3**) cameras at an appropriate distance depending on the spatial resolution required and the specific research question being addressed. It is useful to distinguish the use on single leaves or small plants like *Arabidopsis* from use on whole canopies in the field. Experiments in controlled conditions have allowed identification of individual *Arabidopsis thaliana*

mutants with altered stomatal responses [15], while field studies of breeding lines have also identified genetic differences [4, 5, 16].

2. Set the camera parameters. For acquisition of a default value of emissivity (ε), enter 0.965 into camera settings (*see* **Note 4**). Similarly, the distance of the camera to the subject/target object, air temperature, and humidity need to be measured and entered into the camera settings.
3. The camera parameters such as reflected, atmospheric, and optics temperatures along with the environment conditions (*see* **Note 5** and Subheading 3.3) affect the calculation of the target temperature. The radiation received, and hence the temperature recorded, by the thermal camera depends on (a) the temperature of the object being viewed, (b) the emissivity (ε) of the object's surface, (c) the incoming thermal radiation from the environment (background radiation) reflected by the object (*see* **Note 6**), and (d) any absorption or emission of thermal radiation by the atmosphere between the object and the camera. Most thermal cameras come with software that allows for correction of (c) and (d) given a knowledge of ε and the atmospheric humidity. Luckily, for most close-range applications in plant science the error caused by (d) is small and can usually be neglected.

3.1.2 Acquisition of Images

1. Thermal images should be acquired at times when both stomatal conductance and transpiration tend to be relatively constant and depend upon whether the experiment is under controlled or field conditions.
2. Thermal images generally include both foreground (target of interest) and background regions. It is usually necessary to separate these and only perform further analysis on the pixels representing leaves (*see* **step 2** of Subheading 3.2.2 for details).
3. When imaging in the field, images taken at an oblique angle to the horizontal (as compared with the nadir view) help in maximizing the canopy area in any image, but this can introduce complexities in analysis relating to perspective and varying camera-object distances (*see* **Note 7**).
4. Use of reference surfaces: The identification of a particular canopy or leaf in a group of canopies is easier if a set of markers which make the image processing easier when extracting canopy temperature from a group of similar canopies/areas is used. These markers can be reference surfaces (see Subheading 3.3) or labels to identify or distinguish different plants or pots. It can be useful to ensure overlap of neighboring images to allow better normalization to account for changing environmental conditions and provide increased replication with reduced standard error as discussed in normalization section [5].

3.2 Analysis

3.2.1 Image Processing

The next step after image acquisition is the image analysis. A wide range of softwares are available for image handling and analysis, many of which are specific to the camera used, but the greatest flexibility is available if the original images can be exported into nonproprietary formats for analysis in programs such as Excel (Microsoft Office, 2010), Matlab (The Mathworks, Inc., UK), or Fiji [17]. Nevertheless much useful work, such as identification of object outlines and extraction of object temperature, can usually be achieved in the camera-specific software (e.g., ThermaCAM Researcher (FLIR systems), and Fluke SmartView (Fluke Corporation)).

3.2.2 Separating Leaf and Canopy Temperature from Background

An advantage of using thermal imagers as compared with simple infrared sensors is that the images allow the user to distinguish leaves from background soil. Many thermal cameras come with a built-in visible camera and most of the thermal cameras available in the market come with a data fusion option, where thermal and visible images are available on the camera's LCD for identification and distinguishing foreground and background components while screening (Fig. 2). In subsequent image processing, there are a couple of ways in which the information on leaf temperature may be isolated from a complex thermal image including soil and other objects (*see* **Note 8**).

1. *Overlay with visible image*: The simplest approach is to overlay the thermal image with a visible image of the same scene. A number of methods are available for this that include (1) automated image recognition and alignment algorithms (e.g., [18]); (2) freeware implementations of alignment programs such as the Fiji image analysis platform, which uses ImageJ's interface and plug-ins relevant to biological research [17]; and (3) utilization of the high reflectance of leaves in the near IR in the form of some vegetation index approach (e.g., Ref. 19).
2. *Use of temperature histograms*: An alternative is to make use of the expectation that leaves will generally be substantially cooler than the background soil, and use histogram thresholding to determine which pixels to use (e.g., Ref. 20), possibly with an automated histogram separation method such as the Otsu method [21]. A similar approach has also been used for manual extraction of canopy/leaf temperature eliminating background noise due to soil [5]. In a related approach, Giuliani and Flore [22] used a high temperature background screen to facilitate the use of thresholding to extract canopy temperature.

3.3 Normalization

The surface temperature of plant canopy or leaf depends on the biological factors but is also influenced by the environmental factors including irradiance and wind speed, which are continually changing (*see* **Note 5**). Therefore, it is necessary to isolate treatment dif-

Fig. 2 Example of data fusion from FLIR camera E50 with Picture in Picture option available on the camera's LCD for identification and distinguishing foreground and background component elimination while screening/phenotyping

ferences in temperature from this background variation using a normalization technique. Different normalization techniques can be used as mentioned below, depending on the experimental design and the conditions (whether controlled or field trials).

1. An early normalization was the derivation of the Crop Water Stress Index (CWSI) [23, 24] as shown in Eq. 3:

$$CWSI = (T_{\text{canopy}} - T_{\text{nwsb}}) / (T_{\text{max}} - T_{\text{nwsb}}) \tag{3}$$

   where T_{canopy} is the canopy temperature, T_{nwsb} is the temperature of a non-water stressed reference crop under similar conditions, and T_{max} is an upper temperature for a non-transpiring crop.

2. However, the use of CWSI as a normalization is limited by the fact that T_{nwsb} is site dependent and does not account for variation in net radiation and aerodynamic resistance [25]. Also, it is sometimes hard to have access to a non-transpiring crop reference. Therefore, it has been suggested that an analogous stress index (SI_{cwsi}) could be defined where T_{wet} and T_{dry} are the temperatures of wet and dry (non-transpiring) physical reference, respectively:

$$SI_{\text{cwsi}} = (T_{\text{canopy}} - T_{\text{wet}}) / (T_{\text{dry}} - T_{\text{wet}}) \tag{4}$$

Alternatively, an index (I_{G}) that is proportional to stomatal conductance could be defined [3]:

$$I_{\text{G}} = \frac{T_{\text{dry}} - T_{\text{canopy}}}{T_{\text{canopy}} - T_{\text{wet}}} = g_{1\text{W}}(r_{\text{aW}} + (s / \text{Y})r_{\text{HR}}) \tag{5}$$

where r_{aw} is the boundary layer resistance to water vapor, s is the slope of the curve relating saturation vapor pressure to temperature, γ is psychrometric constant, and r_{HR} is parallel resistance to heat and radiative transfer as defined by Jones and colleagues [3, 20, 25]. An advantage of this index over the *CWSI* or SI_{CWSI} is that I_G is nearly linearly related to stomatal conductance.

3. The physical reference surfaces used must have similar radiative properties to the plant leaves of interest, and ideally should also have similar aerodynamic properties [26, 27]. The appropriate choice of reference surface depends on the scale of observation but studies suggest that real leaves, either sprayed with water or covered in petroleum jelly to stop transpiration, provided the best references because of similar radiometric and aerodynamic properties for single leaf or small plot studies (reviewed in Ref. 14). This can be extended to large areas of well-irrigated reference crop for satellite-scale observations (e.g., Refs. 11, 13, 20, 26).
4. The actual canopy temperature can also lag behind the current equilibrium canopy temperature due to thermal lag in the system. When screening large numbers of genotypes under field conditions, a normalization technique based on using the temperature difference from the image mean has been shown to give highly reproducible results [4, 5]. Figure 3 gives an overview of the process using overlapping images and the calculation of genotype temperature. This overlap strategy and the associated normalization technique have been shown to provide enough power to identify quantitative trait loci (QTLs) [5].

4 Notes

1. It is worth noting that the absolute accuracy of most readily available cameras is only ±1 or ±2 °C, though in most applications the accuracy is not a major limitation as one is concerned with the measurement of temperature differences. This depends on the questions being answered through the experiment. If one is interested in difference between temperature for different genotypes and breeding scenarios, absolute value is not critical. But in case the experiment is designed to understand the energy balance and the morphophysiological relations, absolute temperature may be required as temperatures need to be related to air temperature (not measured by the camera).
2. Different reference surfaces or methods used previously include wet and dry leaf canopies (WDLC), paper references, comparison with air temperature, and calculations made by using

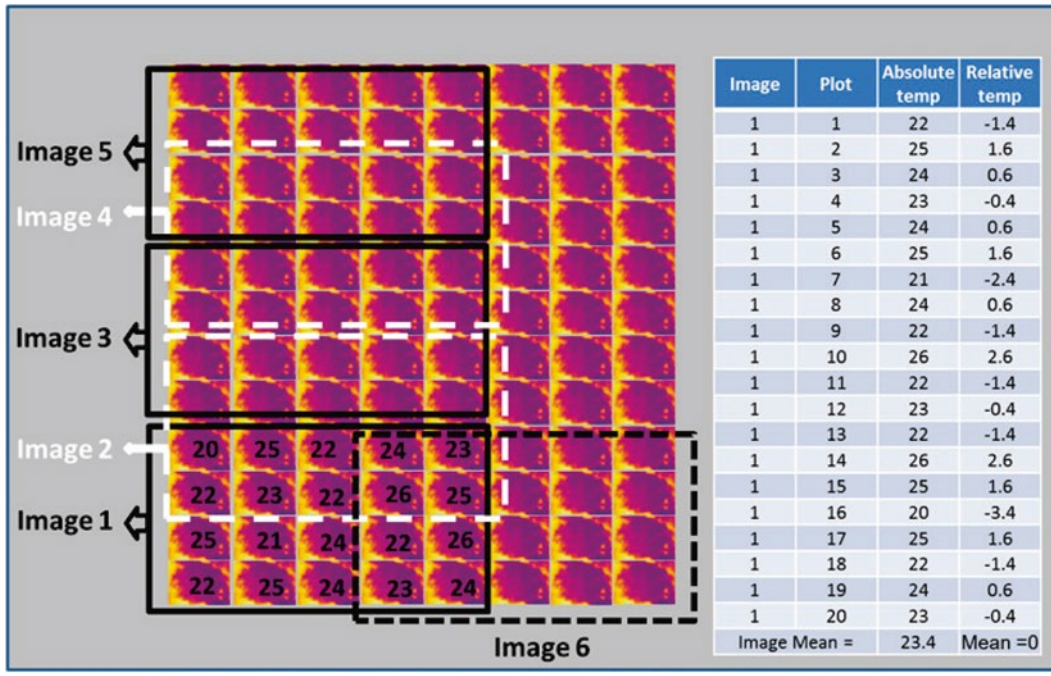

Image	Plot	Absolute temp	Relative temp
1	1	22	-1.4
1	2	25	1.6
1	3	24	0.6
1	4	23	-0.4
1	5	24	0.6
1	6	25	1.6
1	7	21	-2.4
1	8	24	0.6
1	9	22	-1.4
1	10	26	2.6
1	11	22	-1.4
1	12	23	-0.4
1	13	22	-1.4
1	14	26	2.6
1	15	25	1.6
1	16	20	-3.4
1	17	25	1.6
1	18	22	-1.4
1	19	24	0.6
1	20	23	-0.4
Image Mean =		23.4	Mean =0

Fig. 3 Field experimental design showing the image overlap sequence for repeated measurements and table showing an example of normalization method used by Jones and colleagues [4, 5]. The plot number in the table corresponds to the row and column in the image (e.g., row 1, column 1 = plot 1, row 2, column 2 = plot 2)

meteorological data in the form of "wet and dry temperatures." Each of these has its own advantages and disadvantages; for example comparison with air temperature does not take account of other environmental variables, while wet reference surfaces are difficult to maintain under hot conditions.

3. In case the camera is mounted at a height on a tripod where the focus function on camera is inaccessible, firewire cable or USB can be used to connect the camera to computer. The Thermacam Researcher Professional software (FLIR systems) or FLIR IR camera player (FLIR systems) on the computer can be used to manage the live imaging and its acquisition.
4. Choice of value for emissivity (ε): For single leaves, typical emissivities are between 0.93 and 0.98 [28]; in the absence of further precise information a value of 0.965 is recommended. Note that emissivities appropriate to soils tend to be only slightly lower (say 0.94–0.95) though sands may average as low as 0.89. When one views a canopy of leaves from a distance, however, the effective emissivity is higher than that of the component leaves, averaging approximately 0.99 [28].
5. Rate of canopy/leaf transpiration depends on the difference in air-to-leaf vapor pressure. Therefore under high wind, humid, and low irradiance conditions, the temperature difference

between a leaf with open stomata and one with closed stomata is much less as compared to low-wind, low-humidity, and high-irradiance conditions.

6. Correction for the background radiation: It is difficult to estimate the background (incoming to the object) thermal radiation accurately, but one approach to its estimation is to measure the apparent temperature of some crumpled aluminum foil placed in the position of the object (using an emissivity of 1)—the measured temperature is the effective background temperature (T_b) for use in the camera's software (for a leaf within a canopy T_b should be close to canopy temperature, while at the surface of a canopy T_b may be closer to the sky temperature which could be as low as 40 °C for clear sky).
7. In addition to environment, the effect of solar angle and angle of view should be taken into account. Image timing during the day is an important consideration in screening for canopy temperature as direction and angle between the sun and imager causes variation in apparent reflectance with overall reflectance highest when sun is behind the imager and lowest when opposite to imager. Thus imaging from two different angles will provide different results.
8. Field of view (FOV) is critical for image analysis and when collecting temperature data from canopy and elimination of background. Higher resolution or having more number of pixels in the image allows better temperature prediction and background elimination of the concerned object, instead of using low resolution or small number of pixels for temperature prediction making it harder to eliminate the background noise.

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References

1. Wilkinson S, Davies WJ (2002) ABA-based chemical signalling: the co-ordination of responses to stress in plants. Plant Cell Environ 25(2):195–210. doi:10.1046/j.0016-8025.2001.00824.x
2. Jones HG (2004) Irrigation scheduling: advantages and pitfalls of plant-based methods. J Exp Bot 55(407):2427–2436. doi:10.1093/jxb/erh213
3. Jones HG (1999) Use of infrared thermometry for estimation of stomatal conductance as a possible aid to irrigation scheduling. Agric For Meteorol 95(3):139–149. doi:10.1016/s0168-1923(99)00030-1
4. Jones HG, Serraj R, Loveys BR, Xiong L, Wheaton A, Price AH (2009) Thermal infrared imaging of crop canopies for the remote diagnosis and quantification of plant responses to water stress in the field. Funct Plant Biol 36:978–989
5. Prashar A, Yildiz J, McNicol JW, Bryan GJ, Jones HG (2013) Infra-red thermography for

high throughput field phenotyping in *Solanum tuberosum*. PLoS One 8(6):e65816–65811–65819. doi:10.1371/journal.pone.0065816

6. Grant OM, Tronina L, Jones HG, Chaves MM (2007) Exploring thermal imaging variables for the detection of stress responses in grapevine under different irrigation regimes. J Exp Bot 58:815–825. doi:10.1093/jxb/erl153
7. Meron M, Tsipris J, Orlov V, Alchanatis V, Cohen Y (2010) Crop water stress mapping for site-specific irrigation by thermal imagery and artificial reference surfaces. Precis Agric 11: 148–162
8. Amani I, Fischer RA, Reynolds MF (1996) Evaluation of canopy temperature as a screening tool for heat tolerance in spring wheat. J Agron Crop Sci 176:119–129
9. Lindenthal M, Steiner U, Dehne HW, Oerke EC (2005) Effect of downy mildew development on transpiration of cucumber leaves visualized by digital infrared thermography. Phytopathology 95(3):233–240
10. Stoll M, Schultz HR, Baecker G, Berkelmann-Loehnertz B (2008) Early pathogen detection under different water status and the assessment of spray application in vineyards through the use of thermal imagery. Precis Agric 9(6):407–417. doi:10.1007/s11119-008-9084-y
11. Jones HG (2004) Application of thermal imaging and infrared sensing in plant physiology and ecophysiology. In: Callow JA (ed) Advances in botanical research incorporating advances in plant pathology, vol 41. Advances in botanical research. pp 107–163
12. Pinter PJ, Stanghellini ME, Reginato RJ, Idso SB, Jenkins AD, Jackson RD (1979) Remote detection of biological stresses in plants with infrared thermography. Science 205(4406):585–586. doi:10.1126/science.205.4406.585
13. Jones HG (2014) Plants and microclimate: a quantitative approach to environmental plant physiology, 3rd edn. Cambridge University Press, Cambridge
14. Prashar A, Jones HG (2014) Infra-red thermography as a high-throughput tool for field phenotyping. Agronomy 4(3):397–417
15. Merlot S, Mustilli AC, Genty B, North H, Lefebvre V, Sotta B, Vavasseur A, Giraudat J (2002) Use of infrared thermal imaging to isolate *Arabidopsis* mutants defective in stomatal regulation. Plant J 30(5):601–609. doi:10.1046/j.1365-313X.2002.01322.x
16. Rebetzke GJ, Rattey AR, Farquhar GD, Richards RA, Condon AG (2013) Genomic regions for canopy temperature and their genetic association with stomatal conductance and grain yield in wheat. Funct Plant Biol 40(1):14–33. doi:10.1071/fp12184
17. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez J-Y, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9(7):676–682. doi:10.1038/nmeth.2019
18. Wang X, Yang W, Wheaton A, Cooley N, Moran B (2010) Automated canopy temperature estimation via infrared thermography: A first step towards automated plant water stress monitoring. Comput Electron Agric 73(1):74–83, Doi: http://dx.doi.org/10.1016/j.compag.2010.04.007
19. Leinonen I, Jones HG (2004) Combining thermal and visible imagery for estimating canopy temperature and identifying plant stress. J Exp Bot 55(401):1423–1431. doi:10.1093/jxb/erh146
20. Jones HG, Stoll M, Santos T, de Sousa C, Chaves MM, Grant OM (2002) Use of infrared thermography for monitoring stomatal closure in the field: application to grapevine. J Exp Bot 53(378):2249–2260. doi:10.1093/jxb/erf083
21. Jones HG, Sirault XR (2014) Scaling of thermal images at different spatial resolution: the mixed pixel problem. Agronomy 4(3): 380–396
22. Guiliani R, Flore JA (2000) Potential use of infra-red thermometry for the detection of water stress in apple trees. Acta Horticult 537:383–392
23. Jackson RD, Idso SB, Reginato RJ, Pinter PJ (1981) Canopy temperature as a crop water-stress indicator. Water Resour Res 17: 1133–1138
24. Idso SB, Reginato RJ, Jackson RD, Pinter PJ (1981) Foliage and air temperatures - evidence for a dynamic equivalence point. Agric Meteorol 24:223–226
25. Jones HG (1999) Use of thermography for quantitative studies of spatial and temporal variation of stomatal conductance over leaf surfaces. Plant Cell Environ 22(9):1043–1055
26. Leinonen I, Grant OM, Tagliavia CPP, Chaves MM, Jones HG (2006) Estimating stomatal conductance with thermal imagery. Plant Cell Environ29(8):1508–1518.doi:10.1111/j.1365.3040.2006.01528.x
27. Guilioni L, Jones HG, Leinonen I, Lhomme JP (2008) On the relationships between stomatal resistance and leaf temperatures in thermography. Agric For Meteorol 148(11):1908–1912
28. Jones HG, Vaughan RA (2010) Remote sensing of vegetation: principles, techniques, and applications. Oxford University Press, Oxford

Chapter 18

Generating Targeted Gene Knockout Lines in *Physcomitrella patens* to Study Evolution of Stress-Responsive Mechanisms

Monika Maronova and Maria Kalyna

Abstract

The moss *Physcomitrella patens* possesses highly efficient homologous recombination allowing targeted gene manipulations and displays many features of the early land plants including high tolerance to abiotic stresses. It is therefore an invaluable model organism for studies of gene functions and comparative studies of evolution of stress responses in plants. Here, we describe a method for generating targeted gene knockout lines in *P. patens* using a polyethylene glycol-mediated transformation of protoplasts including basic in vitro growth, propagation, and maintenance techniques.

Key words Homologous recombination, Gene targeting, Knockout, *Physcomitrella*, Plant, Stress, Evolution, Protoplast

1 Introduction

Living organisms have to cope with various stresses, both of biotic and abiotic nature. Studies of stress response not only are essential for a better understanding of life and its diversity but also have significant medical and economic implications. Different species respond and adapt very differently from one another. Fight-or-flight strategy used by animals as the first stage of the response to a harmful event is not applicable for plants that are sessile organisms. Plants have their own panoply of stress responses that was shaped by migration of plant ancestors from water habitats to land. At the terrestrial surroundings, they had to develop strategies to respond to much more substantial fluctuations in temperature, light intensity, and quality, including a stronger exposure to the DNA-damaging ultraviolet compound of the light, and now to water availability ranging from drought to flooding. Bryophytes are considered to be the closest to the common ancestor of plants. Among them, the moss *Physcomitrella patens* provides us with powerful

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tools to study functions of genes and together with other model plants allows comparative analyses of evolution of molecular mechanisms of stress response and adaptation [1]. *Physcomitrella* displays many features characteristic to the early land plants including a high tolerance to drought, salinity, osmotic, and other abiotic stresses [2–5].

Physcomitrella is haploid through almost all life cycle. The diploid stage, sporophyte, is short and results in haploid spores. Spores germinate and produce filamentous structures, protonemata. The protonemal filaments, the juvenile stage of gametophyte, can initiate buds that further develop into gametophores, the adult stage of gametophyte. The gametophores have more complex organization, with leaflike structures, rhizoids, female and male sex organs producing gametes, and subsequently diploid zygote that develops into sporophyte.

Physcomitrella is a remarkable model organism. Minimalist anatomy and simple developmental stages of *Physcomitrella* can be easily monitored. This is further facilitated by efficient methods of in vitro growth and propagation [6, 7] that also provide ample source of material for biochemical and molecular analyses. Moreover, *Physcomitrella* is highly amenable to genetic manipulations [8]. Dominant haploid generation of *Physcomitrella* allows fast and simpler recognition of mutant phenotypes [9, 10]. Development of approaches for genetic transformation of *Physcomitrella* yielded not only routine and efficient procedures, such as polyethylene glycol (PEG)-mediated DNA delivery into protoplasts [11], but also led to the realization that it undergoes high frequency of homologous recombination, comparable to that in yeast [12]. Gene targeting through homologous recombination is one of the most efficient means to study gene functions. This technology is far less efficient when applied in plants that have low rates of homologous recombination. Gene targeting is not limited to generation of knockout mutants by gene disruption. It allows also knock-in manipulations, such as introduction of various reporters and tags for visualization of expression of endogenous genes and isolation of molecular complexes. Moreover, endogenous genes or their portions can be replaced by their modified versions, thus providing means for site-directed mutagenesis. Importantly, the *Physcomitrella* genome is sequenced and fully assembled, therefore enabling reverse genetic approaches [1].

Here, we provide a protocol for generating targeted gene knockout lines in *P. patens* using a PEG-mediated transformation of protoplasts including basic techniques for in vitro growth, propagation, and maintenance of *P. patens*. This protocol is routinely used in our lab and is based on the protocols developed earlier [6, 11, 13]. Rich information resources providing further *Physcomitrella* protocols and techniques are currently available online (http://moss.nibb.ac.jp/ and http://biology4.wustl.edu/moss/methods.html).

2 Materials

2.1 Materials and Equipment

1. *Physcomitrella patens*, the "Gransden" strain, freshly grown protonemal tissue.
2. Gene targeting linear DNA vector (Fig. 1), 10–15 µg of purified DNA dissolved in a maximum of 30 µL dH_2O.
3. Laminar airflow hood.
4. Blender suitable for sterilization (*see* **Note 1**).
5. Water bath 45 and 25 °C (room temperature).
6. Hemocytometer.
7. Lighttight box (*see* **Note 2**).
8. Petri dishes, 9 cm in diameter with one vent.
9. Glass Petri dish.
10. Filter paper discs, 5–8 cm in diameter.
11. Sterile cellophane discs, 8 cm in diameter (*see* **Note 3**). To sterilize the cellophane discs separate each other by a disc of filter paper. Assemble ca. 30 cellophane discs in this sandwich way into a glass Petri dish, wrap into an aluminum foil, and autoclave.
12. Sterile wide-bored (or cut tip) blue micropipette tips.
13. Sterile 70 µm nylon mesh attached to sterile 50 mL centrifuge tube.

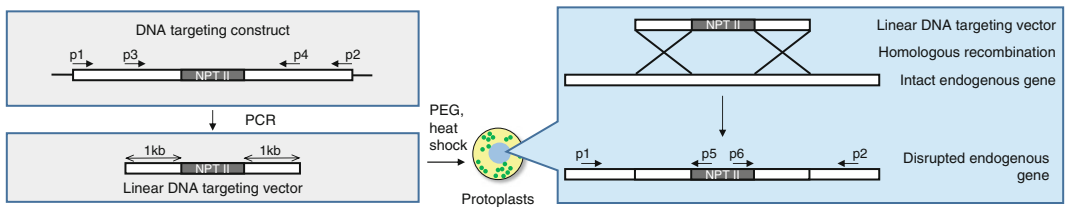

Fig. 1 Strategy of targeted gene disruption by homologous recombination in *Physcomitrella patens* protoplasts. To create the DNA targeting construct, a sufficiently long region of the target gene is amplified by PCR using primers p1 and p2 (outer primers) and integrated into the plasmid vector. The antibiotic resistance selection cassette (in this case neomycin phosphotransferase gene, NPT II) is then ligated into convenient restriction sites within the cloned target gene to replace the coding sequence (*upper left scheme*). The DNA targeting construct is used as a template in a subsequent PCR with primers p3 and p4 (inner primers) to obtain a linear targeting vector. The selection cassette is flanked by about 1 kb regions of homology to the target gene (*lower left scheme*). The linear DNA targeting vector is used for PEG-mediated transformation of *Physcomitrella* protoplasts, where it homologously recombines with the genomic DNA (*upper right scheme*). As a result, the endogenous gene is disrupted by the selection cassette. Stable transformants after the selection process are verified by two PCRs using outer primers in combination with primers to the selection cassette (*lower right scheme*; primer combinations: p1 and p5, p6 and p2). *White rectangles* represent the endogenous gene or the homology regions of the construct and the vector; *gray rectangles* represent the selection cassette

14. Centrifuge 10–15 mL tubes with round bottom and a cap.
15. Surgical tape.

2.2 Media and Solutions

1. Stock solution B (100×): 101 mM $MgSO_4$. Add 25 g of $MgSO_4 \cdot 7H_2O$ (or 12 g of anhydrous $MgSO_4$) to 500 mL of water in a beaker. Dissolve using a magnetic stirrer, transfer to the 1 L graded cylinder, and make up to 1 L with water. Autoclave or filter sterilize. Store at 4 °C.
2. Stock solution C (100×): 184 mM KH_2PO_4, pH 6.5. Weigh 25 g of KH_2PO_4 and dissolve in 500 mL of water as in previous step. Adjust pH to 6.5 with 4 M KOH. Make up to 1 L. Autoclave or filter sterilize. Store at 4 °C.
3. Stock solution D (100×): 1 M KNO_3, 4.5 mM $FeSO_4$. Weigh 101 g of KNO_3 and dissolve in 200 mL of water. Weigh 1.25 g of $FeSO_4 \cdot 7H_2O$ and dissolve separately in 200 mL of water. Adding of H_2SO_4 may help dissolve $FeSO_4 \cdot 7H_2O$. Mix both solutions and make up to 1 L. Autoclave. Store at 4 °C for 2–3 month and discard when iron precipitate forms.
4. Trace element solution (TES, 1000×): 614 mg H_3BO_3, 110 mg $AlK(SO_4)_2 \cdot 12H_2O$, 55 mg $CuSO_4 \cdot 5H_2O$, 28 mg KBr, 28 mg LiCl, 25 mg $Na_2MoO_4 \cdot 2H_2O$, 389 mg $MnCl_2 \cdot 4H_2O$, 55 mg $CoCl_2 \cdot 6H_2O$, 55 mg $ZnSO_4 \cdot 7H_2O$, 28 mg of KI, 28 mg $SnCl_2 \cdot 2H_2O$, 59 mg $NiCl_2 \cdot 6H_2O$. Weigh and separately dissolve all the components in ca. 50 mL of water. Combine the solutions and make up to 1 L. Store at 4 °C.
5. Ammonium tartrate (AT) stock solution (100×): 0.5 M Diammonium (+) tartrate. Weigh 9.21 g of diammonium (+) tartrate and dissolve in 80 mL of water as in previous steps. Make up to 100 mL. Autoclave or filter sterilize. Store at 4 °C.
6. $CaCl_2$ stock solution: 1 M $CaCl_2$. Weigh 11.1 g of $CaCl_2$, dissolve in 80 mL of water, and make up to 100 mL as in previous steps. Autoclave or filter sterilize. Store at room temperature (*see* **Note 4**).
7. Plant agar (*see* **Note 5**).
8. BCD medium (*see* Table 1): Basal minimum medium. Pour ca. 20–25 mL of a medium per a 9 cm Petri dish. Store plates at 4–10 °C.
9. BCD-AT medium (*see* Table 1): BCD medium supplemented with NH_4 as a nitrogen source. Pour ca. 20–25 mL of a medium per a 9 cm Petri dish. Store plates at 4–10 °C.
10. LB broth.
11. Mannitol 8 % (w/v): Weigh 8 g of mannitol and dissolve in 100 mL of water. If sterile solution is needed, autoclave or filter sterilize and store at room temperature.

Table 1
***Physcomitrella patens* media**

	BCD (1 L)	BCD-AT (1 L)	PRM-L (20 mL)	PRM-B (500 mL)	PRM-T (100 mL)	Final concentration
Stock solution B	10 mL	10 mL	200 µL	5 mL	1 mL	1 mM $MgSO_4$
Stock solution C	10 mL	10 mL	200 µL	5 mL	1 mL	1.84 mM KH_2PO_4
Stock solution D	10 mL	10 mL	200 µL	5 mL	1 mL	10 mM KNO_3, 45 µM $FeSO_4$
TES	1 mL	1 mL	20 µL	0.5 mL	0.1 mL	1× TES
AT	–	10 mL[a]	200 µL	5 mL[a]	1 mL	5 mM
Mannitol	–	–	1.2 g	30 g	6 g	6 % (w/v)
Plant agar	5.5 g	5.5 g	–	2.75 g	0.4 g	0.55 % (0.4 % for PRM-T)
H_2O	up to 1 L	up to 1 L	up to 20 mL	up to 495 mL	up to 99 mL	N/A
$CaCl_2$ 1 M	1 mL[b]	1 mL[b]	200 µL	5 mL[b]	1 mL[b]	1 or 10 mM $CaCl_2$
Sterilization	Autoclave	Autoclave	Filter	Autoclave	Autoclave	N/A

[a]Or add appropriate amount of the diammonium (+) tartrate
[b]Add after autoclaving

12. Driselase solution: 1 % (w/v) Driselase (*see* **Note 6**) in 8 % (w/v) mannitol. Add 50 mg Driselase into 5 mL of 8 % (w/v) mannitol solution (not sterile). Dissolve the powder in the centrifuge tube by inverting the tube occasionally and gently for 15 min. Centrifuge at 2500 rpm for 5 min. Filter sterilize the supernatant through a 0.22 µm filter. Prepare Driselase solution in amount of 5 mL per one *Physcomitrella* protonemal homogenate plate.
13. MaMg solution: 0.15 M $MgCl_2$, 8 % (w/v) mannitol, 0.1 % (w/v) MES. Weigh 3.05 g of $MgCl_2 \cdot 6H_2O$, 8 g of mannitol, and 0.1 g of MES. Dissolve all ingredients consecutively in 90 mL of dH_2O. Adjust the pH to 5.6 with 1 M KOH. Make up to 100 mL. Autoclave. Aliquot the solution in 10 mL plastic tubes and store at –20 °C (*see* **Note 7**). Before use thaw by placing the tube in a warm water and vortex until no precipitate is visible. Spin down.
14. PEG-CMS: 0.1 M $Ca(NO_3)_2$, 20 mM HEPES, 7.28 % (w/v) mannitol, 40 % PEG 6000. Weigh 0.236 g of $Ca(NO_3)_2.4H_2O$, 0.0476 g HEPES, and 0.728 g of mannitol and consecutively

dissolve in 5 mL of water. Make up to the volume of 6 mL. Measure the pH. A pH between 7 and 8 is acceptable (ideally pH 7.5). To adjust the pH, use 1 M KOH (usually 30–40 μL). Add PEG, dissolve the solution by incubation at 37 °C in water bath, and shake occasionally. Make up to 10 mL and mix the viscous solution thoroughly (*see* **Note 8**). Leave it for several hours to stabilize the pH. Filter sterilize and dispense in 1 mL aliquots in 1.5 mL microtubes. Store at –20 °C. Before use thaw PEG-CMS by placing tubes in warm water, vortex, and pulse spin (*see* **Note 9**). Dispense 300 μL aliquots into 10 mL centrifuge tubes and centrifuge the tubes briefly. Prepare one tube per transformation.

15. PRM-L (liquid) (*see* Table 1 and **Note 10**): Prepare 2 mL per transformation on the day of transformation.
16. PRM-B (bottom layer) (*see* Table 1 and **Note 10**): Plates with solid medium overlaid with cellophane, prepared 1–2 days before transformation, three plates per transformation. Pour maximum 20 mL of PRM-B into 9 cm Petri dish.
17. PRM-T (top layer) (*see* Table 1 and **Note 10**): Low agarose medium, prepare 10 mL per transformation on the day of embedding the protoplasts.
18. Antibiotics (*see* **Note 11**).

3 Methods

The tissue culture and transformation procedures require sterile conditions and are carried out in the laminar airflow hood.

3.1 Physcomitrella patens Protonemal Tissue Growth, Propagation, and Collection

1. Prepare BCD-AT growth plates and overlay them with sterile cellophane discs (*see* **Note 12**).
2. Take a well-grown (5–7 days old) protonemal homogenate BCD-AT plate (Fig. 2a). Using a sterile spatula, scrape the protonemal tissue off of the cellophane and transfer it to the sterile glass tube of a blender assembly filled with 10 mL of sterile water.
3. Blend the tissue in water until it is cut completely into ca. 0.5 mm small pieces.
4. Using a sterile pipette, inoculate cut tissue suspension onto five cellophane overlaid BCD-AT plates 2 mL each. Make quick circular moves with the plate to evenly distribute the inoculum.
5. To check for a bacterial contamination, add a few drops of the protonemal suspension to the 10 mL tube with 2 mL LB broth. Let it stay at the room temperature for 1–2 days and shake once a day (*see* **Note 13**).

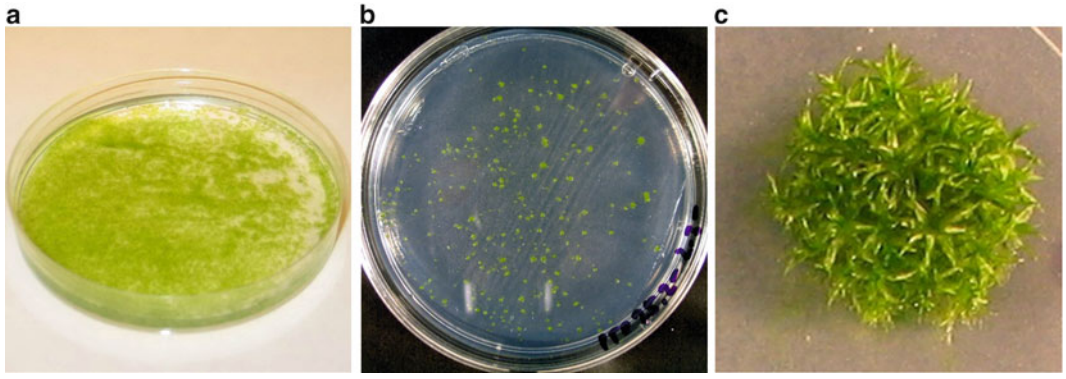

Fig. 2 The moss *Physcomitrella patens* grown in vitro. (**a**) Protonemal homogenate plate, 6 days after incubation on BCD-AT plate. (**b**) Regenerating protoplasts after transformation, 5 days after incubation on PRM. (**c**) Colony with gametophores, incubated for 3 weeks on BCD-AT plate

6. Cultivate the plates for 5–7 days (*see* **Note 14**) at 25 ± 2 °C, discontinuous white light (16-h light/8-h dark, 120 μmol/m^2/s).

3.2 Physcomitrella patens Protoplast Isolation, PEG-Mediated Transformation, and Regeneration

1. Collect the tissue from two 5–7 day old, well-grown BCD-AT cellophane overlaid plates (*see* **Note 15**) with a sterile spatula into a sterile container (e.g., Petri dish) containing 10 mL Driselase solution.
2. Let the tissue digest at room temperature with occasional gentle stirring until the tissue loses its filamentous character (45–90 min) (*see* **Note 16**).
3. Using a sterile pipette transfer the digested protoplast suspension onto a 70 μm filter placed in the corresponding container (*see* **Note 17**). Transfer the collected filtered suspension into the centrifuge tube.
4. Centrifuge at 800 rpm for 4 min, no brakes (*see* **Note 18**).
5. Carefully aspirate the supernatant at once using the pipette and leave ca. 0.1 mL of the liquid over the pellet. Resuspend the protoplasts in residuum very gently by rocking, tilting, and turning the tube. Slowly add 10 mL of 8 % mannitol (pour liquid slowly over the tube wall) and gently mix by rocking the tube. The cells should be evenly resuspended. Repeat **steps 4** and **5**.
6. Set aside 0.2–0.3 mL of protoplast suspension to measure the protoplast density using the counting chamber (hemocytometer).
7. Recover the remaining protoplasts by centrifugation at 800 rpm for 4 min, no brakes (*see* **Note 18**). Meanwhile calculate the protoplast density.

8. To calculate the protoplast density pipette small amount of protoplast suspension to both chambers of the hemocytometer. Count protoplasts in eight 1 mm^2 large squares and calculate the mean value *n*. This gives the density of the protoplasts in 0.1 μL and $n \times 10^5$ corresponds to the total amount of protoplasts in 10 mL of 8 % mannitol. Calculate the amount of MaMg solution to be added to give a final protoplast density of 1.6×10^6/mL.
9. Carefully add calculated amount of MaMg solution to the pellet by slowly pouring it over the tube wall.
10. (Optional) Transfer the resuspended protoplasts from the 10 mL tube to a wider and shallower container (e.g., Petri dish) in order to make further manipulations easier and reduce the risk of contamination.
11. Pipette 300 μL of the protoplast suspension using a wide-bored blue tip into the microtube containing 30 μL DNA solution.
12. Using a Pasteur pipette transfer the mixture of protoplasts and DNA to a 10 mL tube containing 300 μL PEG-CMS. Stir the mixture gently but thoroughly with the pipette tip. Gently suck up and expel the whole mixture once.
13. Heat-shock the protoplasts by placing the tubes in the water bath at 45 °C for 5 min.
14. Take the tubes out from the water bath and cool them in water at room temperature for 10 min.
15. Reduce the concentration of PEG by diluting the mixture with 8 % mannitol. Diluting has to be done slowly over the next 30–60 min in 6–7 steps. Add stepwise twice 300 μL, twice 600 μL, and twice 1 mL aliquots of mannitol and finally top up to the volume of 8 mL. The protoplasts must be always mixed very gently but thoroughly by tilting and rolling the tube. Wait for 3 min between each addition to give the protoplasts enough time to recover.
16. Centrifuge the protoplasts at 800 rpm for 4 min, no brakes (*see* **Notes 18** and **19**).
17. Carefully remove the supernatant (*see* **Note 20**) and gently add 2 mL of PRM-L to each tube. Mix gently; do not shake.
18. Incubate the tubes at 25 °C in the dark overnight (*see* **Note 21**).
19. Next morning, incubate the regenerating protoplasts under the normal growth room light conditions until the PRM-T medium is ready to embed the protoplasts.
20. Prepare PRM-T but do not add $CaCl_2$ yet. Divide it into 10 mL aliquots into the tubes, one tube per transformation. Cool down the PRM-T to 45 °C in the preheated water bath.

21. Centrifuge the protoplasts at 800 rpm for 3 min, no brakes (*see* **Note 18**).
22. Take one of the PRM-T aliquots from the water bath and transfer it to the hood. Add $CaCl_2$ in amount 100 µL per 10 mL of PRM-T.
23. Carefully aspirate the supernatant from the tube with protoplasts and resuspend the protoplasts in residuum very gently by rocking, tilting, and turning the tube. Add 10 mL of PRM-T and pour this on three PRM-B plates (*see* **Note 22**).

3.3 Selection of the Genomic Transformants

An outline of the selection process is shown in Fig. 3.

1. Cultivate the embedded protoplasts 5 days under standard conditions in the growth chamber (Fig. 2b) (*see* **Note 23**).
2. Initiate the first selection by transferring the cellophane with the top agar containing regenerated protoplasts on BCD-AT plate supplemented with appropriate antibiotics. Cultivate for 2–3 weeks under the standard conditions in the growth chamber (*see* **Note 24**).
3. Release the selection. With sterile forceps transfer colonies that show growth on the selective medium to the BCD-AT plates with no antibiotics and no cellophane. Cultivate for 2 weeks under the standard conditions in the growth chamber (*see* **Note 25**).
4. Repeat the selection. Using sterile forceps transfer small part of the protonemal tissue from the edge of each colony (spot inoculum) to BCD-AT medium supplemented with antibiotics and no cellophane. Cultivate for 2 weeks under the standard conditions in the growth chamber.

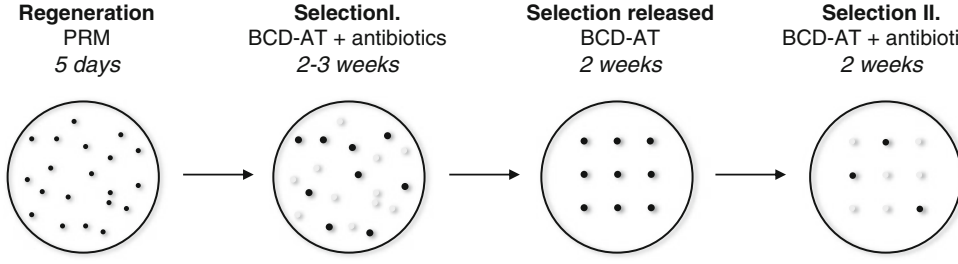

Fig. 3 Outline of the selection of *Physcomitrella* stable transformants. Protoplasts regenerate 5 days on PRM plate. To select the transformed colonies, a cellophane disc with colonies is transferred onto selective BCD-AT plate with antibiotics and incubated for 2–3 weeks. Surviving colonies are transferred on the BCD-AT plate and grown for 2 weeks without antibiotics, allowing unstable transformants (with non-integrated vector) to lose their antibiotic resistance. Spot inocula of all the colonies are then transferred to the selective BCD-AT plate with antibiotics in order to select stable transformants (with targeting DNA integrated into the genomic DNA). *Black dots* represent surviving colonies; *grey dots* represent dying colonies

5. Select stable transformants that survive on the selection medium (*see* **Note 26**).

3.4 Preparation of the Material for Verification and Molecular Characterization of the Targeted Gene Disruption

1. Transfer spot inoculum (small part of the protonemal tissue from the edge of the colony) of the wild type and the lines to be analyzed on the BCD-AT plates with cellophane. Seal the dish with the surgical tape to reduce evaporation. Cultivate under the standard conditions in the growth chamber for 3–4 weeks or longer until the colonies provide sufficient tissue (ca. 0.5–1 cm in diameter) (*see* **Note 27**).
2. To obtain material for molecular analyses from colonies, pick the colony from the cellophane with forceps, dry it briefly with a filter paper, transfer into a 1.5 mL microcentrifuge tube, and freeze in liquid nitrogen.
3. Grind the tissue to a very fine powder using a plastic pestle (cooled in liquid nitrogen) attached to a homogenizer. Keep the tissue frozen by scooping some liquid nitrogen into the tube.
4. To establish a protonemal homogenate subculture from the confirmed transformants transfer the colony (Fig. 2c) on a 1/6 sector of the BCD-AT plate with cellophane, dissect the protonemal part of the colony to as small pieces as possible with forceps, and distribute over the sector of the plate (*see* **Note 28**). Cultivate under standard conditions in the growth chamber for about 2 weeks, until the tissue is dense and has fresh green color. Subculture the protonemal tissue several times (*see* Subheading 3.1, **steps 2–4**) (*see* **Note 29**). The aim is to achieve a yield 500 mg of the tissue per protonemal homogenate plate (*see* **Note 30**).
5. To obtain material for molecular analyses from a protonemal homogenate tissue (Fig. 2a), scrape the protonemal tissue with a sterile spatula off of the cellophane and dry briefly between two layers of filter paper. Repeat drying approx. three times until almost no water comes out of the tissue. Weigh the tissue, transfer a maximum of 100 mg into the 1.5 mL microcentrifuge tube, and freeze in liquid nitrogen. Perform **step 3** to grind the tissue.
6. Extract DNA and RNA using a plant extraction kit (*see* **Note 31**).

3.5 Preparation of the Material for Phenotypic Analyses of the Protonemal Tissue and Gametophores

1. To obtain protonemal tissue suitable for observation, subculture the protonemal tissue on the BCD-AT plates with cellophane (*see* Subheading 3.1, **steps 2–4**). Cultivate for a maximum of 7 days under standard conditions in the growth chamber (*see* **Note 32**).
2. To induce gametophore production, take a little piece (about 1 mm) of the protonemal tissue from freshly grown protonemal homogenate plate (Fig. 2a) with the forceps (*see* **Note**

32). Transfer the spot inoculum to the BCD-AT plate overlaid with cellophane. Cultivate under the standard conditions in the growth chamber for 1 week or until the colonies are about 1 cm in diameter. Transfer the colonies onto the BCD plates, with no cellophane. Cultivate under standard conditions in the growth chamber until the colonies develop gametophores (leafy shoots) with approximately ten leafs (*see* **Note 33**).

3. To compare colonies of different lines, inoculate protonemal spot inoculum (*see* **step 2**) of maximum six lines (including wild type) on one plate containing desired solid media. Arrange the colonies to the circle with approximately the same distance from the edge of the Petri dish and from each other. Cultivate under standard conditions in the growth chamber until the colonies are about 0.5–1 cm in diameter (*see* **Note 34**).

3.6 Short-Term Storage and Regeneration

1. To store *P. patens* as a protonemal homogenate tissue, seal well-grown (5–7 days old) protonemal homogenate plate (Fig. 2a) with parafilm. Store the plate at 4–10 °C and illuminate for 2 h per day with a white light (*see* **Note 35**). To fully regenerate the tissue, subculture the tissue (*see* Subheading 3.1, **steps 2–4**) at least twice. Protonemal tissue plates can be stored for up to 3 months.
2. To store *P. patens* as a colony, take a small piece of the protonemal tissue with sterile forceps or a small piece of the tissue from the edge of the colony (spot inoculum) and transfer to the BCD plate, no cellophane. Cultivate under standard conditions in the growth chamber for 2–3 weeks. Seal the dish with parafilm, store at 4–10 °C and illuminate the plates for 2 h per day with white light (*see* **Note 35**). Plates with colonies can be stored for 2–3 months. Regenerate the tissue by making spot inoculum and transfer to the BCD-AT plates (*see* **Note 36**).

4 Notes

1. For example IKA Ultra Turrax. Previously we used a custom-made blender assembly, consisting of a glass tube and a metallic part with rotating blades. The blender assembly was attached to a drill-like rotor.
2. A cardboard box without holes that closes very tightly is sufficient.
3. Discs can be purchased from a packaging company.
4. $CaCl_2$ cannot be autoclaved added to the medium. Add corresponding amount of the $CaCl_2$ solution to the cooled medium right before pouring it.

5. We use Plant Agar P1001, Duchefa. Use of another agar may require optimization of the final concentration in the medium.
6. We use Driselase from *Basidiomycetes* sp., SIGMA, D8037.
7. Since adjusting pH in small volume might be difficult, making 100 mL of the MaMg solution and freezing is recommended.
8. Proper dissolving of PEG requires repeated thorough mixing and may take time.
9. Make sure that no precipitate is visible.
10. Protoplast regeneration medium (PRM) is BCD-AT medium supplemented with 6 % (w/v) mannitol and higher concentration (10 mM) of $CaCl_2$.
11. According to the antibiotic resistance gene incorporated in the DNA targeting vector we use G418 at the concentration of 40 mg/L.
12. It may take a while to practice this. The media must not be too wet (condensed water) to prevent wrinkling of the cellophane.
13. Clear LB medium means no contamination. If the medium turns cloudy or opaque in 1–2 days, this is a sign of contamination.
14. Cultivate until the plate is homogenously and densely grown by protonemal tissue of intense green color (Fig. 2a).
15. Two plates are optimal to obtain sufficient amount of the tissue (about 1 g) and protoplasts for one transformation.
16. The extent of the digestion can be monitored under the microscope.
17. Filtration can be repeated for better removal of undigested tissue.
18. Since the protoplasts are very fragile, it is recommended to turn the brake off completely. If it takes too long until the centrifuge stops, it is possible to turn the brake on when speed is down to ca. 80 rpm.
19. Alternatively let the protoplasts settle down by leaving the tube standing upright on the bench for 30–60 min.
20. The pellet is very loose.
21. Place the tubes in a lighttight box and incubate in the growth chamber.
22. PRM-T solidifies quickly at room temperature; therefore this step must be done fast.
23. The protoplasts recovery may take 3–6 days. Check under the stereomicroscope if the cells proliferate and the filaments are formed.

24. Colonies originated from untransformed cells turn light brown and die.
25. This step allows non-genomic transformants to lose the non-integrated construct.
26. Do not miss positive transformants showing reduced growth of the colony as an effect of the gene disruption.
27. One colony gives enough tissue for PCR analysis of the gene targeting events, but not for all the subsequent analyses. It is also possible to establish a protonemal homogenate subculture (*see* Subheading 3.4, **step 4**) at this stage, which gives more tissue in shorter time, but is more laborious, especially in case of many positive colonies after the selection process.
28. If an increased amount of the starting tissue is needed, gametophores (leafy shoots) can also be dissected and used as a source of the protonemal tissue.
29. Reduce the amount of water for blending and the number of plates for inoculation during the first round of subculture.
30. Another option is to homogenize the colony in a small amount of water in the homogenizer and inoculate on a BCD-AT plate with cellophane.
31. To confirm targeted insertion event in the genome, PCR amplify and sequence both regions of insertion outward from the selection cassette. Use the outer primers in combination with primers to the selection cassette (Fig. 1). Southern blotting allows detection of multiple insertions in case they occur to the nonhomologous sites of the moss genome. To monitor the transcript production of the targeted gene, conduct the RT-PCR and Northern blotting.
32. Use well-grown tissue that was subcultured at least twice consecutively and grown for 5–7 days under standard conditions in the growth chamber.
33. While growth of gametophores is enhanced by cultivation on BCD medium, the overall growth of the colony is slower than on BCD-AT plates, hence the transfer from BCD-AT to BCD.
34. PEG-mediated transformation can generate polyploids due to protoplast fusion. Polyploid colonies have different shape, more caulonemal tissue, and less gametophores than the wild-type haploid colonies.
35. The most important is regular daily illumination rather than the quality and intensity of the light.
36. Additional options for storage of the *P. patens* lines can be found in the literature.

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References

1. Rensing SA, Lang D, Zimmer AD, Terry A, Salamov A, Shapiro H, Nishiyama T, Perroud PF, Lindquist EA, Kamisugi Y, Tanahashi T, Sakakibara K, Fujita T, Oishi K, Shin IT, Kuroki Y, Toyoda A, Suzuki Y, Hashimoto S, Yamaguchi K, Sugano S, Kohara Y, Fujiyama A, Anterola A, Aoki S, Ashton N, Barbazuk WB, Barker E, Bennetzen JL, Blankenship R, Cho SH, Dutcher SK, Estelle M, Fawcett JA, Gundlach H, Hanada K, Heyl A, Hicks KA, Hughes J, Lohr M, Mayer K, Melkozernov A, Murata T, Nelson DR, Pils B, Prigge M, Reiss B, Renner T, Rombauts S, Rushton PJ, Sanderfoot A, Schween G, Shiu SH, Stueber K, Theodoulou FL, Tu H, Van de Peer Y, Verrier PJ, Waters E, Wood A, Yang L, Cove D, Cuming AC, Hasebe M, Lucas S, Mishler BD, Reski R, Grigoriev IV, Quatrano RS, Boore JL (2008) The *Physcomitrella* genome reveals evolutionary insights into the conquest of land by plants. Science 319(5859):64–69. doi:10.1126/science.1150646, 1150646 [pii]
2. Wang XQ, Yang PF, Liu Z, Liu WZ, Hu Y, Chen H, Kuang TY, Pei ZM, Shen SH, He YK (2009) Exploring the mechanism of *Physcomitrella patens* desiccation tolerance through a proteomic strategy. Plant Physiol 149(4):1739–1750. doi:10.1104/pp. 108.131714
3. Frank W, Ratnadewi D, Reski R (2005) *Physcomitrella patens* is highly tolerant against drought, salt and osmotic stress. Planta 220(3): 384–394. doi:10.1007/s00425-004-1351-1
4. Charron AJ, Quatrano RS (2009) Between a rock and a dry place: the water-stressed moss. Mol Plant 2(3):478–486. doi:10.1093/mp/ssp018
5. Saavedra L, Svensson J, Carballo V, Izmendi D, Welin B, Vidal S (2006) A dehydrin gene in *Physcomitrella patens* is required for salt and osmotic stress tolerance. Plant J 45(2):237–249. doi:10.1111/j.1365-313X.2005.02603.x
6. Knight CD, Cove DJ, Cuming AC, Quatrano RS (2002) Moss gene technology. In: Gilmartin PM, Bowler C (eds) Molecular plant biology, vol 2. Oxford University Press, Oxford, pp 285–301
7. Cove D (2005) The moss *Physcomitrella patens*. Annu Rev Genet 39:339–358. doi:10.1146/annurev.genet.39.073003.110214
8. Quatrano RS, McDaniel SF, Khandelwal A, Perroud PF, Cove DJ (2007) *Physcomitrella patens:* mosses enter the genomic age. Curr Opin Plant Biol 10(2):182–189. doi:10.1016/j.pbi.2007.01.005
9. Ashton NW, Cove DJ (1977) The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants of the moss, *Physcomitrella patens*. Mol Gen Genet 154(1):87–95. doi:10.1007/bf00265581
10. Ashton NW, Grimsley NH, Cove DJ (1979) Analysis of gametophytic development in the moss, *Physcomitrella patens*, using auxin and cytokinin resistant mutants. Planta 144(5):427–435. doi:10.1007/BF00380118
11. Schaefer D, Zryd JP, Knight CD, Cove DJ (1991) Stable transformation of the moss *Physcomitrella patens*. Mol Gen Genet 226(3):418–424
12. Schaefer DG, Zryd JP (1997) Efficient gene targeting in the moss *Physcomitrella patens*. Plant J 11(6):1195–1206
13. Kamisugi Y, Cuming AC, Cove DJ (2005) Parameters determining the efficiency of gene targeting in the moss *Physcomitrella patens*. Nucleic Acids Res 33(19):e173. doi:10.1093/nar/gni172

Chapter 19

Screening Stress Tolerance Traits in *Arabidopsis* Cell Cultures

Imma Pérez-Salamó, Bogáta Boros, and László Szabados

Abstract

Screening for tolerance traits in plant cell cultures can combine the efficiency of microbial selection and plant genetics. Agrobacterium-mediated transformation can efficiently introduce cDNA library to cell suspension cultures generating population of randomly transformed microcolonies. Transformed cultures can subsequently be screened for tolerance to different stress conditions such as salinity, high osmotic, or oxidative stress conditions. cDNA inserts in tolerant cell lines can be easily identified by PCR amplification and homology search of the determined nucleotide sequences. The described methods have been tested and used to identify regulatory genes controlling salt tolerance in *Arabidopsis*. As cDNA libraries can be prepared from any plants, natural diversity can be explored by using extremophile plants as gene source.

Key words In vitro selection, Agrobacterium, Transformation, cDNA library, Gene isolation

1 Introduction

Tolerance to extreme environmental conditions of higher plants is a complex character, a typical multigenic trait, controlled by numerous genes. Unfortunately, most crop species are rather sensitive to drought, soil salinity, or extreme temperatures although certain variability in the degree of tolerance has been identified among the genotypes. Quantitative trait loci (QTLs) associated with salt or drought tolerance have been described in several crops, and are being used to improve stress tolerance. Alternative strategies include the use of interspecific hybridization, in vitro selection, or genetic engineering of crops with regulatory genes, able to alter complex traits [1].

Identification of master regulators of abiotic stress tolerance is a challenging task with considerable economic importance. Genetic approaches have been explored to identify such genes and represent powerful instruments for further traditional and molecular breeding programs. A number of different genetic screens have

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been used to identify plant genes which regulate tolerance to salinity, osmotic, or oxidative stresses [2–5]. Most of these programs were based on mutagenized plant populations generated by physical, chemical, or biological mutagenesis, or employed natural variability as source of genetic variation. As an alternative, functional screening of plant cDNA expression libraries in microbial systems such as yeast was used to isolate regulators of stress responses. As an example, *Arabidopsis* cDNA library was overexpressed in yeast, which was screened for resistance to diamide, leading to the identification of several genes conferring oxidative stress tolerance [6]. cDNA libraries can be prepared from any plant with particular traits and screened in yeast to identify components of particular signaling pathways or novel regulators of stress tolerance. Such strategy was followed to isolate cDNAs from Jatropha curcas or the Pokkali rice, which could confer salt tolerance to yeast [7, 8]. Functional complementation of yeast mutants by *Arabidopsis* cDNAs was employed as screening criteria to identify regulators of sugar and stress signaling [9, 10]. These results suggest that screening at cell level can be an efficient approach to identify stress tolerance genes.

Use of a heterologous microbial system to identify plant regulatory genes has limitations, as important components of the plant signal transduction system or a particular metabolic pathway can be missing from yeast or bacteria. Use of plant cells for such screening purposes can therefore be advantageous, if appropriate selective conditions can be set up. Selection of unicellular cultures or suspensions composed of microcolonies is an attractive possibility as hundreds of thousands or millions of cells or cell clusters can be subjected to selective pressure. In vitro selection can be explored in such traits, which can clearly be recognized in dedifferentiated cultured cells. In vitro selection has been used to recover cell lines tolerant to different type of stress conditions and to generate genotypes with enhanced tolerance to salinity, drought, heavy metals, or waterlogging in numerous plant species including alfalfa [11], tobacco [12], rice [13, 14], maize [15], plum [16], or Bermuda grass [17].

Source of variation in cell cultures can derive from spontaneous or induced variability or introduced by genetic transformation. Somaclonal variation, generated by genetic and epigenetic instability in dedifferentiated callus cultures, was explored in a number of earlier studies as source of variability [18–21]. Extensive somaclonal variation however generates numerous unwanted traits (abnormal growth habit, dwarfism, sterility, etc.), which can question the utility of this approach to recover new cultivars with enhanced stress tolerance. Identification of the genes responsible for stress tolerance of the selected cell lines is also problematic due to the lack of appropriate genetic and/or molecular tool.

Biological tools such as insertion mutagenesis or large-scale transformation of cDNA libraries in plant expression vectors offer an alternative source of variation. An important advantage of such approach is that identification of the mutant alleles or the introduced genes is feasible. T-DNA insertions usually create loss-of-function mutants, most of which are recessive phenotypes [22–25]. Activation tagging with specialized vectors carrying strong enhancer or promoter sequences at the T-DNA border repeats can generate gain-of-function phenotypes by enhancing the transcription of plant genes flanking the T-DNA insertions [26–29]. Overexpression of cDNAs after random library transformation is expected to generate dominant, gain-of-function phenotypes also, which can more easily be recognized in heterozygous cultivated cells [30–35]. Further advantage of the cDNA library transformation is that identification of the inserted genes in selected transformed cell lines is very easy which requires standard molecular technologies only. cDNA insert is amplified from genomic DNA by polymerase chain reaction (PCR) and the identity of the encoded protein is determined by sequencing the PCR fragment and homology search of the sequence [36]. Identification of *Arabidopsis* cDNA clones by screening for salt tolerance of *Arabidopsis* cell cultures, transformed with the COS cDNA library, suggested that plant cell cultures can be employed in proper screens and used to identify novel stress regulators (*see* **Note 1**). Thus, overexpression of the *Arabidopsis* heat-shock factor A4A could confer salt and oxidative stress tolerance not only to cultured cells but also to transgenic plants, affirming the applicability of this approach [35].

2 Materials and Equipment

2.1 Culture Media

1. Liquid MSAR1 medium: Murashige and Skoog (MS) medium containing 2 mg/l IAA, 0.5 mg/l 2,4-D, and 0.5 mg/l IPAR [37, 38].
2. Solid MSAR1 medium to select and maintain cell colonies: MSAR1 medium containing 0.7 % agar.
3. YEB media: 1 l medium contains 5 g peptone, 5 g beef extract, 1 g yeast extract, 5 g sucrose, and pH: 7.2. Solid media contains 20 g/l agar. After autoclaving, add MgSO (final concentration of 2 mM) [39].
4. LB medium: 1 l medium contains 10 g tryptone, 5 g yeast extract, 10 g NaCl, and pH: 7.2. Solid medium has 20 g/l agar.

2.2 Antibiotics

5. In plant culture medium the antibiotics can be used in the following concentrations: 200–400 mg/claforan, 15–20 mg/l hygromycin, 200 mg/l carbenicillin.

6. For agrobacteria the following antibiotics are used (depending on the strains and markers employed): 100 mg/l rifampicin, 100 mg/l kanamycin, 25 mg/gentamycin, 100 mg/l spectinomycin.

2.3 Other Materials

Sterile Petri dishes, Erlenmeyer flasks (500 ml), centrifuge tubes, micropipette tips.

cDNA library in plant expression vector. We used the COS library of *Arabidopsis thaliana* [34] and a cDNA library prepared from salt-induced plants of *Eutrema salsuginea*, cloned in the estradiol-inducible expression cassette of the pER8-GW transformation vector [36]. Other cDNA libraries in different expression vectors can be used. Genetic transformation was performed with the GV3101/pMP90 Agrobacterium strain [39].

2.4 Equipment

1. Standard laboratory equipment for molecular biology.
2. Culture chamber with adjustable temperature control.
3. Gyratory shaker.
4. Sterile laminar air flow cabinet.

3 Methods

Numerous papers and handful of manuals and handbooks describe maintenance and culture of plant cell suspension cultures. Useful hints and advices can be obtained how to initiate, maintain, and handle cell culture of model and crop plant species in the following papers [40–46]. Readers are encouraged to consult these publications prior to initiating a large-scale cell selection experiment.

3.1 Maintenance of Arabidopsis Cell Suspension Culture

The *Arabidopsis* cell suspension culture was maintained in liquid MSAR1 medium. 50 ml cell suspension was cultured in 250 ml Erlenmeyer flasks with continuous shaking (150 RPM). Cultures were subcultured at 7-day interval by transferring 5 ml cell suspension culture into 45 ml fresh culture medium.

Cultures were kept in culture chamber with temperature adjusted to 22 °C and under dim light illumination (10–30 μE).

3.2 Transformation of Cell Suspension Cultures

The advantage of using cDNA libraries for large-scale transformation of plant cells is that the gene source is practically unlimited. cDNA library can be prepared from the same species or from any other related or unrelated plant or any other organism. In order to generate a transformed *Arabidopsis* cell cultures for in vitro selection, two cDNA libraries were used in our laboratory. The Arabidopsis COS library [34] was prepared from different organs and salt-treated seedlings of *A. thaliana* [36]. The halophyte cDNA library derived from salt-treated seedlings of *Eutrema*

salsuginea (unpublished). These libraries were cloned in the pER8-GW expression vector, which controls the transcription of the cDNA clones by the estradiol-inducible XVE promoter. Expression of the inserted cDNA in transformed plant cells in this system therefore depends on the addition of the chemical inducer (*see* **Note 1**). cDNA libraries can however be prepared from any plants or organism, and numerous other plant expression vectors can be used to introduce them into different, transformation-competent plant cell cultures. For preparation of cDNA library, please consult previously published protocols [36]. Strategy of the screening program is depicted in Fig. 1.

1. Inoculate 50 ml YEB medium (containing appropriate antibiotics) with 1 ml fresh starter Agrobacterium culture of the cDNA library. To propagate the COS library in pER8-GW vector 100 mg/l spectinomycin, 22 mg/l gentamycin, and 100 mg/l rifampicin were used. Shake the cultures at 250 rpm, in 28 °C for 4 h. Spin down the Agrobacteria (4000 g/20 min) and resuspend in 3 ml liquid MSAR1 medium. Adjust OD_{600} to 0.8.
2. Fast-growing cell suspension cultures containing single cells or microcolonies should be used for transformation (Fig. 2a). Cell culture is inoculated by the cDNA library 2 days after subculture by adding 2 ml Agrobacterium culture (resuspended in liquid MSAR1 medium) to 50 ml cell suspension. Coincubate cells for 2 h by standing the flask on the bench and subsequently shaking the cultures for 2 days.
3. After coincubation, Agrobacteria are removed by centrifugation of plant cells (100 g/1 min), which are resuspended in fresh MSAR1 medium. Repeat washing and resuspend the cells in fresh culture medium supplemented by 200 mg/l claforan, 200 mg/l carbenicillin, and 15 mg/l hygromycin. Culture the inoculated cell culture by continuous shaking. Composition of antibiotics can be different depending on the bacterial strain, the plant, and bacterial markers used.
4. Cells are subcultured at weekly intervals by adding 10× volume of fresh culture medium containing claforan, carbenicillin, and hygromycin. Transformed cell cultures can be established after 3–5 subcultures and are able to grow in hygromycin-containing culture medium, while the wild-type cell suspension not.

3.3 In Vitro Selection

Numerous selection strategies can be designed which can be used to identify stress tolerance traits in cell level. Selection can be based on growth, survival, color, or differentiation, depending on the trait and the cell culture used. In any case careful optimization of the system is essential to establish the appropriate conditions for efficient selection. Please note that such traits can be selected for, which are expected to function in cultured cells (*see* **Note 2**).

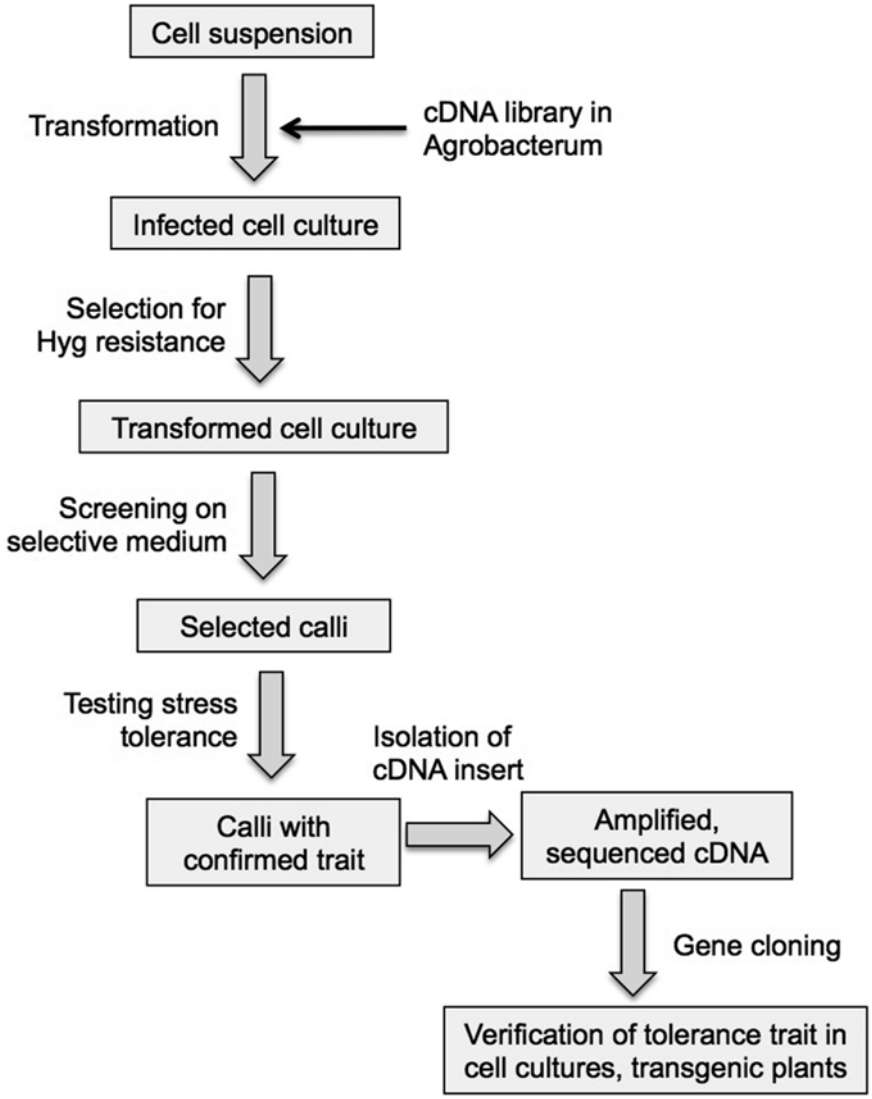

Fig. 1 Work flow of the gene identification program which employs cDNA librarytransformation and in vitro selection of plant cell cultures. Gene identification is done by insert amplification and sequencing of the inserted cDNA

1. Transformation efficiency should first be tested by plating an aliquot of transformed cell suspension onto solidified culture medium containing 400 mg/l claforan and 15 mg/l hygromycin. Typically five Petri plates are used for such testing. Transfer an aliquot of 5 ml cell suspension onto selective culture medium in a 100 mm Petri dish, and disperse it equally on the surface. Incubate the plates for in standard culture conditions. Count the number of growing calli after 3–4 weeks. Non-transformed cell culture should be used as control.

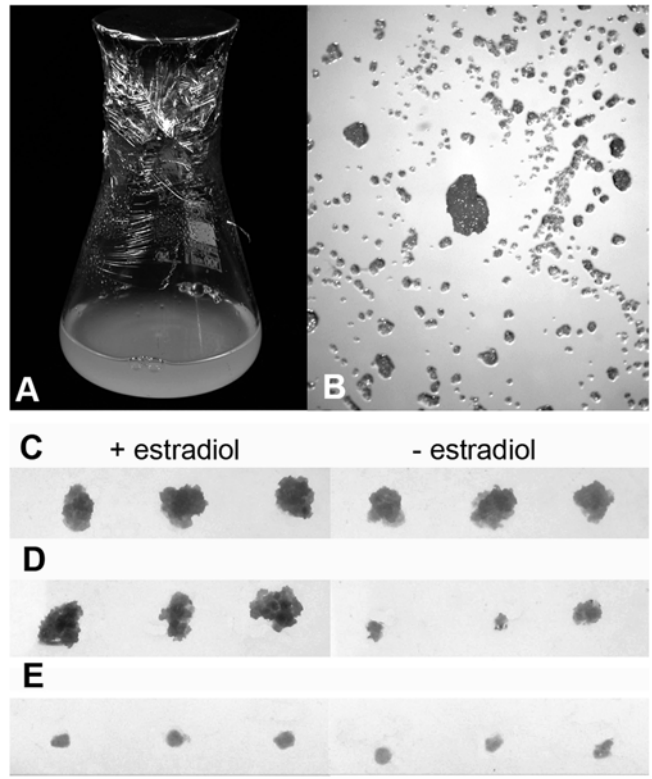

Fig. 2 In vitro screening for salt tolerance in plant cell cultures. (*a*) Established cell suspension culture suitable for large-scale transformation and subsequent screen. (*b*) Growth of transformed microcalli on high-salt medium, supplemented by estradiol, inducer of the chemically regulated XVE expression system. (*c*–*e*) Subcultured microcalli growing on high-salt media (150 mM NaCl) in the presence or absence of 4 μM estradiol. (*c*) Tolerance independent of estradiol. (*d*) Estradiol-dependent salt tolerance. (*e*) Control, non-transformed calli

2. To select for the desired phenotype, transfer transformed cell suspension to selective medium. To select for salt tolerance, agar-solidified medium composed of MSAR1 culture medium supplemented by 400 mg/l claforan, 150 or 175 mg/l NaCl, and 5 μM estradiol was used in our laboratory. If other selection criteria are used, careful testing and optimization of the selection conditions are necessary. In our selection system 8 ml cell culture was plated onto the selective medium in a 150 mm diameter Petri plate. In a typical screening experiment 100 Petri dishes were used (*see* **Note 3**).
3. Even distribution of microcell on the selective medium is critical for efficient selection. To improve plating efficiency, liquid cell suspension can be mixed with warm (40 °C), agar-containing selective medium (containing 0.6 % agar) in a ratio of 3:1 and immediately plated onto the surface of solid selective medium (use 10 ml suspension for 150 mm Petri dish).

Embedding the cells in a thin-layer, low-concentration agar medium will separate evenly the microcalli and improve plating efficiency.

4. Growing colonies are identified visually after 3–4 weeks of incubation (Fig. 2b). Growing calli can be split into two or more pieces of 1 mm diameter, and transferred onto fresh culture medium containing 200 mg/l claforan and 150–175 mg/l NaCl, supplemented with or without 5 μM estradiol. Calli, which showed superior growth in the presence of estradiol, were used to isolate the inserted cDNA (Fig. 2c–e).

3.4 Verification of the Selected Trait

Established cell cultures often lose their regeneration capacity; therefore genetic analysis and verification of the tolerance trait are not possible by traditional genetic methods. Isolation of the cDNA inserts is usually easy from callus cultures by PCR amplification, and the cloned fragments can be used to generate independent transformed cell lines or transgenic plants for verification and further physiological and molecular analysis (*see* **Notes 4** and **5**).

1. Isolate genomic DNA from the selected calli. Numerous methods are available for the preparation of high-quality genomic DNA using either commercial DNA isolation kits or traditional plant DNA isolation protocols [47–49].
2. cDNA inserts are conveniently rescued from the selected calli by PCR amplification of the fragment. Amplify the inserted cDNA using vector-specific PCR primers flanking the cDNA insert. Determine the identity of the cDNA by sequencing the PCR fragment followed by sequence homology search.
3. In order to verify the tolerance trait, clone the amplified cDNA in a plant expression vector. If Gateway cloning system is used, cloning of the PCR fragment is straightforward, as it can be cloned into an entry vector and subsequently transferred to a plant expression destination vector(s) [36].
4. Transform cell suspension cultures with the new vectors using the cell suspension protocol above. Small-scale transformation is sufficient, as a single construct is used. Select for appropriate antibiotic resistance (e.g., hygromycin) as described above.
5. Plate aliquots of transformed, hygromycin-resistant cell suspension onto selective medium containing the same components, which was used for screening. Growth of transformed microcalli on selective medium can indicate that the identified and cloned cDNA is indeed responsible for the tolerance trait. Variation in selection pressure (e.g., using several concentrations of NaCl if salt selection was employed) will provide more reliable data on the degree of tolerance achieved.

6. Gene constructs in plant expression vectors can be used to generate transgenic plants, which are amenable for subsequent molecular and physiological analysis. Testing stress tolerance in whole-plant level is therefore possible using either the same plant species where the cDNA library was originally obtained or heterologous hosts.

4 Notes

1. Transformation of cultured cells with the COS cDNA library will generate cell colonies which, upon addition of the chemical inducer, can produce conditional, dominant, gain-of-function phenotypes. Use of an inducible expression system has the advantage that the phenotype depends on the presence of the inducer, rendering verification of the selected traits more efficient. Once microcalli are identified, the tolerance character can be retested in the presence or absence of the inducer. Causal relationship between the inducer-dependent activation of the inserted cDNA and the observed trait can therefore be easily confirmed. In contrast with this scenario, phenotypes caused by constitutive overexpression of the inserted cDNA or somaclonal variation can be difficult to distinguish.
2. Numerous selection strategies can be designed to identify colonies with phenotypic alterations, which can be identified in cultured cells. Selection can be based on superior growth, survival, or employment of marker genes whose activation is easily detected in cultured cells. Markers such as firefly luciferase (LUC) or green fluorescent protein (GFP) and derivatives offer nondestructive detection, which is advantageous for in vitro selection. Establishment and proper testing of selection criteria is a key for success. Only such traits can be selected for which are expressed in cultured, proliferating cell cultures. Optimal screening conditions should therefore be carefully established in each case.
3. The described protocols are straightforward and technically easy to perform. Nevertheless a successful transformation and in vitro selection program are challenging tasks with numerous sensitive steps. Transformation, selection, and handling of cell suspension cultures require extreme care. Bacterial and fungal contamination is a great danger and can destroy sensitive experiments easily. Experiments usually last for several months, and require handling large number of cultured cells which are prone for contamination. Complete sterility should be maintained during the whole procedure. Always use safe sterile hoods, labcoats, sterilized pipettes separated for handling sterile solutions, etc.

4. Somaclonal variation may generate calli with certain degree of salt tolerance. Such calli are usually able to proliferate in selective conditions in the absence of the estradiol inducer. Some colonies carry the hygromycin resistance; therefore confirmation of the marker trait is insufficient. Care should be taken to test the tolerance trait, as identification of the inserted cDNA from such calli can produce misleading conclusions.
5. Verification of the selected traits requires careful subsequent testing. Although selection and gene identification can be performed in a short time, verification of the obtained results is an essential part of the program. As cultured cells are not amenable for genetic analysis, proper verification can be done by generation of independent transformed cell lines and/or transgenic plants.

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References

1. Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307–319
2. Chinnusamy V et al (2002) Screening for gene regulation mutants by bioluminescence imaging. Sci STKE 2002:1–10
3. Alonso JM, Ecker JR (2006) Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in *Arabidopsis*. Nat Rev Genet 7:524–536
4. Koiwa H et al (2006) Identification of plant stress-responsive determinants in *Arabidopsis* by large-scale forward genetic screens. J Exp Bot 57:1119–1128
5. Papdi C et al (2010) Genetic screens to identify plant stress genes. Methods Mol Biol 639:121–139
6. Kushnir S et al (1995) Characterization of *Arabidopsis thaliana* cDNAs that render yeasts tolerant toward the thiol-oxidizing drug diamide. Proc Natl Acad Sci U S A 92:10580–10584
7. Eswaran N et al (2010) Yeast functional screen to identify genetic determinants capable of conferring abiotic stress tolerance in *Jatropha curcas*. BMC Biotechnol 10:23
8. Kumar R et al (2012) Functional screening of cDNA library from a salt tolerant rice genotype Pokkali identifies mannose-1-phosphate guanyl transferase gene (OsMPG1) as a key member of salinity stress response. Plant Mol Biol 79:555–568
9. Shi J et al (1995) Characterization of a plasma membrane-associated phosphoinositide-specific phospholipase C from soybean. Plant J 8:381–390
10. Kleinow T et al (2000) Functional identification of an *Arabidopsis* snf4 ortholog by screening for heterologous multicopy suppressors of snf4 deficiency in yeast. Plant J 23:115–122
11. McCoy TJ (1987) Characterization of alfalfa (*Medicago sativa* L.) plants regenerated from selected NaCl tolerant cell lines. Plant Cell Rep 6:417–422
12. Rout GR et al (2008) Selection of salt tolerant plants of *Nicotiana tabacum* L. through in vitro and its biochemical characterization. Acta Biol Hung 59:77–92

13. Janardhan Reddy P, Vaidyanath K (1986) In vitro characterization of salt stress effects and the selection of salt tolerant plants in rice (*Oryza sativa* L.). Theor Appl Genet 71:757–760
14. Verma D et al (2013) In vitro selection and field responses of somaclonal variant plants of rice cv PR113 for drought tolerance. Plant Signal Behav 8:e23519
15. Matheka JM et al (2008) In vitro Selection and characterization of drought tolerant somaclones of tropical maize (Zea mays L). Biotechnol Adv 7:641–650
16. Pistelli L et al (2012) Novel Prunus rootstock somaclonal variants with divergent ability to tolerate waterlogging. Tree Physiol 32:355–368
17. Lu S et al (2007) In vitro selection of salinity tolerant variants from triploid bermudagrass (Cynodon transvaalensis x C. dactylon) and their physiological responses to salt and drought stress. Plant Cell Rep 26:1413–1420
18. Wenzel G, Foroughi-Wehr B (1990) Progeny tests of barley, wheat, and potato regenerated from cell cultures after in vitro selection for disease resistance. Theor Appl Genet 80:359–365
19. Guenzi AC et al (1992) Genetic analysis of a grass dwarf mutation induced by wheat callus culture. Theor Appl Genet 84:952–957
20. Kaeppler SM et al (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179–188
21. Wang QM, Wang L (2012) An evolutionary view of plant tissue culture: somaclonal variation and selection. Plant Cell Rep 31:1535–1547
22. Koncz C et al (1992) T-DNA insertional mutagenesis in *Arabidopsis*. Plant Mol Biol 20:963–976
23. Azpiroz-Leehan R, Feldmann KA (1997) T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. Trends Genet 13:152–156
24. Szabados L, Koncz C (2003) Identification of T-DNA insertions in *Arabidopsis* genes. In: Prade RA, Bohnert HJ (eds) Genomics of plants and fungi. Marcel Dekker Inc., New York, pp 255–277
25. Rosso MG et al (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. Plant Mol Biol 53:247–259
26. Weigel D et al (2000) Activation tagging in *Arabidopsis*. Plant Physiol 122:1003–1013
27. Jeong DH et al (2002) T-DNA insertional mutagenesis for activation tagging in rice. Plant Physiol 130:1636–1644
28. Nakazawa M et al (2003) Activation tagging, a novel tool to dissect the functions of a gene family. Plant J 34:741–750
29. Wan S et al (2009) Activation tagging, an efficient tool for functional analysis of the rice genome. Plant Mol Biol 69:69–80
30. LeClere S, Bartel B (2001) A library of *Arabidopsis* 35S-cDNA lines for identifying novel mutants. Plant Mol Biol 46:695–703
31. Ichikawa T et al (2006) The FOX hunting system: an alternative gain-of-function gene hunting technique. Plant J 48:974–985
32. Nakamura H et al (2007) A genome-wide gain-of function analysis of rice genes using the FOX-hunting system. Plant Mol Biol 65:357–371
33. Fujita M et al (2007) Identification of stress-tolerance-related transcription-factor genes via mini-scale Full-length cDNA Over-eXpressor (FOX) gene hunting system. Biochem Biophys Res Commun 364:250–257
34. Papdi C et al (2008) Functional identification of *Arabidopsis* stress regulatory genes using the controlled cDNA overexpression system. Plant Physiol 147:528–542
35. Perez-Salamo I et al (2014) The heat shock factor A4A confers salt tolerance and is regulated by oxidative stress and the mitogen-activated protein kinases MPK3 and MPK6. Plant Physiol 165:319–334
36. Rigo G et al (2012) Transformation using controlled cDNA overexpression system. Methods Mol Biol 913:277–290
37. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue culture. Physiol Plant 15:473–497
38. Mathur J et al (1995) A simple method for isolation, liquid culture, transformation and regeneration of *Arabidopsis thaliana* protoplasts. Plant Cell Rep 14:221–226
39. Koncz C et al. (1994) Specialized vectors for gene tagging and expression studies. In: Gelvin SB (ed) Plant Molecular Biology Manual, Vol B2. Kluwer Academic Publishers, pp. 53-74
40. Ozawa K, Komamine A (1989) Establishment of a system of high-frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.). Theor Appl Genet 77:205–211
41. Ahmed KZ, Sagi F (1993) Culture of and fertile plant regeneration from regenerable embryogenic suspension cell-derived protoplasts of wheat (*Triticum aestivum* L.). Plant Cell Rep 12:175–179
42. Kieran PM et al (1997) Plant cell suspension cultures: some engineering considerations. J Biotechnol 59:39–52

43. Zhong JJ (2001) Biochemical engineering of the production of plant-specific secondary metabolites by cell suspension cultures. Adv Biochem Eng Biotechnol 72:1–26
44. KimSW OMJ (2009) Establishment of plant regeneration and cryopreservation system from zygotic embryo-derived embryogenic cell suspension cultures of *Ranunculus kazusensis*. Methods Mol Biol 547:107–115
45. Mustafa NR (2011) Initiation, growth and cryopreservation of plant cell suspension cultures. Nat Protoc 6:715–742
46. Moscatiello R et al (2013) Plant cell suspension cultures. Methods Mol Biol 953:77–93
47. Offringa R, van der Lee F (1995) Isolation and characterization of plant genomic DNA sequences via (inverse) PCR amplification. Methods Mol Biol 49:181–195
48. Springer NM (2010). Isolation of plant DNA for PCR and genotyping using organic extraction and CTAB. Cold Spring Harb Protoc 2010: pdb prot5515
49. Berendzen K et al (2005) A rapid and versatile combined DNA/RNA extraction protocol and its application to the analysis of a novel DNA marker set polymorphic between *Arabidopsis thaliana* ecotypes Col-0 and Landsberg erecta. Plant Methods 1:4

Chapter 20

Using *Arabidopsis* Protoplasts to Study Cellular Responses to Environmental Stress

Ana Confraria and Elena Baena-González

Abstract

Arabidopsis mesophyll protoplasts can be readily isolated and transfected in order to transiently express proteins of interest. As freshly isolated mesophyll protoplasts maintain essentially the same physiological characteristics of whole leaves, this cell-based transient expression system can be used to molecularly dissect the responses to various stress conditions. The response of stress-responsive promoters to specific stimuli can be accessed via reporter gene assays. Additionally, reporter systems can be easily engineered to address other levels of regulation, such as transcript and/or protein stability. Here we present a detailed protocol for using the *Arabidopsis* mesophyll protoplast system to study responses to environmental stress, including preparation of reporter and effector constructs, large scale DNA purification, protoplast isolation, transfection, treatment, and quantification of luciferase-based reporter gene activities.

Key words Protoplasts, *Arabidopsis thaliana*, Mesophyll, Transient expression system, Stress, Luciferase-based reporters, Promoter activity

1 Introduction

The term protoplast refers to the part of a cell within the cell wall or, in other words, to the spherical structures, sensitive to osmotic shock, that are obtained when cell walls are completely digested [1]. The methods for protoplast isolation were first developed in bacteria [2] and fungi [3, 4] and then transposed to plants, with a paper in Nature in 1960 describing the isolation of protoplasts from tomato root tips by digesting the cell walls with cellulase in a high osmolarity solution [5]. The method was further developed with the addition of pectinase (also called macerozyme) which allowed speeding up the procedure and increasing yield [6]. Even though modifications have been introduced to the original protocol, the principles of plant protoplast isolation remain mostly the same. During the 1980s, methods for direct transformation of protoplasts were developed in multiple labs [7–10], and since then protoplasts have gained ample popularity within the plant community.

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So far, protoplasts have been isolated from a wide variety of plant species and tissues [11–16]. Protoplasts can be quickly prepared from fully differentiated plant material, which retain most leaf responses, and used immediately in cell-based transient expression assays. Mature *Arabidopsis* leaves are most often the material of choice, since they combine the easiness and high yield of mesophyllprotoplast preparation with the possibility of using a large collection of accessions and mutants, hence offering an additional advantage for assessing genetic interactions and for quick transient molecular complementation. Due to the short incubation times, in most cases there is no need to work under sterile conditions. Alternatively, protoplasts can also be isolated from suspension cultures [12, 14]. Although suspension cultures have been successfully used for transient assays [17–19], there are several caveats associated with this system. On one hand, it requires particular conditions depending on the source, as well as tissue culture facilities. Most importantly, long-term maintenance of cells in culture may induce undesirable alterations in the epigenetic landscape [20] and in metabolism, since cultured cells are heterotrophic and require sucrose supplementation. Therefore, the overall physiological and gene expression responses of these undifferentiated cells can be dramatically different from whole leaves.

Transient expression in *Arabidopsis* mesophyll protoplasts can be of particular importance when protein function cannot be deduced from knockout mutant analysis due to redundancy, to pleiotropic effects or to lethality. In these cases, gain-of-function analyses based on the overexpression of the gene of study or of candidate genes may be a more straightforward approach to ascribe function and protoplast assays can provide a convenient screening platform that facilitates more targeted whole plant studies. A reduced number of stable transgenic lines can then be generated to confirm the *in planta* function of the factors selected in the protoplast assays. Recently, the expression of artificial microRNAs (amiRs) against specific transcripts in protoplasts has allowed the application of this technology also to loss-of-function analyses [16, 21].

Several types of transient expression assays have been developed in order to address a broad range of biological problems. Very commonly, such assays are used to study protein localization by fusing the protein of interest to a fluorescent protein, as GFP or YFP. Protein–protein interactions can also be easily assessed by co-immunoprecipitation of differently tagged co-expressed proteins as well as by more specific techniques such as the split-luciferase assay [22, 23] or bimolecular fluorescence complementation [24].

Given that freshly isolated mesophyll protoplasts largely retain the physiological properties of whole plants [13, 25], they have been an important tool also for studying stress responses. Most frequently, reporter-based assays assess differential promoter activity in response to a stress stimulus or to a co-transfected protein

involved in a stress signaling pathway (*see* Subheading 3.4, **step 1** and the references within [13, 26–29]), but reporters can be easily modified in order to evaluate other processes. For example, one can generate promoter truncations and mutations to identify important regulatory regions and motifs [30], exchange the UTR sequence(s) to assess the effect on transcript stability [31], or introduce artificial miRNA binding sites in the UTRs to monitor miRNA activity [32]. Finally, protoplast preparations can be easily scaled up and adapted to yield enough material for a wide range of techniques, from reporter gene assays to high throughput molecular biology procedures, such as microarrays [26, 30, 33, 34] and deep sequencing [35, 36] or coupled to cell sorting to study cell-type specific responses [37–42].

In our laboratory, we mostly use protoplasts to dissect the energy stress signaling pathway. We present the protocol we routinely use to build effector and reporter constructs for protoplast transfection, as well as the procedure for plasmid DNA isolation and for protoplast isolation, transfection, stress treatment and reporter-gene assay quantification. It can be easily adapted to produce other types of reporters and also scaled up according to need. Overall it is an easy protocol to follow that only requires equipment usually found in most labs; it may take some optimization to your own conditions, but the most important to succeed in establishing the technique is to start from consistently healthy plants and to prepare good quality DNA for transfection.

2 Materials

2.1 Effector and Reporter Construction

2.1.1 Effector and Reporter Construction: Cloning

1. Phusion® High-Fidelity DNA polymerase (New England Biolabs, M0530S).
2. Gene and/or promoter-specific primers for cloning specific effectors and/or promoters.
3. Restriction enzymes.
4. T4 DNA ligase.
5. Agarose (low-melting or regular molecular biology grade agarose).
6. Effector DNA–Based on pHBT95 vector ([13]; GenBank EF090408).
7. Reporter DNA–pUC18-derived vector expressing firefly luciferase under a promoter of choice and the *NOS* terminator (e.g., *pRD29A-LUC-NOS* for ABA responses; GenBank EF090409; [13]).
8. Selective medium for bacterial growth.
9. *E. coli* MC1061 strain competent cells.

10. Thermal cycler.
11. Orbital shaker at 37 °C.

2.1.2 Effector and Reporter Construction: Site-Directed Mutagenesis

1. Template plasmid DNA (*see* Subheading 2.1.1).
2. Mutagenesis primers (*see* Subheading 3.1.2, **step 1**).
3. *Pfu* DNA polymerase.
4. dNTP set.
5. *DpnI* restriction enzyme.
6. Thermal cycler.
7. Water bath/incubator at 37 °C.

2.2 Plasmid DNA Purification

2.2.1 Equipment and Materials

1. Refrigerated centrifuge and rotor for 500 mL bottles.
2. Centrifuge with swing-out rotor for 50 mL/15 mL Falcon tubes.
3. Ultracentrifuge (Beckman Coulter Optima TL benchtop ultracentrifuge, TLA100.4 rotor).
4. 500 mL polypropylene copolymer centrifuge bottles with sealing cap.
5. 405 nm 5 mW Mini Laser Module.
6. 2–3 mL syringes.
7. 20G needles.
8. Miracloth (Merck, 475855-1R).
9. OptiSeal™ polypropylene 4.7 mL tubes (Beckman Coulter, 361621).
10. Sterile disposable inoculation loops.

2.2.2 Working Solutions

1. Terrific Broth (TB) medium: 0.05 % w/v TB, 0.5 % v/v glycerol, 100 μg/mL ampicillin. Prepare fresh.
2. Solution I: 10 mM EDTA, pH 8. Store at 4 °C.
3. Solution II: 0.1 N NaOH, 1 % SDS. Prepare fresh. Ensure that NaOH is either fresh or stored in a plastic container.
4. RNaseA (Sigma, R4642)–OPTIONAL.
5. Solution III: 2.5 M CH_3COOK, pH 4.8. Store at 4 °C.
6. Isopropanol.
7. 95 % v/v ethanol.
8. CsCl, ultrapure.
9. RedSafe™ Nucleic Acid Staining Solution (20000×) (Intron Biotechnology, Inc, 21141).
10. *n*-butanol saturated with 1 M NaCl.
11. 70 % v/v ethanol.

2.3 Protoplast Isolation

When incubating protoplasts for up to 16 h, it is unnecessary to work under sterile conditions or to add antibiotics to the incubation medium [21, 43]. It is however important to ensure good constant quality of all solutions for reproducible results.

2.3.1 Equipment and Materials

1. Sterile disposable inoculation loops.
2. Carbon steel single-edge razor blades.
3. Bell-shaped desiccator with vacuum connection.
4. Nylon mesh (70 μm opening).
5. Centrifuge with swing-out rotor for 50 mL Falcon tubes.
6. 0.1 mm depth hemocytometer.

2.3.2 Stock Solutions

All stock solutions should be autoclaved or filter-sterilized; unless stated otherwise, keep at room temperature.

1. 0.8 M mannitol.
2. 2 M KCl.
3. 0.2 M MES–KOH (pH 5.7)–Keep at 4 °C.
4. 1 M $CaCl_2$.
5. 10 % w/v BSA–Keep at −20 °C in 2 mL aliquots to minimize repeated freezing and thawing.
6. 5 M NaCl–Do not filter-sterilize.
7. 1 M $MgCl_2$.
8. Cellulase ("ONOZUKA" R-10) and macerozyme (R-10), Yakult Pharmaceutical Industry Co., Ltd. Aliquot enzymes in 15 mL Falcon tubes and store at −20 °C.

2.3.3 Working Solutions

All working solutions should be prepared with sterile Milli-Q water.

1. Enzyme solution: 0.4 M mannitol, 20 mM KCl, 20 mM MES–KOH (pH 5.7), 1.5 % w/v cellulase, 0.4 % w/v macerozyme, 10 mM $CaCl_2$, 0.1 % BSA. Prepare fresh. See preparation details in Subheading 3.3.
2. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES–KOH (pH 5.7). Filter-sterilize. Keep at room temperature.
3. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES–KOH (pH 5.7). Prepare fresh. Keep at 4 °C until use.

2.4 PEG-Ca^{2+} Transformation

2.4.1 Equipment and Materials

1. Transfection tubes (2 mL round-bottom tubes or 50 mL Falcon tubes, depending on the volume of protoplasts to be transfected).
2. Swing-out rotor for 2 mL round-bottom tubes or 50 mL Falcon tubes.

3. Cell culture plates (6- or 12-well plates) or 100 or 150 mm Petri dishes, depending on the volume of protoplasts transfected.
4. Temperature-controlled chamber with cool white light for protoplast incubation.

2.4.2 Stock Solutions

Unless otherwise stated, all stock solutions should be autoclaved or filter-sterilized and kept at room temperature.

1. Newborn calf serum–Work under sterile conditions. Aliquot original stock and store at –20 °C.
2. 0.8 M mannitol.
3. 2 M KCl.
4. 0.2 M MES–KOH (pH 5.7)–Keep at 4 °C.
5. 1 M $CaCl_2$.

2.4.3 Working Solutions

All working solutions should be prepared with sterile Milli-Q water.

1. 5 % v/v newborn calf serum–Filter-sterilize. Keep at 4 °C.
2. WI solution: 0.5 M mannitol, 4 mM MES–KOH (pH 5.7), 20 mM KCl. Prepare fresh.
3. 40 % PEG–40 % polyethylene glycol 4000, 0.2 M mannitol, 100 mM $CaCl_2$. Prepare fresh, but allow ample time for complete PEG solubilization with gentle rocking before use.

2.5 Luciferase Activity Assay

2.5.1 Equipment and Materials

1. 96-Well plates (OptiPlate-96 Black, Perkin Elmer, 6005270).
2. Luminometer (Berthold Technologies Microlumat Plus LB 96 V).
3. Fluorometer (Perkin Elmer multilabel plate reader Victor^3 1420).

2.5.2 Stock Solutions

Prepare stock solutions with sterile Milli-Q water and, unless stated otherwise, keep at room temperature.

1. 1 M Tris-phosphate (pH 7.8)–Autoclave.
2. 1 M DTT–Prepare fresh.
3. 100 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid–Dissolve in DMSO. The solution needs to be warmed and vortexed heavily to dissolve.
4. 50 % v/v glycerol–Autoclave.
5. 20 % v/v Triton X-100.
6. 100 mM firefly D-luciferin (potassium salt) –Prepare 1 mL aliquots. Keep at –80 °C.
7. 60.9 mM coenzyme A–Keep 1 mL aliquots at –80 °C. Use one whole aliquot to prepare a batch of luciferase substrate, which can then be in turn aliquoted and kept at –80 °C.
8. 0.5 M EDTA.

9. 100 mM ATP.
10. 100 mM $MgSO_4$.
11. 0.5 M Tricine–NaOH (pH 7.8).
12. 10 mM MUG (4-methylumbelliferyl b-D-glucuronic acid dihydrate)–Dissolve 100 mg in 5.7 mL DMSO and dilute with 20 mL water. Store in 200–500 µL aliquots at −80 °C.
13. 1 M Tris–HCl (pH 8.0)–Autoclave. Keep at room temperature.
14. 1 M $MgCl_2$–Filter-sterilize. Keep at room temperature.

2.5.3 Working Solutions

All working solutions should be prepared with sterile Milli-Q water.

1. Lysis buffer: 25 mM Tris-phosphate buffer (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′, *N*′-tetraacetic acid, 10 % v/v glycerol, 1 % v/v Triton X-100. Prepare 2 mL aliquots and keep at −20 °C.
2. Luciferase substrate: 470 µM D-luciferin, 270 µM coenzyme A, 0.1 mM EDTA, 33.3 mM DTT, 530 µM ATP, 2.67 mM $MgSO_4$, 20 mM Tricine–NaOH (pH 7.8). Prepare 2 mL aliquots and keep at −80 °C.
3. MUG working solution: 1 mM MUG in 10 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$. Prepare fresh.
4. 0.2 M Na_2CO_3–Autoclave. Keep at room temperature.

3 Methods

Unless otherwise specified, all steps should be carried out at room temperature. Water refers to sterile Milli-Q water.

3.1 Effector and Reporter Construction

3.1.1 Effector and Reporter Construction: Cloning

Most often, we use the small pUC18-based pHBT95 (EF090408; [13]) and luciferase reporter vectors as backbones for the constructs to be transfected into protoplasts (*see* **Notes 1** and **2**). We build our constructs by traditional cloning, using the unique sites indicated in Fig. 1.

1. Design two primers containing the appropriate restriction sites for: *i)* amplifying the coding region of the desired effector downstream of the constitutive *35S* promoter or an inducible promoter [21, 44, 45]; *ii)* amplifying and fusing the desired promoter to the firefly luciferase (*LUC*) gene (*see* **Note 3**).
2. Amplify your effector and/or promoter of interest with Phusion® or another proofreading DNA polymerase, adjusting the PCR settings to the primers, enzyme, and product requirements. This PCR product is your insert.
3. Digest both insert and vector with the same restriction enzymes, according to the manufacturer's instructions.

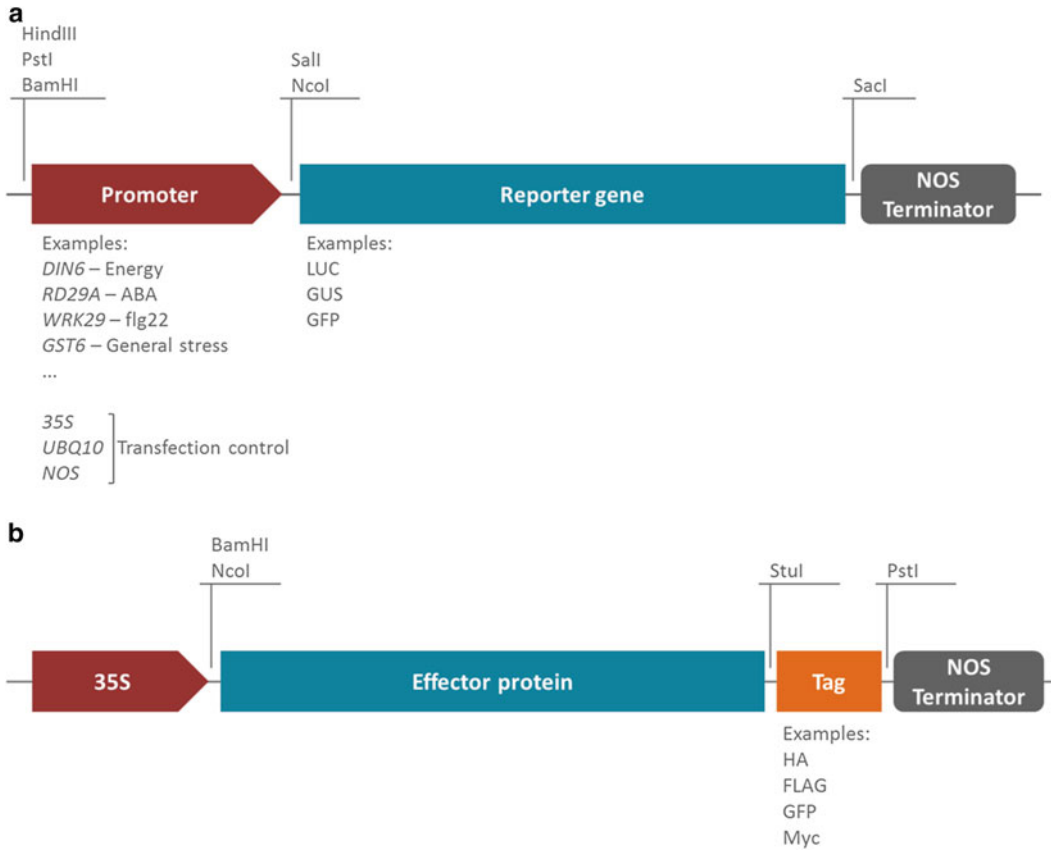

Fig. 1 Unique restriction sites used for building reporter (**a**) and effector (**b**) constructs using the pHBT95 vector

4. Run both insert and vector in an agarose gel and cut bands of interest under UV illumination.
5. If using normal agarose, purify insert and vector from gel. If using low-melting agarose, simply melt the DNA bands before use.
6. Ligate insert to vector following T4 ligase instructions for 2 h to overnight. If using low-melting agarose, carry out ligation at room temperature.
7. Transform ligation into MC1061 competent cells (*see* **Note 4**).
8. Grow transformed cells at 37 °C on plates with selective medium.
9. Purify plasmid DNA at a minipreparation scale.
10. Verify that constructs are correct by restriction enzyme digestion and sequencing.

3.1.2 Effector and Reporter Construction: Site-Directed Mutagenesis

1. Design two fully complementary primers, containing the site to mutate in the middle and extending 12–18 bases to either side of the created mismatch/insertion/deletion.
2. Prepare reaction mix: 2.5 μL 10× *Pfu* buffer, 2.5 μL 2.5 mM dNTPs (each), 10–25 ng primer F, 10–25 ng primer R, 25–50 ng DNA, 0.5 μL *Pfu* DNA polymerase, sterile water up to 25 μL.
3. Split the reaction mix into two 12.5-μL aliquots: one will go through PCR amplification and the other will be frozen and used later as a control for *DpnI* digestion.
4. Carry out the mutagenesis PCR under the following conditions (*see* **Notes 5**–7): 95 °C, 3 min (1 cycle); 95 °C, 30s + 55 °C, 60s + 68 °C, 2 min/kb of plasmid (12–18 cycles).
5. Cool the tubes to room temperature and add 0.5 μL *DpnI* to each reaction, including the ones that were frozen.
6. Incubate reactions at 37 °C overnight (*see* **Note 8**).
7. Use 4 μL of each reaction to transform 50 μL of competent cells.
8. Grow transformed cells at 37 °C on plates with selective medium.
9. Purify plasmid DNA at a minipreparation scale.
10. Sequence to confirm mutation and rule out unintended extra mutations.
11. Subclone the mutated product into a new backbone (*see* Subheading 3.1.1).

3.2 DNA Purification

1. Transform MC1061 competent cells with plasmids containing reporter and/or effector constructs. Grow on plates with selective medium overnight at 37 °C (*see* **Note 4**).
2. Pick a single colony, grow a small culture to saturation and inoculate 200 mL of freshly made TB with 200 μL of the mini culture (*see* **Note 9**).
3. Grow cells in 3-L flasks at 37 °C for 8–16 h at 180–220 rpm (*see* **Note 10**).
4. Centrifuge cells at 2980 × *g* for at least 20 min at 4 °C.
5. Drain the medium and freeze the cell pellet. We store frozen pellets at −20 °C for up to 1 month.
6. Add 40 mL of solution I to the frozen pellet and mix by swirling until clumps are no longer visible.
7. Prepare fresh solution II and (OPTIONAL) add RNAse A to solution III (in a plastic container, add 600 μg of RNAse A to 30 mL of solution III) (*see* **Note 11**).

8. Add 80 mL of solution II and immediately mix thoroughly with circular movements, trying to avoid foaming. Let stand 2 min for full lysis (*see* **Note 12**).
9. Add 30 mL of solution III to the lysed cells and mix gently but thoroughly by inverting approximately ten times.
10. (OPTIONAL, only needed if using RNase): Incubate at room temperature for 20 min.
11. Centrifuge cells at 2980 × *g* for 5–10 min at 4 °C.
12. Filter through a layer of wet Miracloth into clean centrifuge bottles. The lysate should be clear and pass easily through the Miracloth.
13. Add 120 mL isopropanol and mix gently by inverting.
14. Centrifuge cells at 2980 × *g* for 5–10 min at 4 °C. Drain isopropanol.
15. Rinse the pellet gently but quickly with 95 % ethanol. Drain ethanol and let the pellet dry.
16. Resuspend the pellet to a final volume of 4 mL with solution I.
17. Add the 4 mL to a 15 mL tube containing 4.7 g CsCl and mix slowly on a rocking mixer until the salt is fully dissolved.
18. Add 10 μL of RedSafe™ Nucleic Acid Staining Solution (20,000×) and mix until homogenous (*see* **Note 13**).
19. Centrifuge in a swing-out rotor at 1125 × *g* for 5 min.
20. Transfer supernatant to a 4.7 mL OptiSeal™ tube, filling it until the neck line. Prepare balanced tubes for ultracentrifugation.
21. Seal tubes with appropriate cap, using the support rack tool.
22. Centrifuge at ~195,500 × *g* (60,000 rpm) for 13–15 h at 22 °C, with maximum acceleration and minimum deceleration (*see* **Note 14**).
23. After the centrifugation, carefully transfer the tubes to the support rack and uncap them.
24. To visualize plasmid DNA, illuminate one tube at a time from the top with a light that can excite RedSafe™ (405 nm 5 mW Mini Laser Module).
25. Extract the plasmid DNA band with a 2–3 mL syringe and a 20-gauge needle. With the needle still inside discard the rest of the solution and only then remove the needle and transfer the syringe content into a fresh 15-mL tube.
26. Add water up to 3 mL.
27. Add 7 mL of *n*-butanol and shake vigorously. The yellow color from RedSafe™ should transfer to the upper phase (*see* **Note 15**).
28. Add 1 mL of water carefully. This middle layer of water will facilitate removal of the upper organic phase.

29. Remove the upper phase.
30. Repeat twice **steps 26–28**, but using only 4 mL of *n*-butanol each time.
31. Add 3 volumes of 95 % ethanol. Mix by inverting to precipitate the DNA.
32. Centrifuge at 1125 × *g* for 5 min in a swing-out rotor.
33. Wash the DNA pellet twice with 10 mL of 70 % ethanol, vortexing to release the pellet and centrifuging briefly after each wash.
34. Air-dry the pellet.
35. Resuspend plasmid DNA in 300 μL of water and adjust final concentration to 2 μg/μL. Store at –20 °C (*see* **Note 16**).

3.3 Protoplast Isolation

All protoplast centrifugations are performed at room temperature in a swing-out rotor.

1. Prepare fresh enzyme solution–mix water, mannitol, KCl, and MES–KOH, and incubate at 70 °C for 2 min; add the enzymes and solubilize the powder with the help of a sterile disposable inoculation loop; heat at 55 °C for 10 min for complete enzyme solubilization and protease inactivation; add $CaCl_2$ and BSA only after cooling the solution to room temperature (*see* **Note 17**). Filter solution through a 0.45 μm syringe filter into a plastic Petri dish.
2. Pick fully expanded leaves from the second to the fourth pairs of true leaves of *Arabidopsis* plants with 4–5 weeks (*see* **Notes 18–20**).
3. Cut 1–3 leaves at a time with a single edge, carbon steel razor blade into 0.5–1 mm transversal strips, after discarding both the apical and basal portions of the leaf. Cut on a clean paper sheet or Parafilm.
4. As you cut the leaves, transfer the leaf strips using a sterile disposable inoculation loop into the Petri dish and submerge them gently into the enzyme solution.
5. Cover the Petri dish and incubate the leaf strips in the dark under vacuum for 30 min.
6. After vacuum infiltration, incubate the leaf strips in the dark for another 2 h 30 min.
7. Swirl the plate gently to release the protoplasts.
8. Pass the enzyme solution containing the protoplasts through a 70-μm nylon mesh into a 50mL tube on ice (*see* **Note 21**).
9. Add one volume of W5 to the leaf strips remaining to recover additional protoplasts and then transfer it to the tube with the protoplasts filtering through the mesh.

10. Centrifuge protoplasts at 180 × *g* for 3 min.
11. Remove the supernatant with a pipette.
12. Resuspend the protoplast in a small W5 volume (1–2 mL) by swirling the tube gently.
13. Add W5 up to the same total volume (i.e., 2× the initial volume of enzyme solution).
14. Repeat **steps 10–13** once.
15. Repeat **steps 10–12**. Add W5 up to a final total volume of 0.5–0.75 of the initial enzyme solution.
16. Measure protoplast concentration using a hemocytometer and adjust the concentration to 2×10^5 protoplasts/mL.
17. Keep protoplasts on ice for 30 min.
18. Centrifuge protoplasts at 180 × *g* for 3 min, remove the supernatant and resuspend the protoplasts in the same final volume of chilled MMg, so as to keep the adjusted concentration.

3.4 PEG-Ca^{2+} Transformation (See **Note 22***)*

1. Pipet ~20 μg (~10 μL) of CsCl-purified maxiprep plasmid DNAs to transfection tubes, which can be kept at 4 °C for 24 h. Generally, we use 0.5–1.0 μg of an internal transfection control (*UBQ10::GUS*, *35S::GUS* or *NOS::GUS*), 8–10 μg of the luciferase-based reporter and 12–10 μg of controls/effectors. Whenever necessary use control DNA (e.g., empty effector vector) to adjust the total DNA amount per sample to 20 μg (*see* **Note 23**).
2. Coat tissue culture plates for protoplast incubation to minimize cell adhesion to the plastic - cover all the surface with 5 % newborn calf serum, swirl gently, remove well the serum and let the plates dry.
3. Add an appropriate volume of WI to each well of the plate (*see* **Note 24**). Keep lids on until use.
4. Pipet 2×10^4 protoplasts (100 μL) into the previously prepared tubes containing the DNA to transfect. Mix gently by tapping.
5. Add 1 volume (110 μL) of 40 % PEG and mix by tapping (*see* **Note 25**).
6. Incubate for 5 min at RT (*see* **Note 26**).
7. Stop the transfection by adding 2 volumes (440 μL) of W5. Mix by inverting.
8. Centrifuge protoplasts at 180 × *g* for 1–3 min.
9. Remove most of the supernatant (~630 μL).
10. Resuspend the pellet in the remaining solution and transfer to previously prepared plates containing WI. Ensure that the protoplasts are evenly distributed throughout the wells/plates.

12. Incubate the protoplasts under the desired stress and control conditions (*see* Table 1 and **Notes 27** and **28**).
13. Alternatively, incubate the protoplasts first under control light conditions for ~2–3 h in order to allow similar expression of reporter and effector in both control and stress treatments and only then apply stress treatment to protoplasts.
14. To harvest the protoplasts, swirl gently the plates and transfer the cells into fresh tubes.
15. Centrifuge protoplasts at 180 × *g* for 1–3 min.
16. Remove most of the supernatant (i.e., leave approximately 30 μL in each tube) and flash freeze in dry ice or liquid nitrogen.

3.5 Stress Treatments for Protoplasts

See Table 1.

3.6 Luciferase Activity Assay

1. Add lysis buffer to the frozen protoplasts (100 μL per 2×10^4 cells).
2. Vortex briefly and centrifuge at maximum speed on a benchtop centrifuge for 1 min. Keep the lysate on ice.
3. Pipette 20 μL of each lysate into each well of a 96-well plate (or other, according to the luminometer to be used).
4. Assay luciferase activity in a luminometer by automatic injection of 100 μL of luciferase substrate buffer per well (*see* **Notes 29** and **30**).
5. Pipette 10 μL of each lysate into fresh tubes.
6. Add 100 μL of MUG working solution to each tube.
7. Incubate at 37 °C for 2–3 h.
8. Stop reaction by adding 900 μL 0.2 M Na_2CO_3. Mix thoroughly.
9. Transfer 100 μL of each reaction into a well of a 96-well plate and read β-glucuronidase activity in a fluorometer with excitation at 365 nm and emission at 455 nm.
10. Divide luciferase activity by β-glucuronidase activity for calculating normalized relative light units (nRLU).
11. Data can be presented directly as nRLU or alternatively normalized to a control (Fig. 2).
12. We typically have a minimum of three biological replicates (corresponding to three independent protoplast batches), each consisting of a minimum of two independent transfections. From these values, we calculate the mean and standard deviation (SD) or standard error of the mean (SEM) for each sample.

Table 1
Collection of abiotic and biotic stress treatments employed for studying stress responses in protoplasts

Stress	Condition	Type of reporter	References
Cold	0 °C, 4 h + 23 °C, 12 h (maize)[a]	GFP-based; *HVA1* promoter activity	[46]
Salt	0.2 M NaCl, 3 h +13 h after washing (maize)	GFP-based; *HVA1* promoter activity	[46]
Dark	16 h (maize)	GFP-based; *HVA1* promoter activity	[46]
	3–6 h (*Arabidopsis*)	LUC-based; *DIN6, MIR161, MIR775* promoter activity	[30, 47]
ABA	100 μM ABA, 16 h (maize)	GFP-based; *HVA1* promoter activity	[46]
	5–100 μM ABA, 3 h–6 h (*Arabidopsis*)	LUC-based; *RD29A*, *RD29B*, *DIN6, APX2* promoter activity	[27, 48–50]
DCMU[b]	20 μM, 3–6 h	LUC-based; *DIN6, APX2* promoter activity	[30, 50]
Oxidative stress	200 μM H_2O_2, 3 h	LUC-based; *GST6*, *HSP18.2*, *RD29A*, *35S, APX2* promoter activity	[48, 50]
Hypoxia	Submerge protoplasts in 1 mL of buffer in 1.5-mL tube, 3–6 h	LUC-based; *DIN6* promoter activity	[30]
Biotic stress	10–100 nM flg22, 2–4 h	LUC-based; *WRK29, FRK1, GST1, GST6, RD29A* promoter activity	[29, 51, 52]
	10 nM elf18	LUC-based; *FRK1* promoter activity	[29]
	100 nM HrpZ1	LUC-based; *FRK1* promoter activity	[29]
	50 μg/mL peptidoglycan	LUC-based; *FRK1* promoter activity	[29]
	50–200 μg/mL chitin	LUC-based; *FRK1* promoter activity	[29, 53]
	50 μg/mL lipopolysaccharide	LUC-based; *FRK1* promoter activity	[29]
Heat stress	37 °C, 3 h	GFP-based; *HSP18.1-CI* promoter activity	[54]

[a]When cold is used as a stress treatment, keep protoplasts at room temperature throughout the isolation procedure rather than incubating them on ice
[b]3-(3,4-dichlorophenyl)-1,1-dimethylurea

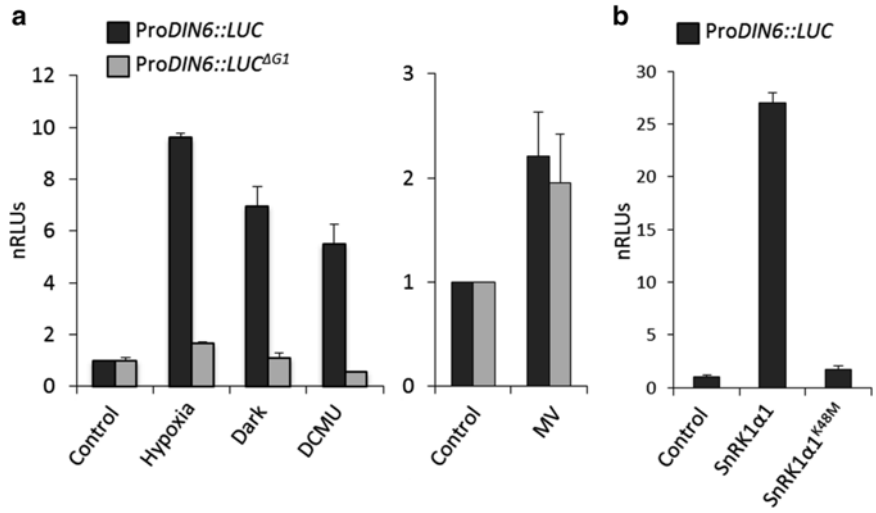

Fig. 2 *DIN6* promoter activity in transiently transfected *Arabidopsis* mesophyll protoplasts. (**a**) The activity of a wild-type version of the *DIN6* (At3g47340) promoter using LUC activity as readout (Pro*DIN6::LUC*) is compared to the activity of a promoter derivative, in which a G-box (G1, CACGTG) most proximal to the TATA box was mutated (CTCGAG; Pro*DIN6::LUCΔG1*). The WT version is activated by different stresses, such as hypoxia (6 h), dark (6 h), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 6 h, 20 μM), whereas the mutated version is non-responsive, presumably due to the lack of the G1 box. On the contrary, both the WT and mutated version are mildly activated by methyl viologen (MV, 3 h, 100 μM) to the same extent, showing that the transcriptional activation is in this case independent of the G1 box. (**b**) The *DIN6* WT promoter is activated by co-transfection with SnRK1α1, but not by an inactive SnRK1α1 variant mutated in the conserved ATP binding pocket (SnRK1α1^{K48M}). The graphs depict normalized luciferase activity values. Bars represent means ± SEM

4 Notes

1. The Sheen lab has donated to the ABRC stock a set of control constructs [13], and we highly recommend the use of this resource for establishing the protoplast transient expression system. On one hand, protoplast transfection efficiency should reach at least 50 % (ideally 80–90 %) and this can be evaluated by transfecting with a GFP marker (pHBT-sGFP(S65T)-NOS; [13]). On the other hand, a set of LUC reporters is available that can respond to specific signals and that can therefore be used [13, 55] as positive controls.
2. We have used with success other vectors besides pHBT95 for protein expression in protoplasts, such as the Gateway-based p35S-HA-GW [24]. Transformation with binary vectors such as pCB302 [56] is also possible, but we consistently find transfection efficiency with these plasmids to be much lower and only use protoplasts in this case to verify constructs before proceeding to plant transformation.

3. When building a reporter for promoter activity, if no prior information on the promoter of interest is available, we generally take the entire intergenic region upstream of the start codon to a maximum of 4–5 kb in the cases where this region is extremely long. For effector proteins, we usually just clone the coding sequence in order to maximize protein expression.
4. We generally use *E. coli* MC1061 for growing cultures for plasmid DNA maxipreps, as the DNA yield and quality are consistently good. However, if the insert to be used harbors repetitive sequences (e.g., the same sequence in sense and antisense direction for hairpin formation), it is recommended to use TOP10 cells, as this strain has reduced DNA recombination and the plasmid DNA yield and quality are also reasonably good.
5. For site-directed mutagenesis, we use an extension temperature of 68 °C, as *Pfu* will not strand displace at 68 °C, but will do so at 72 °C amplifying the original DNA that does not contain the mutation. Therefore, with extension at 68 °C, it is less likely to obtain non-mutated clones.
6. *Pfu* is a proofreading DNA polymerase much slower than regular *Taq*. We often use extension times longer than 10 min without problems.
7. The number of cycles in the PCR should be adjusted to the type of mutation being made: 12 cycles for point mutations; 16 cycles for single amino acid changes; 18 cycles for deletions or insertions.
8. *DpnI* restricts only methylated DNA, i.e., the original plasmid used as a template that does not contain the mutation, but not the newly synthesized product containing the mutation. Digestion time with *DpnI* can be shortened to approximately 8 h, but carrying out overnight digestion can be more effective and help reduce the background.
9. There is no need to autoclave TB when growing the 200-mL cultures for 8 h. We grow these cultures mostly in 3-L flasks to ensure proper aeration.
10. It is important not to exceed these culture times. Bacterial overgrowth will result in decreased quality of plasmid DNA.
11. The use of RNase A in the DNA purification procedure is optional. We have found however that it can decrease the RNA contamination in the final preparation when the CsCl purification is performed in a small rotor such as the Beckman TLA100.4. Do not use glass containers for preparing solutions containing RNase A because it easily binds to glass. If you use RNase, the pellet obtained after isopropanol precipitation will be flaky and will become completely transparent once dried. It

is advised to mark in the bottle where the pellet is to allow easier resuspension later on.

12. In the DNA purification procedure, the lysis of the cells is a critical step that can take some optimization according to the user; one should be neither too harsh nor too gentle. Avoid delays throughout that part of the protocol. If the lysis is not complete the neutralized lysate will be "slimy" and difficult to filter through Miracloth.
13. The CsCl protocol is traditionally done with ethidium bromide. We have adapted it to use RedSafe™ instead of ethidium bromide without detrimental impact on our preparations. When using RedSafe™, we strongly recommend visualization of the plasmid DNA band with the aid of a light that can excite the dye (please check product information with supplier for specific information). In our case, we use a 405 nm 5 mW Mini Laser Module.
14. Larger rotors, such as the Beckman NVT90 rotor, allow better DNA separation; however, we use routinely the smaller TLA100.4 rotor with reproducible and sufficiently good separation.
15. As ethidium bromide, RedSafe™ can be washed off with *n*-butanol. Even though it seems that all the dye is removed in the first wash, we routinely do three washes to obtain a final DNA preparation as pure as possible.
16. Throughout the whole DNA purification procedure, be careful to avoid contamination amongst preparations done simultaneously. In the final resuspension, the use of filter tips is advised as the DNA concentration is particularly high. When the DNA concentration is adjusted to 2 μg/μL, the preparation should be fluid rather than viscous. A simple restriction digestion analysis should be carried out before use for construct confirmation and quality evaluation. The plasmid DNA yield from a 200-mL culture is typically 2–4 mg.
17. β-mercaptoethanol can be added to the enzyme solution for protoplast isolation at a final concentration of 5 mM to prevent protoplast protein oxidation. This is unnecessary in most cases and we do not routinely use it.
18. One of the most important conditions for successful protoplast isolation and transfection is to start from healthy plants. We grow protoplast plants under a 12:12 light–dark cycle (100 μE; 22 °C/18 °C) for 4–5 weeks to maximize vegetative growth and prevent bolting. We pick healthy fully expanded leaves (usually true leaf numbers 5–8) preferably not shaded by other leaves. To ensure plant health we grow the plants in a dedicated walk-in chamber where no older flowering plants (always more susceptible to pests [57]) are allowed, avoiding

excessive humidity in the pots and keeping a plant density of 9 plants per pot (18.5 × 14 × 5.1 cm). We always harvest plants for protoplast isolation at the same time of the day (2 h after the onset of the light period) to avoid potential effects from the circadian clock. We recommend making an initial test of the response at different times of the day/night and to adhere to a particular time once this is established.

19. In order to obtain a good yield of isolated protoplasts, it is important to have enough leaf strips to fill the plate, but not to saturate the enzyme solution. Insufficient starting material can result in poor yield but, from a certain point, excessive starting material will not increase yield and will result in dirtier preparations.
20. An alternative way to isolate protoplasts was recently reported (Tape-*Arabidopsis* Sandwich method [58]), in which the leaf lower epidermal layer is removed to expose mesophyll cells to the enzyme solution, allowing a three-times faster cell wall degradation as compared to the conventional leaf strip method described here [13]. Nevertheless, we have not used this method, as we have deemed important not to introduce alterations in the times of treatments and incubations.
21. The nylon mesh used to filter the initial protoplast preparation should be kept in 95 % ethanol and thoroughly washed with water and briefly rinsed with W5 before use.
22. Other transformation methods, such as electroporation, have been reported [8, 9, 59–61]. However, we only use PEG-Ca^{2+} transformation since it is simpler, cheaper and, most importantly, allows the parallel processing of many more samples than electroporation.
23. A ratio of ~20 μg DNA to 2×10^4 cells is optimal for successful transfections. This amount of cells is sufficient for obtaining a good signal in assays with regular luciferase-based reporters, but can be easily scaled up when needed. The tubes used for transfections should be large enough as to allow easy mixing of protoplasts with PEG; we use either round-bottom 2 mL tubes or 50 mL Falcon tubes, depending on the volume of protoplasts to transfect.
24. It is important that the volume of WI used to incubate the protoplasts after transfection is just sufficient to cover the whole surface of the well/plate with a thin layer of liquid in order to avoid creating a hypoxic environment for the protoplasts. We generally use 500 μL per well in 12-well plates, 1 mL per well in 6-well plates, 5 mL in 100-mm Petri dishes and 12 mL in 150-mm Petri dishes.
25. During transfections, protoplasts have to be consecutively mixed with DNA, 40 % PEG, and W5. Even though proto-

plasts need to be handled gently, it is essential to ensure that cells are always homogeneously mixed with these solutions.

26. When designing the experiments, one should bear in mind that all samples to be compared should be transfected in parallel. With a transfection time of 5 min, it is possible to transfect 10–15 samples, with 30s–20s intervals in between each sample, respectively. Use cut tips for pipetting 40 % PEG during transfections. Additionally, in order to compare reporter activity amongst different samples it is necessary to normalize luciferase activity with that of a co-transfected control reporter driven by a constitutive promoter (in our case β-glucuronidase activity derived from *UBQ10::GUS, NOS::GUS, or 35S::GUS* expression).
27. We describe several short stress treatments used both in our laboratory and described in the literature (3–16 h; *see* Table 1). We are mostly interested in early stress responses, and therefore rarely use incubation times longer than 6 h. Nevertheless, you should always test, and if needed optimize, the stress treatments to your conditions and questions. Longer incubation times of up to several days have been employed, e.g., in the context of the circadian clock as well as for silencing via artificial miRNAs [16, 21, 43], and might be required for assessing secondary/later responses to stress. The duration of the treatment will also depend on whether the output is LUC activity based on promoter activation or for example direct kinase activation, which might require just a few minutes of incubation under stress (e.g., [62, 63]). The output will also determine whether cells are directly subjected to stress conditions or whether they are pre-incubated under control conditions before being subjected to stress (to allow equal protein accumulation when for example kinase activity is measured from control and stress conditions).
28. If possible, the protoplasts should be incubated in a dedicated chamber with temperature control and the same light quality as the plant growth chamber. We incubate the protoplasts without agitation.
29. There are commercially available firefly luciferase assay buffers (e.g., Promega). However, we routinely prepare our own firefly luciferase assay buffer, which reduces costs without compromising the sensitivity of the assay.
30. Even though luminescence can be easily assayed using a sensitive charge-coupled device (CCD) camera, we routinely use a plate reader luminometer, as it allows more precise quantitative measurements. Moreover, plate readers are often equipped with an injection system that supplies the luciferin substrate automatically at defined time points, ensuring similar integration times for all measurements.

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References

1. Brenner S, Dark FA, Gerhardt P, Jeynes MH, Kandler O, Kellenberger E, Klieneberger-Nobel E, McQuillen K, Rubio-Huertos M, Salton MRJ, Strange RE, Tomcsik J, Weibull C (1958) Bacterial protoplasts. Nature 181(4625):1713–1715
2. Weibull C (1953) The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. J Bacteriol 66(6): 688–695
3. Eddy AA, Williamson DH (1957) A method of isolating protoplasts from yeast. Nature 179(4572):1252–1253
4. Bachmann BJ, Bonner DM (1959) Protoplasts from neurospora crassa. J Bacteriol 78: 550–556
5. Cocking EC (1960) A method for the isolation of plant protoplasts and vacuoles. Nature 187(4741):962–963
6. Power JB, Cocking EC (1969) A simple method for the isolation of very large numbers of leaf protoplasts by using mixtures of cellulase and pectinase. Biochem J 111(5):33P
7. Krens FA, Molendijk L, Wullems GJ, Schilperoort RA (1982) In vitro transformation of plant protoplasts with Ti-plasmid DNA. Nature 296(5852):72–74
8. Fromm M, Taylor LP, Walbot V (1985) Expression of genes transferred into monocot and dicot plant cells by electroporation. Proc Natl Acad Sci U S A 82(17):5824–5828
9. Riggs CD, Bates GW (1986) Stable transformation of tobacco by electroporation: evidence for plasmid concatenation. Proc Natl Acad Sci U S A 83(15):5602–5606
10. Negrutiu I, Shillito R, Potrykus I, Biasini G, Sala F (1987) Hybrid genes in the analysis of transformation conditions : I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol Biol 8(5):363–373. doi:10.1007/BF00015814
11. Pandey S, Wang X-Q, Coursol SA, Assmann SM (2002) Preparation and applications of *Arabidopsis thaliana* guard cell protoplasts. New Phytol 153(3):517–526. doi:10.1046/j.0028-646X.2001.00329.x
12. Davey MR, Anthony P, Power JB, Lowe KC (2005) Plant protoplasts: status and biotechnological perspectives. Biotechnol Adv 23(2):131–171. doi:10.1016/j.biotechadv.2004.09.008, S0734-9750(04)00096-5 [pii]
13. Yoo SD, Cho YH, Sheen J (2007) *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2(7):1565–1572. doi:10.1038/nprot.2007.199, doi:nprot.2007.199 [pii]
14. Miao Y, Jiang L (2007) Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells. Nat Protoc 2(10):2348–2353. doi:10.1038/nprot.2007.360, nprot.2007.360 [pii]
15. Eeckhaut T, Lakshmanan PS, Deryckere D, Van Bockstaele E, Van Huylenbroeck J (2013) Progress in plant protoplast research. Planta 238(6):991–1003. doi:10.1007/s00425-013-1936-7
16. Li JF, Zhang D, Sheen J (2014) Epitope-tagged protein-based artificial miRNA screens for optimized gene silencing in plants. Nat Protoc 9(4):939–949. doi:10.1038/nprot.2014.061, nprot.2014.061 [pii]

17. Tran LS, Nakashima K, Sakuma Y, Simpson SD, Fujita Y, Maruyama K, Fujita M, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2004) Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive cis-element in the early responsive to dehydration stress 1 promoter. Plant Cell 16(9):2481–2498. doi:10.1105/tpc.104.022699tpc.104.022699 [pii]
18. De Sutter V, Vanderhaeghen R, Tilleman S, Lammertyn F, Vanhoutte I, Karimi M, Inze D, Goossens A, Hilson P (2005) Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J 44(6):1065–1076. doi:10.1111/j.1365-313X.2005.02586.x, TPJ2586 [pii]
19. Vanden Bossche R, Demedts B, Vanderhaeghen R, Goossens A (2013) Transient expression assays in tobacco protoplasts. Methods Mol Biol 1011:227–239. doi:10.1007/978-1-62703-414-2_18
20. Kwiatkowska A, Zebrowski J, Oklejewicz B, Czarnik J, Halibart-Puzio J, Wnuk M (2014) The age-dependent epigenetic and physiological changes in an *Arabidopsis* T87 cell suspension culture during long-term cultivation. Biochem Biophys Res Commun 447(2):285–291. doi:10.1016/j.bbrc.2014.03.141, S0006-291X(14)00601-9 [pii]
21. Li JF, Chung HS, Niu Y, Bush J, McCormack M, Sheen J (2013) Comprehensive protein-based artificial microRNA screens for effective gene silencing in plants. Plant Cell 25(5):1507–1522. doi:10.1105/tpc.113.112235, tpc.113.112235 [pii]
22. Fujikawa Y, Kato N (2007) Split luciferase complementation assay to study protein-protein interactions in *Arabidopsis* protoplasts. PlantJ52(1):185–195.doi:10.1111/j.1365-313X.2007.03214.x, TPJ3214 [pii]
23. Li JF, Bush J, Xiong Y, Li L, McCormack M (2011) Large-scale protein-protein interaction analysis in *Arabidopsis* mesophyll protoplasts by split firefly luciferase complementation. PLoS One 6(11), e27364. doi:10.1371/journal.pone.0027364PONE-D-11-13286 [pii]
24. Ehlert A, Weltmeier F, Wang X, Mayer CS, Smeekens S, Vicente-Carbajosa J, Droge-Laser W (2006) Two-hybrid protein-protein interaction analysis in *Arabidopsis* protoplasts: establishment of a heterodimerization map of group C and group S bZIP transcription factors. Plant J 46(5):890–900. doi:10.1111/j.1365-313X.2006.02731.x, TPJ2731 [pii]
25. Sheen J (2001) Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. Plant Physiol 127(4):1466–1475
26. Boudsocq M, Willmann MR, McCormack M, Lee H, Shan L, He P, Bush J, Cheng SH, Sheen J (2010) Differential innate immune signalling via Ca(2+) sensor protein kinases. Nature 464(7287):418–422. doi:10.1038/nature08794, nature08794 [pii]
27. Rodrigues A, Adamo M, Crozet P, Margalha L, Confraria A, Martinho C, Elias A, Rabissi A, Lumbreras V, Gonzalez-Guzman M, Antoni R, Rodriguez PL, Baena-Gonzalez E (2013) ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in *Arabidopsis*. Plant Cell 25(10):3871–3884. doi:10.1105/tpc.113.114066, tpc.113.114066 [pii]
28. Wehner N, Hartmann L, Ehlert A, Bottner S, Onate-Sanchez L, Droge-Laser W (2011) High-throughput protoplast transactivation (PTA) system for the analysis of *Arabidopsis* transcription factor function. Plant J 68(3):560–569. doi:10.1111/j.1365-313X.2011.04704.x
29. Wu S, Lu D, Kabbage M, Wei HL, Swingle B, Records AR, Dickman M, He P, Shan L (2011) Bacterial effector HopF2 suppresses *Arabidopsis* innate immunity at the plasma membrane. Mol Plant Microbe Interact 24(5):585–593. doi:10.1094/MPMI-07-10-0150
30. Baena-Gonzalez E, Rolland F, Thevelein JM, Sheen J (2007) A central integrator of transcription networks in plant stress and energy signalling. Nature 448(7156):938–942
31. Remy E, Cabrito TR, Batista RA, Hussein MA, Teixeira MC, Athanasiadis A, Sa-Correia I, Duque P (2014) Intron Retention in the 5′UTR of the Novel ZIF2 Transporter Enhances Translation to Promote Zinc Tolerance in *Arabidopsis*. PLoS Genet 10(5), e1004375. doi:10.1371/journal.pgen.1004375PGENETICS-D-13-02254 [pii]
32. Martinho C, Confraria A, Elias A, Crozet P, Rubio-Somoza I, Weigel D, Baena-Gonzalez E (2015) Dissection of miRNA pathways using *Arabidopsis* mesophyll protoplasts. Mol Plant 8(2):261–275
33. Babu M, Griffiths JS, Huang TS, Wang A (2008) Altered gene expression changes in *Arabidopsis* leaf tissues and protoplasts in response to Plum pox virus infection. BMC Genomics 9:325. doi:10.1186/1471-2164-9-325, 1471-2164-9-325 [pii]
34. Leonhardt N, Kwak JM, Robert N, Waner D, Leonhardt G, Schroeder JI (2004) Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell 16(3):596–615. doi:10.1105/tpc.019000tpc.019000 [pii]

35. Chang YM, Liu WY, Shih AC, Shen MN, Lu CH, Lu MY, Yang HW, Wang TY, Chen SC, Chen SM, Li WH, Ku MS (2012) Characterizing regulatory and functional differentiation between maize mesophyll and bundle sheath cells by transcriptomic analysis. Plant Physiol 160(1):165–177. doi:10.1104/pp.112.203810, 112.203810 [pii]
36. Obulareddy N, Panchal S, Melotto M (2013) Guard cell purification and RNA isolation suitable for high-throughput transcriptional analysis of cell-type responses to biotic stresses. Mol Plant Microbe Interact 26(8):844–849. doi:10.1094/MPMI-03-13-0081-TA
37. Bargmann BO, Birnbaum KD (2010) Fluorescence activated cell sorting of plant protoplasts. J Vis Exp 36: doi:1673 [pii] 10.3791/1673
38. Dinneny JR, Long TA, Wang JY, Jung JW, Mace D, Pointer S, Barron C, Brady SM, Schiefelbein J, Benfey PN (2008) Cell identity mediates the response of *Arabidopsis* roots to abiotic stress. Science 320(5878):942–945. doi:10.1126/science.1153795, 1153795 [pii]
39. Galbraith DW, Janda J, Lambert GM (2011) Multiparametric analysis, sorting, and transcriptional profiling of plant protoplasts and nuclei according to cell type. Methods Mol Biol699:407–429.doi:10.1007/978-1-61737-950-5_20
40. Gifford ML, Dean A, Gutierrez RA, Coruzzi GM, Birnbaum KD (2008) Cell-specific nitrogen responses mediate developmental plasticity. Proc Natl Acad Sci U S A 105(2):803–808. doi:10.1073/pnas.0709559105, 0709559105 [pii]
41. Yadav RK, Girke T, Pasala S, Xie M, Reddy GV (2009) Gene expression map of the *Arabidopsis* shoot apical meristem stem cell niche. Proc Natl Acad Sci U S A 106(12):4941–4946. doi:10.1073/pnas.0900843106, 0900843106 [pii]
42. Yadav RK, Tavakkoli M, Xie M, Girke T, Reddy GV (2014) A high-resolution gene expression map of the *Arabidopsis* shoot meristem stem cell niche. Development 141(13):2735–2744. doi:10.1242/dev.106104, 141/13/2735 [pii]
43. Kim J, Somers DE (2010) Rapid assessment of gene function in the circadian clock using artificial microRNA in *Arabidopsis* mesophyll protoplasts. Plant Physiol 154(2):611–621. doi:10.1104/pp.110.162271, pp.110.162271 [pii]
44. Kovtun Y, Chiu WL, Zeng W, Sheen J (1998) Suppression of auxin signal transduction by a MAPK cascade in higher plants. Nature 395(6703):716–720. doi:10.1038/27240
45. Yanagisawa S, Yoo SD, Sheen J (2003) Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature 425(6957):521–525. doi:10.1038/nature01984, nature01984 [pii]
46. Sheen J (1996) Ca2+-dependent protein kinases and stress signal transduction in plants. Science 274(5294):1900–1902
47. Confraria A, Martinho C, Elias A, Rubio-Somoza I, Baena-Gonzalez E (2013) miRNAs mediate SnRK1-dependent energy signaling in *Arabidopsis*. Front. Plant Sci 4:197. doi:10.3389/fpls.2013.00197
48. Kovtun Y, Chiu WL, Tena G, Sheen J (2000) Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc Natl Acad Sci U S A 97(6): 2940–2945
49. Fujii H, Chinnusamy V, Rodrigues A, Rubio S, Antoni R, Park SY, Cutler SR, Sheen J, Rodriguez PL, Zhu JK (2009) In vitro reconstitution of an abscisic acid signalling pathway. Nature 462(7273):660–664. doi:10.1038/nature08599, nature08599 [pii]
50. Xiang L, Le Roy K, Bolouri-Moghaddam MR, Vanhaecke M, Lammens W, Rolland F, Van den Ende W (2011) Exploring the neutral invertase-oxidative stress defence connection in *Arabidopsis thaliana*. J Exp Bot 62(11):3849–3862. doi:10.1093/jxb/err069, err069 [pii]
51. Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature 415(6875):977–983
52. Wang Y, Li J, Hou S, Wang X, Li Y, Ren D, Chen S, Tang X, Zhou JM (2010) A Pseudomonas syringae ADP-ribosyltransferase inhibits *Arabidopsis* mitogen-activated protein kinase kinases. Plant Cell 22(6):2033–2044. doi:10.1105/tpc.110.075697, tpc.110.075697 [pii]
53. Liu T, Liu Z, Song C, Hu Y, Han Z, She J, Fan F, Wang J, Jin C, Chang J, Zhou JM, Chai J (2012) Chitin-induced dimerization activates a plant immune receptor. Science 336(6085):1160–1164. doi:10.1126/science.1218867, 336/6085/1160 [pii]
54. Li M, Berendzen KW, Schoffl F (2010) Promoter specificity and interactions between early and late *Arabidopsis* heat shock factors. Plant Mol Biol 73(4-5):559–567. doi:10.1007/s11103-010-9643-2
55. Niu Y, Sheen J (2012) Transient expression assays for quantifying signaling output. Methods Mol Biol 876:195–206. doi:10.1007/978-1-61779-809-2_16

56. Xiang C, Han P, Lutziger I, Wang K, Oliver DJ (1999) A mini binary vector series for plant transformation. Plant Mol Biol 40(4): 711–717
57. Bush J, Jander G, Ausubel FM (2006) Prevention and control of pests and diseases. Methods Mol Biol 323:13–25. doi:10.1385/1-59745-003-0:13
58. Wu FH, Shen SC, Lee LY, Lee SH, Chan MT, Lin CS (2009) Tape-*Arabidopsis* Sandwich—a simpler *Arabidopsis* protoplast isolation method. Plant Methods 5:16. doi:10.1186/1746-4811-5-16, 1746-4811-5-16 [pii]
59. Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell 2(10):1027–1038. doi:10.1105/tpc.2.10.10272/10/1027 [pii]
60. Sheen J (1991) Molecular mechanisms underlying the differential expression of maize pyruvate, orthophosphate dikinase genes. Plant Cell 3(3):225–245. doi:10.1105/tpc.3.3.225, 3/3/225 [pii]
61. Shen J, Fu J, Ma J, Wang X, Gao C, Zhuang C, Wan J, Jiang L (2014) Isolation, culture, and transient transformation of plant protoplasts. Curr Protoc Cell Biol 63:2.8.1–2.8.17. doi:10.1002/0471143030.cb0208s63
62. Teige M, Scheikl E, Eulgem T, Doczi R, Ichimura K, Shinozaki K, Dangl JL, Hirt H (2004) The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. Mol Cell 15(1):141–152. doi:10.1016/j.molcel.2004.06.023S1097276504003491 [pii]
63. Boudsocq M, Barbier-Brygoo H, Lauriere C (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in *Arabidopsis thaliana*. J Biol Chem 279(40):41758–41766. doi:10.1074/jbc.M405259200M405259200 [pii]

Chapter 21

Construction of Artificial miRNAs to Prevent Drought Stress in *Solanum tuberosum*

Anna Wyrzykowska*, Marcin Pieczynski*, and Zofia Szweykowska-Kulinska

Abstract

The use of artificial microRNAs (amiRNAs) is still a relatively new technique in molecular biology with a wide range of applications in life sciences. Here, we describe the silencing of the *CBP80/ABH1* gene in *Solanum tuberosum* with the use of amiRNA. The CBP80/ABH1 protein is part of the Cap Binding Complex (CBC), which is involved in plant responses to drought stress conditions. Transformed plants with a decreased level of CBP80/ABH1 display increased tolerance to water shortage conditions. We describe how to design amiRNA with the Web MicroRNA Designer platform in detail. Additionally, we explain how to perform all steps of a procedure aiming to obtain transgenic potato plants with the use of designed amiRNA, through callus tissue regeneration and *Agrobacterium tumefaciens* strain LBA4404 as a transgene carrier.

Key words Artificial microRNA (amiRNA), *Solanum tuberosum*, Drought stress, *CBP80/ABH1*, Transformation, *Agrobacterium tumefaciens*

1 Introduction

The discovery that miRNAs regulate gene expression in plants and animals along with subsequent functional studies has had an enormous impact on our understanding of the relation between the function and expression of different genes. Mature microRNAs are usually single stranded 21-nucleotide long RNA molecules. They are the products of microRNA genes whose expression is driven by RNA polymerase II [1]. In most cases, plant microRNA genes are independent transcription units that contain no open reading frame. MicroRNA gene transcripts, also called pri-miRNAs, have a cap structure at their 5′ end and are polyadenylated at the 3′ end [2]. The transcripts are able to form hairpin structures. During

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microRNA biogenesis, the pri-miRNA is cleaved by the DCL1 (Dicer Like-1) protein that produces a shortened precursor referred to as the pre-miRNA [3]. In the next stage, a duplex of microRNA/microRNA* is diced out wherein the RNA molecules contain phosphates at their 5′ ends and two unpaired nucleotides at the 3′ end, which terminates with a free 3′ hydroxyl group [3].

Since the discovery of microRNA molecules studies have focused mainly on their biogenesis and function. Numerous proteins which participate in this process in *Arabidopsis* have been discovered and described, including DNA-dependent RNA polymerase II [1], DCL1 (Dicer Like-1) [3], DCL3 (Dicer Like-3) [4], HYL1 (Hyponastic Leaves 1) [5], SE (Serrate) [6], DDL (Dawdle) [7], CBP20 (Cap Binding Complex 20) [8], CBP80/ABH1 (Cap Binding Complex 80/ABA Hypersensitive 1) [8], HASTY [9] and HEN1 (Hua Enhancer 1) [10], SDN1 (Small RNA Degrading Nuclease 1) [11], AGO1 (Argonaute 1) [12], NOT2 (Mediator and At-Negative on TATA less 2) [13], and DRB4 (Double-stranded RNA binding protein 4) [14, 15]. Recent studies also identified several new components involved in miRNA biogenesis. They are EMU (Erecta mRNA Under-expressed) [16], FCA [17], TGH (TOUGH) [18], STA1 (STABILIZED1) [19], SIC (SICKLE) [20], and MOS2 [21], RACK1 (RECEPTOR FOR ACTIVATED C KINASE 1) [22]. However, their function in miRNA biogenesis is still not well understood.

MicroRNA molecules are involved in the regulation of gene expression at the posttranscriptional level. Mature microRNA molecules are incorporated into the RISC (RNA-induced silencing complex) complex and this complex then binds to the target mRNA according to base-pairing complementarity between the microRNA and the target mRNA [23]. Transcript cleavage is catalyzed by the AGO1 protein, which is a principal component of the RISC complex [12]. Cleavage of the target mRNA leads to its degradation and reduces the level of protein encoded by the target gene. Although the primary mechanism of gene expression regulation by microRNAs is the slicing of the target mRNA, it should be mentioned that there are numerous examples where the microRNA molecules block the translation process [24]. In this way, microRNAs also contribute to reduce the level of target gene protein. Up to now, there are 427 microRNA molecules described in *A. thaliana* (www.mirbase.org) [25].

After describing and understanding the mechanisms of gene expression regulation by microRNAs, the next stage of research involves using microRNAs to modify the expression level of a gene of interest [26]. For this purpose artificial microRNA (amiRNA) genes are designed and introduced into plants, which are able to generate non-natural mature microRNA molecules. A very important parameter in designing artificial microRNA is the energy of hybridization between the amiRNA and its targeted mRNA. One should also consider the possibility of accidentally disturbing the

expression of other genes that results from artificial microRNA production. Like natural miRNAs, artificial miRNAs can regulate the expression of more than one mRNA. Therefore, when designing each amiRNA the possibility of silencing the expression of other genes has to be excluded. Additionally, it should be noted that the process from artificial microRNA design to confirming its functionality usually takes a long time and, depending on the studied plant, can take half a year or more.

Plant transformations are traditionally carried out using *Agrobacterium tumefaciens*. This bacterium has the natural ability to introduce its DNA fragment (the T-DNA) into the genome of the host plants [27, 28]. Transformations of *A. thaliana* are usually carried out by the floral dip method with the use of the *A. tumefaciens* AGl1 strain [29]. During the transformation procedure only closed inflorescences are subjected to the bacterial suspension to enable transgene introduction into the plant cell genome during the meiotic divisions. Transformation efficiency varies in the range of 1–3 %, as counted by the number of transgenic plants compared to the number of seeds obtained from the transformed plants.

Due to the very low efficiency of self-pollination of potato, its genetic transformation via the floral dip method would be extremely difficult. Instead, cells within leaves and internodes from plants grown in vitro are usually transformed. Plant fragments, wounded with a scalpel in order to induce mitotic divisions, are exposed to the bacteria. In the case of potato, the LBA4404 strain of *A. tumefacien* is usually used [30]. The regeneration of whole plants from many callus tissues grown from transformed plant cells then takes place. Depending on the potato varieties, this transformation process has different efficiency and duration. For example, the transformation of the Desiree variety takes 3–4 months and its efficiency could be over 40 % if counted by the number of independent transgenic plants obtained compared to the number of explants used.

2 Materials

2.1 Bacteria and Plasmids

1. *Escherichia coli* strain DH5α: *supE44 Δlac169(Φ80lacZΔM15) hsdR17 recA endA1 gyrA96 thi-1 recA1*.
2. *Agrobacterium tumefaciensstrain* LBA4404: rif^R pAL4404 with disarmed Ti pAL4404 plasmid and rifampicin resistance (to a concentration of 100 μg/ml).
3. *pGEM-T Easy* plasmid (Promega).
4. *pART27* plasmid [31].
5. *pHannibal* plasmid [32].
6. *pRS300* plasmid [33].

2.2 Plant Material

1. *Solanum tuberosum*, variety Desiree

2.3 Media for In Vitro S. tuberosum Culture

1. ½ MS (Murashige–Skoog) medium: 2.2 g/L MS medium concentrate (Duchefa Biochemie B V, Netherlands), 15 g/L sucrose, 8 g/L agar, supplemented or not with 0.05 mg/mL kanamycin. Dissolve MS medium concentrate and sucrose in distilled water, adjust pH to 5.5–5.6 with 0.1 M KOH, add agar, and autoclave at 121 °C for 20 min. For selection of transgenic lines, add appropriate amount of a stock solution of kanamycin (sterilized with the use of 0.22-μm pore diameter filters) after cooling medium to around 50 °C.
2. MCI medium: 2.2 g/L MS medium concentrate (Duchefa Biochemie B V, Netherlands), 16 g/L glucose, 8 g/L agar, 5 mg/L α-naphthaleneacetic acid (NAA), 0.1 mg/L 6-benzylaminopurine (BAP), 0.05 mg/mL kanamycin, 0.5 mg/mL biotaksym (Bioton, Poland). Dissolve MS medium concentrate and glucose in distilled water, adjust pH to 5.5–5.6 with 0.1 M KOH, add agar, and autoclave at 121 °C for 20 min. Add appropriate amount of stock solutions of plant hormones and antibiotics (sterilized with the use of 0.22-μm pore diameter filters) only after cooling medium down to around 50 °C.
3. GR_2 medium containing auxins: 2.2 g/L MS medium concentrate (Duchefa Biochemie B V, Netherlands), 16 g/L glucose, 20 μg/L gibberellic acid (GA_3), 20 μg/L α-naphthaleneacetic acid (NAA), 2 mg/L zeatin riboside, 0.05 mg/mL kanamycin, 0.5 mg/mL biotaksym (Bioton, Poland). Dissolve MS medium concentrate and glucose in distilled water, adjust pH to 5.5–5.6 with 0.1 M KOH, add agar, and autoclave at 121 °C for 20 min. Add appropriate amount of stock solutions of plant hormones (dissolved in ethanol in the case of GA_3) and antibiotics (sterilized with the use of 0.22-μm pore diameter filters) only after cooling medium down to around 50 °C.

2.4 Bacterial Culture Media

1. Liquid LB (Luria–Bertani) medium: 10 g/L Bacto tryptone, 5 g/L yeast extract, 10 g/L NaCl. Autoclave at 121 °C for 20 min.
2. Solid LB (Luria–Bertani) medium: Add 15 g of agar in 1000 mL of liquid LB medium and autoclave at 121 °C for 20 min. After cooling down to around 50 °C, add selected antibiotics and pour medium into petri dishes under sterile conditions.

2.5 Antibiotics Used during Culture of Different Bacterial Species

See Table 1

2.6 Enzymes

1. *Taq* DNA Polymerase [10 U/μL] (Fermentas).
2. *Pfu* DNA Polymerase [2.5 U/μL] (Fermentas).

Table 1
Antibiotics used during culture of different bacterial species

Antibiotic	Concentration in medium [mg/mL]	Bacteria
Ampicillin	0.05	*E. coli*
Biotaksym/Cefotaxime sodium	0.5	*A. tumefaciens*
Kanamycin	0.05	*E. coli*, *A. tumefaciens*
Rifampicin	0.1	*A. tumefaciens*

3. SuperScript III Reverse transcriptase [200 U/μL] (Invitrogen).
4. Turbo DNase [2 U/μL] (Applied Biosystems).
5. T4 DNA Ligase [5 U/μL] (Fermentas).
6. CIAP alkaline phosphatase [1 U/μL] (Fermentas).
7. *BamH*I [10 U/μL] (Fermentas).
8. *Not*I [10 U/μL] (Fermentas).
9. *Xho*I [10 U/μL] (Fermentas).

2.7 Software

1. ContigExpress program from the Vector NTI software suite (Promega).

2.8 Buffers and Solutions

1. 2× HSE Loading Buffer (for DNA): 4 M urea, 50 % sucrose, 50 mM EDTA, 0.1 % bromophenol blue, 0.1 % xylene cyanol.
2. Loading Buffer for RNA: 10 mM Tris–HCl pH 7.4, 95 % formamide, 5 mM EDTA, 0.1 % bromophenol blue, 0.1 % xylene cyanol.
3. 10× TBE Buffer: 10 mM Tris–HCl pH 8.0, 95 % boric acid, 20 mM EDTA.
4. 1 % agarose gel: Dissolve 1 g of agarose in 100 mL of 1× TBE Buffer by heating in a microwave. After cooling down to about 50 °C, add 5 μL of 10 mg/L ethidium bromide per 100 mL of gel.
5. RNase-free water (H_2O_{DEPC}): 0.1 % DEPC. Autoclave twice at 121 °C for min and shake for 12–16 h at 37 °C before use.

2.9 Oligonucleotides

See Tables 2, 3, 4, 5, and 6.

3 Methods

3.1 Obtaining the cDNA Sequence of the CBP80/ABH1 Gene

Potato is a tetraploid plant and therefore carries four slightly different nucleotide sequence alleles of the *CBP80/ABH1* gene. Thus, when planning to design a single artificial microRNA molecule capable of down-regulating the expression of all alleles of the

Table 2
Primers used for preparation of expression constructs generating artificial microRNAs

Primer 1	5′-CTGCAAGGCGATTAAGTTGGGTAAC-3′
Primer 6	5′-GCGGATAACAATTTCACACAGGAAACAG-3′
Primer 2 -amiRNA80.1	5′-GATAACGGTACAGGCAGGCCGACTCTCTCTTTTGTATTCC-3′
Primer 3-amiRNA80.1	5′-GAGTCGGCCTGCCTGTACCGTTATCAAAGAGAATCAATGA-3′
Primer 4 -amiRNA80.1	5′-GAGCCGGCCTGCCTCTACCGTTATCACAGGTCGTGATATG-3′
Primer 5 -amiRNA80.1	5′-GATAACGGTAGAGGCAGGCCGGCTCTACATATATATTCCT-3′
Primer 2 -amiRNA80.2	5′-GATAACGGTACAGGCAGCCGGACTCTCTCTTTTGTATTCC-3′
Primer 3 -amiRNA80.2	5′-GAGTCCGGCTGCCTGTACCGTTATCAAAGAGAATCAATGA-3′
Primer 4 -amiRNA80.2	5′-GAGTACGGCTGCCTGAACCGTTTTCACAGGTCGTGATATG-3′
Primer 5 -amiRNA80.2	5′-GAAAACGGTTCAGGCAGCCGTACTCTACATATATATTCCT-3′

Table 3
Primers used for cDNA amplification and sequencing of the *CBP80/ABH1* gene

F80ST	5′-GGAATGAGTAGTTGGCGG-3′
R80ST	5′-TAAATCATTCCTCCAGAGGTC-3′
cbp80-1R	5′-CAGTGAAAGAAACAAAGCCTC-3′
cbp80-1 F	5′-GTGCCTTCCTTGGGGTGGTGC-3′
cbp80-2 F	5′-CTGCCTGTACCGTTCAGATATG-3′
cbp80-2R	5′-GGGCAGTTTTCCTACCTTTCAC-3′

Table 4
Primers used for preparation of probes for northern blotting analysis

Probe-80.1	5′-GTCGGCCTGCCTGTACCGTTA-3′
Probe-80.2	5′-GTCCGGCTGCCTGTACCGTTA-3′

Table 5
Primers used for cDNA amplification of the *CBP80/ABH1* and cyclophilin genes by qPCR

Fcbp80	5′-TCCTTCAAATAAAACTGAGGATC-3′
Rcbp80	5′-CCTGGCAGAGCCTTGC-3′
F_cyclofilin	5′-CTCTTCGCCGATACCACTCC-3′
R_cyclofilin	5′-TCACACGGTGGAAGGTTGAG-3′

Table 6
Primers used for amplification of the potato actin gene cDNA fragment (accession number: ABB55389)

FACT1ST	5′-GGAAACATTGTGCTCAGTGGTGG-3′
RACT3ST	5′-CTCTGCCTTTGCAATCCACATC-3′

CBP80/ABH1 gene, one must first know their mRNA sequences. The next step is to identify regions of the cDNA with identical sequence, which might be a target for artificial microRNA molecules.

The *CBP80/ABH1* cDNA from the potato variety Desiree was amplified using primers F80ST and R80ST (Fig. 1). PCR was performed using the following thermal profile: 5 min of 95 °C; 30 cycles of 30 s 95 °C, 30 s 60 °C, 120 s 72 °C; 5 min 72 °C using *Pfu* DNA polymerase and primers listed in Table 3. cDNA prepared from total RNA using SuperScript III reverse transcriptase was used as a template, and the reaction products cloned into the pGEM T-Easy vector. In order to identify the sequence of mRNAs derived from four alleles of the *CBP80/ABH1* gene, at least 12 independent cDNA clones should be sequenced (*see* **Note 1** and Table 3). The sequences of the individual cDNA clones were assembled together using a ContigExpress program of the Vector NTI software suite. It should be noted that the same type of analysis could be performed using commercially or freely available programs such as CAP3 [34]. The complete sequence of the *CBP80/ABH1* cDNA clones were aligned with one another using the Clustal W2 program (currently available upgraded version of ClustalW2 is Clustal Omega—www.ebi.ac.uk/Tools/msa/clustalo/) [35].

Based on the prepared cDNA sequence alignment, we were able to identify mRNA of all four alleles of the *CBP80/ABH1* gene. In one of the alleles we found two deletions (83 bp and 200 bp) and one insertion (4 bp). These identified mutations give rise to the appearance of a premature stop codon. Furthermore, all identified alleles differ only by a single nucleotide change [36]. Selected cDNA sequence fragments of the *CBP80/ABH1* gene, identical in all four alleles, were then used as a target sequence for the artificial microRNA designing.

3.2 Design of Artificial microRNA Molecules

In order to design artificial microRNA molecules (Table 2), we used the platform Web MicroRNA Designer 2 (WMD2) [26, 33]. Currently, there is an available upgraded version of the site, WMD3 (www.wmd3.weigelworld.org) (*see* **Note 2**). Selected fragments of the cDNA sequences of the *CBP80/ABH1* gene in fasta format should be introduced into the "target gene" window in the "Designer" bookmark. Additionally, one should fill in the remain-

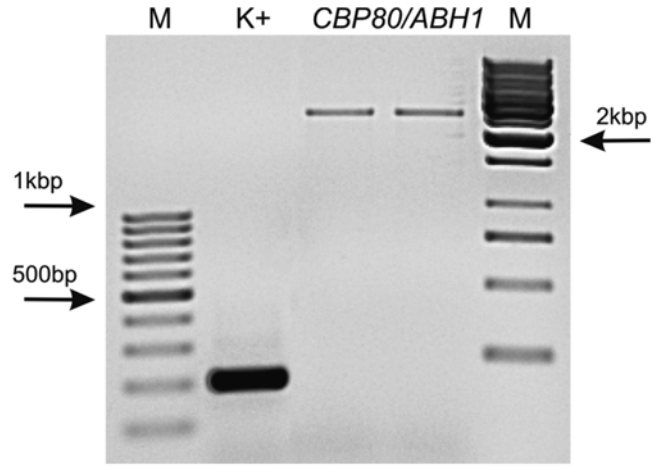


Fig. 1 Electrophoresis in a 1 % agarose gel of PCR products derived from *CBP80/ABH1* cDNA amplification from Desire plants. As a control (K+) the part of cDNA of Actin gene was amplified (Table 6). M—molecular mass marker. Reproduced from Pieczynski Marcin's PhD thesis (on-line access: www.repozytorium.amu.edu.pl)

ing windows: "Genome"—select the genome release you are interested in (in this case *S. tuberosum*), "Minimum number of included targets "—define the minimum number of target sequences for which the artificial microRNA will be designed, "accepted off-targets"—define the acceptable mRNAs derived from the genome of interest, which may be affected by designed artificial microRNA, "Description"—define a project name for the artificial microRNAs designing. Finally, enter the email address to which the results with the characteristics of the designed artificial microRNA molecules should be sent. This email from WMD3 will contain a list of designed artificial microRNA molecules and their characteristics. For each amiRNA, the hybridization energy assigned to the target mRNA as well as to the fully complementary sequence and a number of identified off-targets will be indicated. In addition, all designed molecules will be listed taking into account additional criteria: the number and location of a mismatch between the amiRNA and mRNA sequence, amiRNA 5′ end complementarity to the target sequence, and the number of mRNAs with 5 or less mismatches when aligned to amiRNA. On the basis of all the previously mentioned parameters, amiRNAs will be listed and colored according to the most favorable parameters (green), the less favorable characteristics (yellow and orange) until the least favorable (red color) of the list. It should be noted that the red color does not indicate that the molecules are not functional, only that they may potentially be less specific.

During the final selection of the molecule, one should take into account all the parameters (*see* **Note 3**). In addition, please note that the introduction of an amiRNA gene into a plant genome

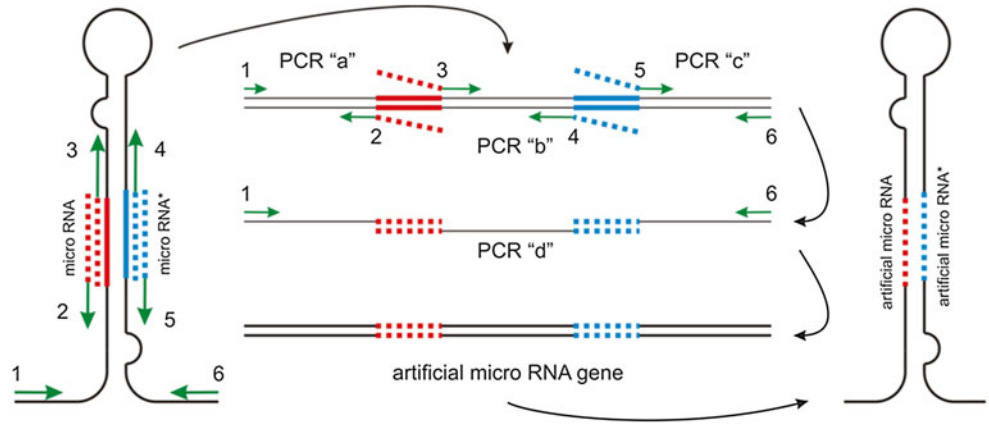
and the subsequent verification of its functionality is a time consuming process. It is therefore a good idea to select more than one amiRNA candidate for further research.

For further research with the potato *CBP80/ABH1* gene, two amiRNA molecules: amiRNA80.1—5′-TAACGGTACAGGCA GGCCGAC-3′ and amiRNA80.2—5′- TAACGGTACAGGCAG CCGGAC-3′, were selected. The selected molecules were characterized by the following parameters: perfect match hybridization energy (energy of target mRNA hybridization to fully complementary sequence; kcal/mol) of −48.71 and −51.44; hybridization energy (energy of target mRNA hybridization to microRNA sequence) of −43.55 and −44.83; and identified off-targets numbers 9 and 6, respectively.

3.3 Construction of the Artificial microRNA Gene

In order to obtain a gene capable of generating artificial microRNAs, the pRS300 plasmid containing the microRNA 319a gene derived from *Arabidopsis thaliana* Col-0 was used and primers were designed based on the selected amiRNA using the WMD2 site, bookmark "Oligo" (*see* **Note 4** and Table 2) [33]. Additionally, the pRS300 plasmid has a multitude of additional restriction sites that are useful when cloning an artificial microRNA gene. For all PCR reactions performed, to obtain an amiRNA gene sequence carrying amiRNA and amiRNA* always use a DNA-dependent DNA polymerase characterized by a low number of errors during the synthesis of the new strand with proofreading activity (*Pfu* DNA polymerase or *Tgo*). Introduction of the amiRNA and amiRNA* sequence into the microRNA 319a gene sequence was carried out in four consecutive PCR reactions (Fig. 2). Reactions "a," "b," and "c" were carried out using plasmid pRS300 as the template and primers 1, 2, 3, 4, 5, and 6. Primers 1 and 6 are universal for this procedure, whereas primers 2, 3, 4 and 5 are unique to a given amiRNA and amiRNA* molecule and were designed using the tab "Oligo" from WMD2 (Fig. 3, *see* **Note 5**).

PCR reactions were performed according to the recommended profile temperature: 2 min 95 °C; 24 cycles of 30 s 95 °C, 30 s 55 °C, 40 s 72 °C; 7 min 72 °C. When carrying out the "b" reaction, the annealing temperature was lowered to 50 °C. It is very important that the products of the "a," "b," and "c" reactions are purified individually by elution from the gel. In this way, the reaction products can be purified from the pRS300 plasmid template, where the presence of contamination makes it practically impossible to obtain the appropriate reaction products. Reaction products of "a," "b," and "c" were used as a template in the "d" reaction, which also uses primers 1 and 6 (Fig. 3). The reaction was performed according to the recommended temperature profile: 2 min 95 °C; 24 cycles of 30 s 95 °C, 30 s 55 °C, 90 s 72 °C; 7 min 72 °C. A product of the "d" reaction was cloned into the pGEM T-Easy vector and sequenced to confirm the accuracy of the artificial microRNA gene sequence.



	Forward primer	Reverse primer	Template
PCR "a"	1	2	pRS300
PCR "b"	3	4	pRS300
PCR "c"	5	6	pRS300
PCR "d"	1	2	products of PCRs "a", "b" and "c"

Fig. 2 Schematic representation of the "step-by-step" preparation of a gene construct containing artificial microRNA. Primers 1–6 were used in subsequent PCR reactions. Scheme based on Schwab and Ossowski [26] and www.wmd3.weigelworld.org

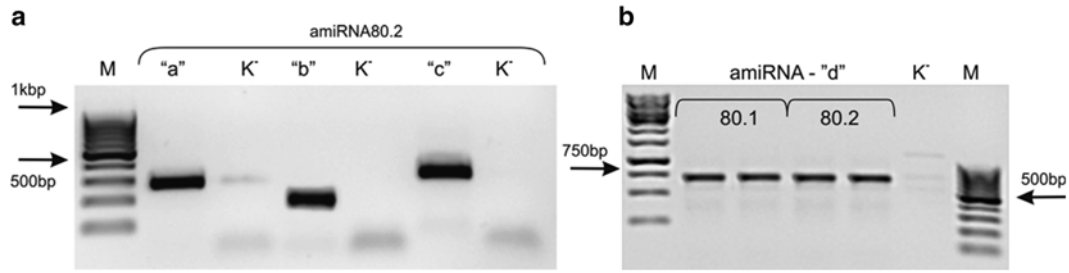


Fig. 3 Electrophoresis of PCR products from subsequent PCR reactions with different primers ("a"–"d"), during which the exchange of microRNA and microRNA* from the MIR319a gene to the artificial microRNA was made. a—reactions "a," "b," and "c", b—reaction "d", K⁻—negative control, M—molecular mass marker. Reproduced from Pieczynski Marcin's PhD thesis (on-line access: www.repozytorium.amu.edu.pl)

3.4 Cloning into the Binary Vector

In further experimental work, the pHannibal and pART27 vectors were used. pHannibal is dedicated to the preparation of an expression construct able to generate RNA hairpin structures [32], whereas pART27 vector is a binary vector used for plant genetic transformation [31]. The "d" reaction products, containing the amiRNA gene sequence, were digested with the *Xho*I and *BamH*I restriction enzymes for cloning into the pHannibal vector. pHannibal was also prepared for cloning by digestion with the above

restriction enzymes (*see* **Note 6**). The restriction enzymes *Xho*I and *BamH*I were chosen to maintain the correct orientation of the amiRNA gene according to the orientation of CaMV promoter and the OCS terminator, which are located in the vector. At the same time, the use of restrictase allowed the removal of the intron sequence from pHannibal vector. The presence of the amiRNA gene in the pHannibal vector was confirmed by restriction digestion using the *Not*I enzyme (*see* **Note 7**). In the next stage the expression cassette, containing the CaMV promoter—amiRNA gene—OCS terminator, was recloned into the pART27 binary vector using the *Not*I restriction enzyme. When cloning the expression cassette into the pART27 vector it is possible to confirm its presence in the vector by an α-complementation assay. The correctness of the expression cassette final sequence was confirmed by sequencing.

While carrying out the described research work, we used *E. coli* DH5α strain cells to obtain a large quantity of recombinant pHannibal and pART27 plasmids at each stage. Bacteria were grown at 37 °C in standard LB medium, liquid or solidified by adding 1.5 % agar. To select *E. coli* cells carrying the pHannibal or pART27 vector, the antibiotics ampicillin (0.05 mg/mL final concentration) and spectinomycin (0.1 mg/mL final concentration), respectively, were used. Transformation of *E. coli* was performed by subjecting the cells to heat shock. Isolation of plasmid DNA from the *E. coli* suspension was carried out using the standard alkaline lysis method [37].

3.5 *A. tumefaciens* and Potato Transformation

3.5.1 Agrobacterium Transformation

1. After 48 h of growth of *A. tumefaciens* in 20 mL of LB medium, centrifuge the culture at 2500 × g at 4 °C for 8 min. Discard the supernatant and resuspend the bacterial pellet in 200 μL of 20 mM $CaCl_2$ (*see* **Note 8**).
2. Separate the cell suspension into two eppendorf tubes with 100 μL per tube.
3. Add 1 μg of plasmid DNA suspended in 40 μL of water to one of the tubes and add 40 μL of water to the second, which will be used as a negative control of transformation.
4. Place the tubes in liquid nitrogen for 8 s until boiling stops and then incubate at 37 °C for 5 min (*see* **Note 9**) [38].
5. To each eppendorf tube containing bacteria add 1 mL of liquid LB medium. Incubate for 4 h at 28 °C with shaking (200 rpm).
6. After incubation, spread bacteria on petri dishes with solid LB medium containing the antibiotics rifampicin (0.1 mg/mL) and spectinomycin (0.1 mg/mL) (*see* **Note 10**). Incubate at 28 °C for 48 h. Sealing of the plates is not necessary.
7. Pick one colony and transfer to a Falcon tube with 10 mL of LB medium containing antibiotics, and incubate 24 h at 28 °C with shaking (100 rpm).

8. Split the culture in two: use 4 mL for plasmid isolation and restriction enzyme analysis to compare band pattern and ensure you are using the clone with the correct insert (*see* **Note 11**), and use 6 mL to prepare glycerol (50 %) stocks (*see* **Note 12**).

3.5.2 Agrobacteria Preparation

1. For plant transformation, *A. tumefaciens* from a glycerol stock or a colony on plate can be used. Transfer bacteria with binary plasmid (in this case pART27) to 5 mL of LB medium with the same antibiotics (rifampicin and spectinomycin, Table 1) and grow overnight on shaker (200 rpm) at 28 °C.
2. Transfer the inoculum to 50 mL of LB medium with antibiotics and grow another 24 h under the same conditions.
3. Centrifuge bacterial suspension at 700–1000 × g for 15 min after transferring to Falcon tube. Discard the supernatant and resuspend bacterial pellet in 15 mL of MS medium.
4. Spread 100 μL from the 15-mL suspension on plates with solid ½ MS medium.

3.5.3 Plant Transformation

1. Place plant leaves from in vitro culture on a fresh empty plate and perform several incisions on each leaf with a scalpel (*see* **Note 13**).
2. Place the incised leaves upside down on plates prepared earlier with solid ½ MS medium and bacteria (*see* **Note 13**). Incubate for 2 days at RT in darkness. Parafilm sealing of the plates is obligatory.
3. Transfer the leaves to plates containing MCI medium with spectinomycin and Biotaksym (0.5 mg/mL) (*see* **Note 10** and Table 1). Incubate plates for 10 days at 21 °C.
4. Transfer explants to plates with GR_2 medium containing auxins and the same antibiotics as in **step 3** (Table 1).
5. Every 14 days transfer explants to freshly prepared plates with GR_2 medium (*see* **Note 14**). Formation of callus tissue occurs after 4–6 weeks (Fig. 4).
6. Carefully excise regenerated shoots (1.5–3 cm) and transfer to glass tubes (*see* **Note 15**) filled to a height of 4–5 cm with solid ½ MS medium containing antibiotic (0.05 mg/mL kanamycin), close with plugs of cotton wool, and seal with Parafilm M.
7. After plant transgenic line selection (Fig. 5), analyze transgene expression levels and its functionality (Figs. 6 and 7).
8. The regenerated shoot from one explant should be considered as an independent transgenic line (*see* **Note 16**).
9. Plant transgenic lines obtained after tuberization in a green house and subject them to drought stress conditions (Fig. 8a). Measurements of relative water content (RWC) should indicate that the transgenic lines show improved drought tolerance (Fig. 8b).

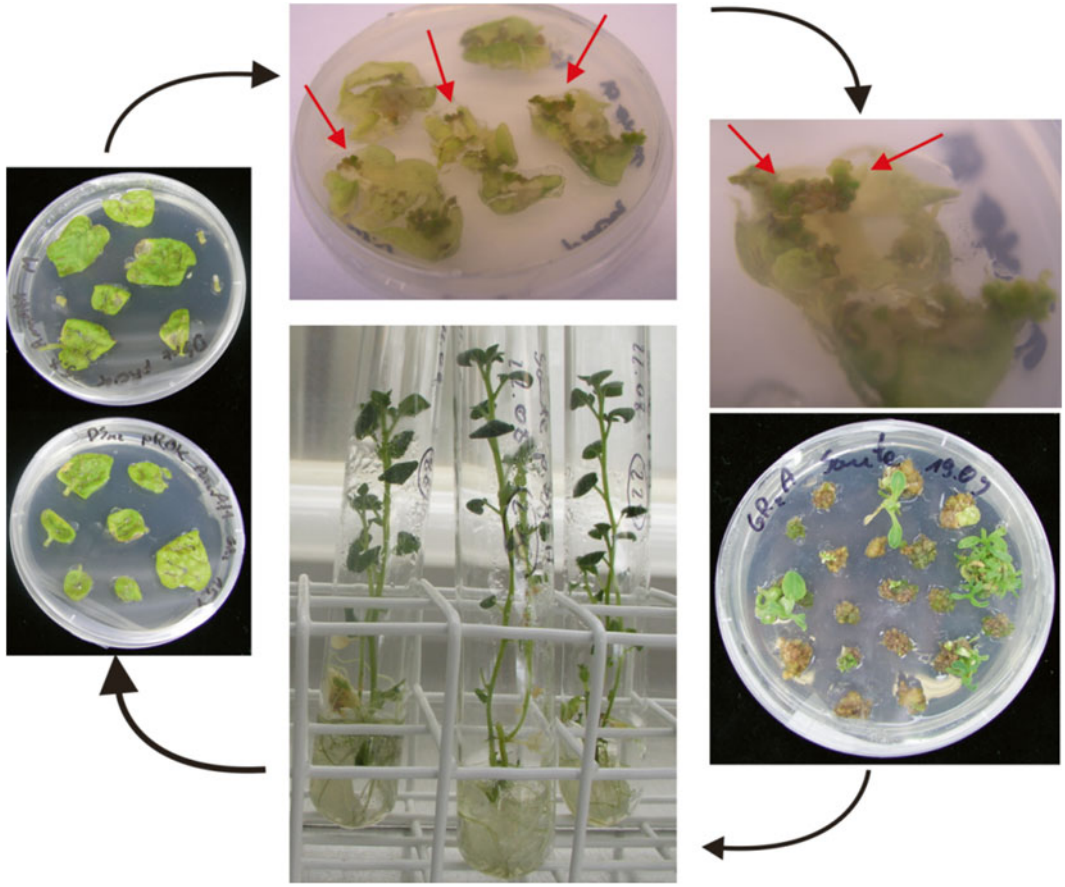


Fig. 4 Schematic representation of in vitro plant transformation. Leaves from micro-plants were transformed with *A. tumefaciens*. Callus tissue was then regenerated and, from the callus tissue, new regenerated shoots. After transferring the shoots to glass tubes, transgenic micro-plants were obtained

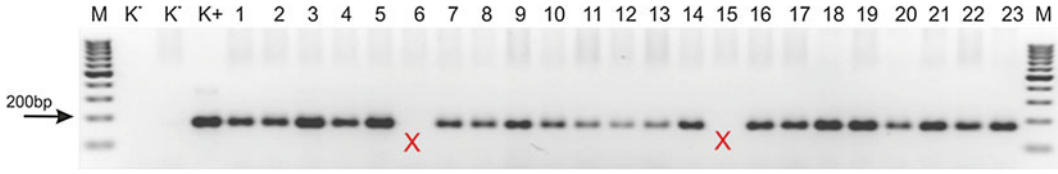


Fig. 5 Electrophoresis in 1 % agarose gel of PCR products of the transgene in transformed plant lines containing the amiRNA80.2 construct (1–23). K^+—positive control of PCR reaction with pART27 containing expression cassette used as a template. Numbers represent subsequent transgenic plant lines. K^-—negative PCR control without template and/or with gDNA from non-transformed plants. M—molecular marker. The lines without the transgene are marked with a *red X*. Reproduced from Pieczynski Marcin's PhD thesis (on-line access: www.repozytorium.amu.edu.pl)

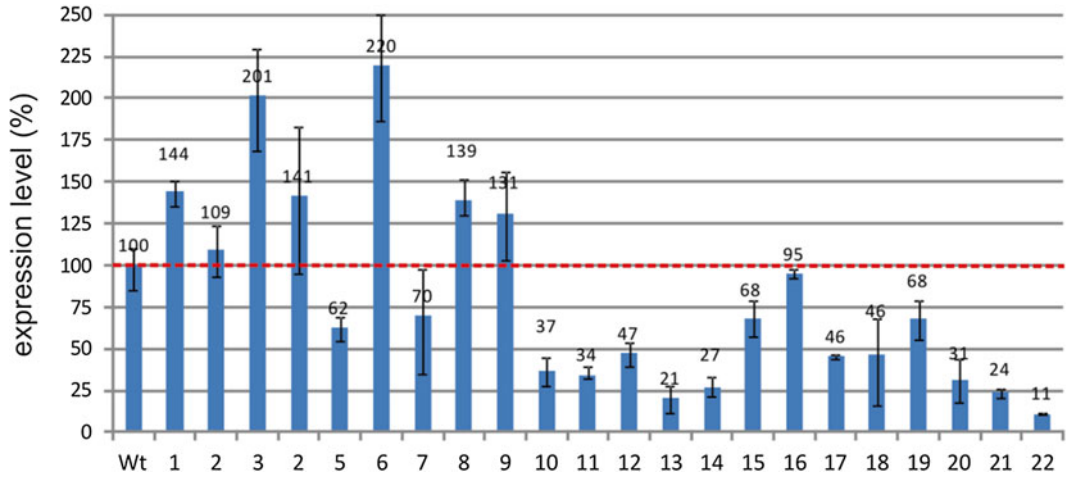


Fig. 6 Expression level of the *CBP80/ABH1* mRNA in the obtained transgenic plant lines (1–22) transformed with the amiRNA80.2 construct. Wt—expression level of the *CBP80/ABH1*mRNA in a non-transformed plant was set at 100 %. Reproduced from Pieczynski Marcin's PhD thesis (on-line access: www.repozytorium.amu.edu.pl)

4 Notes

1. In order to identify the mRNA sequence derived from four different alleles of the gene of interest, a significant number of clones need to be sequenced. Below is the probability of obtaining the mRNA sequence of all four alleles, depending on the number of sequenced clones:
   - Number of sequenced clones (each of the four mRNA alleles represented by at least one clone):
     10 clones sequenced—probability 78 %
     13 clones sequenced—probability 90 %
     16 clones sequenced—probability 96 %
   - Number of sequenced clones (each of the four mRNA alleles represented by at least two clones):
     10 clones sequenced—probability 22 %
     13 clones sequenced—probability 53 %
     20 clones sequenced—probability 90 %
2. In order to design artificial microRNA molecules for potato, Web MicroRNA Designer 2 platform was used for the *CBP80/ABH1* gene silencing. Currently, the WMD2 platform has been replaced by a new version—Web MicroRNA Designer 3

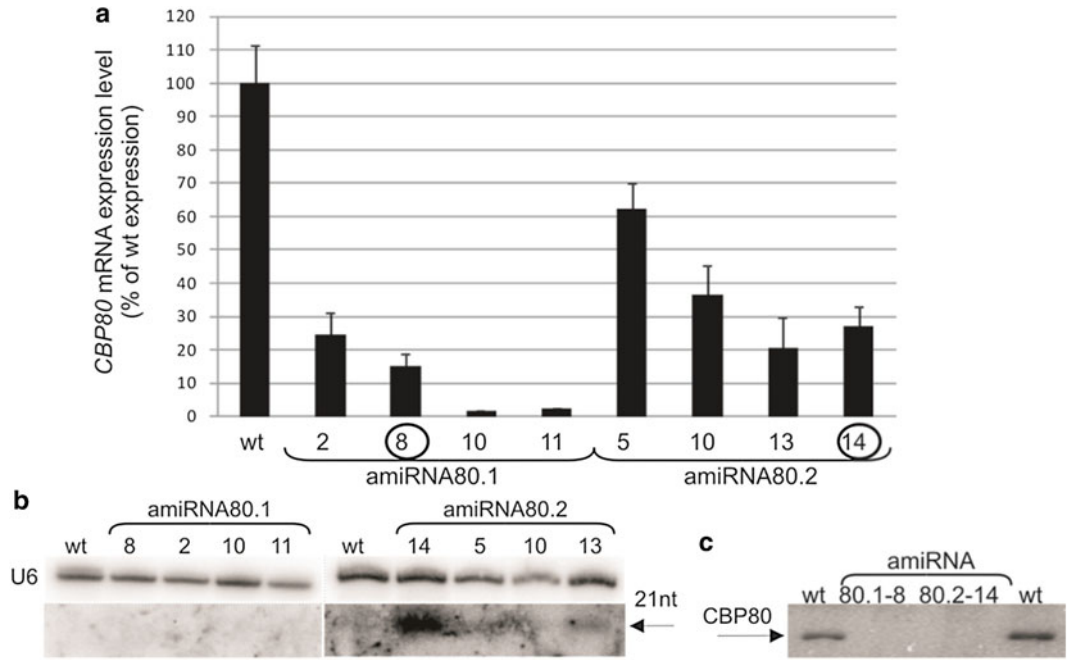


Fig. 7 (**a**) qPCR measurements of *CBP80/ABH1* mRNA levels in selected transgenic lines (calculated as a percentage of *CBP80/ABH1* mRNA expression in non-transformed plants; Table 5). Circles indicate selected transgenic lines that were further analyzed. (**b**) Detection of the artificial miRNAs amiR80.1 and amiR80.2 in the Desiree plants and selected transgenic lines using Northern hybridization (Table 4). (**c**) CBP80/ABH1 protein detection in the non-transformed plants and amiR80.1-8 and amiR80.2-14 transgenic lines using Western blots. Reproduced from Pieczynski et al. with permission from John Wiley and Sons [32]

(http://wmd3.weigelworld.org). When designing artificial microRNA molecules using the present protocol, please note that some information in the text (e.g., sequence of amiRNA molecules and their hybridization to target mRNA parameters) refers only to the use of WMD2 platform.

3. We recommend a thorough analysis of the potential effects of potential off-targets expression disturbances. The target sequence to which an amiRNA is designed may be either conserved and present in a large number of genes or unique. Therefore, there is a possibility that the designed amiRNA will disturb the expression of genes with similar function to the destination target (for example genes belonging to one gene family or genes encoding proteins with similar functional domains). Unfortunately, after the final artificial microRNA molecule selection it is hard to control the level of mRNAs other than the target mRNA. Only after analysis using the obtained transgenic plant lines will the extent of expression of the other mRNAs be determined.

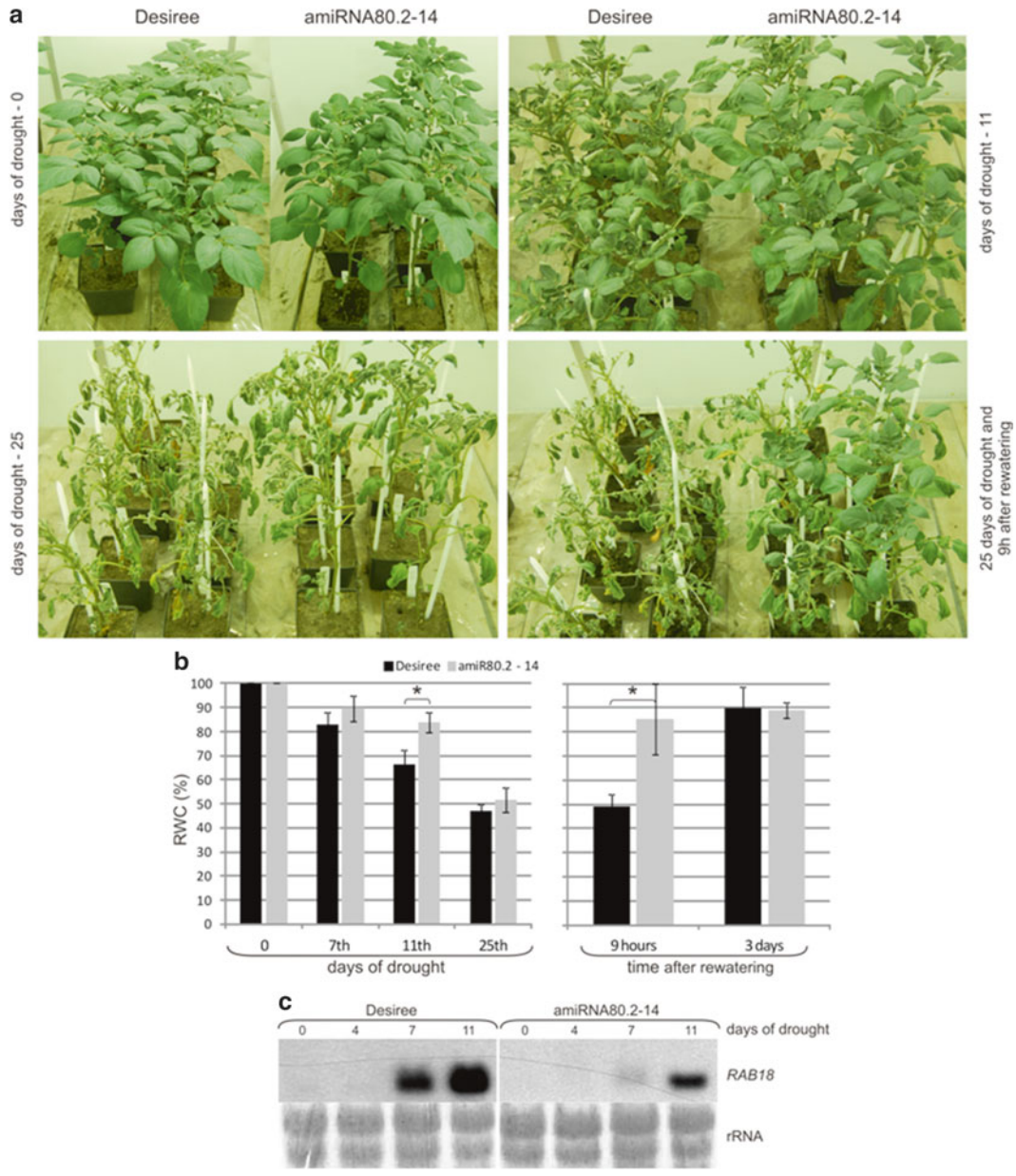


Fig. 8 (**a**) Six-week-old, well-watered plants were subjected to water stress. Desiree and amiRNA80.2-14 plants before the application of water stress (*upper-left panel*) and after 11 and 25 days without watering are shown. Desiree and amiRNA80.2-14 plants 9 h after rewatering (*lower-right panel*) are shown. (**b**) The RWC in leaves from the Desiree and transgenic amiRNA80.2-14 potato plants after drought stress. The RWC was measured at 0, 7, 11, and 25 days after the application of drought stress (*left panel*). The measurements taken at various time points during drought stress are presented as a percentage of the RWC of the plant at day 0. The *right panel* shows the RWC upon restarting irrigation after 9 h and 3 days. The leaves were detached and weighed to determine the initial FW (fresh weight), SW (saturated weight) and DW (dry weight) values. (**c**) Northern blot hybridization of the *Arabidopsis* RAB18 cDNA probes bound to potato RNAs isolated from the Desiree and amiR80.2-14 plants after 0, 4, 7, and 11 days without watering. Reproduced from Pieczynski et al. with permission from John Wiley and Sons [32]

4. The pRS300 plasmid contains the microRNA 319a gene sequence from *A. thaliana*. Note that during the preparation of the artificial microRNA expression construct it is possible to use practically any microRNA gene. However, if a different microRNA gene other than the *A. thaliana* miRNA319a is used, the microRNA* molecule in addition to the 2, 3, 4 and 5 primers sequences will need to be designed. When designing these, the example of primers that could be designed using the "Oligo" tab should be closely followed.
5. The WMD3 platform is used to design an artificial microRNA molecule and primer sequences used for amiRNA introduction into the microRNA 319a gene derived from *A. thaliana*. The miRNA* sequence is designed along with the primers and its sequence is part of the 4 and 5 primers which are used in the "b" and "c" PCR reactions.
6. When cloning a PCR product into the pHannibal vector, it is strongly recommended to dephosphorylate the 5′ ends of the plasmid DNA after restriction digestion and prior to the ligation reaction.
7. The pHannibal vector contains two *NotI* restriction sites that divide the vector into two fragments with lengths of 2966 bp and 2858 bp, respectively. These fragments differ in length only by 108 bp and migrate in the agarose gel electrophoresis practically as a single band. Cloning a DNA fragment of about 700 bp into the pHannibal vector causes the recombinant pHannibal vector after digestion with *NotI* to generate two different length bands in gel electrophoresis analysis.
8. Other methods of transformation such as electroporation or Triparental conjugation [39, 40] can also be performed.
9. Proper protective gloves and glasses should be used when handling liquid nitrogen. After freezing, immediately place samples into a 37 °C incubator.
10. Use of selection antibiotic depends on the type of plasmid you work with. Make sure to decide on the right type. Rifampicin prevents other bacteria from growing in the medium, which might occur due to the long reproducing time of *A. tumefaciens*. Spectinomycin selects Agrobacterium cells containing the pART27 plasmid. Biotaksym belongs to the antibiotic group of cephalosporins and allows the removal of agrobacteria when they are no longer needed after plant transformation.
11. Sometimes rearrangements can occur in the transgene sequence during the transformation. Therefore, it is important before plant transformation to check the sequence for which the analysis of the digestion pattern with restriction enzymes is helpful. Mutations in a transgene nucleotide sequence can inactivate/

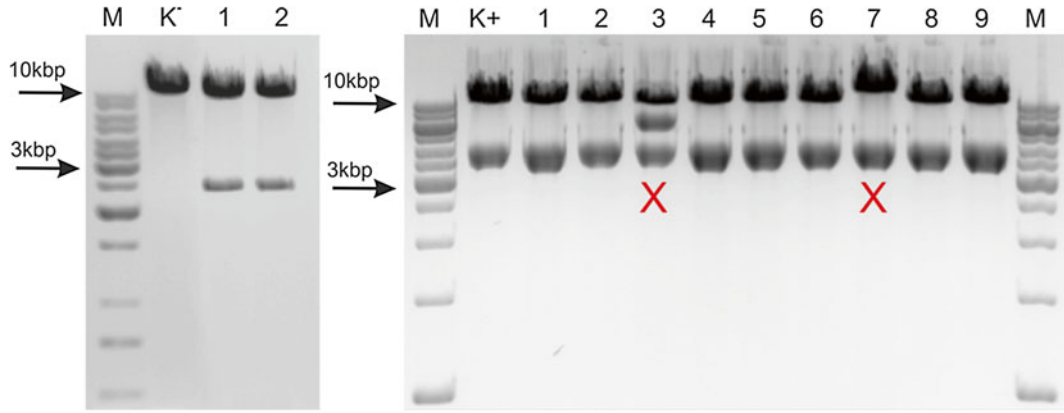


Fig. 9 Electrophoresis in 1 % agarose gel of *Not*I restriction enzyme digestion products. The *left panel* shows the pART27 plasmid with cloned expression cassette isolated from *E. coli* (1–2) and used for transformation of Agrobacterium cells. The *right panel* shows the products of *Not*I digestion from different *A. tumefaciens* colonies (1–9) containing the pART27 plasmid with the cloned expression cassette. Plasmids with sequence rearrangements are marked with a *red X*. K^-—pART27 plasmid without the expression cassette, K^+—pART27 plasmid containing the expression cassette, M—DNA molecular marker. Reproduced from Pieczynski Marcin's PhD thesis (online access: www.repozytorium.amu.edu.pl)

activate restriction sites in the transgene so the differences in length and pattern of bands on the agarose gel can be observed (Fig. 9). The sequence of the transgene may also be checked by classical DNA sequencing. As plasmid isolation from *A. tumefaciens* has a low efficiency, it may be helpful to introduce a binary plasmid, isolated from Agrobacterium, back into *E. coli* for propagation in a larger amount. After overnight growth, plasmids can be isolated from *E. coli* for re-sequencing.

12. Glycerol stocks are the best method for long-term storage of transformed *A. tumefaciens*. Use 300 µL 50 % (v:v) glycerol with 700 µL of culture medium, freeze in liquid nitrogen, and store at −80°C.
13. For plant transformation, leaf excision (10 × 10 mm) or stem internodal (without growing points) fragments (10 mm in length) may be used.
14. It has been reported that growth regulators and antibiotics can degrade in the presence of light, so subculturing every 2 weeks will allow the appropriate level of these components to be maintained.
15. Other vessels can be used for in vitro culture such as Vitro Vent Vessels (Duchefa), vented Magenta GA7 vessels or Suncaps (Sigma). We use customized glass tubes ~15 cm in height and of ~ 2.5 cm diameter.
16. The random integration of the transgene into different sites of the plant genome can alter the expression pattern of the trans-

gene [30]. As a consequence during the field trials the transgenic potato lines can exhibit unspecific phenotypic changes. It is thus very important to maintain a careful cataloging system and to confirm the initial data obtained from microplants with greenhouse or field trials on tuber generated plants.

References

1. Kim YJ, Zheng B, Yu Y et al (2011) The role of Mediator in small and long noncoding RNA production in *Arabidopsis thaliana*. EMBO J 30:814–822
2. Xie Z, Allen E, Fahlgren N et al (2005) Expression of *Arabidopsis* MIRNA Genes. Plant Physiol 138:2145–2154
3. Schauer SE, Jacobsen SE, Meinke DW et al (2002) DICER-LIKE1: blind men and elephants in *Arabidopsis* development. Trends Plant Sci 7:487–491
4. Schmitz RJ, Hong L, Fitzpatrick KE et al (2007) DICER-LIKE 1 and DICER-LIKE 3 redundantly act to promote flowering via repression of FLOWERING LOCUS C in *Arabidopsis thaliana*. Genetics 176:1359–1362
5. Szarzynska B, Sobkowiak L, Pant BD et al (2009) Gene structures and processing of *Arabidopsis thaliana* HYL1-dependent pri-miRNAs. Nucleic Acids Res 37:3083–3093
6. Raczynska KD, Stepien A, Kierzkowski D et al (2014) The SERRATE protein is involved in alternative splicing in *Arabidopsis thaliana*. Nucleic Acids Res 42:1224–1244
7. Yu B, Bi L, Zheng B et al (2008) The FHA domain proteins DAWDLE in *Arabidopsis* and SNIP1 in humans act in small RNA biogenesis. Proc Natl Acad Sci U S A 105:10073–10078
8. Kmieciak M, Simpson CG, Lewandowska D et al (2002) Cloning and characterization of two subunits of *Arabidopsis thaliana* nuclear cap-binding complex. Gene 283:171–183
9. Park MY, Wu G, Gonzalez-Sulser A et al (2005) Nuclear processing and export of microRNAs in *Arabidopsis*. Proc Natl Acad Sci U S A 102:3691–3696
10. Yu B, Yang Z, Li J et al (2005) Methylation as a crucial step in plant microRNA biogenesis. Science 307:932–935
11. Ramachandran V, Chen X (2008) Degradation of micro RNAs by a family of exoribonucleases in *Arabidopsis*. Science 321:1490–1492
12. Baumberger N, Baulcombe DC (2005) *Arabidopsis* ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. Proc Natl Acad Sci U S A 102:11928–11933
13. Wang L, Song X, Gu L et al (2013) NOT2 proteins promote polymerase II-dependent transcription and interact with multiple MicroRNA biogenesis factors in *Arabidopsis*. Plant Cell 25:715–727
14. Rajagopalan R, Vaucheret H, Trejo J et al (2006) A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. Genes Dev 20:3407–3425
15. Pouch-Pélissier MN, Pélissier T, Elmayan T et al (2008) SINE RNA induces severe developmental defects in *Arabidopsis thaliana* and interacts with HYL1 (DRB1), a key member of the DCL1 complex. PLoS Genet. doi:10.1371/journal.pgen.1000096
16. Furumizu C, Tsukaya H, Komeda Y (2010) Characterization of EMU, the *Arabidopsis* homolog of the yeast THO complex member HPR1. RNA 16:1809–1817
17. Jung JH, Seo PJ, Ahn JH et al (2012) *Arabidopsis* RNA-binding protein FCA regulates microRNA172 processing in thermosensory flowering. J Biol Chem 287:16007–16016
18. Calderon-Villalobos LIA, Kuhnle C, Dohmann EMN et al (2005) The evolutionarily conserved TOUGH protein is required for proper development of *Arabidopsis thaliana*. Plant Cell 17:2473–2485
19. Lee B, Kapoor A, Zhu J et al (2006) STABILIZED1, a stress-upregulated nuclear protein, is required for pre-mRNA splicing, mRNA turnover, and stress tolerance in *Arabidopsis*. Plant Cell 18:1736–1749
20. Zhan X, Wang B, Li H et al (2012) *Arabidopsis* proline-rich protein important for development and abiotic stress tolerance is involved in microRNA biogenesis. Proc Natl Acad Sci U S A 109:18198–18203
21. Wu X, Shi Y, Li J et al (2013) A role for the RNA-binding protein MOS2 in microRNA maturation in *Arabidopsis*. Cell 23:645–657
22. Speth C, Willing EM, Rausch S et al (2013) RACK1 scaffold proteins influence miRNA abundance in *Arabidopsis*. Plant J 76:433–445
23. Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209–216

24. Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M et al (2008) Widespread translational inhibition by plant miRNAs and siRNAs. Science 320:1185–1190
25. Kozomara A, Griffiths-Jones S (2014) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 42:68–73
26. Schwab R, Ossowski S (2006) Highly specific gene silencing by artificial microRNAs in *Arabidopsis*. Plant Cell 18:1121–1133
27. Matzke AJ, Chilton MD (1981) Site-specific insertion of genes into T-DNA of the Agrobacterium tumor-inducing plasmid: an approach to genetic engineering of higher plant cells. J Mol Appl Genet 1:39–49
28. Duckely M, Hohn B (2003) The VirE2 protein of Agrobacterium tumefaciens: the Yin and Yang of T-DNA transfer. FEMS Microbiol Lett 223:1–6
29. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735–743
30. Millam S (2007) Potato (*Solanum tuberosum* L.). In: Wang K (ed) Methods in molecular biology. Humana Press Inc., Totowa, NJ, pp 25–35
31. Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol Biol 20:1203–1207
32. Wesley SV, Helliwell C, Smith N et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590
33. Ossowski S, Schwab R, Weigel D (2008) Gene silencing in plants using artificial microRNAs and other small RNAs. Plant J 53:674–690
34. Huang X, Madan A (1999) CAP3: A DNA Sequence Assembly Program. Genome Res. doi:10.1101/gr.9.9.868
35. Larkin MA, Blackshields G, Brown NP et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947–2948
36. Pieczynski M, Marczewski W, Hennig J et al (2012) Down-regulation of CBP80 gene expression as a strategy to engineer a drought-tolerant potato. Plant Biotechnol J 11:459–469
37. Ausubel FM, Brent R, Kingston RE et al (2003) Current protocols in molecular biology. John Wiley & Sons Inc., Hoboken, NJ
38. Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of Agrobacterium tumefaciens after freeze-thaw transformation and drug selection. Biotechniques 16:664–668
39. Mersereau M, Pazour GJ, Das A (1990) Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene 90:149–151
40. Wise AA, Liu Z, Binns AN (2006) Three methods for the introduction of foreign DNA into Agrobacterium. Methods Mol Biol 343:43–53

Part IV

Plant–Pathogen Interactions

Chapter 22

Virus-Induced Gene Silencing for Gene Function Studies in Barley

Maria Barciszewska-Pacak, Artur Jarmołowski, and Andrzej Pacak

Abstract

Virus-Induced Gene Silencing (VIGS) creates a natural antiviral defense in plants. However, it has been also a powerful tool for endogenous gene silencing in dicot and monocot plants by exploitation of recombinant viruses, harboring silencing inducing sequences. The Barley Stripe Mosaic Virus (BSMV) based VIGS system is an efficient and rapid RNAi approach that is routinely applied in functional genomics studies of cereals. We present here a protocol for BSMV VIGS application in barley based on mechanical inoculation of the plants with in vitro transcribed recombinant BSMV RNAs as the silencing triggers.

Key words Virus-induced gene silencing (VIGS), Virus, Barley stripe mosaic virus (BSMV), Posttranscriptional gene silencing (PTGS), RNA interference (RNAi), Functional genomics, Barley

1 Introduction

Posttranscriptional gene silencing (PTGS) was discovered in the 1990s of the twentieth century through the observation of the so called co-suppression phenomenon described in *Petunia hybrida* [1, 2]. Based on further studies, PTGS was found to occur at the RNA level; therefore, it was also called RNA silencing [3]. PTGS is triggered by double-stranded RNA molecules (dsRNAs) that are homologous to a DNA fragment of the gene to be silenced. In plants, these dsRNAs are cleaved into smaller RNA fragments by members of the Dicer-like endonuclease family (DCLs). Products of this digestion are called small interfering RNAs (siRNAs) and are responsible for gene silencing [4–6]. The single-stranded siRNAs are incorporated into the RNA-induced silencing complex (RISC) [7–9], which participates in silencing of specific RNA targets by their cleavage. Among dsRNA molecules that can induce gene silencing, the intermediates of plant (+) ssRNA virus replication [10–15] as well as secondary structures of viral genomes can serve as substrates for the cleavage catalyzed by DCLs [16–18, 14]. There are four plant DCLs, in *Arabidopsis*, DCL1, DCL2, DCL3,

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DOI 10.1007/978-1-4939-3356-3_22,

DCL4, each of them producing different small RNAs in various silencing pathways, although plant (+) ss RNA virus siRNAs may be the product of action of both DCL2 and DCL4 [19]. In RISC, the slicer activity is actually performed by Argonaute proteins, AGOs [20–22]: in *Arabidopsis* AGO1 recruits viral siRNAs in *Cucumber mosaic virus* (CMV) infected plants [23].

1.1 RNA-Silencing as a Specific Plant Immune System against Viruses

The size of viral-derived small RNAs, vsRNAs [14], which are siRNAs found in the infected plants, depends on the virus type. Usually, vsRNAs are 21 nt long but sometimes a mix of 21 and 24-nt virus siRNAs can be found in infected plants. However, in some cases exclusively 22 nt siRNAs or preferably 24 nt siRNAs are also produced during plant infection [24]. The RISC with incorporated vsRNAs directs cleavage of viral RNAs, therefore leading to their degradation in plants [25]. Plant viruses strongly induce but on the other hand are also the targets of VIGS [17]. The viral siRNAs are produced locally as well as systemically [5, 18]. During primary infection, VIGS (Virus-Induced Gene Silencing) derived vsRNAs move ahead of the developing infection, and induce RNA silencing in non-infected parts of a plant before the virus reaches uninfected plant organs. In this way a plant is protected against viral spreading, and a recovery phenotype can be observed in non-infected leaves. Additionally, secondary viral infections become more difficult due to the viruses sequence similarity and in accordance with a phenomenon known as cross-protection [26].

In VIGS, DCLs act on dsRNA replication intermediates, which are (+) and (−) RNA strand hybrids also called the replicative forms, and/or on stem fragments of hairpins within single-stranded genomic RNAs [14]. The effects of primary VIGS may be amplified by the host RNA-dependent RNA polymerase, RdRp [14]. The RdRp uses vsRNAs for complementary RNA synthesis, creating new dsRNA molecules, which are additional sources of virus derived siRNAs. This has been proved *in planta* by detection of viral small RNAs coming from viral genome regions different from those inducing the primary VIGS [27]. The *A. thaliana* RNA-dependent RNA polymerase RDR6, as well as the SDE3 RNA helicase are both necessary for the secondary siRNAs synthesis [28]. However, vsRNAs do not always trigger the secondary VIGS. In the presence of aberrant RNAs derived from sense transgenes or viral genomes, the already mentioned RDR6 and SDE3, together with AGO1 and the RNA binding protein SGS3 (Suppressor of Gene Silencing 3), produce dsRNAs which are processed into small RNAs and in this way the antiviral silencing signals start to be amplified [14]. The SGS3 involvement in small RNAs generation is based on its binding to and protection of RNAs from degradation before their conversion into dsRNAs by RDR [29].

RdRp is also very important for the amplification of antiviral silencing response with regard to RDR6- and SDE3-dependent

RNA silencing spreading between cells and over long distances. This is called non-cell-autonomous silencing. The systemic silencing starts first by its induction through a local infection, then the silencing signal spreads via the plant vascular system and finally leaves the vasculature, amplifying and moving through plasmodesmata from cell to cell of a leaf. It is believed that systemic silencing leads to systemic antiviral response, making the non-infected tissues immune to the virus. Systemic spreading of VIGS may be therefore at the basis of recovery and the cross-protection phenomenon [28, 14], as already mentioned.

1.2 siRNA Generation during Viral Infection

Among the four *A. thaliana* DCL proteins, DCL1 is responsible for miRNA production, DCL3 products direct chromatin modifications, and DCL2 and DCL4 take part in the antiviral response of infected plants [19]. *Arabidopsis* infected by *Tobacco rattle virus* (TRV) generates virus-specific 21- and 24-nt long siRNAs, but when a plant is infected with *Turnip crinkle virus* (TCV) only 22-nt siRNAs are produced. 21-nt siRNAs from TRV are produced by DCL4, while 24-nt siRNAs from the same virus are produced by DCL3. However, only 21-nt TRV siRNAs, similarly to 22-nt siRNAs from TCV, direct virus destruction [19]. Elimination of the activity of DCL4 in TRV infected plants leads to 22-nt siRNAs production via DCL2 action. TCV 22-nt siRNAs are also products of DCL2 activity due to the inactivation of DCL4 by the P38 TCV suppressor protein. Since endogenously encoded trans-acting siRNA (tasiRNA) production is DCL4-dependent, a lack of tasiRNAs is observed in plants expressing P38 [19]. TRV and TCV viruses efficiently amplify in double *dcl2-dcl4 Arabidopsis* mutants. Although both DCL2 and DCL4 produce antiviral siRNAs, DCL4 seems to play a more important role than DCL2, but when absent is fully replaced by DCL2. The DCL proteins are therefore functionally redundant, but also hierarchically organized in terms of antiviral activities [19, 24]. It seems that DCL activity would be enough for viral RNA degradation, but it is worth to mention that the antiviral VIGS response is actually based on active RISC complexes programmed by virus specific siRNAs [17].

1.3 RNA Silencing Suppressors

There are plenty of mechanisms to counteract RNA silencing, which evolved in different viruses. Basically, they depend on viral suppressor proteins, which gained these activities additionally to their other functions. These are mainly: (a) coat proteins like TCV P38; (b) movement proteins like P25 of *PotatovirusX* (PVX), 2b of *Cucumber mosaic virus* (CMV), P19 of *Tomato bushy stunt virus* (TBSV), γb of *Barley Stripe mosaic virus* (BSMV); (c) replication proteins like P130 of *Tomato mosaic virus* (ToMV); (d) replication enhancer proteins like P21 of *Beet yellows virus* (BYV); (e) viral pathogenicity factors, which help in virus systemic accumulation rather than replication, like HcPro (helper component proteinase)

of *Potato virus Y* (PVY), and the already mentioned P19 and γb [30, 14]. *Citrus tristeza virus* (CTV) encodes three different silencing suppressors [31].

Viral silencing suppressors act in several different ways. They may inhibit silencing components, like P21 from BYV [32] or P19 from *Tombusvirus*, which strongly bind to 21-nt long double stranded siRNAs, but weakly to 19–20 nt duplexes [33]. Although HcPro interacts with an endogenous calmodulin-related negative regulator of gene silencing rgsCaM (regulator of gene silencing CaM) [34], it acts also via interactions with ds siRNA [32]. On the other hand, the 2b protein of CMV inhibits slicer activity of AGO1 in RISC reconstitution experiments [23]. Recently, it has been shown that apart from antiviral siRNAs binding, TBSV P19 exhibits also a miR168-mediated AGO1 control to cope with the RNA silencing based host defense [35]. AGO proteins are also targets of the viral suppressors TCV P38, NSs from *Tomato spotted wiltvirus* (TSWV) and P1 from *Sweet potato mild mottle virus* (SPMMV). These three viral suppressors of RNA silencing (VSRs) often mimic host-encoded glycine/tryptophan (GW)-containing proteins and bind Argonaute proteins counteracting the plant antiviral defense [36–38]. The third strategy of viral suppressors of RNA silencing is modification of the host transcriptome, e.g., geminivirus *African cassava mosaic virus* (ACMV) uses the transcriptional activator protein TrAP to induce the expression of Werner exonuclease-like1 (WEL1), leading to the inhibition of transgene-induced RNA silencing in *Arabidopsis*. However, a positive regulator of this silencing, the Werner syndrome-like exonuclease (WEX) also exists, and the TrAP-induced WEL1 accumulation likely causes inhibition of suppression via competition for different factors necessary for proper functionality of WEX [39]. Finally, some vsRNAs come from viral genome secondary structure regions, that are not accessible to RISC but are simply cleaved by DCL proteins. The vsRNAs are loaded into RISC but are unproductive because they lost the silencing inducing sequences due to a viral replicase mistakes, omitting hairpin type structures. These defective interfering RNAs are therefore not targeted against any transcripts [14].

1.4 VIGS as a Tool in Functional Genomics

Although VIGS is a natural antiviral defense mechanism *in planta*, it has been regarded first as a tool for endogenous gene silencing by using of recombinant viruses that harbor silencing inducing sequences [40]. The VIGS approach is based on cloning of a short target gene sequence into the viral vector and introducing an infectious viral RNA or DNA containing sequence from a gene of interest into a young plant. A few weeks after VIGS induction, silencing of endogenous target mRNA occurs together with viral replication inhibition [40]. The VIGS induction can be performed in several different ways. These are mechanical inoculations of young leaves with the viral transcripts generated in vitro or the application of

binary vectors possessing cDNAs encoding genomic viral RNAs. The latter method can be used as high-throughput VIGS, where short fragments of gene candidates are cloned into viral genome cDNA under the 35S CaMV promoter in a binary vector. Having transformed Agrobacterium, the agroinoculation of a plant is performed and 2–3 weeks after the loss-of-function phenotypes can be analyzed [41, 40].

For testing the efficiency of viral RNAi vectors, the silencing of reporter genes is usually applied. One of them is the phytoene desaturase gene (*PDS*) encoding an enzyme of β-carotenoids biosynthesis [42]. *PDS* silencing leads to chlorophyll destruction upon intensive light and the easily visible photobleaching phenotype can be monitored [43, 16].

There are many examples of successful VIGS applications in dicotyledonous and monocotyledonous plants. Initially, TMV based VIGS vectors were widely used for *PDS* silencing in *Nicotiana benthamiana*. A hybrid vector containing a coat protein (CP) from ToMV was used, which helped to make the silencing insert more stable than in the case of the initial vector containing the doubled subgenomic promoter of the TMV CP gene [44, 45]. Later, TMV VIGS vectors were improved by the application of short direct inverted repeats targeting *PDS*, and also *GFP* [16]. The second type of virus that has been exploited intensively in the VIGS approach is PVX [46]. The PVX vector happened to be more stable than the TMV based one, but PVX possessed one disadvantage: a narrower range of host plants than TMV. Unfortunately, in both these VIGS systems the disease symptoms of the inoculated plants make the PTGS phenotypes difficult to distinguish from viral infection symptoms. The viruses also do not infect meristems, therefore in these tissues VIGS based on PVX or TMV cannot be used [12, 47]. Nevertheless, PVX VIGS vectors were widely used and helped in the identification of tobacco genes involved in *N*-mediated resistance against TMV [48] as well as of *HSP90* involvement in the *N. benthamiana* resistance to *Pseudomonas syringae* [49]. TRV VIGS vectors were a breakthrough in viral RNAi vector construction [47], mainly due to the fact that TRV infects meristems and roots, spreads easily within a plant and causes mild infection symptoms [40, 50].

In monocots, an efficient VIGS system is based on BSMV [51, 43, 16, 52, 53]. Laborious and difficult transformation of the monocots has been at least partially overcome by using the BSMV based VIGS in monocots. The BSMV *βa* coat protein gene can be deleted from the BSMV genome, which makes the silencing effect more pronounced but causes more extensive infection symptoms [51, 43, 16, 52]. BSMV VIGS vectors each possess a single virus RNA cDNA under the T7 RNA polymerase promoter to enable in vitro transcription of viral RNAs. The silencing inducing sequence is cloned into the 3′ UTR of BSMV RNAγ cDNA, downstream of the *γb* gene [43]. In 2003, application of the BSMV

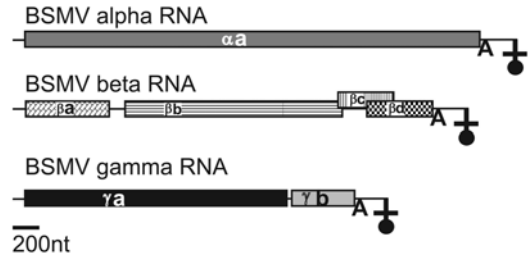

Fig. 1 A scheme of the three BSMV ND18 genomic RNAs. RNAα (GenBank accession U35767; 3787 nt) encodes the 1138 amino acid αa protein; RNAβ (GenBank accession U35770; 3239 nt) encodes four proteins: 198 amino acid coat protein—βa, 512 amino acid βb protein, 131 amino acid βc protein, and 155 amino acid βd protein; RNAγ (GenBank accession M16577; 2791 nt) encodes 648 amino acid γa protein and 152 amino acid γb protein. The RNA components are drawn to scale. The 3′ ends of BSMV RNAs possess tRNA-like structures. A, internal polyA sequence

VIGS system and use of short (40–60 nt) direct inverted repeats as silencing inserts enhanced VIGS leading to the stronger phenotype of *PDS* silencing in barley [16]. In 2006, Ding and colleagues presented another VIGS system for monocots, based on *Brome mosaic virus* (BMV) [54]. The system was based on different BMV strains, F-BMV and R-BMV (Russian strain) from which the BMV in vitro transcribed RNAs 1, 2, 3 were derived. Later the VIGS system based on the only BMV Russian strain was tested in barley with a distinct construct and compared to the already described BSMV VIGS system. Again, the latter proved to be the most efficient silencing system for monocots [55].

BSMV is a (+) ss RNA virus containing a tripartite RNA genome consisting of RNAs α, β, γ and a subgenomic RNA created on the RNAγ [12] (Fig. 1). Each of the four RNAs possesses the 5′ cap structure and a 3′ terminal tRNA-like structure [56]. RNA α encodes the αa protein with methyltransferase and helicase motifs [57]. The β*a* gene encodes a CP and a triple-gene β block *b, c, d*, TGB proteins involved in virus spreading within an infected plant [58]. RNA γ possesses the γ*a* gene for a protein with the RdRp motif, and the γ*b* gene encoding a protein acting as an RNA silencing suppressor [12]. BSMV replicates in proplastid and chloroplast vesicles, and virions are located in cytoplasmic inclusions of chloroplasts [59].

2 Materials

1. *MluI, SpeI* restriction enzymes and the corresponding buffers.
2. 0.5 M EDTA pH 8.0.
3. 3 M sodium acetate pH 5.2.
4. 100 % ethanol.
5. DEPC-treated H_2O (RNase-free H_2O).

6. mMESSAGE mMACHINE®T7, High Yield Capped RNA Transcription Kit, Ambion, Life Technologies.
7. Optional: TURBO DNase [2 U/μl], Ambion, Life Technologies.
8. Optional: DNase Inactivation Reagent, Ambion, Life Technologies.
9. Carborundum (silicon carbide, SiC) used as an abrasive powder.
10. Inoculation FES buffer (77 mM glycine, 60 mM K_2HPO_4, 22 mM $Na_4P_2O_7 \times 10H_2O$, 1 % w/v bentonite) [60].

3 Methods

3.1 In Vitro Synthesis of the Infectious BSMV RNAs

1. For linearization of viral cDNA containing plasmids, (described previously, [60]) prepare 10 μg of each of the plasmids containing BSMV RNAs, α, β, γ cDNAs, to restriction enzyme digestions in 50-μl reaction volumes, as shown in Table 1.
2. Incubate samples at 37 °C for 2 h.
3. For precipitation of linearized plasmids, add to plasmid linearization reactions 1/20 volume (2.5 μl) of 0.5 M EDTA pH 8.0, 1/10 volume (5 μl) of 3 M sodium acetate pH 5.2, and 2 volumes (100 μl) of 100 % ethanol.
4. Mix and incubate at −20 °C for 15 min.
5. Centrifuge at 4 °C for 15 min at 18,000*g*.
6. Discard supernatant and add 750 μl of 70 % ethanol to the pellet.
7. Centrifuge at 4 °C for 5 min at 18,000*g*.
8. Discard supernatant.
9. Centrifuge again at 4 °C for 2 min at 18,000*g* and remove the remaining ethanol.
10. Air-dry the pellet for 5 min.
11. Dissolve the pellet in 15 μl of DEPC-treated H_2O.
12. Measure plasmid concentration spectrophotometrically (NanoDrop ND-1000, NanoDrop, Wilmington, DE, U.S.A.).
13. Use mMESSAGE mMACHINE®T7, High Yield Capped RNA Transcription Kit (Ambion, Life Technologies) for in vitro transcription of the BSMV RNAs (*see* **Note 1**).
14. Prepare three eppendorf tubes for the three different BSMV RNAs, α, β, γ, as shown in Table 2 (*see* **Note 2**).
15. Incubate at 37 °C for 2 h.
16. Optional: The synthesized RNAs amount exceeds the amount of linearized plasmids in the samples, therefore plasmid DNA

Table 1
Restriction enzyme digestions scheme for the BSMV cDNA containing plasmids

	Plasmid α	**Plasmid β**	**Plasmid γ**
Plasmid DNA	10 μg (up to 30 μl)	10 μg (up to 30 μl)	10 μg (up to 30 μl)
10× buffer	5 μl of *MluI* 10× buffer	5 μl of *SpeI* 10× buffer	5 μl of *MluI* 10× buffer
Restriction enzyme	2 μl of *MluI* [10 U/μl]	2 μl of *SpeI* [10 U/μl]	2 μl of *MluI* [10 U/μl]
BSA	No need to be added	To be added up to [100 μg/ml]	No need to be added
H_2O	13 μl	13 μl	13 μl

Table 2
In vitro transcriptions scheme for the BSMV α, β and γ RNAs

	RNA α	**RNA β**	**RNA γ**
Linearized plasmid DNA	0.5 μg	0.5 μg	0.5 μg
10× buffer for T7 RNA Pol	1 μl	1 μl	1 μl
Enzyme mix (T7 RNA Pol)	1 μl	1 μl	1 μl
2× NTP/CAP	5 μl	5 μl	5 μl
RNase-free H_2O	up to 10 μl	up to 10 μl	up to 10 μl

removal can be skipped. In the case this step is needed, add 1 μl of TURBO DNase [2 U/μl] (Ambion, Life Technologies) to the in vitro transcription reaction mixes and incubate at 37 °C for 15 min, then add 0.1 volume of DNase Inactivation Reagent (Ambion, Life Technologies) to inactivate DNase and incubate 5 min at room temperature (RT) mixing occasionally. Centrifuge at room temperature for 1.5 min at 10,000*g* and transfer the transcribed RNA (supernatant) to a new eppendorf tube.

17. For the quality and quantity control of the RNAs obtained, carry out 1 % agarose gel electrophoresis (Fig. 2) (*see* **Notes 3** and **4**). For this purpose, mix 0.5 μl of each of transcription reactions with 4.5 μl of RNase-free H_2O and 5 μl of 2× RNA loading dye, and load on the gel. Remember to load on a gel a molecular mass marker (DNA marker can be also used) next to the RNA samples.

3.2 Mechanical Inoculations of Plants with the BSMV Transcripts

1. Choose barley cultivar susceptible to BSMV infection. Apply for example barley Black Hulless or Clansman cultivars [60, 16, 61].
2. Mix three BSMV transcripts: RNAα, RNAβ and RNAγ (Fig. 2) containing silencing inducing sequence in a 1:1:1 ratio.

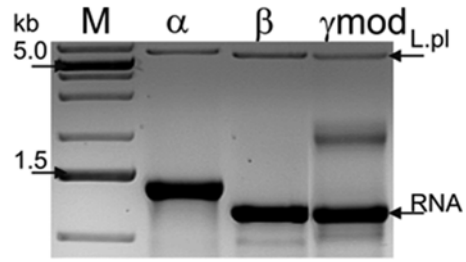

Fig. 2 1 % Agarose gel electrophoresis of in vitro transcribed BSMV RNAs α, β and modified RNAγ containing the 375-nt silencing inducing sequence. DNase treatment was not performed after the in vitro transcriptions and 0.5 μl of each transcription reaction was loaded on the gel. The DNA molecular mass marker GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific) was used. RNAs separate between 1.0 and 1.5 kb bands. L.pl indicates linearized plasmids remaining in the reaction mixtures. The plasmids as well as any unspecific products visible (lane γmod) do not hamper plant inoculation and then viral infection

3. Take a 1-week-old barley seedling (Fig. 3a), sprinkle the first emerging leaf with carborundum (Fig. 3b) and then rub it gently with the fingertips of the hands wearing gloves (Fig. 3c) (*see* **Note 5**).
4. Drop 5 μl of FES buffer and then 3 μl of BSMV RNAs mix on a fingertip of the glove-wearing hand, and rub the mixture gently alongside the leaf (Fig. 3d, e) [60].
5. Always perform mock inoculations of control plants by using FES buffer only.
6. Observe the barley phenotype after inoculation (*see* **Note 6**). BSMV infection affects barley growth (Pacak, unpublished data). Analyze very carefully any phenotypical changes together with gene silencing effects at both the RNA and protein levels. Compare results not only to mock control but also to barley plants treated with a neutral BSMV non-silenced control, e.g., BSMV-GFP control (*see* **Notes 7** and **8**). Avoid any viral contamination during inoculation procedure (*see* **Note 9**).

4 Notes

1. The kit contains 5′ cap structure analogs necessary to receive in vitro transcribed infectious BSMV RNA molecules.
2. 10 μl reaction volumes are sufficient for inoculation of 10 plants. The reaction volumes can be scaled up or down.
3. All reagents necessary for agarose gel and 1× TBE buffer preparation must be RNase-free. Use diethyl pyrocarbonate, DEPC-treated water (0.1 % v/v; after DEPC treatment, incu-

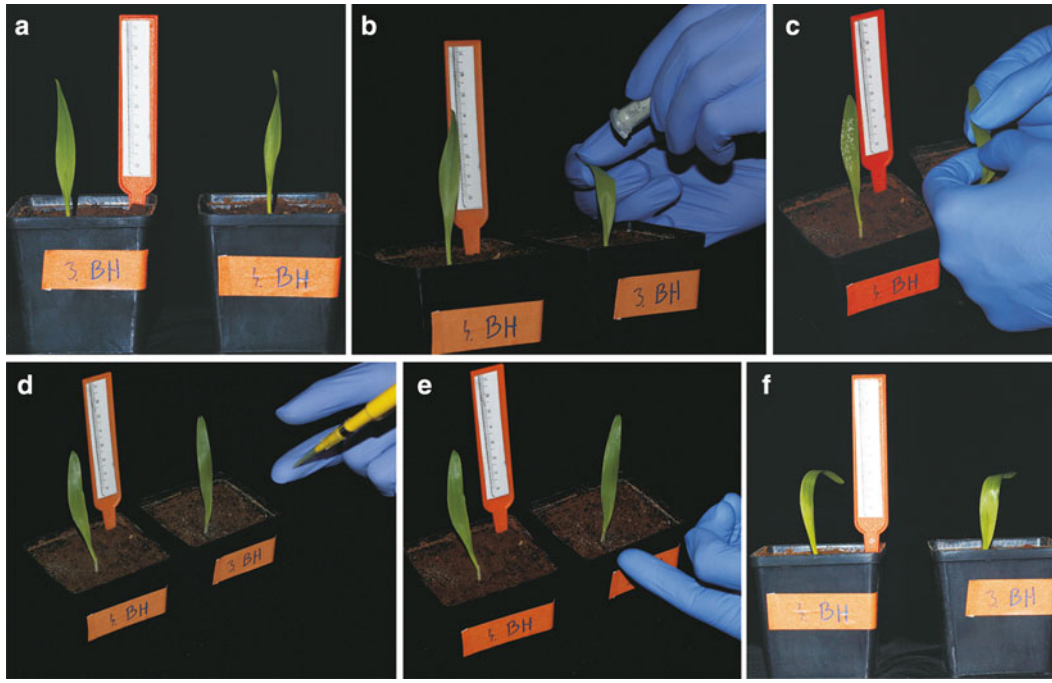

Fig. 3 Consecutive steps during barley inoculation. (**a**) One-week-old barley seedlings. (**b**) Carborundum powder application on leaf no 1. (**c**) Gentle rubbing of the carborundum with fingertips. (**d**, **e**) FES buffer and BSMV RNAs mixing on fingertip. (**f**) Barley plants just after inoculation

bate water with DEPC overnight at room temperature, then autoclave it twice to remove free DEPC). DEPC is a harmful, potentially carcinogen reagent.

4. An electrophoresis tank should be rinsed with 3 % H_2O_2 prior to use.
5. Inoculate the first leaf when the second leaf has just started to emerge (6, 7 days after sowing). However, note that Holzberg and colleagues inoculated the second barley leaf [43]. After inoculation leaves have wilted phenotype (Fig. 3f).
6. First symptoms of BSMV infection are visible 4–6 days after inoculation as stripes of discoloration occur on the emerging third leaf (Fig. 4).
7. The reporter gene silencing phenotype, e.g., *PDS* silencing, should be well visible 10 days post infection (dpi). The most pronounced phenotypical changes are visible on leaf number 3, not on the inoculated and the second leaves of the infected plant (Fig. 5); see also [60]. However, Lacomme and colleagues observed that the strongest *PDS* silencing caused a photobleaching phenotype on the fourth barley leaves [16]. In the case of inoculation of the second leaf, the

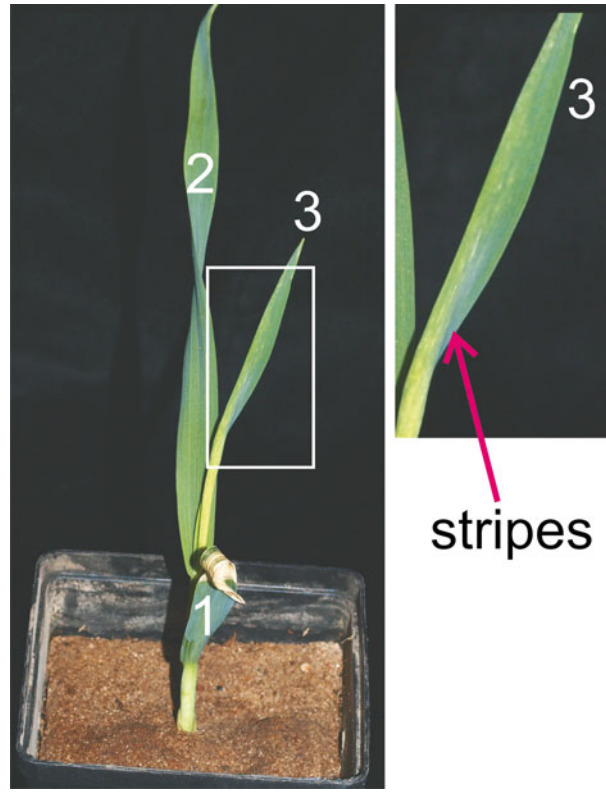

Fig. 4 Barley cv. Black Hulless 6 days after inoculation of the first leaf (1). Numbers 1, 2 and 3 indicate consecutive barley leaves (counting from youngest to the oldest). The red arrow points at visible stripes of discoloration indicating BSMV infection. The picture was taken 13 days after sowing

strongest *PDS* silencing phenotype was observed on the fifth leaf [43].

8. Interestingly, it is not necessary to have 100 % identity of the silencing-inducing sequence inserted into the BSMVγ RNA and the target endogenous mRNA. Using a 393 bp silencing inducing fragment of barley *PDS* (GenBank AY062039.1; $BSMV_{PDS400}$ [60]) inserted in the sense orientation into the BSMVγ RNA it was possible to induce *PDS* silencing in both diploid and hexaploid wheat (377 nucleotide identities per 393 nucleotides of the silencing *PDS* insert compared to *PDS* from *Triticum aestivum*, GenBank DQ270236.1) (Fig. 6). Note that gene silencing triggered by the BSMV VIGS system is transient due to the instability of the silencing inducing sequence within the viral RNA. Four weeks after inoculation with $BSMV_{PDS400}$ there is almost no intact *PDS400*

Fig. 5 *PDS* silencing photobleaching phenotype observed on the third leaf of the infected barley plant (**b**) compared to mock inoculated barley (**a**). Four consecutive barley cv. Black Hulless leaves (1, 2, 3, 4) were collected 14 dpi. (**a**) Mock inoculated barley (buffer only). (**b**) Barley inoculated with the in vitro transcribed BSMV RNAs including RNAγ containing *PDS* silencing inducing fragment in the sense orientation ($BSMV_{PDS400}$, [60])

fragment in the infected plant [60]. It was recently reported that using modified BSMV RNAγ and also RNAβ it is possible to obtain more stable gene silencing effects [62].

9. Owing to the work with modified viruses, all glass, mortars, and pestles must be soaked with antiviral reagents such as Virkon for ~24 h and then autoclaved.

Acknowledgements

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Fig. 6 Leaves of different monocot plants inoculated with RNAα, RNAβ, and the same RNAγ=BSMV$_{PDS400}$ [60] molecule 14 dpi. Although the *PDS* silencing sequence is derived from barley, the photobleaching phenotypes are visible in diploid *Triticum monococcum* (**a**), hexaploid *Triticum aestivum* cv. Amaretto wheat (**b**), barley, Black Hulless cultivar (**c**). Numbers 2, 3, 4 indicate consecutive leaves

References

1. Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. Plant Cell Online 2(4):279–289
2. Van der Krol AR, Mur LA, Beld M, Mol J, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell Online 2(4):291–299
3. Baulcombe D (2002) Viral suppression of systemic silencing. Trends Microbiol 10(7):306–308
4. Hamilton A, Voinnet O, Chappell L, Baulcombe D (2002) Two classes of short interfering RNA in RNA silencing. EMBO J 21(17):4671–4679
5. Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286(5441):950–952
6. Plasterk RH (2002) RNA silencing: the genome's immune system. Science 296(5571):1263–1265
7. Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404(6775):293–296
8. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song J-J, Hammond SM, Joshua-Tor L, Hannon GJ (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305(5689):1437–1441

9. Matranga C, Tomari Y, Shin C, Bartel DP, Zamore PD (2005) Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123(4):607–620
10. Baulcombe D (2002) RNA silencing. Curr Biol 12(3):R82–R84
11. Cerutti H (2003) RNA interference: traveling in the cell and gaining functions? Trends Genet 19(1):39–46
12. Hull R (2001) Matthews' plant virology. Academic, New York
13. Kalantidis K, Psaradakis S, Tabler M, Tsagris M (2002) The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. Mol Plant-Microbe Interact 15(8):826–833
14. Voinnet O (2005) Induction and suppression of RNA silencing: insights from viral infections. Nat Rev Genet 6(3):206–220, http://www.nature.com/nrg/journal/v6/n3/suppinfo/nrg1555_S1.html
15. Waterhouse PM, Wang M-B, Lough T (2001) Gene silencing as an adaptive defence against viruses. Nature 411(6839):834–842
16. Lacomme C, Hrubikova K (2003) Enhancement of virus-induced gene silencing through viral-based production of inverted-repeats. Plant J 34(4):543–553
17. Pantaleo V, Szittya G, Burgyán J (2007) Molecular bases of viral RNA targeting by viral small interfering RNA-programmed RISC. J Virol 81(8):3797–3806
18. Szittya G, Molnar A, Silhavy D, Hornyik C, Burgyan J (2002) Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. Plant Cell 14(2):359–372
19. Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, Voinnet O (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science 313(5783):68–71
20. Brodersen P, Voinnet O (2006) The diversity of RNA silencing pathways in plants. Trends Genet 22(5):268–280
21. Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115(2):199–208
22. Song J-J, Liu J, Tolia NH, Schneiderman J, Smith SK, Martienssen RA, Hannon GJ, Joshua-Tor L (2003) The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat Struct Mol Biol 10(12):1026–1032, http://www.nature.com/nsmb/journal/v10/n12/suppinfo/nsb1016_S1.html
23. Zhang X, Yuan Y-R, Pei Y, Lin S-S, Tuschl T, Patel DJ, Chua N-H (2006) Cucumber mosaic virus-encoded 2b suppressor inhibits *Arabidopsis* Argonaute1 cleavage activity to counter plant defense. Genes Dev 20(23):3255–3268
24. Waterhouse PM, Fusaro AF (2006) Viruses face a double defense by plant small RNAs. Science 313(5783):54–55
25. Ruiz-Ferrer V, Voinnet O (2007) Viral suppression of RNA silencing: 2b wins the Golden Fleece by defeating Argonaute. Bioessays 29(4):319–323. doi:10.1002/bies.20556
26. Ratcliff FG, MacFarlane SA, Baulcombe DC (1999) Gene silencing without DNA: RNA-mediated cross-protection between viruses. Plant Cell 11(7):1207–1215
27. Vaistij FE, Jones L, Baulcombe DC (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell Online 14(4):857–867
28. Himber C, Dunoyer P, Moissiard G, Ritzenthaler C, Voinnet O (2003) Transitivity-dependent and-independent cell-to-cell movement of RNA silencing. EMBO J 22(17):4523–4533
29. Yoshikawa M, Peragine A, Park MY, Poethig RS (2005) A pathway for the biogenesis of trans-acting siRNAs in *Arabidopsis*. Genes Dev 19(18):2164–2175
30. Silhavy D, Burgyán J (2004) Effects and side-effects of viral RNA silencing suppressors on short RNAs. Trends Plant Sci 9(2):76–83
31. Lu R, Folimonov A, Shintaku M, Li W-X, Falk BW, Dawson WO, Ding S-W (2004) Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. Proc Natl Acad Sci U S A 101(44):15742–15747
32. Lakatos L, Csorba T, Pantaleo V, Chapman EJ, Carrington JC, Liu YP, Dolja VV, Calvino LF, López-Moya JJ, Burgyán J (2006) Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. EMBO J 25(12):2768–2780
33. Vargason JM, Szittya G, Burgyán J, Hall TMT (2003) Size Selective Recognition of siRNA by an RNA Silencing Suppressor. Cell 115(7):799–811, http://dx.doi.org/10.1016/S0092-8674(03)00984-X
34. Anandalakshmi R, Marathe R, Ge X, Herr J, Mau C, Mallory A, Pruss G, Bowman L, Vance VB (2000) A calmodulin-related protein that

suppresses posttranscriptional gene silencing in plants. Science 290(5489):142–144
35. Várallyay É, Oláh E, Havelda Z (2014) Independent parallel functions of p19 plant viral suppressor of RNA silencing required for effective suppressor activity. Nucleic Acids Res 42(1):599–608
36. Azevedo J, Garcia D, Pontier D, Ohnesorge S, Yu A, Garcia S, Braun L, Bergdoll M, Hakimi MA, Lagrange T (2010) Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev 24(9):904–915
37. de Ronde D, Pasquier A, Ying S, Butterbach P, Lohuis D, Kormelink R (2014) Analysis of Tomato spotted wilt virus NSs protein indicates the importance of the N-terminal domain for avirulence and RNA silencing suppression. Mol Plant Pathol 15:185–195
38. Giner A, Lakatos L, García-Chapa M, López-Moya JJ, Burgyán J (2010) Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. PLoS Pathog 6(7), e1000996
39. Trinks D, Rajeswaran R, Shivaprasad P, Akbergenov R, Oakeley EJ, Veluthambi K, Hohn T, Pooggin MM (2005) Suppression of RNA silencing by a geminivirus nuclear protein, AC2, correlates with transactivation of host genes. J Virol 79(4):2517–2527
40. Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP (2004) Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant J 39(5):734–746
41. Baulcombe DC (1999) Fast forward genetics based on virus-induced gene silencing. Curr Opin Plant Biol 2(2):109–113
42. Matthews PD, Luo R, Wurtzel ET (2003) Maize phytoene desaturase and ζ-carotene desaturase catalyse a poly-Z desaturation pathway: implications for genetic engineering of carotenoid content among cereal crops. J Exp Bot 54(391):2215–2230
43. Holzberg S, Brosio P, Gross C, Pogue GP (2002) Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant J 30(3):315–327
44. Kumagai M, Donson J, Della-Cioppa G, Harvey D, Hanley K, Grill L (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proc Natl Acad Sci 92(5):1679–1683
45. Rabindran S, Dawson WO (2001) Assessment of recombinants that arise from the use of a TMV-based transient expression vector. Virology 284(2):182–189
46. Ruiz MT, Voinnet O, Baulcombe DC (1998) Initiation and maintenance of virus-induced gene silencing. Plant Cell Online 10(6):937–946
47. Ratcliff F, Martin-Hernandez AM, Baulcombe DC (2001) Technical advance: tobacco rattle virus as a vector for analysis of gene function by silencing. Plant J 25(2):237–245
48. Peart JR, Mestre P, Lu R, Malcuit I, Baulcombe DC (2005) NRG1, a CC-NB-LRR protein, together with N, a TIR-NB-LRR protein, mediates resistance against tobacco mosaic virus. Curr Biol 15(10):968–973
49. Lu R, Malcuit I, Moffett P, Ruiz MT, Peart J, Wu AJ, Rathjen JP, Bendahmane A, Day L, Baulcombe DC (2003) High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. EMBO J 22(21):5690–5699
50. Valentine T, Shaw J, Blok VC, Phillips MS, Oparka KJ, Lacomme C (2004) Efficient virus-induced gene silencing in roots using a modified tobacco rattle virus vector. Plant Physiol 136(4):3999–4009
51. Hein I, Barciszewska-Pacak M, Hrubikova K, Williamson S, Dinesen M, Soenderby IE, Sundar S, Jarmolowski A, Shirasu K, Lacomme C (2005) Virus-Induced Gene Silencing-Based Functional Characterization of Genes Associated with Powdery Mildew Resistance in Barley. Plant Physiol 138(4):2155–2164. doi:10.1104/pp. 105.062810
52. Scofield SR, Huang L, Brandt AS, Gill BS (2005) Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. Plant Physiol 138(4):2165–2173. doi:10.1104/pp. 105.061861
53. Shen Q-H, Saijo Y, Mauch S, Biskup C, Bieri S, Keller B, Seki H, Ülker B, Somssich IE, Schulze-Lefert P (2007) Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. Science 315(5815):1098–1103
54. Ding XS, Schneider WL, Chaluvadi SR, Mian MR, Nelson RS (2006) Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Mol Plant-Microbe Interact 19(11):1229–1239
55. Pacak A, Strozycki PM, Barciszewska-Pacak M, Alejska M, Lacomme C, Jarmolowski A, Szweykowska-Kulinska Z, Figlerowicz M (2010) The brome mosaic virus-based recombination vector triggers a limited gene silencing response depending on the orientation of the

inserted sequence. Arch Virol 155(2):169–179. doi:10.1007/s00705-009-0556-9

56. Agranovsky A, Dolja V, Atabekov J (1982) Structure of the 3′ extremity of barley stripe mosaic virus RNA: Evidence for internal poly (A) and a 3′-terminal tRNA-like structure. Virology 119(1):51–58
57. Petty IT, French R, Jones RW, Jackson AO (1990) Identification of barley stripe mosaic virus genes involved in viral RNA replication and systemic movement. EMBO J 9(11):3453–3457
58. Petty I, Jackson A (1990) Mutational analysis of barley stripe mosaic virus RNA β. Virology 179(2):712–718
59. Torrance L, Cowan G, Gillespie T, Ziegler A, Lacomme C (2006) Barley stripe mosaic virus-encoded proteins triple-gene block 2 and γb localize to chloroplasts in virus-infected monocot and dicot plants, revealing hitherto-unknown roles in virus replication. J Gen Virol 87(8):2403–2411
60. Bruun-Rasmussen M, Madsen CT, Jessing S, Albrechtsen M (2007) Stability of Barley stripe mosaic virus-induced gene silencing in barley. Mol Plant-Microbe Interact 20(11):1323–1331
61. Pacak A, Geisler K, Jorgensen B, Barciszewska-Pacak M, Nilsson L, Nielsen T, Johansen E, Gronlund M, Jakobsen I, Albrechtsen M (2010) Investigations of barley stripe mosaic virus as a gene silencing vector in barley roots and in Brachypodium distachyon and oat. Plant Methods 6(1):26
62. Kawalek A, Dmochowska-Boguta M, Nadolska-Orczyk A, Orczyk W (2012) A new BSMV-based vector with modified β molecule allows simultaneous and stable silencing of two genes. Cell Mol Biol Lett 17(1):107–123

Chapter 23

Methods for Long-Term Stable Storage of *Colletotrichum* Species

Kei Hiruma and Yusuke Saijo

Abstract

In this chapter we describe methods for long-term preservation of ascomycete genus *Colletotrichum* species. *Colletotrichum* species employ a hemibiotrophic infection strategy and cause clear anthracnose diseases on various host plants including the model plant *Arabidopsis thaliana*. Their infection proceeds in a highly synchronized manner, which is helpful for the dissection of the fungus-plant interactions at the molecular level. Gene engineering methods, including efficient protocols for targeted gene disruption, and whole-genome sequences are available for several *Colletotrichum* species. Thus, these pathogens provide us with model systems to address the molecular mechanisms underlying hemibiotrophic fungal pathogenicity.

We describe how to prepare glycerol stocks or filter paper fungal stocks for long-term preservation of *Colletotrichum* species. These two methods are easily handled, and provide a stable preservation for at least a few years.

Key words *Colletotrichum* species, Glycerol stocks, Filter paper stocks

1 Introduction

The large ascomycete genus *Colletotrichum* is one of the most economically important groups of plant pathogens, causing anthracnose disease on a wide range of crops [1]. After the penetration from a black melanized structure, termed appressoria, *Colletotrichum* species transiently establish a biotrophic interaction phase with the host, which is illustrated by invagination of the host plasma membranes. Then, on as-yet-undefined cues, the fungi switch to a necrotrophic phase, thereby destroying and killing the host cells [2]. *Colletotrichum* species can be cultured axenically and are competent for transgenic approaches by the means of Agrobacterium- or PEG-mediated transformation procedures. This greatly facilitates mutagenic approaches to critically determine gene function by targeted gene disruption. Furthermore, the

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whole-genome sequences of four pathogenic *Colletotrichum* species have been published recently [3, 4].

Here we describe two methods to store *Colletotrichum* species for a long term. In our experience, long-term stable storage is crucial for plant inoculation experiments with *Colletotrichum* fungi because their sequential subculturing (even several times) from one plate to another often reduces pathogenicity.

2 Materials

1. PDA medium: 39 g/L Potato dextrose agar (Difco).
2. Mathur's medium: 2.8 g/L glucose, 1.2 g/L $MgSO_4/7H_2O$, 2.7 g/L KH_2PO_4, 2.2 g/L mycological Peptone, 3 % Bacto Agar.
3. Glycerol: 25 % glycerol diluted in distilled water (DW).
4. Filter paper.
5. Paper envelopes.

3 Methods

3.1 Glycerol Stocks

This method allows us to preserve *Colletotrichum* fungi for about 3 years in glycerol stocks at −80 °C. We normally receive a plate or tube with a nutrient medium in which fungi grow from fungal stock centers. We describe this method based on the assumption that *Colletotrichum* species grow in a plate with a nutrient medium (described as "master plate" below).

1. From the master plates for *Colletotrichum* species (Fig. 1a), transfer one piece of the mycelium (grown on nutrient agar media) to the center of new PDA or Mathur's medium plates.
2. Incubate the plates at 25 °C without light until the fungal colonies spread to the edge of the Petri dishes (Fig. 1a). To avoid bacterial contamination, addition of 100 μg/mL rifamycin and 125 μg/mL streptomycin to the medium may be needed.
3. For spore preparation, cut out three pieces of mycelium (2 × 2 mm) with a sterilized knife from the edge of colonies on PDA or Mathur's medium.
4. Place them on PDA or Mathur's medium (100 mL) in 300 mL flasks (Fig. 1b).
5. Add 2 mL of distilled water (DW) to the flasks and briefly shake them by hand so that water spreads over the medium surface in the flasks.
6. Incubate the flasks for 1–2 weeks at 25 °C under continuous light, which often results in better spore formation. Orange

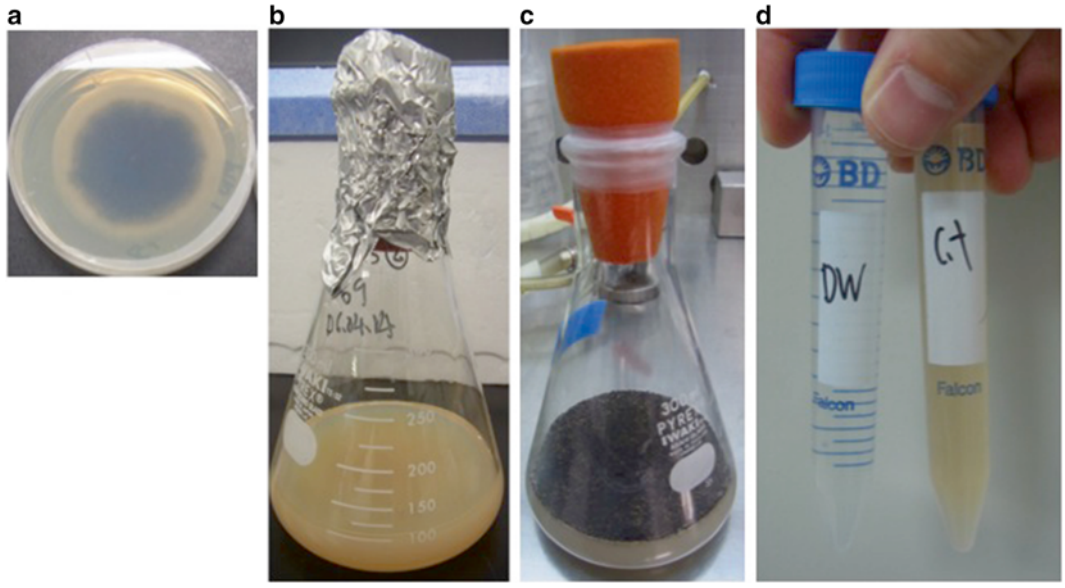

Fig. 1 Mycelial growth and conidiation of *Colletotrichum* species. (**a**) A colony of *Colletotrichum* species (the mycelium should be picked from the edge). (**b**) A flask with 100 mL Mathur's media. (**c**) Growth of *Colletotrichum* species in Mathur's media after 1-week incubation. (**d**) Spores of *Colletotrichum* species. The solution is pigmented by the presence of spores

dots on the media during the incubation period indicate that spores are already produced (Fig. 1c, *see* **Note 1**).

7. After 1 week or so, add 4 mL distilled water (DW) to the flasks and then shake them by hand. If you have spores, the suspension will look pigmented (Fig. 1d, *see* **Note 1**).
8. Transfer the spore suspension to new 1.5 or 2 mL tubes, and add an equal volume of autoclaved 50 % glycerol to prepare glycerol stocks (final concentration: 25 % glycerol).
9. Freeze these stocks in liquid nitrogen.
10. Keep the tubes at −80 °C. These cell stocks can be kept stably for a few years at least.

3.2 Filter Paper Fungal Stocks

Alternatively, filter paperdiscs can be stored in which fungal mycelium has grown. In this case, the stocks are stored at −20 °C. It may be safer to prepare the stocks by this method in addition to the glycerol stocks in case of trouble with the −80 °C freezer for the aforementioned glycerol stocks.

1. Autoclave (121 °C 15 min) filter paper discs (3 × 3 mm).
2. Place 20 sterilized paper discs on Petri dishes containing PDA or Mathur's medium.
3. Place some pieces of mycelium on the center of the plates.

4. Incubate the plates until the fungus has covered/impregnated the discs.
5. Pick up the discs with the fungi from the plates with fire-flamed forceps.
6. Incubate discs in a clean bench for a few days for drying up.
7. Wrap discs up in sterilized paper envelopes (5 × 5 cm), which should be kept at −20 °C.

4 Notes

1. White aerial hyphae may be noticed in the flasks. In general, this suggests that the fungi have not produced a lot of spores. The aerial hyphae are formed mainly when the flasks are not sufficiently aerated (you may see a lot of water on the side of the flasks). In these cases, it is worth trying to change the stopper to increase the aeration.

References

1. Agrios GN (1988) Plant pathology, 3rd edn. Academic, San Diego, CA
2. Perfect SE, Hughes HB, O'Connell RJ, Green JR (1999) *Colletotrichum*: a model genus for studies on pathology and fungal-plant interactions. Fungal Genet Biol 27(2–3):186–198
3. O'Connell RJ, Thon MR, Hacquard S, Amyotte SG, Kleemann J, Torres MF, Damm U, Buiate EA, Epstein L, Alkan N, Altmuller J, Alvarado-Balderrama L, Bauser CA, Becker C, Birren BW, Chen Z, Choi J, Crouch JA, Duvick JP, Farman MA, Gan P, Heiman D, Henrissat B, Howard RJ, Kabbage M, Koch C, Kracher B, Kubo Y, Law AD, Lebrun MH, Lee YH, Miyara I, Moore N, Neumann U, Nordstrom K, Panaccione DG, Panstruga R, Place M, Proctor RH, Prusky D, Rech G, Reinhardt R, Rollins JA, Rounsley S, Schardl CL, Schwartz DC, Shenoy N, Shirasu K, Sikhakolli UR, Stuber K, Sukno SA, Sweigard JA, Takano Y, Takahara H, Trail F, van der Does HC, Voll LM, Will I, Young S, Zeng Q, Zhang J, Zhou S, Dickman MB, Schulze-Lefert P, Loren V, van Themaat E, Ma LJ, Vaillancourt LJ (2012) Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. Nat Genet 44(9):1060–1065
4. Gan P, Ikeda K, Irieda H, Narusaka M, O'Connell RJ, Narusaka Y, Takano Y, Kubo Y, Shirasu K (2013) Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. New Phytol 197(4):1236–1249

Chapter 24

Plant Inoculation with the Fungal Leaf Pathogen *Colletotrichum higginsianum*

Kei Hiruma and Yusuke Saijo

Abstract

After *Colletotrichum* storage methods in Chapter 23, we describe here experimental methods for the inoculation of *Colletotrichum higginsianum* (*C.h.*) on *Arabidopsis* leaves. We put a particular focus on the methods for lesion measurements after the drop-inoculation of the leaves and on *C.h.* biomass measurements in the leaves by RT-qPCR analysis. As an option, we also briefly describe methods for counting *C.h.* entry ratio.

Key words *Colletotrichum higginsianum*, *Arabidopsis*, Lesion measurements, Biomass measurements, RT-qPCR

1 Introduction

A wide range of *Colletotrichum higginsianum* (*C.h.*) isolates infect *Arabidopsis* [1, 2]. The fungi penetrate the host epidermal cells from black melanized appressoria in a synchronized manner, and then transiently form biotrophic intracellular hyphae (in the biotrophic phase) before switching to the destructive necrotrophic phase. Recently the whole-genome sequences and transcriptome data obtained at several time points after the inoculation have been published [3]. With these accumulating data sets, this pathosystem offers a model for studying plant-fungus interactions on both sides.

We focus on methods for inoculation of *C.h.* on *Arabidopsis* plants. Several methods have been developed to assess *C.h.* growth in leaves. First, a lesion measurement method is based on the assumption that lesion diameters correlate well with the degree of leaf colonization by *C.h.*, although one needs expertise to distinguish lesions formed as a result of hypersensitive response (HR) cell death of the host [4].

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Earlier studies determined *C.h.* biomass in leaves by using RT-qPCR with *C.h.*-specific *actin* primers [5, 6]. Because this method clearly distinguishes the lesions formed by successful *C.h.* infection from those formed by strong HR cell death responses of the plant, it better represents the growth of *C.h.* biomass in leaves.

We describe *C.h.* inoculation protocols for both lesion size and biomass measurements. In addition, it would be informative to measure the *C.h.* entry rate to evaluate the contribution of pre-invasive basal defenses to *C.h.* growth [7, 8]. For this reason, we also briefly describe a *C.h.* inoculation protocol for the entry counting.

This protocol can be applicable to other *Colletotrichum* species with minor modifications.

2 Materials

1. Glycerol or paper stocks of *C. higginsianum*.
2. An erythrocytometer.
3. 4-week-old *Arabidopsis* plants or 10-day-old seedlings grown at 22–25 °C with a 16-h photoperiod and 70 μmol/m^2 s light intensity.
4. *C.h.*-specific *Actin* primers (sequences can be found in, e.g., [5] or [6]).
5. An RT-qPCR machine.
6. A light microscope.

3 Methods

3.1 Spore Preparation

To conduct stable infection experiments, it is important to ascertain the *C.h.* spore concentration in the inoculum by counting spores as described below.

1. Streak *C.h.* onto a PDA or Mathur's plate from the glycerol stocks with a sterilized tip. In the case of starting from *C.h.* filter paper discs, take one piece of the discs and place it on the nutrient medium (PDA or Mathur's) in Petri dishes. Then incubate the plates until the fungal colonies almost reach to the edge (for about 1 week).
2. Add a few pieces of the mycelium to 100 mL of PDA or Mathur's medium. Then add 2 mL of distilled water (DW) and incubate for around 1 week as described in Chapter 23 (Fig. 1a).
3. Add approximately 10 mL of DW to the flasks and shake them by hand, before transferring the spore suspensions to 15 mL Falcon tubes.

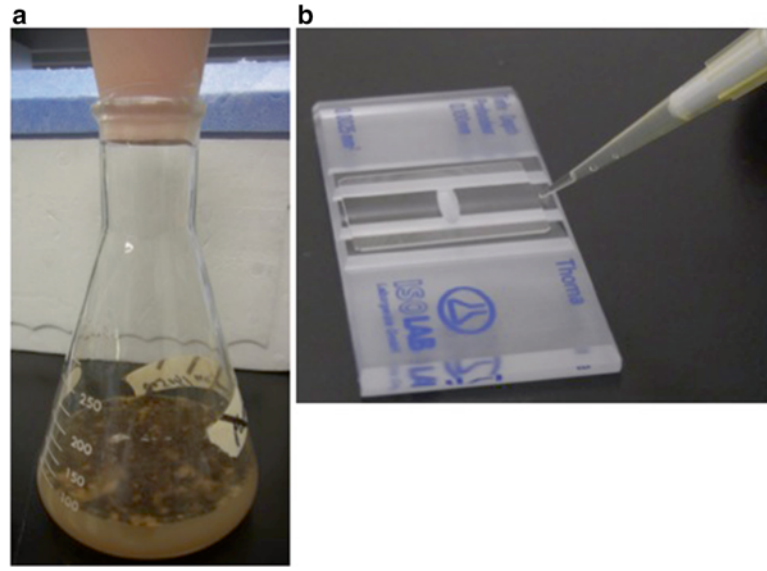

Fig. 1 Growth of *Colletotrichum higginsianum* and spore counting with an erythrocytometer. (**a**) The growth of *Colletotrichum higginsianum* (*C.h.*) in Mathur's medium after a 2-week incubation. *Black* in addition to *orange* mycelium can be seen. (**b**) Applying a *C.h.* spore suspension to an erythrocytometer

4. Centrifuge the Falcon tubes at $760 \times g$ for 3 min to precipitate the spore pellet.
5. After centrifugation, remove the supernatant by decanting or pipetting (depending on the size of the pellet).
6. Add 2 mL of DW to the tubes to resuspend the pellet.
7. Determine the spore concentration with an erythrocytometer (Fig. 1b). In case a high concentration is expected, make a dilution series (ranging from 1/10 to 1/50).
8. Repeat the measurement at least twice to minimize technical variations.

3.2 Inoculation for Lesion Measurements

1. Drop-inoculate 4-week-old plant leaves with 5 μL (2.5×10^5 spores/mL) of suspension (two spots per leaf) (*see* **Note 1**). As a positive control *pad3* plants, which showed much greater lesions on leaves upon *C.h.* infection than wild-type plants do [2], can be used.
2. Keep the inoculated plants in a container with high humidity for 4–5 days. Make sure to keep high humidity during the incubation so as not to let the droplets dry. Spraying water on the wall of a container before closing should be sufficient to keep high humidity.
3. Take pictures of the lesions with a ruler (Fig. 2).

Fig. 2 Lesion formation upon *C.h.* drop-inoculation. *C.h.* was drop-inoculated (two spots) on a Col-0 *Arabidopsis* leaf and then incubated for 5 days under high humidity

4. Measure the lesion diameter in both X and Y directions on the pictures by using a software such as ImageJ [9]. At least 40 lesions (20 leaves) per genotype are needed for proper data analysis.
5. Calculate the average for both X and Y directions.
6. Calculate the standard deviation (SD) or standard error (SE) from at least 40 replicates with statistics.
7. Repeat at least two additional independent experiments to confirm the conclusion.

3.3 Inoculation for Quantification of Living C.h. Biomass

1. Spray-inoculate 4-week-old plants with *C.h.* suspension at 1×10^5 spore/mL (*see* **Note 1**).
2. Incubate plants for 3.5 days with high humidity (*see* **Note 2**).
3. Collect five inoculated leaves per 2 mL tube, and prepare at least four tubes per genotype.
4. Extract RNA from the leaves according to the standard protocol and then subject RNA to DNase treatment (also important for eliminating DNA from dead hyphae).
5. Perform reverse transcriptase reactions using 500–1000 ng of total RNA.

6. Determine relative expression levels for *C.h.* actin normalized with a housekeeping gene (Plant ActinAB [6] or expressed protein [4]) by RT-qPCR. The use of several housekeeping genes might be preferable to confirm the results.
7. Calculate the average with SD or SE from at least four replicates from different plants.
8. Repeat at least two additional independent experiments to draw the conclusion (*see* **Note 3**).

3.4 C.h. Entry Rate Counting

1. Drop-inoculate 10-day-old cotyledons with 2 μL of *C.h.* suspension at 1×10^5 spore/mL (*see* **Note 4**).
2. Incubate plant material for 2.5 days with high humidity.
3. Measure the entry rate by normal light microscopy (*see* **Note 5**). The entry rate (%) is calculated using the following formula: Number of black melanized appressoria with formation of intracellular biotrophic hyphae/number of total black melanized appressoria. Include at least 100 appressoria per cotyledon and 5 cotyledons in the measurement (*see* **Note 5**).
4. Calculate the average with SD or SE from the entry rate from five cotyledons.
5. Repeat at least two different experiments to confirm the results.

4 Notes

1. An optimum *C.h.* spore concentration can change depending on the laboratory conditions. It is recommended to test several different spore concentrations to find a condition in which the lesion symptom becomes detected around 4 days post-infection (dpi).
2. In our conditions, *C.h.* form black melanized appressoria around 10 h post-infection (hpi), and form intracellular biotrophic hyphae from the appressoria around 2 dpi. Then, thin necrotrophic hyphae start to emerge around 3–3.5 dpi, which is followed by disease symptoms that become apparent typically around 4–5 dpi.
3. One of the major disadvantages of biomass measurements is that the results could be influenced by the degree of plant cell death progress upon *C.h.* infection. So it is crucial to harvest plant samples before the majority of the *C.h.* switch to the destructive necrotrophic phase, namely before discernible lesion formation.
4. For measuring the *C.h.* entry rate, the results tend to be highly variable with 4-week-old plant leaves. We thus recommend the use of 10-day-old seedlings for this purpose.

5. Intracellular hyphae are detected in the epidermal cells in a clearly different focal plane from that for the detection of the black melanized appressoria. It is convenient to first focus on black melanized appressoria and then to change the focus to observe intracellular biotrophic hyphae clearly.

References

1. O'Connell R, Herbert C, Sreenivasaprasad S, Khatib M, Esquerre-Tugaye MT, Dumas B (2004) A novel *Arabidopsis-Colletotrichum* pathosystem for the molecular dissection of plant-fungal interactions. Mol Plant Microbe Interact 17(3):272–282
2. Narusaka Y, Narusaka M, Park P, Kubo Y, Hirayama T, Seki M, Shiraishi T, Ishida J, Nakashima M, Enju A, Sakurai T, Satou M, Kobayashi M, Shinozaki K (2004) RCH1, a locus in *Arabidopsis* that confers resistance to the hemibiotrophic fungal pathogen *Colletotrichum higginsianum*. Mol Plant Microbe Interact 17(7):749–762
3. O'Connell RJ, Thon MR, Hacquard S, Amyotte SG, Kleemann J, Torres MF, Damm U, Buiate EA, Epstein L, Alkan N, Altmuller J, Alvarado-Balderrama L, Bauser CA, Becker C, Birren BW, Chen Z, Choi J, Crouch JA, Duvick JP, Farman MA, Gan P, Heiman D, Henrissat B, Howard RJ, Kabbage M, Koch C, Kracher B, Kubo Y, Law AD, Lebrun MH, Lee YH, Miyara I, Moore N, Neumann U, Nordstrom K, Panaccione DG, Panstruga R, Place M, Proctor RH, Prusky D, Rech G, Reinhardt R, Rollins JA, Rounsley S, Schardl CL, Schwartz DC, Shenoy N, Shirasu K, Sikhakolli UR, Stuber K, Sukno SA, Sweigard JA, Takano Y, Takahara H, Trail F, van der Does HC, Voll LM, Will I, Young S, Zeng Q, Zhang J, Zhou S, Dickman MB, Schulze-Lefert P, Loren V, van Themaat E, Ma LJ, Vaillancourt LJ (2012) Lifestyle transitions in plant pathogenic *Colletotrichum* fungi deciphered by genome and transcriptome analyses. Nat Genet 44(9):1060–1065
4. Ross A, Yamada K, Hiruma K, Yamashita-Yamada M, Lu X, Takano Y, Tsuda K, Saijo Y (2014) The *Arabidopsis* PEPR pathway couples local and systemic plant immunity. EMBO J 33(1):62–75
5. Narusaka M, Shirasu K, Noutoshi Y, Kubo Y, Shiraishi T, Iwabuchi M, Narusaka Y (2009) RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. Plant J 60(2):218–226
6. Hiruma K, Fukunaga S, Bednarek P, Pislewska-Bednarek M, Watanabe S, Narusaka Y, Shirasu K, Takano Y (2013) Glutathione and tryptophan metabolism are required for *Arabidopsis* immunity during the hypersensitive response to hemibiotrophs. Proc Natl Acad Sci U S A 110(23):9589–9594
7. Huser A, Takahara H, Schmalenbach W, O'Connell R (2009) Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis. Mol Plant Microbe Interact 22(2):143–156
8. Hiruma K, Nishiuchi T, Kato T, Bednarek P, Okuno T, Schulze-Lefert P, Takano Y (2011) *Arabidopsis* ENHANCED DISEASE RESISTANCE 1 is required for pathogen-induced expression of plant defensins in non-host resistance, and acts through interference of MYC2-mediated repressor function. Plant J 67(6):980–992
9. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Methods 9(7):671–675

Chapter 25

Tracing Plant Defense Responses in Roots upon MAMP/DAMP Treatment

Kei Hiruma and Yusuke Saijo

Abstract

This chapter describes how to apply microbe-associated molecular pattern (MAMP) or damage-associated molecular pattern (DAMP) solutions to *Arabidopsis* roots to trace defense responses in the root. Plants sense the presence of microbes via the perception of MAMPs or DAMPs by surface-localized pattern recognition receptors. The mechanisms governing plant root immunity are poorly characterized compared with those underlying plant immunity in the leaf, despite the fact that plant roots constantly interact with countless microbes living in soils that carry potential MAMPs and could stimulate the production of plant-derived DAMPs during colonization. To understand how a plant root immune system detects and reacts to the potential sources of a stimulus, we describe a simple method to monitor activation of root immunity upon MAMP/DAMP treatment by measuring relative expression of defense-related genes by RT-qPCR.

Key words *Arabidopsis* roots, DAMPs, Defense-related genes, MAMPs, Plant immunity

1 Introduction

Roots are constantly surrounded by a diversity of microbes carrying potential microbe-associated molecular patterns (MAMPs) that can activate plant immunity through their recognition by surface-localized pattern recognition receptors [1]. Recently it has been increasingly reported that plant endogenous elicitors generated upon perturbations of host cellular processes, termed damage-associated molecular patterns (DAMPs), also activate defense responses in similar ways to MAMPs and have been postulated as an amplifier for MAMP-triggered signaling [2, 3]. As most of the plant-inhabiting microbes are harmless and some of them are even beneficial to plants, plant roots may develop a sophisticated immune system to avoid its activation against these microbes, with a governing principle that is still poorly understood. To address this it is first necessary to analyze in detail MAMP/DAMP-triggered immunity in roots.

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In this chapter, we describe how to apply MAMPs/DAMPs to elicit defense responses in *Arabidopsis* roots. Briefly, sterilized *Arabidopsis* seeds are germinated and grown vertically in a 0.5 MS medium with sucrose. Seven-day-old seedlings are then transferred to new 0.5 MS medium (with a different nutrient content if necessary) and incubated for 2 more days. Nine days after germination the plant roots are subjected to MAMP or DAMP solutions. Finally, RT-qPCR analysis detects defense-related gene induction upon MAMP/DAMP treatment. In principle, this method can also be applied to inoculate root-inhabiting microbes on *Arabidopsis* roots to examine defense responses.

2 Materials

1. 0.5 MS plus Suc medium: half-strength MS, 25 mM sucrose.
2. 0.5 MS medium: half strength MS.
3. 70 % Ethanol.
4. 100 % Ethanol.
5. Flg22: 1 mM stock [4].
6. Chitin: 10 mg/mL stock [5].
7. Pep1: 1 mM stock [6].

3 Methods

3.1 Seed Sterilization

1. Add 1 mL of 70 % ethanol to 1.5- or 2-mL tubes containing *Arabidopsis* seeds.
2. Incubate for 10 min with gentle mixing.
3. Remove 70 % ethanol and add 100 % ethanol to rinse the seeds on a clean bench.
4. Remove 100 % ethanol and wash seeds at least three times with distilled water (DW).

3.2 Seed Sowing on 0.5 MS Plus Suc Medium

1. Sow seeds on 0.5 MS plus Suc agar plates.
2. Grow plants vertically for 7 days at 22 °C and 12-h light/12-h dark.
3. Transfer to a plate with new medium, such as sucrose-free 0.5 MS, and grow the seedlings for a further 2–3 days (*see* **Note 1**).

3.3 Application of MAMPs or DAMPs to Roots

1. Apply a few droplets of MAMP/DAMP solution (e.g., flg22, chitin at defined concentrations) onto the roots (Fig. 1). Keep the plates horizontally to prevent the spread of the MAMP/DAMP solution to the above ground parts of the seedlings (Fig. 2).

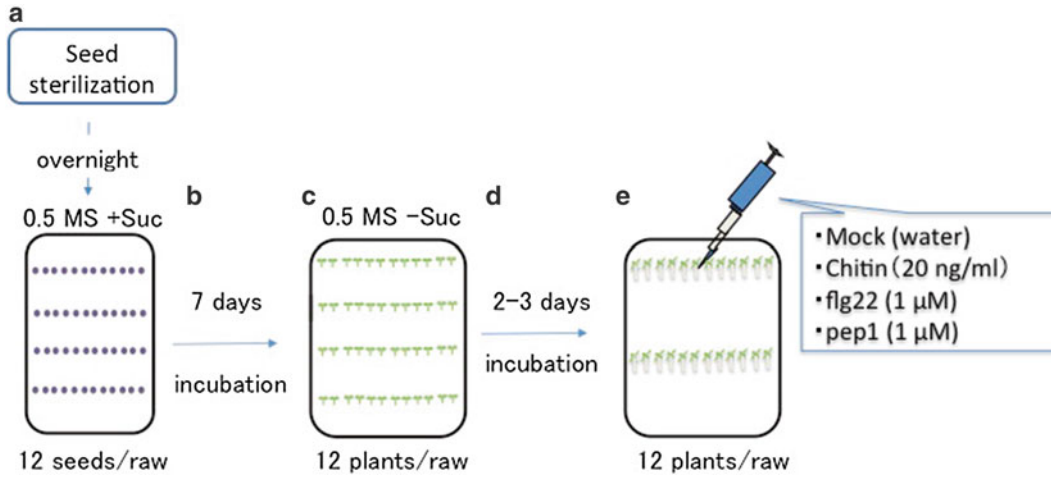

Fig. 1 Workflow for MAMP or DAMP treatment. (**a**) Seed sterilization with ethanol. (**b**) Incubation of plants on 0.5 MS plus 25 mM sucrose (0.5 MS + Suc) for 1 week. (**c**) Transfer 7-day-old plants to new medium, such as 0.5 MS without sucrose (0.5 MS – Suc). (**d**) Incubation of plants on sucrose-free 0.5 MS (0.5 MS – Suc) for 2–3 days. (**e**) Application of elicitor solutions to the roots

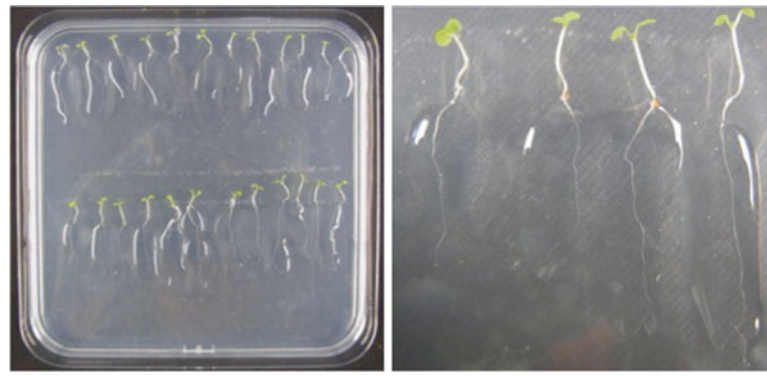

Fig. 2 *Arabidopsis* seedlings grown on plates for elicitor treatments on roots

2. Incubate the seedlings for the time periods to be tested. Make sure to keep the solution on the roots for at least the first few hours after application of the solution. It seems better to keep the roots wet with additional solution on roots during the incubation time.
3. Collect and freeze roots in 2-mL tubes with metal beads until use (metal beads are later used for homogenizing the samples).

3.4 Monitoring the Expression of Defense-Related Genes

1. Extract RNA in a standard reagent with RNase-free DNase. Frozen root samples should be completely homogenized by a crusher. Repeat this crushing step at least three times. Before adding an RNA extraction buffer, it is also important to keep

temperature in tubes as low as possible with liquid nitrogen to avoid RNA degradation. Normal RNA extraction methods or kits with DNase treatment are all applicable for extracting RNA.

2. Perform reverse transcriptase reactions using approximately 200 ng of total RNA. In case only a small amount of RNA is available, this step can be conducted with a minimum of 100 ng.
3. Measure relative expression levels for defense-related genes by RT-qPCR. We have shown that *CYP71A12* and *PROPEP3* are activated upon all the MAMP/DAMP treatments. For housekeeping genes, *ActinAB* [7] or an expressed protein [8] would be suitable.

4 Notes

1. Plant defense responses to MAMP/DAMP treatments could be influenced by the nutrient content. Therefore, use an appropriate nutrient medium that fits the purpose of your experiments. When roots are inoculated with root-inhabiting microbes the presence of sucrose often causes the overgrowth of the microbes in the medium. It is therefore preferable to use a nutrient medium without sucrose.

References

1. Boller T, Felix G (2009) A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol 60:379–406
2. Yamaguchi Y, Huffaker A (2011) Endogenous peptide elicitors in higher plants. Curr Opin Plant Biol 14(4):351–357
3. Fontana MF, Vance RE (2011) Two signal models in innate immunity. Immunol Rev 243(1):26–39
4. Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 5(6):1003–1011
5. Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc Natl Acad Sci U S A 104(49):19613–19618
6. Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA (2010) PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in *Arabidopsis*. Plant Cell 22(2):508–522
7. Hiruma K, Fukunaga S, Bednarek P, Pislewska-Bednarek M, Watanabe S, Narusaka Y, Shirasu K, Takano Y (2013) Glutathione and tryptophan metabolism are required for *Arabidopsis* immunity during the hypersensitive response to hemibiotrophs. Proc Natl Acad Sci U S A 110(23):9589–9594
8. Ross A, Yamada K, Hiruma K, Yamashita-Yamada M, Lu X, Takano Y, Tsuda K, Saijo Y (2014) The *Arabidopsis* PEPR pathway couples local and systemic plant immunity. EMBO J 33(1):62–75

Chapter 26

Analysis of the Immunity-Related Oxidative Bursts by a Luminol-Based Assay

Marco Trujillo

Abstract

The rapid production of reactive oxygen species (ROS) in response to biotic and abiotic cues is a conserved hallmark of plant responses. The detection and quantification of ROS generation during immune responses is an excellent readout to analyze signaling triggered by the perception of pathogens. The assay described here is easy to employ and versatile, allowing its use in a multitude of variations. For example, ROS production can be analyzed using different tissues including whole seedlings, roots, leaves, protoplasts, and cultured cells, which can originate from different ecotypes or mutants. Samples can be tested in combination with any ROS-inducing elicitors, such as the FLS2-activating peptide flg22, but also lipids or even abiotic stresses. Furthermore, early (PAMP-triggered) and late (effector-triggered) ROS production induced by virulent and avirulent bacteria, respectively, can also be assayed.

Key words Reactive oxygen species (ROS), NADPH oxidase, Immunity, flg22, *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*)

1 Introduction

The enhanced production of ROS is a hallmark response of plants to diverse stress stimuli. The production of ROS in response to pathogen attack, also known as the oxidative burst, was first described by Doke [1]. Doke described the production of superoxide ($O_2^{\bullet -}$) in potato tubers triggered by the infection of the oomycete *Phytophthora infestans* and also in potato tuber protoplasts in response to hyphal cell wall components [1, 2]. The generation of ROS has since been proposed to have various functions, such as antimicrobial activity, cross-linking of cell wall polymers at sites of attempted penetration by fungi, as a local signal to mediate stomatal closure, or as systemic secondary messengers to activate immune responses in distal tissues [3–5].

Early studies showed that the ROS production during an infection can be biphasic (Fig. 1). Virulent as well as avirulent pathogens trigger a general but relatively weak first oxidative burst that

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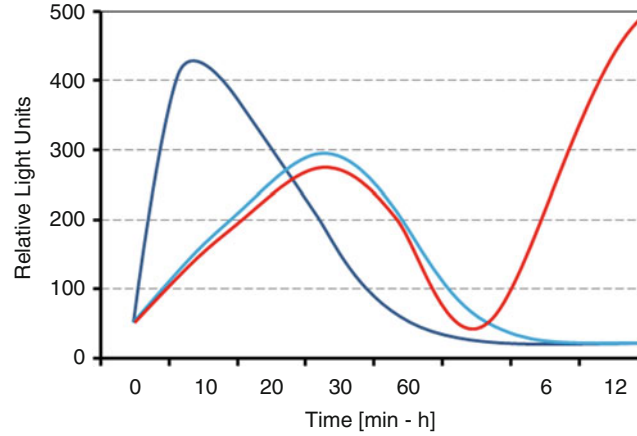

Fig. 1 ROS burst patterns during immune responses. The ROS production pattern differs depending on whether it is triggered by a purified minimal elicitor, such as flg22, or by pathogens. Leaf discs treated with flg22 react with a fast production of ROS which peaks at approximately 10 min after elicitation (*dark blue*). The response to virulent bacteria results in a weaker and delayed ROS generation, which peaks at approximately 30 min and is characteristic for the initiation PTI (*light blue*). The initial ROS burst of plants inoculated with avirulent bacteria is similar to that of virulent bacteria. By contrast, only avirulent bacteria activate a second massive production of ROS (*red*), which is characteristic for ETI

takes place within the first hour after infection. However, only avirulent pathogens trigger a second, massive, and prolonged oxidative burst, which is restricted to specific host cultivars and pathogen race combinations (Fig. 1) [4]. The early oxidative burst was first described as being a "general unspecific reaction." However, it has become clear that this reaction is the result of the perception of pathogen-associated molecular patterns (PAMPs) by plasma membrane located receptor-like kinases (RLKs). The best described example is the flagellin-sensitive 2 (FLS2) receptor of the bacterial flagellin [6, 7]. Activation of RLKs by the perception of PAMPs is the initial step in the activation of ROS production and PAMP-triggered immunity (PTI) [3, 5].

The second oxidative burst, on the other hand, is dependent on the recognition of pathogen virulence factors, so-called effectors, which are perceived directly or indirectly, by a resistance (R)-gene product, generally a nucleotide-binding leucine-rich repeat (NB-LRR) protein [8]. These intracellular sensors mediate the activation of effector-triggered immunity (ETI). The second stronger ROS production is a hallmark of the hypersensitive response (HR) which includes cell death [8].

Several sources for ROS production have been proposed or identified. However, it is important to differentiate between extracellular (apoplastic) and the intracellular generation of ROS [3]. In *Arabidopsis thaliana* (hereafter *Arabidopsis*), the apoplastic ROS

production triggered by RLKs such as FLS2 is completely dependent on the NADPH oxidase respiratory burst homologue (RBOH)D [9], but may also rely after NADPH oxidase activation on the cell wall peroxidases (PRX)33 and 34 [10]. NADPH oxidases catalyze the generation of superoxide, which can either spontaneously or enzymatically be converted into hydrogen peroxide (H_2O_2) by superoxide dismutases or peroxidases. By contrast, the second ROS burst triggered during ETI is likely to include sources such as chloroplasts, peroxidases, and mitochondria [3].

ROS production, or to be precise, hydrogen peroxide generation, can be quantified using luminol. Luminescence from luminol requires its activation by an oxidant which in this case is the indirect NADPH oxidase-dependent generation hydrogen peroxide and hydroxide ions. In the presence of the horseradish peroxidase, which acts as a catalyst, the hydrogen peroxide is decomposed to form oxygen and water. The reaction of luminol with the hydroxide ion results in the formation of a dianion. The oxygen released from the hydrogen peroxide subsequently reacts with the luminol dianion. As a result, an unstable organic peroxide is generated, electrons change from the excited to the ground state, and finally energy is emitted in the form of a photon which can be quantified by a luminescence detector.

2 Materials

2.1 Media for Plants and Bacteria

1. Medium for *Arabidopsis* seedlings: Half-strength Murashige and Skoog (MS) salts with vitamins, sucrose 0.5 % [w/v], MES 500 mg/l pH 5.6.
2. King's B medium for *Pseudomonas*: Add to 1 L distilled water, 10 g proteose peptone no. 2 (Difco), 1.5 g anhydrous K_2HPO_4, 15 g glycerol, 5 ml $MgSO_4$ (1 M stock, sterile), antibiotics (as needed). Add water to first three ingredients, adjust the pH to 7.0 with HCl, and bring volume to 1 L. Autoclave and then add 5 ml of sterile 1 M $MgSO_4$ and antibiotics (as needed).

2.2 Solutions and Chemicals

1. Reaction solution: Prepare a 500× horseradish peroxidase stock solution of 10 mg/ml in water. Make 50 µl aliquots and store at −20 °C. Prepare a 500× stock solution of 15 mg/ml luminol in DMSO and store at −20 °C. Example: To prepare 10 ml of a 20 µg/ml peroxidase and 30 µg/ml luminol reaction solution mix 20 µl of each of the stock solutions with 10 ml of water.
2. Peptide elicitors, such as flg22, are diluted from a 10 mM stock solution in water.

2.3 Equipment

1. Microtiter plate reader, e.g., Tecan Infinite F200 PRO fitted with a luminometer.

3 Methods

3.1 Plant Material

1. Stratify *Arabidopsis* seeds for 3 days at 4 °C and subsequently grow under short day conditions (8-h light/16-h dark), 65 % humidity, 21 °C, and 120–150 μmol/m^2 s.
2. To analyze leaves use adult plant material, sow *Arabidopsis* seeds directly onto soil and grow plants for 5–7 weeks. Use fully expanded leaves of the same age (*see* **Note 1**).
3. To analyze seedlings, sterilize approximately 100 *Arabidopsis* seeds with chlorine gas using 100 ml of commercially available bleach and add carefully 5 ml of 37 % HCl. Close the desiccator and sterilize for 3–4 h. Subsequently, ventilate seeds for 30 min and submerge in half-strength MS in an appropriate vessel such as a petri-dish or multiwall plate. After 10 days, pass two to three seedlings into a well from a 96-well plate, also containing half-strength MS such that they stay submerged, and grow for another 2 days before measurement.
4. To analyze roots, sterilize seeds as described above and grow seedlings vertically on solid half-strength MS medium for approximately 14 days.

3.2 Bacteria

1. Streak out the desired strain of bacteria, e.g., *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*, empty vector with Kan resistance) [11] from a glycerol stocks (stored at −80 °C) onto King's B medium agar plates containing 50 μg/ml kanamycin and 30 μg/ml rifampicin 1 day before the assay.
2. Incubate inoculated KMB plates for 24 h at 28 °C.
3. For elicitation, scrape bacteria from plates and wash twice in sterile water by centrifugating at 5000 × *g* for 10 min and resuspending the pellet.
4. Measure the OD_{600} employing spectrophotometer to quantify bacteria of a 1:10 dilution, to ensure measurement within the linear range.
5. The final bacterial solution can be adjusted to an OD_{600} of 0.1 (approx. 1×10^6 colony-forming units/ml). Ideal concentration of the bacterial suspension may need to be determined individually for different strains.

3.3 Sample Preparation

1. Leaves: Prepare leaf discs from fully expanded leaves (*see* **Note 2**) using a sharp biopsy puncher of 5 mm and swiftly place them with the abaxial side in contact with the water to avoid desiccation (critical, *see* **Note 3**). Leaf discs can additionally be sliced in half to increase the reaction area. Well plates of all sorts are suitable but 96-well plates allow more replicates.

2. Seedlings: 10 days after germination, pass two to three seedlings into a well from a 96-well plate, also containing half-strength MS such that they stay submerged. Allow to grow for another 2 days before measurement.
3. Roots: Prepare root tissue by cutting into in 0.5 cm long pieces using a new scalpel blade and place two to three pieces per well in a 96-well plate. Use the lower half of the roots and exclude the meristematic tissue area at the tip.

3.4 Measurement

1. Prepare samples as described above 1 day before assaying ROS production.
2. Float samples overnight at room temperature in 150 μl of sterilized tap water.
3. Immediately before starting the assay, prepare the reaction solution containing 20 μg/ml peroxidase and 30 μg/ml luminol.
4. Carefully remove the water in which the samples were incubated overnight without damaging sample tissue (critical).
5. Swiftly add 120 μl of the aqueous reaction solution using a multichannel pipet to avoid desiccation (critical) and incubate for 20 min at room temperature. Be careful not to stress the samples mechanically.
6. Add 120 μl of reaction solution containing double concentration of elicitor (e.g., peptide or bacteria) using a multichannel pipet and mix carefully (*see* **Note 4**).
7. Place samples in the microtiter plate reader or photon counting camera and wait for 1 min before starting to allow autofluorescence to cease.
8. Start monitoring luminescence (*see* **Note 5**).

4 Notes

1. The main drawback of the ROS assay is its variability. However, there are several steps that can be taken which will help to considerably increase reproducibility. Most important are the growth conditions; these need to be identical, especially light conditions. Use seeds harvested from plants grown simultaneously. It is imperative that plants do not have any sort of infection or wounding. Also, infestation with dark-winged fungus gnats, which produce larvae that will feed on leaves and roots, will result in considerable variability between samples.
2. We always obtained the most consistent results with plants older than 6 weeks and grown under short day. However,

attention must be paid to that there are no senescent leaves present on the plants.

3. The measured luminescence that is produced by the reaction of luminol with hydrogen peroxide in leaf discs originates from the edges resulting from cutting. Two points are pivotal: first, avoid desiccation of the edges and second, always use very sharp biopsy punchers which will reduce wounding responses.
4. Duration of the measurement is dependent on the elicitor, the bacteria strain, and the ROS burst type. Most elicitors which are perceived by RLKs, including flg22, elf18, but also chitin and oligogalacturonides, induce the production of ROS very quickly. Within 5–10 min, the ROS burst peaks and ebbs off in the next 50 min. However, when using virulent bacteria such as *Pst*, PAMP-dependent ROS production is triggered after 30–60 min and ebbs off within the next 30 min (Fig. 1) [12, 13]. By contrast, when measuring the oxidative burst which is triggered by the recognition of effectors by R-proteins such as NB-LRRs, ROS production starts 6–8 h after inoculation.
5. Luminol-based measurement of ROS production can be carried out with a variety of equipment that is able to detect luminescence. However, the bottleneck is always the sensitivity. Two settings may help to improve sensitivity: (1) increased binning when using a camera, although at the cost of a reduced resolution, and (2) increasing the integration time, which results in an increased dwelling time on each sample.

Acknowledgment

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References

1. Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. Physiol Plant Pathol 23:345–357
2. Doke N (1983) Generation of superoxide anion by potato tuber protoplasts during the hypersensitive response to hyphal cell wall components of Phytophthora infestans and specific inhibition of the reaction by suppressors of hypersensitivity. Physiol Plant Pathol 23:359–367
3. Wrzaczek M, Brosche M, Kangasjarvi J (2013) ROS signaling loops—production, perception, regulation. Curr Opin Plant Biol 16(5):575–582. doi:10.1016/j.pbi.2013.07.002
4. Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48:251–275
5. Kadota Y, Sklenar J, Derbyshire P, Stransfeld L, Asai S, Ntoukakis V, Jones JD, Shirasu K, Menke F, Jones A, Zipfel C (2014) Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. Mol Cell 54(1):43–55. doi:10.1016/j.molcel.2014.02.021
6. Gomez-Gomez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 5(6):1003–1011
7. Chinchilla D, Bauer Z, Regenass M, Boller T, Felix G (2006) The *Arabidopsis* receptor kinase

FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell 18(2): 465–476

8. Bent AF, Mackey D (2007) Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Annu Rev Phytopathol 45:399–436. doi:10.1146/annurev.phyto.45.062806.094427
9. Mersmann S, Bourdais G, Rietz S, Robatzek S (2010) Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. Plant Physiol 154(1):391–400. doi:10.1104/pp. 110.154567
10. Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP (2012) The apoplastic oxidative burst peroxidase in *Arabidopsis* is a major component of pattern-triggered immunity. Plant Cell 24(1):275–287. doi:10.1105/tpc.111.093039
11. Chen Z, Kloek AP, Boch J, Katagiri F, Kunkel BN (2000) The Pseudomonas syringae avrRpt2 gene product promotes pathogen virulence from inside plant cells. Mol Plant Microbe Interact 13(12):1312–1321. doi:10.1094/MPMI.2000.13.12.1312
12. Trujillo M, Ichimura K, Casais C, Shirasu K (2008) Negative regulation of PAMP-triggered immunity by an E3 ubiquitin ligase triplet in *Arabidopsis*. Curr Biol 18(18):1396–1401
13. Smith JM, Heese A (2014) Rapid bioassay to measure early reactive oxygen species production in *Arabidopsis* leave tissue in response to living Pseudomonas syringae. Plant Methods 10(1):6. doi:10.1186/1746-4811-10-6

Chapter 27

Quantitative Analysis of Microbe-Associated Molecular Pattern (MAMP)-Induced Ca^{2+} Transients in Plants

Fabian Trempel, Stefanie Ranf, Dierk Scheel, and Justin Lee

Abstract

Ca^{2+} is a secondary messenger involved in early signaling events triggered in response to a plethora of biotic and abiotic stimuli. In plants, environmental cues that induce cytosolic Ca^{2+} elevation include touch, reactive oxygen species, cold shock, and salt or osmotic stress. Furthermore, Ca^{2+} signaling has been implicated in early stages of plant–microbe interactions of both symbiotic and antagonistic nature. A long-standing hypothesis is that there is information encoded in the Ca^{2+} signals (so-called Ca^{2+} signatures) to enable plants to differentiate between these stimuli and to trigger the appropriate cellular response. Qualitative and quantitative measurements of Ca^{2+} signals are therefore needed to dissect the responses of plants to their environment. Luminescence produced by the Ca^{2+} probe aequorin upon Ca^{2+} binding is a widely used method for the detection of Ca^{2+} transients and other changes in Ca^{2+} concentrations in cells or organelles of plant cells. In this chapter, using microbe-associated molecular patterns (MAMPs), such as the bacterial-derived flg22 or elf18 peptides as stimuli, a protocol for the quantitative measurements of Ca^{2+} fluxes in apoaequorin-expressing seedlings of *Arabidopsis thaliana* in 96-well format is described.

Key words Ca^{2+} measurements, Aequorin, Microbe-associated molecular pattern (MAMP)

1 Introduction

Ca^{2+}fluxes are elements in the signal transduction of many stimuli that are perceived by plant cells. These biotic or abiotic signals may be of endogenous or exogenous origin. For instance, Ca^{2+} fluxes are induced by bacterial and fungal microbe-associated molecular patterns (e.g., flagellin, elongation factor-Tu, peptidoglycan, chitin), by plant peptide hormones and by symbiosis signals. Similarly, abiotic stimuli such as osmotic stress, salinity, oxidative stress, and mechanical cues trigger Ca^{2+} fluxes [1–6]. Ca^{2+} signals are decoded

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by the cells in which they are produced, but may also be involved in the transduction of systemic signals throughout the plant as for example in response to changes in salinity or herbivore attacks [7, 8]. These manifold functions and important roles place Ca^{2+} signaling in the centre of attention when studying the responses of plants to their environment.

A variety of probes can be employed to examine changes in intracellular Ca^{2+} concentrations. One of the most widely employed is aequorin, the luciferase from *Aequorea victoria*, which can be heterologously expressed in plants [9, 4]. Aequorin contains three EF-hand motifs that bind Ca^{2+} ions with high affinity, leading to conformational changes, which result in the oxidation and subsequent decarboxylation of the prosthetic group coelenterazine (CTZ). The resulting coelenteramide is present in an excited state and will emit light at a wavelength of 468 nm when relaxing to the ground state. In order to reconstitute the holoprotein, CTZ needs to be added to plants expressing apoaequorin. Due to its hydrophobic nature, it will freely diffuse into cells and bind to the apoprotein. Differently mutagenized versions of aequorin and chemical derivatives of coelenterazine are available, enabling researchers to probe Ca^{2+} fluxes spanning five orders of magnitude. However, not all CTZ-aequorin combinations are suitable and absolute changes in Ca^{2+} concentrations can only be calculated when the combination of native CTZ and native aequorin is used in experiments, due to the lack of standard curves for the other combinations [10]. Therefore, this protocol is restricted to the measurements performed with the original pMAQ2 lines engineered by Knight et al. [9] in conjunction with native CTZ.

In the present protocol, whole seedlings are used for measurements. Readers are advised that the aequorin measurements presented in this protocol do not permit spatial resolution but report a "global" Ca^{2+} response from the whole plant. Nevertheless, measurements performed in this way are sensitive to detect even small differences, as demonstrated by reactive oxygen species feedback to Ca^{2+} signaling [11] and is amenable to high throughput measurements in 96-well or even 384-well plate format [12]. Thus, aequorin-dependent Ca^{2+} transient measurement of whole seedlings is a powerful tool for the analysis of plants' reactions to environmental stimuli, such as MAMPs.

2 Materials

2.1 Plant Material

Seeds from *A. thaliana* pMAQ2 plants (Col-0 background, cytoplasmatic aequorin under control of CaMV 35S promoter) [9] or other lines expressing aequorin [13].

2.2 Seed Sterilization Components

1. Cell culture plates for plant culture: e.g., 24-well suspension culture plates (Greiner Bio-One, Frickenhausen, Germany).
2. Gas-permeable plate sealing membranes 80 × 140 mm: e.g., BREATHseal® (Greiner Bio-One) or breathe-EASIER® (Diversified Biotech, Dedham, Massachusetts, USA).
3. Filter paper (thin).
4. Plastic racks for cell culture plates.
5. Vacuum desiccator with socket, approximately 18.5 L volume, with fitting ceramic plate (Fig. 1).
6. Two gas-washing bottles, 1 L volume (Fig. 1).
7. Rubber tubing.
8. Vacuum pump.
9. Sodium hypochlorite solution (with 12 % available chlorine).
10. Hydrochloric acid, fuming, 37 %.

2.3 Plant Culture Components

1. Half-strength Murashige and Skoog (MS) medium, 0.25 % (w/v) sucrose: Weigh in 2.207 g of MS medium salt mixture (e.g., from Duchefa Biochemie, Haarlem, The Netherlands), 2.5 g of sucrose, and 0.1952 g of MES and dissolve in 900 mL ultrapure water. Adjust to pH 5.7 with potassium hydroxide (KOH). Adjust volume to 1 L and sterilize medium by autoclaving.
2. Gas-permeable adhesive tape: e.g., Leukopor® (BSN medical, catalog number 0248200).

2.4 Seedling Transfer and Reconstitution Components

1. Ultrapure water.
2. Pipetting reservoir.
3. Cushioned forceps (*see* **Note 1**) (Fig. 2a).
4. 200 µL pipette tips.
5. 96-well microplates for luminescence measurements (*see* **Note 2**).
6. Coelenterazine stock solution: CTZ stocks are prepared by dissolving solid coelenterazine in methanol to achieve a 10 mM stock solution. Different CTZ variants are available from Biosynth, PJK, Invitrogen, or Sigma (e.g., native CTZ, PJK GmbH, Kleinblittersdorf, Germany; CTZ-h, Biosynth, Staad, Switzerland). Store CTZ stock solutions in opaque (light protection) vessels at –20 °C for short time storage or –80 °C for long time storage.

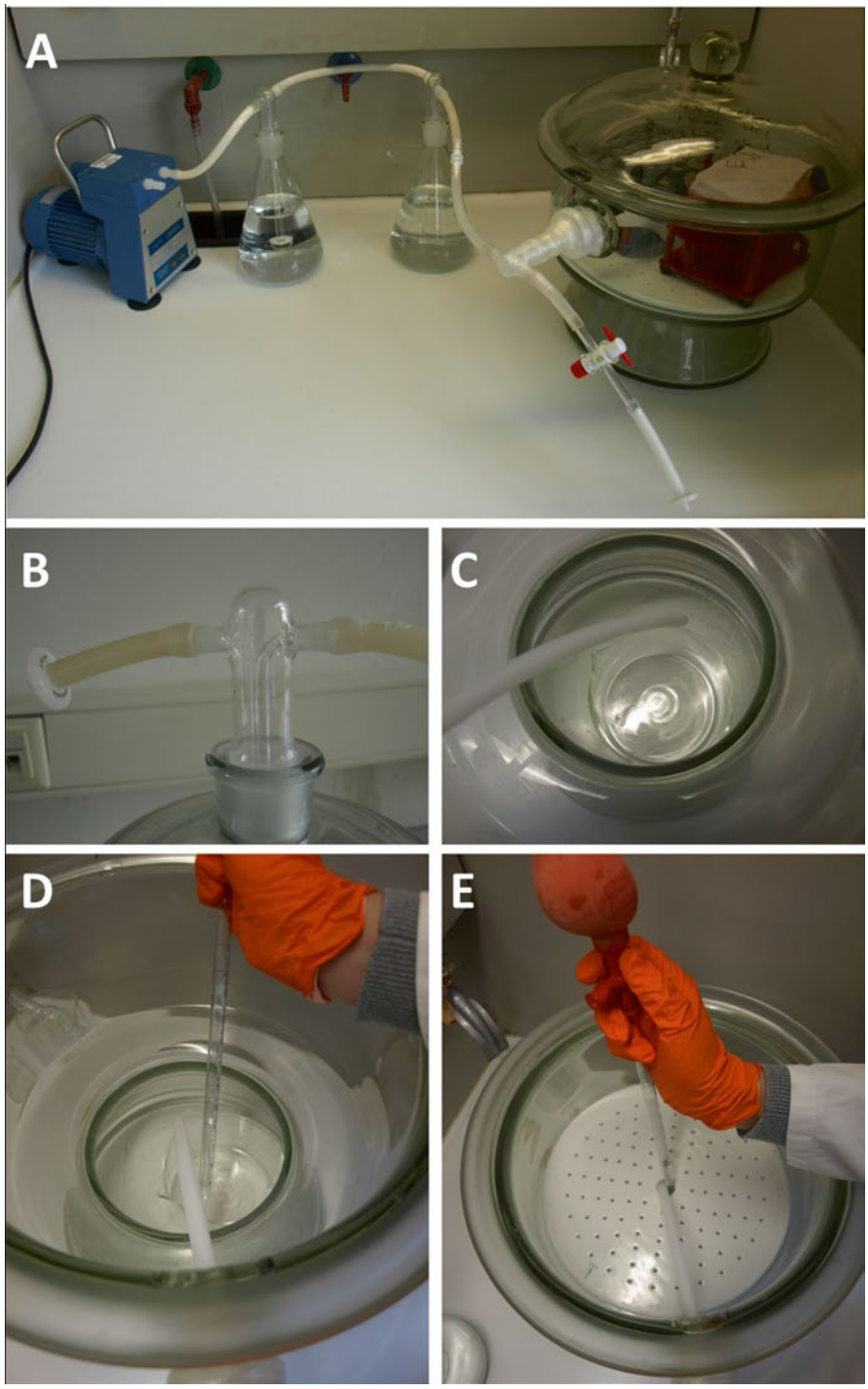

Fig. 1 Seed sterilization with chlorine gas. (**a**) Setup for gas-phase seed sterilization. (**b**) Air inlet/outlet for the desiccator can be fitted with any standard 0.2 μm filter to maintain sterility. Note this may be integrated in the covers of smaller desiccator (as shown here) or in the side of larger desiccators (as shown in **a**). (**c**) Placement of beaker in the bottom of the desiccator. Note position of outlet tubing next to beaker. (**d**) Pipetting of sodium hypochlorite solution with the ceramic plate removed. (**e**) Pipetting of HCl (into the beaker below) through central opening of ceramic plate

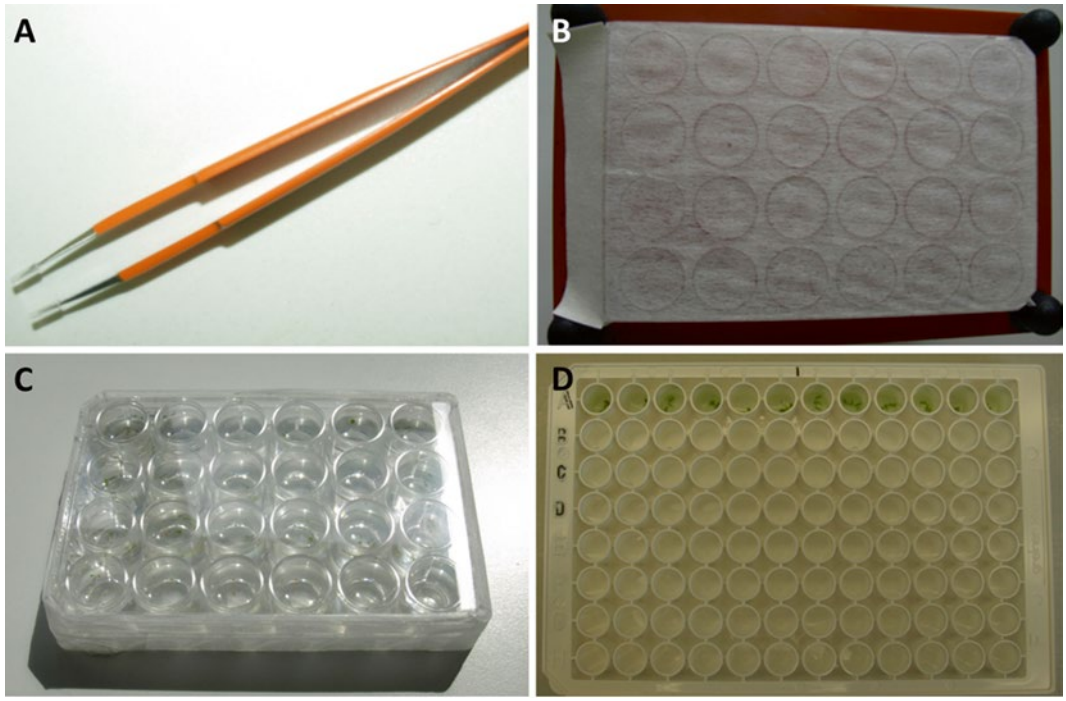

Fig. 2 Plant culture and seedling transfer materials. (**a**) Cushioned forceps. Note plastic pipette tips fitted on tips of forceps to reduce damage when handling delicate seedlings. (**b**) 24-well cell culture plate sealed with gas-permeable sealing membrane. (**c**) 24-well culture plate with germinating seedlings. Note gas-permeable tape was used to seal and reduce microbial contamination. (**d**) 96-well microplate with transferred seedlings

2.5 Ca^{2+} Measurement Components

1. MAMP stock solutions: AtPep-1, flg-22, and elf-18 synthetic peptides can be stored as 1 mM stock solutions in ultrapure water. Chitin from ground shrimp shells (Sigma-Aldrich, catalog number C9752) can be stored as 20 mg/mL in ultrapure water. All elicitor stock solutions should be stored as aliquots at −20 °C in vessels with low protein binding characteristics (e.g., Protein LoBind tubes®, Eppendorf, Hamburg, Germany). Prepare 3× concentrated working solutions from these stocks in ultrapure water immediately before use.
2. PCR 12-tube strips.
3. Discharge solution (2 M $CaCl_2$, 20 % (v/v) ethanol): Weigh in 294.04 g of $CaCl_2 \times 2H_2O$ and dissolve in 700 mL ultrapure water. Make up volume to 800 mL with ultrapure water and add 200 mL 100 % ethanol (p.a.).
4. Luminometer microplate reader with automatic reagent dispenser: e.g., Luminoskan® Ascent, Varioskan® Flash multimode reader (Thermo Fisher Scientific, Waltham, Massachusetts, USA), GloMax®-96 microplate Luminometer (Promega, Madison, Wisconsin, USA).

3 Methods

3.1 Seed Sterilization with Chlorine Gas

1. Under a fume hood, prepare the following setup (Fig. 1a): Connect the vacuum desiccator to two gas-washing bottles each filled with 800 mL of water and connect the whole setup to a vacuum pump. The outlet of the desiccator should be fashioned in a way so that chlorine gas with a higher density compared to air may be removed from the desiccator; i.e., a rubber tube should be fixed to the outlet that reaches to the bottom of the desiccator. This is to promote complete removal of chlorine gas with the pump. Furthermore, the influx of fresh air to the desiccator has to be enabled, so that there is no formation of vacuum while pumping out the chlorine gas (*see* **Note 3** and Fig. 1a, b).
2. Remove the lids of 24-well cell culture plates under sterile conditions under a laminar flow bench. The lids should be stored in the bench or any other sterile environment until ready to be reused in Subheading 3.2 below.
3. Load 15–20 seeds per well: Place some seeds on thin filter paper and slightly tap paper to load the seeds into the wells. Take care to avoid carryover of plant debris, as this will hamper sterilization and allow fungal/bacterial growth during liquid culture (*see* **Note 4**).
4. Seal plates with gas-permeable sealing membranes, load plates to racks.
5. Put a small beaker (at least 70 mL volume) into the bottom of the desiccator. Fill it with 45 mL of sodium hypochlorite (*see* **Note 5** and Fig. 1c, d). Put the ceramic plate into the desiccator so that cell culture plates can be positioned above the beaker. Thread the outlet tube through the central opening of the ceramic plate (Fig. 1e). Position the tube in a way so that it is next to the beaker with sodium hypochlorite (Fig. 1c–e).
6. Through the central opening of the ceramic plate, add 15 mL of 37 % hydrochloric acid into the beaker (Fig. 1e). Be careful, the reaction starts immediately! Quickly put racks with cell culture plates into the desiccator and close it. Close the fume hood and sterilize for 3–4 h.
7. After sterilization, start the pump to remove chlorine gas (Fig. 1a). Let it run for at least 3 h to remove most of the chlorine gas. Keep the fume hood closed during pumping.
8. Stop the pump and open the desiccator. Let it stand open overnight under the closed fume hood to let remaining chlorine gas dissipate.

9. Remove plates. Discard solution in beaker and the water from the gas-washing bottles according to your local waste disposal regulations.

3.2 Liquid Culture

1. Under a laminar flow bench, remove sealing membranes (Fig. 2b) and discard.
2. Add 2 mL of half-strength MS, 0.25 % sucrose medium to each well.
3. Put lids onto plates and secure in place with gas-permeable adhesive tape (Fig. 2c).
4. Incubate plates at 4 °C in the dark for at least 2 days to stratify seeds.
5. Put plates into growth cabinet under long day conditions (16 h light), 21 °C for 8–10 days.

3.3 Transfer of Seedlings and Holoaequorin Reconstitution

1. With a multichannel pipette, add 75 µL of ultrapure water to each well of a 96-well plate.
2. Transfer the seedlings one by one into the 96-well plate (*see* **Note 6**, Fig. 2d) and make sure they are completely (including the roots) covered with water.
3. Prepare and load CTZ working solution (3 mL of CTZ working solution are required per 96-well plate, including spare volume). To prepare 3 mL of 40 µM CTZ working solution, add 3 mL of ultrapure water to a pipetting reservoir. While slightly tilting the reservoir to collect the water on one side, add 12 µL of 10 mM CTZ stock solution and mix immediately by slightly shaking the reservoir. Directly transfer 25 µL of this mix to each well of the microplate with a multichannel pipette (hence, the end concentration of CTZ we routinely use is 10 µM, *see* **Note 7**). Cover the plate and incubate in the dark at ambient temperature for 4–12 h (*see* **Note 7**).

3.4 Ca^{2+} Measurements in 96-Well Plates with Manual Elicitor Injection

1. Prepare elicitor working solutions: Dilute thawed stock solutions to achieve 3× concentrated working solutions in ultrapure water. Equilibrate to room temperature to avoid "cold shock" reaction when adding to seedlings. Peptide elicitors are usually used at final working concentrations of 100 nM to 1 µM, whereas shrimp shell chitin is used at a concentration of 50–200 µg/mL.
2. Switch on plate reader and computer, open the software and select the area to be measured (*see* **Note 8**).
3. Fill a 50-mL vessel (e.g., a Greiner tube) with discharge solution and place in vessel carrier, put aspirate tubing into vessel. Place dispenser head into external waste container and prime

the dispenser with 1500 µL discharge solution. Gently wipe the dispenser head, remove blind plug, and inject into correct dispenser port (*see* **Notes 9** and **10**). Depending on your machine, make sure that the dispenser height is adjusted correctly to the height of the microplate you are using; otherwise the machine may be severely damaged (*see* **Note 11**).

4. Pipet 60 µL of MAMP working solutions into single tubes of a PCR 12-tube strip. Thus, a 12-channel pipette can be used to conveniently load different elicitors at any desired combination while minimizing dead volume (*see* **Note 12**).
5. Put the microplate into the plate reader. Remove the lid; otherwise, some machines may be severely damaged! You may want to seal the remaining wells not chosen for measurements with tape to avoid evaporation of liquid.
6. Start the measurement. Perform at least ten measurements per well at 6-s intervals with 300 ms measuring time to record background luminescence. Let the machine eject the plate. Add 50 µL of your MAMP working solution to each well of a row with a multichannel pipette and immediately resume the measurement. Perform 300 measurements per well at 6-s intervals with 300 ms measuring time. This covers a 30 min (300 × 6 s) overall measurement time per sample; other desired time periods can be achieved by adjusting the number of measurements per well. After elicitor measurement, program the machine to inject 150 µL of discharge solution and measure the discharge well by well in "monitor" mode (continuous measurement) for 1–2 min. Perform the measurement of the whole plate in this way row by row (*see* **Notes 9** and **13**).
7. After the run, take out the microplate. Remove the dispenser head from the port and place it into an external waste container. Close the port with the blind plug. Rinse aspirate tubing with distilled water and put it into a vessel with distilled water. Flush the pump, tubing, and dispenser with at least 50 mL of distilled water (*see* **Note 10**). Finally, place aspirate tubing in 20 % ethanol and fill tubing with ethanol. Dry the dispenser head carefully and place it into the dispensing head holder. Switch off the machine.

3.5 Data Analysis

Analysis of data acquired in Ca^{2+} measurements is conveniently achieved with the FlagScreen script for R available on the homepage of the Leibniz Institute of Plant Biochemistry (http://www.ipb-halle.de/datenbanken/flagscreen/) [12]. Basically, it offers two modes of analysis. The program can be used to plot the rate k (or L/L_{max}), which is the ratio of luminescence counts per second at a given measurement point i (Li), and the sum of the total remaining counts from point $i+1$ including the end of the

measurement and discharge (L_{max}) (Fig. 3a). For experimental setups performed with measurement intervals other than 1 s, k needs to be corrected by multiplying L_{max} with the measuring interval. In the case of the current protocol, L_{max} has to be multiplied with the 6 s interval applied in the measurements. Therefore, for the protocol at hand, k is calculated in the following way:

$$k_i = L_i \Big/ 6 \times \left(\sum_{i+1}^{n} L \right)$$

Alternatively, the program can be used to calculate the Ca^{2+} concentration (Fig. 3a) directly. However, it has to be noted that until now, a calibration curve for the calculation of Ca^{2+} concentrations has only been developed for native coelenterazine used with native aequorin [10]. Therefore, values of Ca^{2+} concentrations calculated in the fashion described above will only be meaningful if the correct aequorin-CTZ combination has been used in the measurements.

The calculation of the Ca^{2+} concentration is performed as described by Rentel and Knight [14]:

$$p\mathrm{Ca} = 0.332588(-\log k_i) + 5.5593$$

We recommend measuring multiple seedlings to obtain the average Ca^{2+} concentrations. Values calculated in this way can be plotted conveniently by using the FlagScreen script or in any spreadsheet calculation software. Thus, the method described here can reveal distinct cytosolic Ca^{2+} signatures induced by various microbe-derived or damage-derived elicitor signals (Fig. 3b). Alternatively, when the apoaequorin transgene is introduced into selected mutant backgrounds, it can reveal the contribution of the mutated genetic component in Ca^{2+} signaling. For instance, reactive oxygen species produced through the respiratory burst oxidase homolog D, RBOHD, is required for the second peak of the flg22-induced Ca^{2+} signature (Fig. 3c).

4 Notes

1. To prepare cushioned forceps, take a pair of style 5 forceps (e.g., from Rubis® or A. Dumont & Fils®) and put the cut points of 1–10 μL pipette tips (preferably long tips; e.g., Gel Saver II tips from Kisker Biotech®) onto the tips of the tweezers. Carefully tap the forceps points down onto a hard surface in order to secure the pipette tips in place. Cushioned forceps reduce damage inflicted from handling of the seedlings (Fig. 2a).

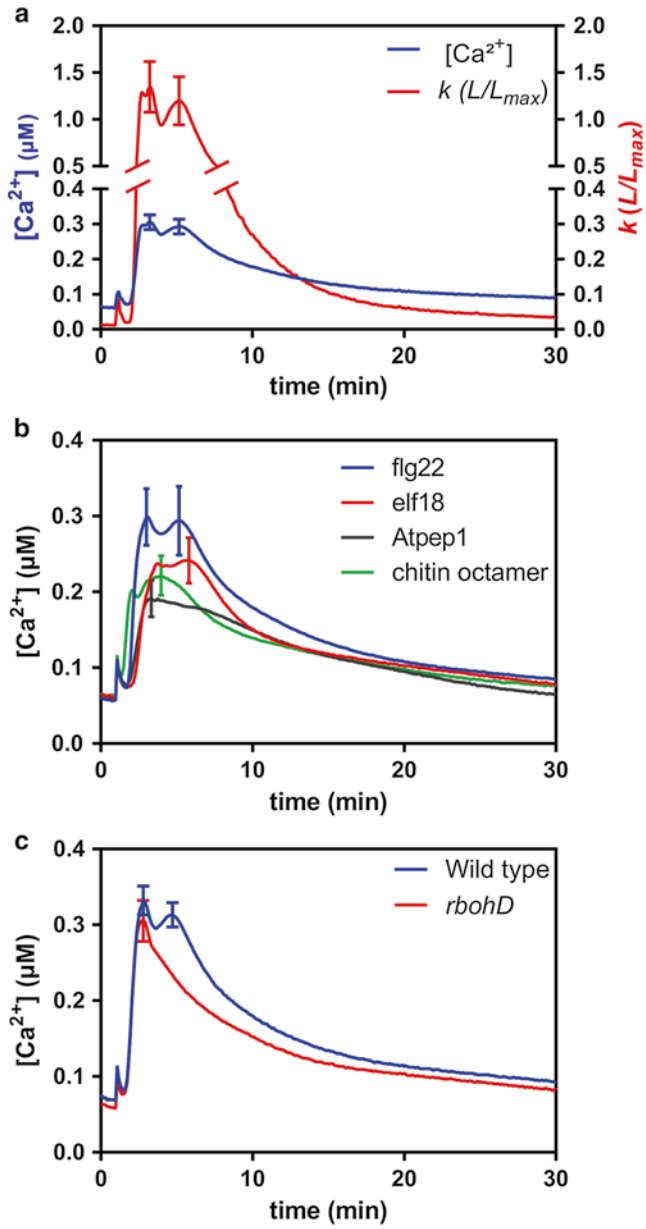

Fig. 3 Calcium transients recorded with pMAQ2 lines. (**a**) Calcium transients can be plotted as the rate constant k (i.e., L/L_{max}) or, if calibration curves are available, as actual cellular calcium concentrations [Ca^{2+}]. (**b**) Different elicitors generate distinct calcium signatures in pMAQ2 seedlings. All measurements were performed with 1 µM of the indicated elicitors. (**c**) Feedback of ROS on Ca^{2+} signature can be analyzed with calcium measurements. Flg22-induced calcium transients were compared between wild type (pMAQ2) and *rbohD* (*respiratory burst oxidase homolog D*) mutant seedlings. For further information on measurements performed in (**b**) and (**c**), *see* [11, 13]. Measurements in (**a**) and (**c**) were performed with 1 µM flg22, $n \geq 25$. Error bars represent 2× SE

2. Generally, all microplates specified for luminescence measurements can be used but low protein binding properties are desirable to reduce elicitor sequestration. We recommend Lumitrac® 200 medium binding F-bottom chimney well 96-well white plates (Greiner Bio-One). While half-area microplates can be used, seedling transfer is easier with full-area plates. All volumes cited in the Ca^{2+} measurement protocol are calculated for full-area plates and will have to be adjusted accordingly when using half-area plates.
3. Chlorine gas sterilization is a simple and convenient way to sterilize seeds directly inside the vessel in which they will be cultured. Cell culture plates with sterilized seeds that have been sealed with gas-permeable sealing membranes can also be stored for some time. However, due to the strongly oxidizing properties of chlorine gas, precautions need to be taken to avoid injuries. Sterilization is therefore performed in a desiccator under a fume hood and the chlorine gas is removed before the desiccator is being opened. Proper protective clothing has to be worn when performing seed sterilization.
4. Seeds should be already pre-sieved to remove most of the siliques and other plant debris. Using blotting paper for seed transfer helps reducing husk in the culture. If husk falls into a well of the culture plate, it can be easily removed with a pipette tip. Do not load too many seeds, as this will hamper sterilization success and favor fungal growth. Use well-dried seeds. Freshly harvested seeds can be loaded into the 24-well plates, sealed with gas-permeable seal and dried overnight at 37–50 °C. If seeds are not dry, HCl will develop upon contact with chlorine gas on the seed surface and damage seeds.
5. The amount of hypochlorite and HCl needed depends on the size of the desiccator and may have to be adjusted accordingly. Generally, the ratio is 3 parts hypochlorite and 1 part HCl. Hypochlorite needs to be in slight excess, as otherwise HCl vapor will damage the seeds. Be sure to let the chlorine gas dissipate completely overnight under a fume hood as chlorine traces sticking on the plastic will acidify the culture medium and affect seedling growth.
6. Seedlings have to be transferred in a healthy state to the microplates they will be measured in. Therefore, infliction of stress by handling should be minimized. In order to reduce possible bacterial contaminations, seedlings should be transferred under a laminar flow bench. When loading seedlings to microplates it is advisable to handle the seedlings with cushioned tweezers in one hand and untangling the roots with a yellow pipette tip (200 µL) in the other hand. Seedlings can be gripped carefully at the hypocotyl. Seedlings are thus trans-

ferred into microplates one by one. Generally, seedlings of the same size should be chosen for analysis, although normalization during data analysis will compensate signal variations, which are based on different seedling sizes. For best results, seedlings that have their first rosette leaves just emerging should be chosen.

7. Holoprotein reconstitution can be performed with different amounts of CTZ. The final concentration for reconstitution should be between 2 and 20 μM. In experimental setups with manual elicitor injections it is usually desirable to perform the holoprotein reconstitution overnight before the measurement. In setups with automated injection, the reconstitution can be performed for 4–6 h and measurement can be performed overnight. Keep in mind that CTZ is not stable for prolonged periods of time in the presence of oxygen and reconstituted aequorin will be constantly consumed. Therefore, reconstitution should not be prolonged excessively.
8. For measurements with manual injection of elicitors, it is best to perform measurements row by row. Thus, the time which elapses for each row between elicitor injection and resumption of measurements is the same for each seedling. Furthermore, the time which elapses between elicitor treatment and discharge is reduced, which is favorable for accuracy. In setups with automatic elicitor injection, 2 rows (24 wells) can generally be measured at the same time. When more rows are measured at the same time, the measurement interval has to be changed accordingly at the expense of accuracy. Whereas background and transient measurements can be performed in short intervals, the discharge response is so fast that the peak can easily be missed by interval measurements. However, the peak height is essential for the normalization procedure during data analysis. Therefore, discharge measurements should be performed one well at a time in one continuous measurement.
9. Due to the speed of the discharge response (*see* **Note 8**), the dispenser injecting the discharge solution should be inserted into the port directly beneath the measuring position of the microplate reader. Thus, the measurement can start at the moment of injection without the necessity of an additional moving step. In Luminoskan® instruments, this is port M, whereas in Varioskan® instruments, both L1 and L2 can be employed, depending on which dispenser is used. Consult the manual of your machine to find out which port to use.
10. Discharge solution has a low surface tension due to the high ethanol content. Therefore, drops of solution may spill from the dispenser head at times. Regrettably, $CaCl_2$ causes metallic

corrosion and can therefore cause damage in the machine. In order to avoid this, the instrument should therefore be checked and cleaned at regular intervals and possible spills removed immediately.

11. All safety guidelines applying for your instrument must be followed. Make sure that the plate layout fits the microplate you are using. Make sure you prime the dispensers before starting a measurement with the specified volume of solution. Make sure dispensers and tubing are certified for the solutions you are using.
12. Peptide MAMPs are extremely sticky and containers for MAMPs should not be reused for another MAMP. Ca^{2+} transients in plants may be triggered at very low concentrations. Therefore, precautions should be taken not to contaminate the elicitor solutions: As an example, pipetting from (narrow) 15-mL reaction vessels can lead to the contamination of the neck of the pipette, causing carryover of elicitors into other tubes. This may result in the misinterpretation of Ca^{2+} transients.
13. Luminescence as a function of Ca^{2+} influx can easily be measured in plate readers. Generally, the measurement can be divided into three phases: The background measurement, the MAMP treatment with measurement of the Ca^{2+} transient, and the discharge step, in which all aequorin is released to determine its total amount for subsequent data normalization. The whole measurement can be automated when plate readers with two or more dispensers are available to inject the elicitor and discharge solution. For reasons of time constraints, background and discharge measurements may have to be shortened in automated measurement setups.

References

1. Dodd AN, Kudla J, Sanders D (2010) The language of calcium signalling. Annu Rev Plant Biol 61:593–620. doi:10.1146/annurev-arplant-070109-104628
2. Haruta M, Sabat G, Stecker K, Minkoff BB, Sussman MR (2014) A peptide hormone and its receptor protein kinase regulate plant cell expansion. Science 343(6169):408–411. doi:10.1126/science.1244454
3. Kudla J, Batistic O, Hashimoto K (2010) Calcium signals: the lead currency of plant information processing. Plant Cell 22:541–563. doi:10.1105/tpc.109.072686
4. Mithofer A, Mazars C (2002) Aequorin-based measurements of intracellular Ca^{2+}-signatures in plant cells. Biol Proced Online 4:105–118. doi:10.1251/bpo40
5. Sanders D, Brownlee C, Harper JF (1999) Communicating with calcium. Plant Cell 11(4):691–706
6. Seybold H, Trempel F, Ranf S, Scheel D, Romeis T, Lee J (2014) Ca^{2+} signalling in plant immune response: from pattern recognition receptors to Ca^{2+} decoding mechanisms. New Phytol 204:782–790. doi:10.1111/nph.13031
7. Choi WG, Toyota M, Kim SH, Hilleary R, Gilroy S (2014) Salt stress-induced Ca^{2+} waves are associated with rapid, long-distance root-to-shoot signaling in plants. Proc Natl Acad Sci U S A 111(17):6497–6502. doi:10.1073/pnas.1319955111
8. Mousavi SA, Chauvin A, Pascaud F, Kellenberger S, Farmer EE (2013) GLUTAMATE RECEPTOR-LIKE genes

mediate leaf-to-leaf wound signalling. Nature 500(7463):422–426. doi:10.1038/nature12478
9. Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature 352(6335):524–526. doi:10.1038/352524a0
10. Bonora M, Giorgi C, Bononi A, Marchi S, Patergnani S, Rimessi A, Rizzuto R, Pinton P (2013) Subcellular calcium measurements in mammalian cells using jellyfish photoprotein aequorin-based probes. Nat Protoc 8(11):2105–2118. doi:10.1038/nprot.2013.127
11. Ranf S, Eschen-Lippold L, Pecher P, Lee J, Scheel D (2011) Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. Plant J 68(1):100–113. doi:10.1111/j.1365-313X.2011.04671.x
12. Ranf S, Grimmer J, Pöschl Y, Pecher P, Chinchilla D, Scheel D, Lee J (2012) Defense-related calcium signaling mutants uncovered via a quantitative high-throughput screen in *Arabidopsis thaliana*. Mol Plant 5(1):115–130. doi:10.1093/mp/ssr064
13. Mehlmer N, Parvin N, Hurst CH, Knight MR, Teige M, Vothknecht UC (2012) A toolset of aequorin expression vectors for in planta studies of subcellular calcium concentrations in *Arabidopsis thaliana*. J Exp Bot 63(4):1751–1761. doi:10.1093/jxb/err406
14. Rentel MC, Knight MR (2004) Oxidative stress-induced calcium signaling in *Arabidopsis*. Plant Physiol 135(3):1471–1479. doi:10.1104/pp. 104.042663

Chapter 28

Rapid Assessment of DNA Methylation Changes in Response to Salicylic Acid by Chop-qPCR

Stephanie Rausch and Sascha Laubinger

Abstract

Methylation of cytosines plays an important role in epigenetic regulation of gene expression. Several methods exist to determine the methylation status of DNA. Here, we describe a rapid and cost-effective method called Chop-qPCR to determine dynamic changes in the DNA methylation patterns, as they occur for instance in response to environmental stresses.

Key words Chop-qPCR, DNA methylation, Methylation-dependent restriction enzyme, McrBC, Epigenetics, Biotic stress, *Arabidopsis thaliana*

1 Introduction

Several epigenetic mechanisms define DNA accessibility and therefore transcriptional activity. One of these epigenetic layers of gene regulation is the methylation of cytosine to 5-methylcytosine. This direct covalent modification of DNA is evolutionarily ancient and is present in prokaryotic and eukaryotic organisms, where it is strongly associated with gene silencing [1].

In contrast to animals, where DNA methylation is mostly found in a CpG context, plant genomic DNA exhibits methylation also in CpHpG and CpHpH contexts (where H is any base except G). In the model plant *Arabidopsis thaliana*, three main types of DNA methyltransferases are known to catalyze DNA methylation in CpG, CpHpG, and CpHpH contexts, respectively. The enzyme METHYLTRANSFERASE 1 (MET1) is responsible for CpG methylation and functions in the maintenance of cytosine methylation [2]. A plant-specific enzyme, called CHROMOMETHYLASE 3 (CMT3), is responsible for DNA methylation in CpHpG context. CpHpH methylation is performed by DOMAINS REARRANGED METHYLASE 2 (DRM2). DRM2 is recruited to the DNA via the RNA-directed DNA methylation pathway

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(RdDM) leading to methylation of the targeted DNA sequence [3]. In *Arabidopsis*, also four DNA demethylases are known (DEMETER (DME), REPRESSOR OF GENE SILENCING 1 (ROS1), DEMETER-LIKE 1 (DML1), and DML2), which remove methylated cytosine by a base-pair excision pathway [4–6].

DNA methylation ensures transcriptional repression of transposable elements and repeats [7, 8], it affects gene expression [1], it is important for the selection of polyadenylation signals [9, 10], and it plays an important role in genomic imprinting during early plant development [2]. DNA methylation has been reported to be regulated dynamically. For example, it is involved in dynamic defense regulation against abiotic and biotic stresses [6, 11, 12].

Different methods were developed to determine the methylation status of the DNA of interest: (1) bisulfite sequencing, (2) affinity enrichment and (3) chop-qPCR [13]. For bisulfite sequencing, DNA is desaminated by adding sodium bisulfite to convert all unmethylated cytosines into uracils, which appear as thymines in later PCR application. Methylated cytosines are protected against deamination, which allows distinguishing between methylated and unmethylated cytosines. Bisulfite treatment can be combined with PCR analysis to determine the methylation status of a specific region of interest. Alternatively, bisulfite treated DNA can be sequenced by e.g., Illumina® technology, providing entire methylome maps of a genome at a single-base resolution. Bisulfite sequencing is still time consuming and relatively expensive, especially when interested in monitoring methylation changes in a huge number of samples.

Another possibility to analyze DNA methylation is to concentrate methylated DNA fragments by using methyl-binding proteins or 5-methylcytosine-specific antibodies. After increasing the amount of methylated DNA, DNA can be analyzed by next-generation sequencing approaches. Data obtained by affinity enrichment provides rapid, genome-wide information but do not contain any information about the methylation status of individual cytosines.

In contrast to bisulfite sequencing and affinity enrichment, Chop-qPCR is a relatively easy to perform and an inexpensive method to determine the degree of methylation in a genomic region of interest. Briefly, for Chop-qPCR DNA is treated with methylation-sensitive restriction enzymes and analyzed with quantitative real-time PCR (qPCR). With Chop-qPCR, one is able to assess DNA methylation levels of a specific genomic region of interest, and its changes during for example exposure to stress. Chop-qPCR does not provide genome-wide information and cannot report on the methylation status of individual cytosine (except if one uses specific restriction enzymes). All methods described here have advantages and disadvantages and one has to consider

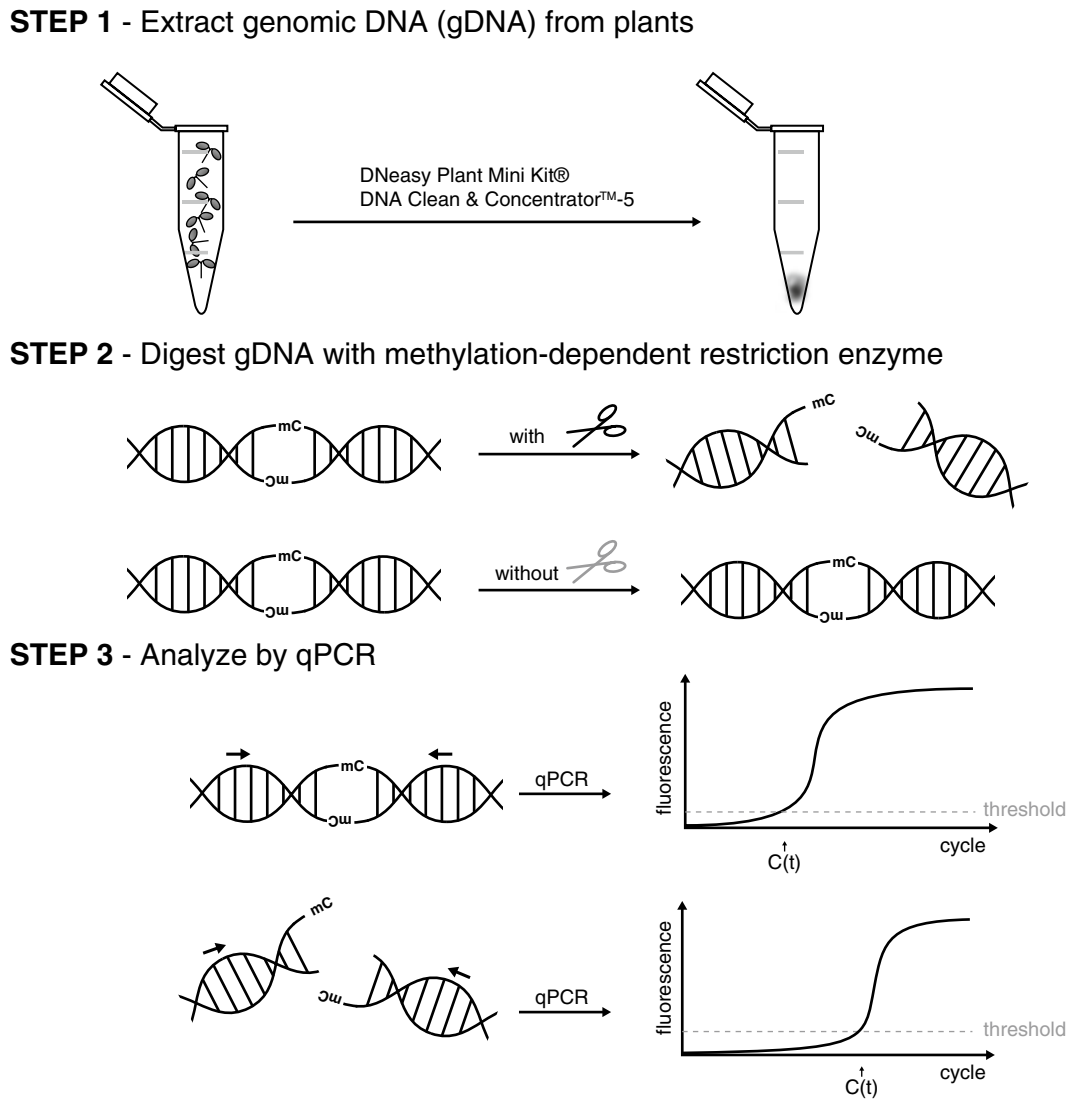

Fig. 1 An overview of the different steps of DNA methylation analysis by Chop-qPCR

individually which experimental setup suits best the biological question.

This protocol describes how to perform Chop-qPCR and is essentially divided into three steps: (1) extraction of genomic DNA (gDNA), (2) methylation-dependent restriction digest, and (3) quantitative PCR (qPCR) (Fig. 1).

In short, gDNA is extracted from different samples, cleaned and concentrated. Afterwards, the gDNA is digested with the methylation-dependent restriction enzyme McrBC, which leads to the removal of all DNA fragments containing methylated cytosines (*see* **Note 1**). The herein used McrBC is a methylation-dependent endonuclease that unspecifically cuts fully and semi-methylated

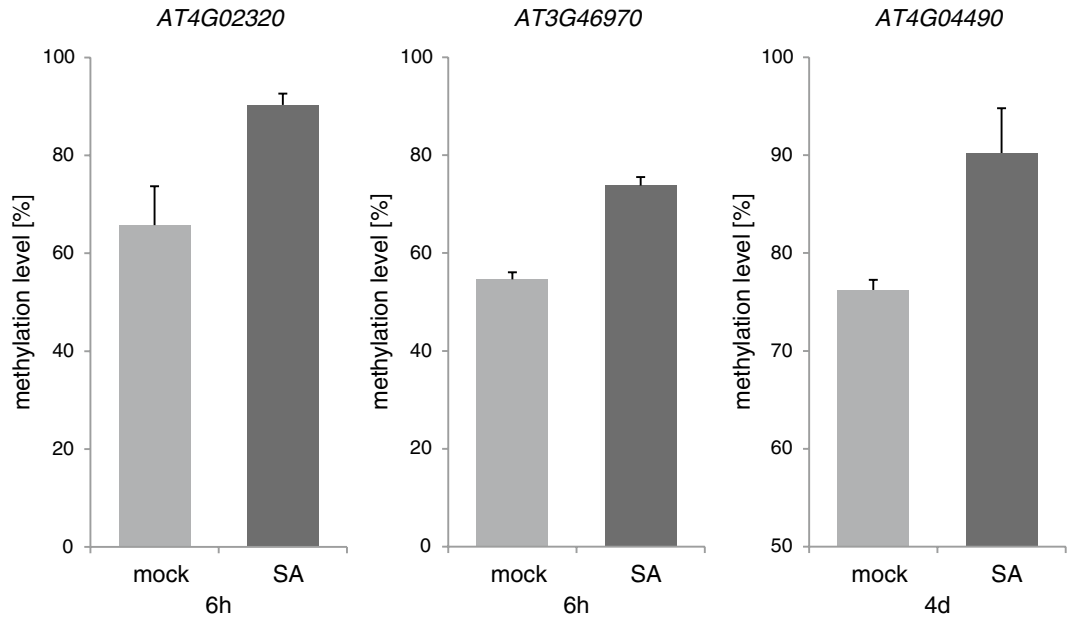

Fig. 2 Chop-qPCR analysis reveals changes in DNA methylation in three different genes after SA treatment. Error bars indicate the standard error of the mean of three biological replicates

doubled-stranded DNA. Mock- and McrBC-treated DNA fragments are used as a template for qPCR, so one is able to determine the methylation level quantitatively. By comparing mock- and McrBC-treated samples via qPCR, the relative DNA methylation status can be determined. As an example for Chop-PCR, we quantified the methylation level of three genes, which were reported to be differentially methylated after treatment with salicylic acid [11] (Fig. 2).

2 Material

2.1 Plant Material

Seeds were sterilized by washing three times in 80 % ethanol with 0.05 % Silwet L-77. After washing, seeds were dried on a sterile filter paper and transferred to square plates with 50 ml of ½ Murashige and Skoog media with PhytoAgar. Plates were stratified after sowing at 4 °C in the dark for 3 days and grown in a plant incubator for 10 days under continuous light.

2.2 Salicylic Acid Treatment

1. Salicylic acid (Duchefa Biochemie; prepare 1 M stock solution in 100 % ethanol).
2. Silwet L-77.
3. Ethanol (100 %).

4. Sterile water.
5. Spray diffusers (Roth).

2.3 Genomic DNA Extraction

1. DNeasy® Plant Mini Kit (Qiagen, 69104).
2. DNA Clean & Concentrator™-5-5 (Zymo research, D4014).
3. Bench-top centrifuge.
4. Heating block or water bath (65 °C).
5. Ice.
6. Mortar and pestle or mixer mill and zirkonoxid beads (Ø 3 mm).
7. 1.5-ml reaction tubes (Safe-Lock Tubes).
8. Spectrophotometer to measure DNA concentration.

2.4 Oligonucleotides

Oligonucleotides for the analysis have to be suitable with the qPCR analysis. Therefore, design your oligonucleotides so that they span between 70 and 200 base pairs of your region-of-interest. Primers for this study (listed in Table 1) were designed with CLC Main Workbench (CLC bio; Version 6.9). If bisulfite sequencing data from your region-of-interest are available, the primer should keep a certain distance from the McrBC cutting site; approximately 30 base pairs from putative cutting site (*see* **Note 2**).

2.5 McrBC Digest

1. PCR stripes or tubes.
2. Methylation-dependent restriction enzyme McrBC (New England Biolabs, #M0272L).
3. Spectrophotometer to measure DNA concentration.
4. Incubator (e.g., PCR machine).

2.6 Quantitative Real-Time PCR (qPCR)

1. Maxima SYBR Green qPCR Master Mix (2×), ROX solution provided (Thermo Scientific).
2. Oligonucleotides.
3. 384-well plate.
4. Adhesive seal for PCR plates.

Table 1
Oligonucleotides used for qPCR analysis

Gene	Forward primer sequence 5′→3′	Reverse primer sequence 5′→3′
AT4G02320	AGGAGAGCTGTTAAGAAGTG	AAGTGGCCAAATTAACCATG
AT3G46970	CCTTGATGGGGCTAATGTTG	GAGTGACATACCAGTCCGTC
AT4G04490	TCTCCTCAAACTCGACAAGA	AACAACCACAACGCTCCATG

5. DNase-free water (provided with Maxima SYBR Green qPCR Master Mix).
6. Centrifuge to spin-down PCR plate.
7. Real-time PCR machine.
8. Agarose GTQ.
9. System to cast/run agarose gels.

3 Methods

3.1 Plant Treatment with the Phytohormone Salicylic Acid

Plants were sprayed with 1 mM salicylic acid (SA) and 0.01 % Silwet L-77 or the same amount of the solvent (ethanol and 0.01 % Silwet L-77) until the leaf surfaces are completely covered with drops. Treatment was repeated once a day for 4 days. Seedlings were harvested directly before the first treatment and 6 h or 4 days after treatment with SA. The harvested plant material was immediately frozen in liquid nitrogen until further usage.

3.2 Extraction of gDNA from Plant Samples

1. Genomic DNA was extracted with two kits, namely DNeasy® Plant Mini Kit (Qiagen, 69104) and DNA Clean & Concentrator™-5-5 (Zymo research, D4014) (*see* **Note 3**).
2. Grind plant material with liquid nitrogen using a mortar and pestle (large sample volumes). Use a spatula to transfer the plant powder to a reaction tube. Small sample volumes can be ground with a mixer mill (2 times 30 s with a frequency of 30/s) and two zirconoxid beads (per reaction tube). Take care that the sample is ground to a fine powder. To avoid thawing of the plant material, precool tubes, mortars, pestles, and spatulas in liquid nitrogen.
3. Fill around 50–100 mg ground plant material (that is about 100–200 μl fine plant powder) into a 1.5-ml reaction tube and extract gDNA using the DNeasy® Plant Mini Kit (Qiagen).
4. Add 400 μl of Buffer AP1 and 4 μl of RNase A to the ground leaf material. Vortex briefly and incubate the sample at 65 °C for 10 min to digest away all RNA. While incubating invert the sample every 2 min. Keep Buffer AP1 and RNase A separated and mix freshly.
5. Add 130 μl of Buffer P3, mix well by pipetting and put the reaction tube on ice for 5 min.
6. Centrifuge the samples at 20,000 × *g* at room temperature for 5 min to precipitate residual leaf material.
7. Transfer the lysate into a QIAshredder spin column placed in a 2-ml collection tube.
8. Centrifuge the samples at 20,000 × *g* at room temperature for 2 min.

9. Pipet the flow-through into a new 1.5-ml reaction tube. Make sure that you do not transfer pelleted material.
10. Add 1.5× volume of Buffer AW1 and mix well. Make sure that you add ethanol to Buffer AW1 before first usage.
11. Add 650 µl of the mixture to the DNeasy® Mini spin column with a 2-ml collection tube and centrifuge at 6000 × *g* at room temperature for 1 min.
12. Discard the flow-through and repeat this step with the remaining sample volume.
13. Wash the bound gDNA by adding 500 µl of Buffer AW2. Make sure that you add ethanol to Buffer AW2 before first usage. Centrifuge at room temperature at 20,000 × *g* and discard the flow through for 2 min.
14. Repeat previous washing step.
15. Transfer the spin column into a new 2-ml reaction tube and spin down at room temperature with 20,000 × *g* for 1 min to remove residual wash buffer.
16. Transfer the spin column into a new 1.5-ml reaction tube.
17. Elute bound gDNA with 100 µl water (sterile-filtered). After adding water directly onto the matrix, incubate the spin column at room temperature for 2–5 min and spin down at 6000 × *g* for 2–5 min.
18. Repeat the previous step in order to get a total of 200 µl gDNA.

3.3 Concentration of gDNA

The concentration of gDNA is usually relatively low when using the DNeasy® Plant Mini Kit. It is therefore convenient to concentrate the gDNA using the DNA Clean & Concentrator™-5 (Zymo research, D4014).

1. To 200 µl gDNA, add 400 µl of DNA Binding Buffer (volume ratio of 1:2 gDNA:DNA Binding Buffer) and vortex briefly.
2. Transfer the mixture into the Zymo-Spin™ Column placed in a collection tube.
3. Centrifuge (full speed) at room temperature for 30 s and discard the flow-through.
4. Add 200 µl of Wash Buffer and spin down for 30 s (full speed, room temperature) and discard the flow through.
5. Repeat washing step.
6. Transfer Zymo-Spin™ Column into a 2-ml reaction tube and centrifuge for 30 s (full speed, room temperature) to remove residual ethanol.
7. Place Zymo-Spin™ Column into a new 1.5-ml reaction tube and add 20 µl of water (sterile filtered) directly onto the col-

umn matrix, incubate at room temperature for 2–5 min and centrifuge for 30 s (full speed) to elute the bound gDNA from the matrix.

8. Determine DNA concentration photometrically. The concentration should range between 10–30 ng/μl.

3.4 Enzymatic Digestion of Methylated DNA

The restriction enzyme McrBC is an endonuclease that recognizes fully or half-methylated DNA with the recognition sequence RmC(N40-3000)RmC. According to the manufacturer's instructions, McrBC (New England Biolabs, #M0272L) cuts approximately 30 base pairs from the methylated cytosine distributed over several base pairs. Unmethylated DNA will not be cleaved by McrBC.

Normally, 50 ng of gDNA is digested with 5 U McrBC using the recommended 10× NEB buffer 2 (New England Biolabs; #B7002S), 100× GTP (New England Biolabs; #N0419S; 100 mM) and 100× BSA (New England Biolabs; #B9000S; 20 mg/ml), all of which are provided together with enzyme. To prevent degradation of GTP due to repeated freeze–thaw cycles, prepare smaller aliquots of 10× GTP. The BSA solution comes as a 100× stock solution. Prepare 10× BSA aliquots, which makes pipetting easier and more accurate.

1. For each gDNA sample prepare two separate reactions, a McrBC-treated and a mock-treated sample. Digestion of the gDNA can be performed in single PCR tubes or PCR stripes. Pipet all reactions on ice.

   Digest setup:

x μl	50 ng gDNA
5 μl	10× NEB Buffer 2 (New England Biolabs; #B7002S)
5 μl	10× GTP (New England Biolabs; #N0419S; diluted to 10 mM)
5 μl	10× BSA (New England Biolabs; #B9000S; diluted to 2 mg/ml)
0.5 μl	McrBC (10,000 U/ml; New England Biolabs; #M0272L)
34.5 - *x* μl	Fill up to 50 μl with DNase-free water

2. Mix well and incubate the reaction in a PCR cycler with a heated lid (110 °C) using the following settings:

37 °C	8 h	Digest
65 °C	20 min	Inactivate enzyme

3. For short-term storage, keep treated gDNA at room temperature or 4 °C. gDNA can be frozen at −20 °C until it will be used for qPCR analysis. Avoid repeated freeze–thaw cycles, as this may cause gDNA degradation. Up to ten freeze–thaw cycles were tested, which in our hands did not cause any problems during later application.

3.5 Quantitative Real-Time PCR

After digestion, the amount of gDNA is quantified by real-time PCR. Therefore, qPCR reactions using a SYBR Green qPCR Master Mix are mixed together according to Table 2. The SYBR Green allows photometrical measurement of double stranded DNA at a wavelength of 530 nm.

For all samples, perform three technical replicates to minimize pipetting errors. For each oligonucleotide pair, a no-template control should be included (that is when template DNA in the reaction mixture is replaced by the same amount of water). The "no-template" reports on unspecific amplification and contaminations. Calculate the amplification efficiency for each oligonucleotide pair. To do so, prepare serial 1:5 dilution of your undigested gDNA template, at least four different concentrations have to be included (e.g., 1. undiluted, 2. 1:5 dilution, 3. 1:25 dilution, and 4. 1:125 dilution). Each experimental setup should be repeated with three independent biological replicates.

1. To minimize pipetting errors, prepare a master mix on ice containing all components of the reaction except for the template DNA and distribute the master mix into each well of the PCR plate.
2. Add the template DNA to the reaction mixture.
3. Seal plate with adhesive seal.
4. Briefly spin down the plate to collect all drops at the bottom of the wells.
5. Transfer the plate into the qPCR thermocycler and start the following cycling protocol. After initial denaturation and activation of the hot-start DNA polymerase, the program cycles 40 times and after each cycle the SYBR Green fluorescence is

Table 2
qPCR reaction setup

5 µl	Maxima SYBR Green qPCR Master Mix (2×)
0.25 µl	Forward oligonucleotide (10 µM)
0.25 µl	Reverse oligonucleotide (10 µM)
1 µl	gDNA template (equates 1 ng of the original DNA sample) (*see* **Note 4**)
3.5 µl	Nuclease-free water

determined photometrically at 530 nm. After the 40 cycles the melting temperature is determined. For each individual oligonucleotide pair, the melting curve should have a peak at a defined temperature. A broader peak or several peaks suggest the amplification of unspecific PCR product or the formation of oligonucleotide dimers.

6. Check the size of the resulting PCR products on a 2–3 % agarose gel to further ensure that the correct genomic region was amplified.

   Thermal cycling conditions: heat lid at 110 °C during the complete PCR run.

Temperature [°C]	Incubation time	Cycles
95 °C	5 min	1
95 °C	15 s	40
55 °C	30 s	
72 °C	20 s	
	Measure fluorescence photometrically at 530 nm	
Determine melting curve:		
95 °C	30 s	1
Increase temperature from 55 to 95 °C: +1 °C, 5 s		
measure fluorescence photometrically at 530 nm after each +1 °C step		

7. Data analysis is performed using a relative quantification method. This is only possible when the standard curve of amplification revealed a PCR efficiency of 90–110 % (*see* **Note 5**).
8. The cycle threshold values (C(t)) automatically generated by the Real time PCR machine are used to perform data analysis.
9. Calculate the average C(t)-values for the technical replicate.
10. Based on the pipetted dilution series, the amplification efficiency can be determined. The relationship between the averaged C(t) values and the logarithm of the dilution should be linear, because in a perfect PCR the DNA amount doubles every cycle. By plotting the log(dilution series) versus the C(t) values and by linear regression, one can calculated the slope (m). Depending on the slope for each oligonucleotide pair, the PCR amplification efficiency in percentage can be determined using the following equation:

$$\text{Efficiency}[\%] = [10^{-1/m} - 1] \times 100$$

The amplification efficiency should lie between 90 and 110 %.

The relative methylation level of the examined sequences are determined by the ΔΔC(t) method and followed by a conversion in percentage of methylation.

11. First, calculate the ΔΔC(t) values:

$$\Delta\Delta C(t) = \Delta C(t)\text{undigestedsample} - \Delta C(t)\text{digestedsample}$$

12. Afterwards determine the fold-change $= 2^{\Delta\Delta C(t)}$
13. Finally, convert the values into the percentage of methylation

$$\text{Methylation}[\%] = (1 - 2^{\Delta\Delta C(t)}) \times 100$$

The complete formula to determine the percentage of methylation is as follows:

$$\text{Methylation}[\%] = (1 - 2^{\Delta C(t)\text{undigestedsample} - \Delta C(t)\text{digestedsample}}) \times 100$$

4 Notes

1. It is also possible to use other methylation-dependent or methylation-sensitive enzymes to analyze different cytosine methylation contexts, e.g., HpaII. As all enzymes have different recognition sites, make sure that your chosen enzyme is suitable for the sequence context of the methylated cytosine you are interested in.
2. It could take some time to find the perfect primer pairs as several moderately differentially methylated cytosines or a strongly affected cytosine has to lie in the amplified region. Additionally, all differentially methylated cytosines should be modified in the same direction; otherwise you will not be able to find differences in the methylation level.
3. gDNA can be also extracted with a traditional phenol–chloroform extraction followed by ethanol precipitation. To avoid unnecessary contact with toxic components, we decided to use the two described kits to extract gDNA.
4. For this study, a gDNA template concentration of 1 ng was used. For more abundant genes, e.g., transposons or other multiple copy genes, one can further dilute the template.
5. If your primer efficiencies are below 90 % or higher than 110 %, design new primers. Primers with a bad efficiency will create biases to your analysis.

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References

1. Law JA, Jacobsen SE (2010) Establishing, maintaining and modifying DNA methylation patterns in plants and animals. Nat Rev Genet 11(3):204–220. doi:10.1038/nrg2719
2. Saze H, Mittelsten Scheid O, Paszkowski J (2003) Maintenance of CpG methylation is essential for epigenetic inheritance during plant gametogenesis. Nat Genet 34(1):65–69. doi:10.1038/ng1138
3. Cao X, Jacobsen SE (2002) Locus-specific control of asymmetric and CpNpG methylation by the DRM and CMT3 methyltransferase genes. Proc Natl Acad Sci U S A 99(Suppl 4):16491–16498. doi:10.1073/pnas.162371599
4. Gong Z, Morales-Ruiz T, Ariza RR, Roldan-Arjona T, David L, Zhu JK (2002) ROS1, a repressor of transcriptional gene silencing in *Arabidopsis*, encodes a DNA glycosylase/lyase. Cell 111(6):803–814
5. Morales-Ruiz T, Ortega-Galisteo AP, Ponferrada-Marin MI, Martinez-Macias MI, Ariza RR, Roldan-Arjona T (2006) DEMETER and REPRESSOR OF SILENCING 1 encode 5-methylcytosine DNA glycosylases. Proc Natl Acad Sci U S A 103(18):6853–6858. doi:10.1073/pnas.0601109103
6. Choi CS, Sano H (2007) Abiotic-stress induces demethylation and transcriptional activation of a gene encoding a glycerophosphodiesterase-like protein in tobacco plants. Mol Genet Genomics 277(5):589–600. doi:10.1007/s00438-007-0209-1
7. Zilberman D, Gehring M, Tran RK, Ballinger T, Henikoff S (2007) Genome-wide analysis of *Arabidopsis thaliana* DNA methylation uncovers an interdependence between methylation and transcription. Nat Genet 39(1):61–69. doi:10.1038/ng1929
8. Zhang X, Yazaki J, Sundaresan A, Cokus S, Chan SW, Chen H, Henderson IR, Shinn P, Pellegrini M, Jacobsen SE, Ecker JR (2006) Genome-wide high-resolution mapping and functional analysis of DNA methylation in *Arabidopsis*. Cell 126(6):1189–1201. doi:10.1016/j.cell.2006.08.003
9. Wang X, Duan CG, Tang K, Wang B, Zhang H, Lei M, Lu K, Mangrauthia SK, Wang P, Zhu G, Zhao Y, Zhu JK (2013) RNA-binding protein regulates plant DNA methylation by controlling mRNA processing at the intronic heterochromatin-containing gene IBM1. Proc Natl Acad Sci U S A 110(38):15467–15472. doi:10.1073/pnas.1315399110
10. Lei M, La H, Lu K, Wang P, Miki D, Ren Z, Duan CG, Wang X, Tang K, Zeng L, Yang L, Zhang H, Nie W, Liu P, Zhou J, Liu R, Zhong Y, Liu D, Zhu JK (2013) *Arabidopsis* EDM2 promotes IBM1 distal polyadenylation and regulates genome DNA methylation patterns. Proc Natl Acad Sci U S A. doi:10.1073/pnas.1320106110
11. Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR (2012) Widespread dynamic DNA methylation in response to biotic stress. Proc Natl Acad Sci U S A 109(32):E2183–E2191. doi:10.1073/pnas.1209329109
12. Yu A, Lepere G, Jay F, Wang J, Bapaume L, Wang Y, Abraham AL, Penterman J, Fischer RL, Voinnet O, Navarro L (2013) Dynamics and biological relevance of DNA demethylation in *Arabidopsis* antibacterial defense. Proc Natl Acad Sci U S A 110(6):2389–2394. doi:10.1073/pnas.1211757110
13. Laird PW (2010) Principles and challenges of genomewide DNA methylation analysis. Nat Rev Genet 11(3):191–203. doi:10.1038/nrg2732

Chapter 29

Determining Nucleosome Position at Individual Loci After Biotic Stress Using MNase-qPCR

Margaux Kaster and Sascha Laubinger

Abstract

Nucleosome occupancy in promoter and genic regions can severely influence the transcription levels. Few methods have been established to investigate the nucleosome occupancy along the DNA. In this chapter we describe a detailed protocol to analyze the nucleosome occupancy at a specific locus using MNase-pPCR.

Key words MNase-qPCR, Nucleosome occupancy, Nuclei isolation, Epigenetics, Biotic stress, *Arabidopsis*

1 Introduction

Plants constantly have to cope with a variety of stresses without being able to avoid them. But plants developed a complex signaling network to perceive and properly respond to abiotic and biotic stresses. Most signaling pathways eventually result in reprogramming plant gene expression. These changes in gene expression can be facilitated through various mechanisms, for instance, through activation or repression of transcriptional regulators, or through epigenetic mechanisms such as DNA methylation or posttranslational modifications of histone tails [1]. All these epigenetic modifications affect the chromatin structure.

DNA that is tightly packed around histone-forming nucleosomes (dense chromatin, heterochromatin) is less accessible to regulatory proteins, such as transcription factors or polymerases, and is considered as transcriptionally inactive. On the contrary, nucleosomes are more loosely associated with the DNA in euchromatic regions, which are transcriptionally active. This degree of DNA packaging around nucleosomes is not irrevocable and can dynamically change under certain stress situations or during development. All eukaryotes employ ATP-dependent chromatin

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remodeling factors that affect nucleosome distribution to influence transcriptional activity of genes [2–5].

Because of its important function in gene regulation, nucleosome distribution and remodeling have gained attraction and a few methods have been established to determine nucleosome occupancy. Among them are for example formaldehyde-assisted isolation of regulatory element sequencing (FAIRE-seq), DNase I hypersensitive site sequencing (DNase-seq), and micrococcal nuclease sequencing (MNase-seq) [6].

FAIRE-seq is based upon the fact that cross-linking in regions of condensed chromatin is stronger than in nucleosome-free regions. Therefore, plant material is cross-linked and then purified through phenol-chloroform-isoamyl alcohol extraction. The nucleosome-free DNA will then be solved in the aqueous phase, in contrast to the DNA associated with nucleosomes. DNA enriched in the aqueous phase can be analyzed using next-generation sequencing techniques, which provides maps of open chromatin. The advantage of this method is that it does not require isolation of nuclei as compared to other methods. The disadvantage is that it requires cross-linking, the DNA needs to be sonicated before purification, and it produces relatively high background signals [7–9]. DNase I is a nonspecific endonuclease that preferentially cleaves open chromatin, creating small fragments that can be used for library preparation and sequencing [6, 10, 11]. The regions preferentially cleaved by DNase I are called hypersensitive sites and often correlate with regulatory elements of transcriptional units [12]. MNase is an endo-exonuclease from the microorganism *Staphylococcus aureus* [13]. MNase cleaves free DNA, omitting the DNA that is wrapped around histone octamers (Fig. 1). MNase and DNase I treatments are in principle very similar, since both methods rely on differential enzymatic digestion of chromatin. However, the difference is that DNase I maps open chromatin, whereas MNase maps DNA tightly bound to nucleosomes [6]. In comparison, DNase I and MNase both have a certain bias; however mapping of open chromatin using the DNase I method is restricted to "active" regions, whereas MNase assay maps all regions of open chromatin. Recently, a new method has been published called assay for transposase-accessible chromatin using sequencing (ATAC-seq). For this method the hyperactive Tn5 transposase is used to fragment and tag nucleosome-free DNA at the same time [9]. ATAC-seq was developed for genome-wide analysis, but the method has not yet been adapted for the analysis of single loci.

Here, we describe a step-by-step protocol to determine nucleosome density by MNase treatment. The protocol provided here allows the analysis of single loci by tiled qPCR, but it can be easily adapted for whole-genome analyses of nucleosome density (MNase-seq). In a nutshell, for MNase treatment plant nuclei containing intact nucleosomes are purified and treated with MNase. After DNA extraction, 150 bp DNA fragments protected by

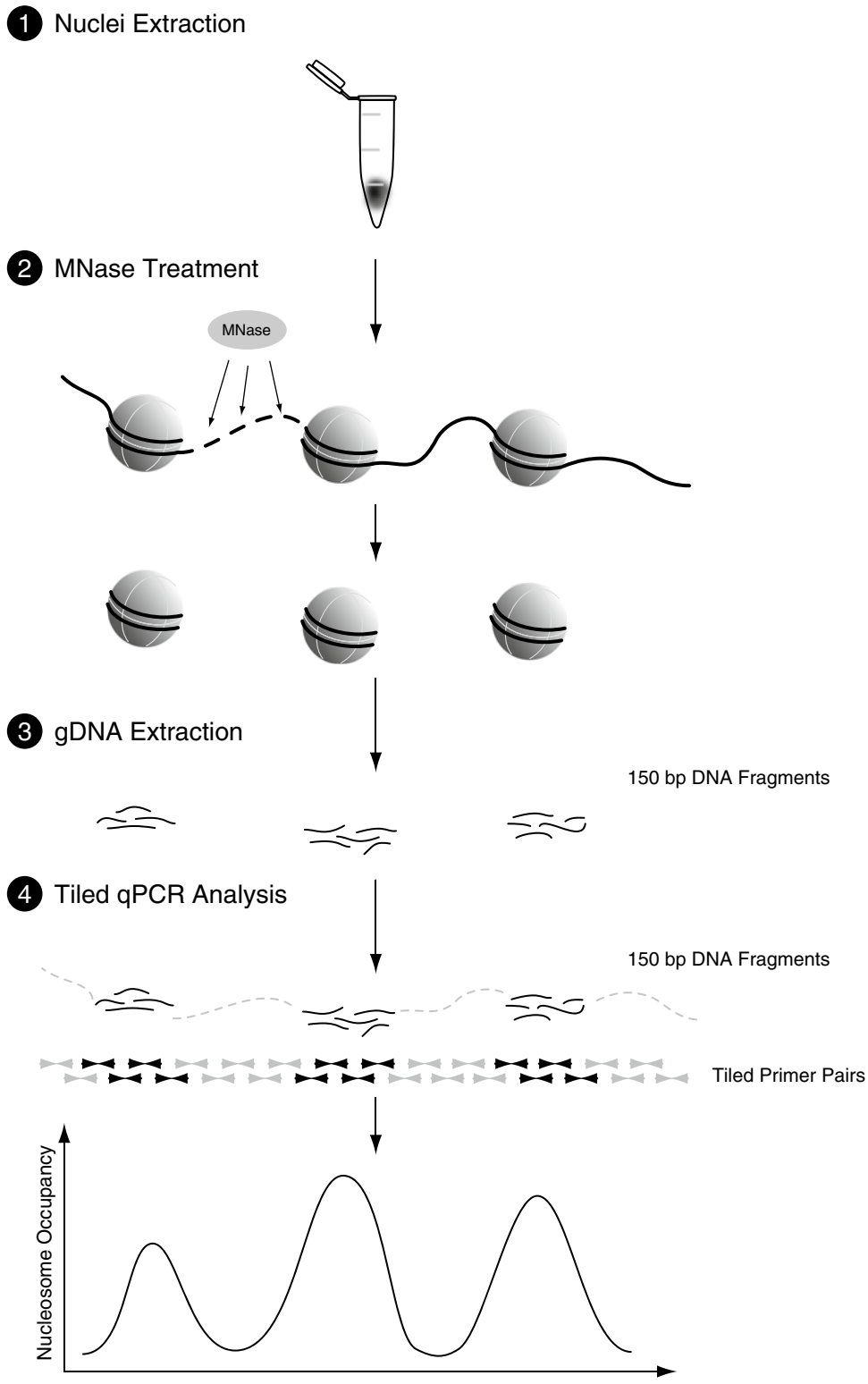


Fig. 1 Workflow of the MNase-qPCR experiment. For MNase experiments four steps need to be performed: (1) nuclei extraction, (2) MNase treatment, (3) gDNA extraction, and (4) tiled qPCR analysis

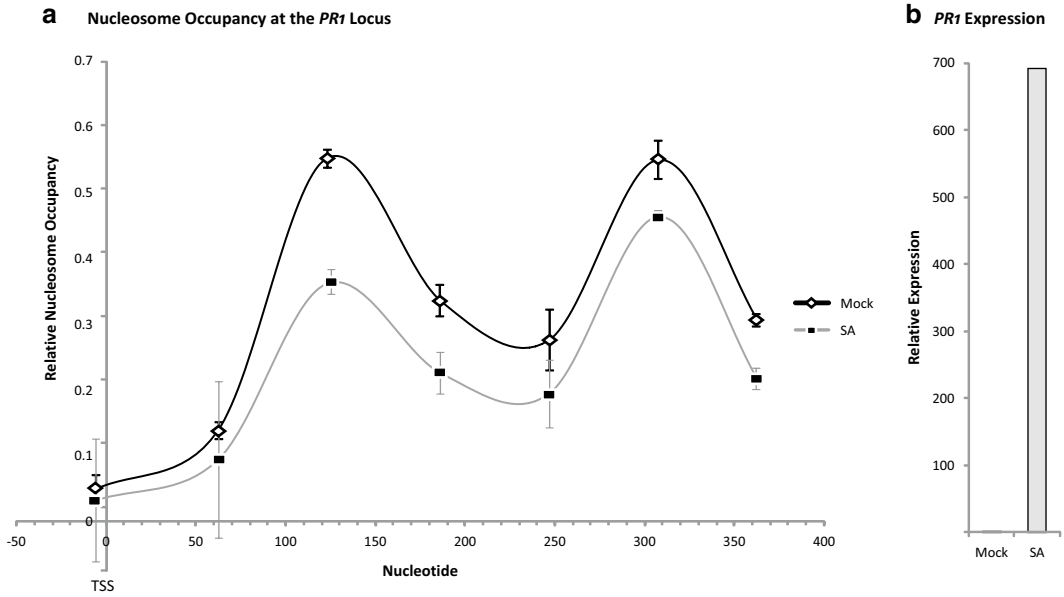


Fig. 2 Nucleosome occupancy at the *PR1* locus with (SA) and without (Mock) salicylic acid treatment. Seedlings were grown under continuous light and treated either with 1 mM SA or with mock solution on 3 consecutive days. Plant material was harvested 2 h after the last treatment (14 days). Nucleosome occupancy was determined via MNase treatment and tiled qPCR analysis. *Error bars* denote the standard error of the mean (three independent biological experiments)

nucleosomes are visualized by gel electrophoresis. These fragments can be analyzed by tiled qPCR to look at specific loci (MNase-qPCR, Fig. 1) or by deep-sequencing, as described above.

We applied MNase-qPCR to analyze the nucleosome occupancy in the gene body at the *pathogenesis-related protein 1* (*PR1*) locus after salicylic acid (SA) treatment. PR1 is one of the early regulators of pathogen response and is known to be highly expressed upon attack by various pathogens as well as upon SA treatment (Fig. 2). We were able to show that the first two nucleosomes after the transcription start site (TSS) have a reduced occupancy after SA treatment (Fig. 2). This is in concert with the current view that higher expression rates correlate with lower nucleosome occupancy [4, 14–16].

2 Materials

2.1 Plant Growth

1. MS medium.
2. Phyto agar.
3. Plates.
4. Clean bench.
5. *Arabidopsis thaliana* (Col-0).

6. 80 % Ethanol + Silwet.
7. 100 % Ethanol.
8. Plant incubator.
9. Salicylic acid (Duchefa Biochemie, prepare 1 M stock solution in 100 % ethanol).
10. Spray diffusers (Roth, N145.1).
11. Liquid nitrogen.

2.2 Nuclei Extraction

1. Mortar and pestle.
2. Liquid nitrogen.
3. 50-ml reaction tubes.
4. Honda buffer: 0.44 M Sucrose, 1.25 % Ficoll, 2.5 % Dextran T40, 20 mM HEPES-KOH pH 7.4, 10 mM $MgCl_2$, 0.5 % Triton X-100, 5 mM DTT, 1 mM Pefabloc, cOmplete (EDTA-free, 1 tablet per 50 ml; Roche).
5. Miracloth (Merck Millipore).
6. Centrifuge with cooling function.
7. 1.5-ml reaction tubes.
8. Benchtop centrifuge with cooling function.
9. MNase reaction buffer: 20 mM Tris–Cl pH 8, 5 mM NaCl, 2.5 mM $CaCl_2$.
10. Photometer for DNA quantification.

2.3 MNase Treatment

1. Micrococcal Nuclease (Takara, Cat. # 2910A).
2. STOP buffer: 100 mM EDTA, 100 mM EGTA.
3. Heating block.
4. Proteinase K.
5. RNase.

2.4 gDNA Extraction

1. Hood.
2. 1.5-ml safe-lock reaction tubes.
3. Phenol-chloroform-isoamyl alcohol (in a 25:24:1 ratio, *see* **Note 1**).
4. Chloroform.
5. 3 M Sodium acetate.
6. Agarose.
7. System to cast/run agarose gels.
8. 10× TBE buffer: 890 mM Tris base; 890 mM boric acid; 20 mM EDTA; pH 8.3.
9. GeneJET Gel Extraction Kit.
10. Photometer for DNA quantification.

2.5 Primer Design

PCRTiler v1.42 (free online tool).

The tiled primer sets that were used for this experiment are shown in Table 1 below.

2.6 qPCR Analysis

1. SYBR Green qPCR Master Mix.
2. PCR frame plate.
3. Optical adhesive seal for PCR plates.
4. Real-time PCR detection system.

3 Methods

3.1 Plant Growth

Sterilize plants with ethanol and grow them on half-strength MS medium. Treat plants either with mock solution or with 1 mM salicylic acid (SA) on days 12, 13, and 14 after germination. Harvest the plant material after 14 days and snap-freeze in liquid nitrogen.

3.2 Nuclei Extraction

1. To be able to analyze nucleosome occupancy it is necessary to isolate nuclei before MNase treatment. For nuclei isolation grind 2 g of plant material (seedlings) to a fine powder using precooled mortar and pestle and collect the powder in a precooled 50-ml reaction tube. Avoid thawing of the plant material.
2. Resuspend the powder in 20 ml of Honda buffer.
3. Filter suspension through two layers of Miracloth (Merck Millipore, Catalogue No. 475855).
4. Centrifuge the filtrate in a precooled centrifuge (4 °C) at 2500 × *g* for 15 min.
5. Carefully resuspend the pellet in 1 ml of Honda buffer.
6. Centrifuge in a precooled centrifuge (4 °C) at 2500 × *g* for 10 min.
7. Repeat **steps 5** and **6** until the pellet is white or at least not intense green.
8. Resuspend the pellet in 1 ml of MNase reaction buffer (*see* **Note 2**).
9. Centrifuge in a precooled centrifuge (4 °C) at 2500 × *g* for 5 min.
10. Resuspend pellet in 660 µl of MNase reaction buffer and measure DNA concentration using a Nanodrop. The concentration can have a range of 50–150 ng/µl.
11. Nuclei can now be stored at −80 °C overnight or longer.

3.3 MNase Treatment

1. If samples were stored at −80 °C, thaw nuclei samples on ice.
2. Of the 660 µl from the nuclei isolation, transfer 160 µl to a new reaction tube and add up the volume to 500 µl with

Table 1
List of primer sets used for this experiment

Name	Position relative to TSS	Forward	Reverse
Gypsy-1	−273	CCACCCATCGATGTCTAAGTT	CCACCTTATCTTCAGTTAAACGATTC
Gypsy-2	−176	GATAAGGTGGGCGATTTACG	ATCCATCGTTGGAATCTCCT
Gypsy-3	−73	TACACATTCCTCAGCCGTTG	CCTATCTCCCAGCTAGCAACC
Gypsy-4	1	ACTGGTTGCTAGCTGGGAGA	CCAGTGTTGGTTCTCCTTGG
Gypsy-5	53	TGGAGAAAGTGTTCTCGGATAAA	TTGATCTGAATAGTTGCATTGACA
Gypsy-6	101	GCGAAGTTGCTGTGAACAAA	TACATTGAGTTTGGCCGATG
Gypsy-7	186	CGGCCAAACTCAATGTAAGC	TCCCTCTTCTAGAGGTTTTGTCC
Gypsy-8	248	TGTGTAGGTAAACAAGTTTTGAGGA	TCAGCAAGTTTGTCAGTTCCA
PR1-1	−934	TGCTTAGAATCACAGATTCATATCAGG	CAGTATACCTAATTTTGTACCGCCTTC
PR1-2	−861.5	GTAAATGATATACGAAGGCGGTACAA	TTTTTCCAAACACTAATACGCAGTT
PR1-3	−686	TTTTGGATAAATCTCAATGGGTGA	TGCCTATGGCTGAAAAGTCC
PR1-4	−647.5	CTACGTCACTATTTTACTTACGTCAT	CTTAGTGTTTCATGCATATGAGTATC
PR1-5	−503	TATTTGAAAATTGACTGTAG	AAAATGTTTTGAAGATATCTTCCTG
PR1-6	−431	TGTCCGATACGATTTTTCTG	GTGTTTTTTTTTTCTTCATGATTCTAATTTAAG
PR1-7	−353	TAGAAGACTTAAATTAGAATCATG	GTAAGTATACCCTAAATGAAG
PR1-8	−299.5	GACATGAAACAACTATATACAATG	TATTGTTTCGTATCGGTAGCTTTG
PR1-9	−221.5	CAACAATGGCAAAGCTACCG	TGCAATTGTCCAAATGAATAGAAG
PR1-10	−173	GTAAGGACAAGATTGACAAAAAAATAG	GTAAATACATGGTCCATGTGTGAG
PR1-11	−106	GAATATTACTAAAATACTCACACATG	GTAAACATCGCTTATATAGAGATTTG

(continued)

Table 1
(continued)

Name	Position relative to TSS	Forward	Reverse
PR1-12	−6	CGATGTTTACGAACCCCAAA	GGGAAGAACAAGAGCACCT
PR1-13	62.5	TGAATTTTACTGGCTATTCTCG	GCCTGGTTGTGAACCCTTAGA
PR1-14	123	AAAGCTCAAGATAGCCCACA	TAGGCTGCAACCCTCTCG
PR1-15	185.5	GGTAGGCGTAGGTCCCAT	CGTAAGGCCCACCAGAGT
PR1-16	247	TAAGAGGCAACTGCAGAC	CACATGTTCACGGCGGAG
PR1-17	307.5	TAGCGGTGACTTGTCTGG	TGACCACAAACTCCATTGC
PR1-18	362	TAACTACAACTACGCTGC	CCTCACTTTGGCACATCC

Table 2
Pipetting scheme for the MNase treatment

	Sample	Control
Nuclei	500 µl	160 µl
MNase reaction buffer	–	340 µl
MNase enzyme	0.5 µl for 70–80 ng/µl DNA	–
Incubate at 37 °C for 8 min		
Stop buffer	50 µl	50 µl
10 % SDS	50 µl	50 µl
Proteinase K	40 µg	40 µg
Incubate at 65 °C for 1 h		
RNase	1 U	1 U
Incubate for at 37 °C 1 h and then at 4 °C overnight		

MNase reaction buffer. This is your undigested control. Apart from the addition of the MNase enzyme treat the control exactly as your MNase-treated sample (*see* **Note 3**).

3. To approximately 70–80 ng DNA, add 0.5 µl micrococcal nuclease (final concentration 0.01–0.02 units/µl) to your sample (*see* **Note 4**).
4. Incubate the sample (with MNase) and the control (without MNase) at 37 °C for 8 min (*see* **Note 4**).
5. Terminate the reaction by adding 50 µl of STOP buffer, 50 µl 10 % SDS, and 40 µg proteinase K.
6. Incubate samples at 60 °C for 1 h.
7. Incubate samples with 1 U RNase (10 µg/µL) at 37 °C for 1 h and then at 4 °C overnight.
8. A pipetting scheme for the MNase treatment is shown in Table 2 below.

3.4 gDNA Extraction

While working with phenol or chloroform always work under the hood and wear gloves and goggles at all times. Avoid direct contact or inhalation.

1. Use the entire reaction volume of the MNase treatment for DNA extraction.
2. Transfer each sample to a 2-ml safe-lock reaction tube (*see* **Note 5**).
3. Add 1 vol. of phenol-chloroform-isoamyl alcohol (approximately 610 µl). Vortex the samples for 30 s.

4. Centrifuge at 12,000 × *g* for 5 min.
5. Transfer the aqueous (upper) phase to a new reaction tube.
6. Repeat **steps 3–5** two to three times. The interphase between aqueous and organic phase should be clear.
7. Transfer the aqueous phase to a new reaction tube and add 1 vol. of chloroform.
8. Vortex the samples for 30 s.
9. Centrifuge at 12,000 × *g* for 5 min.
10. Repeat **steps 7–8** at least two times to get rid of residual phenol.
11. Transfer aqueous phase to a new reaction tube.
12. Add 1/10 vol. NaAc (3 M) and 2 vol. of 100 % ethanol to precipitate the DNA.
13. Incubate samples at −20 °C for 2 h.
14. Centrifuge for 30 min at maximum speed.
15. Remove the supernatant without disturbing the pellet.
16. Wash the pellet by adding 200 µl 70 % ethanol. Do not resuspend.
17. Spin down samples for 5 min at maximum speed.
18. Discard the supernatant and leave pellets at room temperature until they are dry (no residual ethanol left). Do not dry the pellet for too long; otherwise it will not go in solution.
19. Resuspend pellets in 50 µl water. If pellets are hard to resuspend, incubate samples at 60 °C for 10–15 min.
20. Cast a 2 % agarose gel and load all volume of your sample (not the controls).
21. Excise the 150-bp band corresponding to the mononucleosomal fraction (Fig. 3) and extract with the GeneJET Gel Extraction Kit.
22. The DNA can be stored at −20 °C for several weeks.

3.5 Primer Design

For nucleosome occupancy analysis via qPCR, tiled oligonucleotides spanning the region of interest have to be designed. Each amplicon should have an average size of 100 bp and overlap by 20 bp. We opted to use an online primer design tool called PCRTiler v1.42 (http://pcrtiler.alaingervais.org:8080/PCRTiler/), but amplicons can be designed also manually. For the experiment shown here, we used the DNA sequences 2 kb upstream and 500 bp downstream of the TSS of the *PR1* locus (At2g14610) for oligonucleotide design using the PCRTiler v1.42.

3.6 qPCR Analysis

DNA protected by nucleosomes during the MNase treatment can easily be amplified during qPCR. In comparison, DNA regions devoid of nucleosomes are largely removed by MNase and hence

5 µl	Maxima SYBR Green qPCR Master Mix (2×)
1 µl	Template DNA (3 ng/µl)
3 µl	Nuclease-free water (is included in the qPCR Kit)

0.5 µl	Forward oligonucleotide (10 µM)
0.5 µl	Reverse oligonucleotide (10 µM)
	or
1 µl	Primer mix (10 µM each)

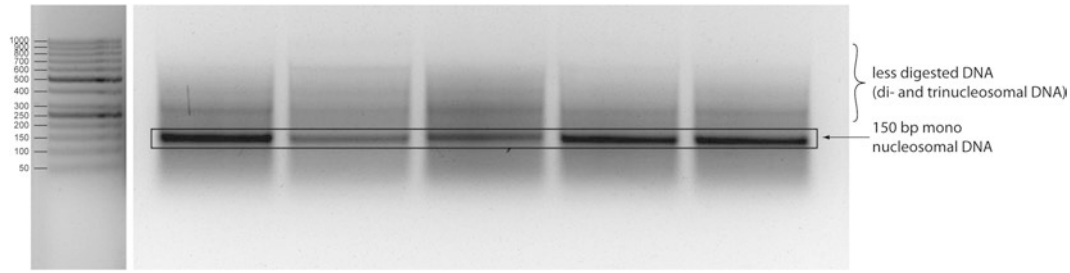


Fig. 3 Electrophoretic analysis of genomic DNA after MNase treatment

cannot get properly amplified. To analyze the nucleosome occupancy of a gene of interest a tiled qPCR analysis can be performed (Fig. 2). To be able to compare treated versus mock-treated samples it is crucial to make sure that input DNA amounts are identical. Therefore an internal control should be included to which input DNA amounts can be normalized (in this case *gypsy-like retrotransposon*, At4g07700). As additional controls, the undigested DNA and a "no-template control" should be used and for each reaction three technical replicates should be prepared.

1. For standard qPCR reactions, a master mix containing the Maxima SYBR Green qPCR Master Mix (2×), primers, and water is prepared and the template is then added to the reaction. However, in this case it is more practical to prepare the master mix containing the template DNA and then add the primers. Keep in mind that different master mixes for each template (controls, etc.) are needed. After preparing, distribute the master mix into the PCR plate.
   Example of a single reaction (upscale for preparation of a master mix):
   Example of primers added individually to the reaction or as a primer mix (preparation of a master mix for the primer mix is recommended, see **Note 6***)*:

Table 3
PCR programm and melting curve settings

Cycle	Temp	Time
PCR program		
1	95 °C	5 min
2	95 °C	10 s
3	55 °C	30 s
4	72 °C	20 s
Repeat steps 2–4 39 times and do photometric measurement at 530 nm after each cycle		
Melting curve		
5	95 °C	30 s
6	Ramp from 55 °C to 95 °C (+1 °C each cycle)	5 s
Photometric measurement at 530 nm after each cycle		

2. For the "no-template control," add nuclease-free water instead of template.
3. To calculate PCR amplification efficiency it is important to make serial dilutions of undigested wild-type DNA. For this experiment four serial 1:5 dilutions were used.
4. Seal the PCR plate with an optical adhesive seal.
5. To make sure that all samples are collected at the bottom of the well perform a short spin (500 × *g* for 30 s).
6. Incubate the plate in a qPCR thermocycler using the three-step program shown below with a photometric measurement at 530 nm step after each amplification cycle (*see* **Note 7**). At the end perform a melting curve analysis with the settings shown below in Table 3 (*see also* **Note 8**):

3.7 Data Evaluation

For further analysis cycle threshold (Ct) values need to be determined. The Ct value determines the cycle in which the sample passed the threshold of detectability. Threshold and resulting Ct values generated by the Bio-Rad CFX384 program were used in this experiment. Calculations for one oligonucleotide pair are shown below.

1. Calculate the average Ct value of the three technical replicates for each sample.
2. To determine the PCR amplification efficiency, a standard curve of the dilution series needs to be plotted. For this, as exemplified in Table 4 below, calculate the logarithm of the dilution series and plot them versus the average corresponding Ct values.

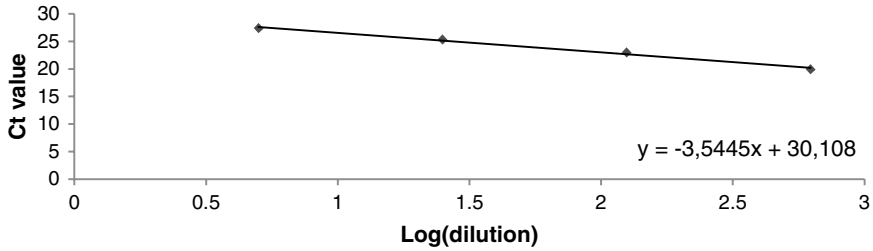
Table 4
Exemplary calculations to determine the primer efficiency

Serial 1:5 dilution	Log(dilution)	Ct values	Average Ct values
625	2.7959	20.18	20.11
		19.97	
		20.19	
125	2.0969	23.16	23.23
		23.04	
		23.49	
25	1.3979	25.33	25.45
		25.36	
		25.65	
5	0.6990	27.41	27.56
		27.34	
		27.93	

3. This will result in a linear regression line of which the slope m represents the amplification efficiency. Normal PCR efficiency should be around 90–110 %:

$$E = 10\left(-\frac{1}{\text{slope}}\right)$$

For our example the graph would be as follows:



The resulting slope is then

$$m = -3.5445$$

With the slope you can now calculate the PCR efficiency:

$$E = 10\left(-\frac{1}{-3.5445}\right) = 1.926$$

Table 5
Exemplary caluclation to determine the relative nucleosome occupancy

Name	Ct values	Average Ct	$\Delta Ct = 2^{(average\ Ct(sample) - average\ Ct(control))}$	ΔCt normalized over primer *E*	Normalization to gypsy locus	Normalization to highest value
Mock	23.17	23.15	0.32	0.34	0.34	1.00
Mock	23.17					
Mock	23.11					
Mock (control)	21.57	21.53				
Mock (control)	21.53					
Mock (control)	21.48					
SA	23.88	23.86	0.16	0.18	0.19	0.58
SA	23.71					
SA	23.99					
SA gDNA (control)	21.29	21.25				
SA gDNA (control)	21.26					
SA gDNA (control)	21.21					

To get percentage values subtract the *E* value from 1 and multiply this by 100.

4. The fraction of input was then calculated as

$$2^{(Ct(sample)-Ct(control))} \times \text{Primer } E$$

normalizing each sample to the undigested genomic DNA (Ct(control)) and normalized over the according primer efficiency. At the end all values are normalized over that of gypsy-like retrotransposon +101 loci for each sample and to the overall highest value, as shown below in Table 5.

4 Notes

1. Depending on the pH, phenol can be used to extract DNA and/or RNA. Therefore it is important to use phenol that is suitable for DNA extraction.
2. If another reaction buffer is used it should be taken into consideration that the MNase enzyme activity is strictly Ca^{2+} dependent.
3. The difference in concentration is deliberate since the MNase-treated samples will be gel extracted in the end and therefore a higher concentration is needed.
4. Too long digestion times will completely digest the DNA, and too short will give no mononucleosomal fraction. This is also influenced by the amount of input DNA/nuclei. It is possible that the reaction time or DNA amount need to be adjusted individually.
5. Using safe-lock tubes might avoid leakage of phenol and is important for your own safety.
6. Before adding the primers it is useful to prepare primer mixtures for each amplicon to decrease the workload and pipetting errors.
7. If you experience problems with PCR amplification, for instance of AT-rich regions, try to decrease the elongation temperature during qPCR to 60 °C. Especially in promoter regions this has shown to be very effective.
8. The melting curves should all have a peak at one temperature. More than one peak implies the formation of oligonucleotide dimers or unspecific PCR products during amplification.

References

1. Dowen RH, Pelizzola M, Schmitz RJ, Lister R, Dowen JM, Nery JR, Dixon JE, Ecker JR (2012) Widespread dynamic DNA methylation in response to biotic stress. Proc Natl Acad Sci U S A 109(32):E2183–2191. doi:10.1073/pnas.1209329109, 1209329109[pii]
2. Farrona S, Hurtado L, Bowman JL, Reyes JC (2004) The *Arabidopsis thaliana* SNF2 homolog AtBRM controls shoot development and flowering. Development 131(20):4965–4975. doi:10.1242/dev.01363
3. Sang Y, Silva-Ortega CO, Wu S, Yamaguchi N, Wu MF, Pfluger J, Gillmor CS, Gallagher KL, Wagner D (2012) Mutations in two non-canonical *Arabidopsis* SWI2/SNF2 chromatin remodeling ATPases cause embryogenesis and stem cell maintenance defects. Plant J. doi:10.1111/tpj.12009
4. Han SK, Sang Y, Rodrigues A, Biol F, Wu MF, Rodriguez PL, Wagner D (2012) The SWI2/SNF2 chromatin remodeling ATPase BRAHMA represses abscisic acid responses in the absence of the stress stimulus in *Arabidopsis*. Plant Cell 24(12):4892–4906. doi:10.1105/tpc.112.105114
5. Jerzmanowski A (2007) SWI/SNF chromatin remodeling and linker histones in plants. Biochim Biophys Acta 1769(5-6):330–345. doi:10.1016/j.bbaexp.2006.12.003
6. Zentner GE, Henikoff S (2012) Surveying the epigenomic landscape, one base at a time. Genome Biol 13(10):250. doi:10.1186/gb4051
7. Simon JM, Giresi PG, Davis IJ, Lieb JD (2013) A detailed protocol for formaldehyde-assisted isolation of regulatory elements (FAIRE). Current protocols in molecular biology Chapter

21:Unit21.26. doi:10.1002/0471142727.mb2126s102
8. Giresi PG, Kim J, McDaniell RM, Iyer VR, Lieb JD (2007) FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. Genome Res 17(6):877–885. doi:10.1101/gr.5533506
9. Buenrostro JD, Giresi PG, Zaba LC, Chang HY, Greenleaf WJ (2013) Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods 10(12):1213–1218. doi:10.1038/nmeth.2688, nmeth.2688[pii]
10. Weintraub H, Groudine M (1976) Chromosomal subunits in active genes have an altered conformation. Science 193(4256):848–856
11. Boyle AP, Davis S, Shulha HP, Meltzer P, Margulies EH, Weng Z, Furey TS, Crawford GE (2008) High-resolution mapping and characterization of open chromatin across the genome. Cell 132(2):311–322. doi:10.1016/j.cell.2007.12.014, S0092-8674(07)01613-3[pii]
12. Berr A, Shafiq S, Shen WH (2011) Histone modifications in transcriptional activation during plant development. Biochim Biophys Acta 1809(10):567–576. doi:10.1016/j.bbagrm.2011.07.001
13. Alexander M, Heppel LA, Hurwitz J (1961) The purification and properties of micrococcal nuclease. J Biol Chem 236:3014–3019
14. Clark DJ, Felsenfeld G (1991) Formation of nucleosomes on positively supercoiled DNA. EMBO J 10(2):387–395
15. Eberharter A, Becker PB (2002) Histone acetylation: a switch between repressive and permissive chromatin—second in review series on chromatin dynamics. Embo Rep 3(3):224–229. doi:10.1093/embo-reports/kvf053
16. Kwon CS, Lee D, Choi G, Chung WI (2009) Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in *Arabidopsis*. Plant J 60(1):112–121. doi:10.1111/j.1365-313X.2009.03938.x, TPJ3938[pii]

Chapter 30

Phosphoprotein Enrichment Combined with Phosphopeptide Enrichment to Identify Putative Phosphoproteins During Defense Response in *Arabidopsis thaliana*

Ines Lassowskat, Wolfgang Hoehenwarter, Justin Lee, and Dierk Scheel

Abstract

Phosphoprotein/peptide enrichment is an important technique to elucidate signaling components of defense responses with mass spectrometry. Normally, proteins can be detected easily by shotgun experiments but the low abundance of phosphoproteins hinders their detection. Here, we describe a combination of prefractionation with desalting, phosphoprotein and phosphopeptide enrichment to effectively accumulate phosphorylated proteins from leaf tissue of stressed *Arabidopsis* plants.

Key words Phosphoproteins, Phosphopeptides, Enrichment, PAPE, MOAC, Defense response, *Arabidopsis thaliana*, MAPK

1 Introduction

Phosphorylation is an importantposttranslationalprotein modification (PTM) with regulatory roles in diverse cellular signaling pathways like cell differentiation, development, cell cycle control, metabolism and environmental stress responses [1–3]. It is estimated that 30 % of all proteins are phosphorylated at any given time and state [4] and approximately 4 % of the *Arabidopsis* genome encodes serine threonine protein kinases, indicating the extent of the phosphoproteome and the far-reaching impact of this PTM in plants [5]. Beside its different roles in the regulation of protein synthesis, gene expression and apoptosis, phosphorylation events exhibit a pivotal role in defense responses [6]. An example is the reversible activation of mitogen-activated protein kinase (MAPK)-mediated phosphorylation signaling cascades upon stress or other environmental signals [7–9] like pathogen attack. The corresponding downstream targets of such cascades are, to a great extent, unknown. For further understanding of

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defense mechanisms in plants, more knowledge about signaling cascades is important.

Mass spectrometry based proteomics is ideally suited for the analysis of protein phosphorylation in the cell. The peptide mass shift incurred by the covalent attachment of a phosphate moiety is easily measured and can be precisely mapped with MS/MS peptide sequencing. Tens of thousands of individual peptide measurements in a single LC-MS analysis of a protein extract can allow the detection of a similarly high number of phosphorylation events concomitantly giving a detailed picture of in vivo phosphorylation. Quantification of the phosphopeptide ion signal can give a quantitative picture of protein phosphorylation and kinase/phosphatase activity and can be used to determine site specific phosphorylation stoichiometries.

Protein phosphorylation in the cell in particular of signaling components is reversible, highly transient and often fractional. This necessitates the enrichment of the phosphoproteins/peptides to exceed the limit of detection of even the most sensitive mass spectrometers. Immobilized metal oxide affinity chromatography (MOAC)-based enrichment of phosphopeptides using TiO_2, ZrO_2, or other amphoteric ion exchangers is the most popular means of phosphopeptide enrichment and has been used to identify tens of thousands of phosphopeptides in yeast and human cell lines [10–12]. Phosphoprotein enrichment procedures have also been employed but without this resounding success. In plants the number of phosphopeptides identified in proteomics studies is much more modest. One reason is presumably the higher dynamic range of protein abundance because of super abundant proteins especially of the photosynthetic apparatus. Another is that the plant cell wall and the abundance of secondary metabolites and phenolic compounds often require harsher cell disruption and protein extraction procedures which are detrimental to the stability of the high energy phosphate bond. Therefore, plant biochemists have begun to appreciate the value of a combined strategy for phosphoprotein/peptide enrichment for phosphoproteomics research in plants [13].

In previous work done in our laboratory, it could be shown that a reduction of the RuBisCO content via ammonium sulfate (AS) precipitation had a positive effect on the preparation of 2D-PAGE, as well as the enrichment of phosphoproteins [14]. As a further improvement for phosphoprotein analysis, we now incorporated the MOAC method [15] to the AS-based RuBisCO removal step, which, by itself, already acts as prefractionation/enrichment of phosphoproteins (Fig. 1). This led to a facile, but efficient, phosphoproteome analysis procedure, which we termed prefractionation-assisted phosphoprotein enrichment (PAPE, [16]). In other work a two step enrichment procedure combining MOAC enrichment of phosphoproteins followed by TiO_2MOAC

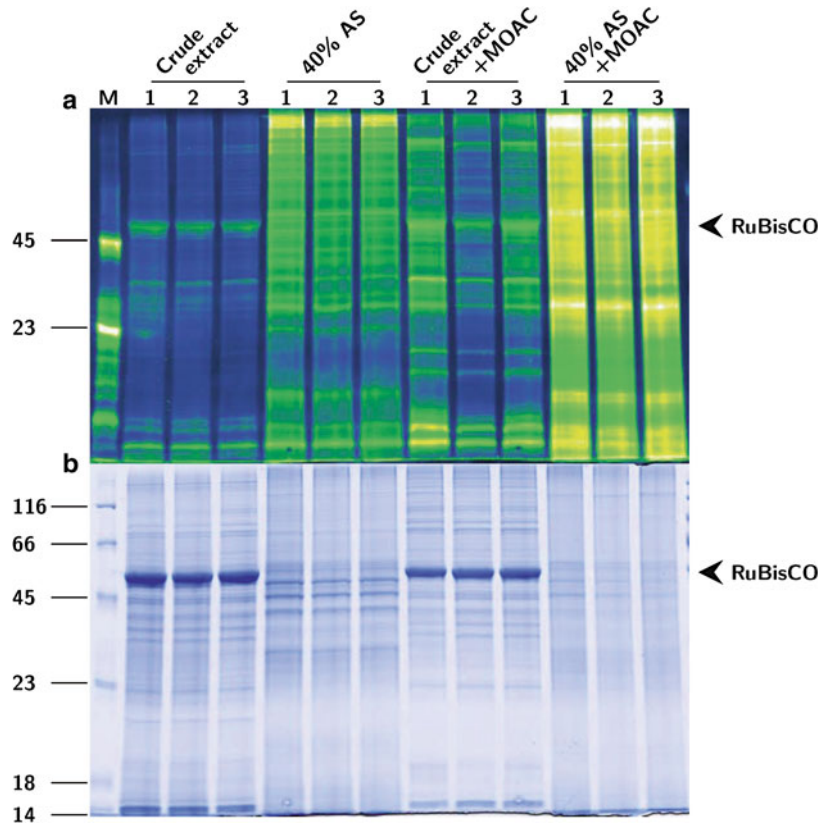

Fig. 1 Prefractionation-assisted phosphoprotein enrichment (PAPE). SDS-PAGE of different extraction and phosphoprotein enrichment steps (10 μg each). Each step was performed three times (lanes labeled 1, 2, and 3). Visualization of proteins was achieved with (**a**) Pro-Q Diamond phosphoprotein staining in false-color representation. Pro-Q Diamond (Life Technologies, Darmstadt, Germany) staining was carried out according to a modified protocol [18]. Fluorescent images were obtained using the Typhoon scanner (GE Healthcare, Hercules, USA) with the settings: 532 nm excitation, 580 nm band pass emission filter and the photo multiplier tube at 550. ImageJ software (National Institute of Health, Bethesda, MD, USA) was used for false color representation. Total protein (**b**) was visualized with Novex® Colloidal Blue Staining Kit (Life Technologies, Darmstadt, Germany). Peppermint Stick™Phosphoprotein Molecular Weight Standard (Life Technologies, Darmstadt, Germany) was used as the molecular weight marker. Protein molecular weights are indicated on the *left-hand margin. Black arrows* mark the position of the large subunit of RuBisCO

enrichment of phosphopeptides was developed [17]. Here we present detailed protocols that are of use to establish and perform two- or conceivably three-step enrichment of the phosphoproteins/peptides for LC-MS analysis of in vivo protein phosphorylation in plants (Fig. 2).

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a resistivity of 18 MΩ·cm at 25 °C) and

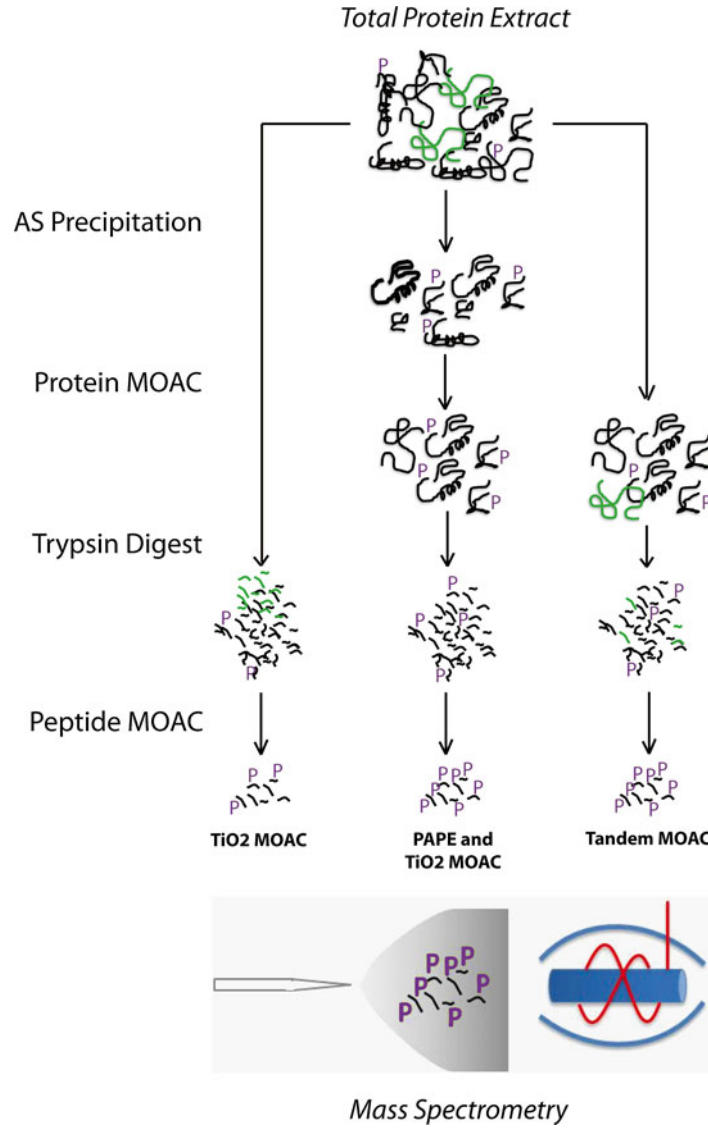

Fig. 2 Schematic of possible combinations of phosphoprotein/peptide enrichment procedures in plant phosphoproteomics. Phosphate moieties are *colored mauve*, RuBisCO *green*. The possible combinations of AS precipitation, protein, and peptide MOAC are shown. AS precipitation and protein MOAC is called PAPE, to which phosphopeptideenrichment using TiO_2 can be added

analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

2.1 Protein Extraction

1. Extraction buffer: 100 mM HEPES–KOH, pH 7.5, 5 % (v/v) glycerol, 5 mM EDTA. Weigh 11.9 g HEPES and transfer to graduated cylinder. Add 0.93 g EDTA and 25 mL glycerol.

Add water to a volume of 400 mL. Mix and adjust pH with KOH. Make up to 500 mL with water. Store at 4 °C.

2. β-mercaptoethanol: Store at 4 °C.
3. Protease and phosphatase inhibitors: Store according to manufacturer's guideline.
4. 0.45 μm cellulose mixed ester (CME) filter.

2.2 Prefractionation

1. Ammonium sulfate: Grind crystals to fine powder (*see* **Note 1**).
2. Wash solution 1: 20 % (v/v) 50 mM Tris–HCl, pH 7.5, 80 % (v/v) acetone. Weigh 0.3 g Tris and transfer to graduated cylinder. Add water to a volume of 40 mL. Mix and adjust pH with HCl. Make up to 50 mL with water. Add 200 mL acetone to a total volume of 250 mL. Store at –20 °C.
3. Wash solution 2: 100 % (v/v) acetone. Store at –20 °C.

2.3 Phosphoprotein Enrichment

1. $Al(OH)_3$ (Sigma-Aldrich, St. Louis, USA) (*see* **Note 2**).
2. Incubation buffer: 8 M urea, 200 mM aspartate, 200 mM glutamate, 30 mM 2-(N-morpholino)ethanesulfonic acid (MES), 20 mM imidazole, 0.25 % (w/v) (3-[Cholamidopropyl)-dimethylammonio]-propan-sulfonate (CHAPS), pH 6.1 (*see* **Note 3**). Adjust pH with HCl. Store at 4 °C.
3. Elution buffer: 8 M urea, 100 mM potassium pyrophosphate (TKPP), pH 9.0 (*see* **Note 4**). Store at 4 °C.
4. Centrifugal filter devices, 3 kDa cutoff.
5. 2D Clean-up Kit (GE Healthcare, Hercules, USA; *see* **Note 5**). Store according to manufacturer's instructions.
6. Shotgun buffer: 8 M urea, 50 mM Tris–HCl, pH 8.5. Aliquot and store at –20 °C.
7. 2D-Quant Kit (GE Healthcare, Hercules, USA) for protein quantitation (*see* **Note 6**). Store according to manufacturer's instructions.

2.4 In-solution Digestion

1. Tris-stock solution: 0.4 M Tris–HCl, pH 7.8. Store at 4 °C.
2. Reducing solution: 30 mg Dithiothreitol (DTT), 750 μL H_2O, 250 μL Tris stock solution (200 mM DTT, 100 mM Tris). Prepare freshly before use.
3. Alkylating solution: 36 mg Iodoacetamide (IAA), 750 μL H_2O, 250 μL Tris stock solution (200 mM DTT, 100 mM Tris). Prepare freshly before use.
4. Diluting solution: 50 mM ammonium dihydrogen carbonate, pH 8.5.
5. Trypsin solution. Prepare and store according to manufacturer's instructions.
6. C18-columns or -Spin Tips self-made or commercially available (*see* **Note 7**).

2.5 Phosphopeptide Enrichment

1. Glygen™ Top Tips containing TiO_2.
2. Spin columns, 10 μm polyethylene filters, caps.
3. Equilibration buffer: 50 % (v/v) acetonitrile (ACN), 2.5 % (v/v) trifluoroacetic acid (TFA) in H_2O, phthalic acid-saturated (~500 mg of phthalic acid in 20 mL of equilibration buffer).
4. Wash buffer 1: 50 % (v/v) ACN, 0.1 % (v/v) TFA in H_2O.
5. Wash buffer 2: 0.1 % (v/v) TFA in H_2O.
6. Elution buffer: 5 % (v/v) ammonia in H_2O.

3 Methods

Carry out all procedures at room temperature unless otherwise specified. Plants are sprayed with a bacterial suspension containing 5×10^8 c.f.u./mL bacteria (e.g., *Pseudomonas syringae* DC3000) with 0.04 % Silwet L-77 to initiate the defense response.

3.1 Protein Extraction

1. Grind leaf material to a fine powder in liquid nitrogen. Weigh material in proper sized reaction tube (*see* **Note 8**).
2. Prepare 3 times extraction buffer working solution according to the amount of leaf material (*see* **Note 9**). Mix extraction buffer with 0.1 % (v/v) β-mercaptoethanol, Protease Inhibitor and Phosphatase Inhibitor according to manufacturer's instructions (*see* **Note 10**). Store on ice.
3. Add 3 volumes of extraction buffer working solution to ground leaf material. Mix vigorously. Mix every reaction tube vigorously 10 times alternating for 20 s. Place on ice in between (*see* **Note 11**). Mix all reaction tubes horizontally for another 10 min at 4 °C (*see* **Note 12**).
4. Centrifuge at 4 °C and $3220 \times g$ for 15 min. Transfer supernatant to new reaction tube and repeat centrifugation step (*see* **Note 13**).
5. Filter supernatant through 0.45 μm CME filter. Store filtered supernatant on ice.

3.2 Precipitation with Ammonium Sulfate (AS)

1. Determine volume of filtered supernatant to first decimal place. Calculate appropriate mass of AS to gain 40 % end concentration in solution. Use following calculation: $m(\mathrm{AS})[\mathrm{g}] = (V[\mathrm{ml}] / 1000) \times 226$
2. Transfer supernatant to proper sized reaction tube and place with magnetic stirrer on ice on a multiple stirring device (*see* **Note 14**). Stir gently (400 rpm).

3. Add the AS in portions within 15 min. Add within 1 min small amounts of AS to each reaction tube. Then leave it stirring for 1 min. At the end leave it stirring for 2 min (*see* **Note 15**).
4. Stop stirring and incubate for 30 min at 4 °C (*see* **Note 16**).
5. Centrifuge at 4 °C and 3220 × *g* for 15 min. Discard supernatant and repeat centrifugation step. Remove leftovers of supernatant with pipette (*see* **Note 17**).
6. Add proper volume of wash solution 1. Mix vigorously and centrifuge at 4 °C and 3220 × *g* for 10 min. Discard supernatant (*see* **Note 18**).
7. Repeat **step 6** once.
8. Repeat **step 6** once with wash solution 2.
9. Dry pellet for 5–10 min (*see* **Note 19**).
10. Dissolve pellet in appropriate amount of incubation buffer (*see* **Note 20**).
11. Determine protein concentration with 2D-Quant Kit according to manufacturer's instructions (*see* **Note 21**).

3.3 Phosphoprotein Enrichment

All centrifugation steps are done at 15 °C and 18,500 × *g*.

1. Weigh 40 mg of $Al(OH)_3$ in 2 mL reaction tube. Equilibrate with 1.8 mL incubation buffer and centrifuge. Discard supernatant (*see* **Note 22**).
2. Load 1.5 mL of sample with concentration of 0.5 mg/mL and mix vigorously. Incubate by rotating for 30 min (*see* **Note 23**). Centrifuge and discard supernatant (*see* **Note 24**).
3. Add 1.8 mL of incubation buffer and mix vigorously for 1 min. Centrifuge and discard supernatant.
4. Repeat 3 times.
5. Centrifuge again and remove leftovers of supernatant with pipette (*see* **Note 25**).
6. Add 800 µL of elution buffer and mix vigorously. Incubate rotating for 45 min. Centrifuge and collect supernatant with pipette in new reaction tube.
7. Repeat **step 6** with 400 µL elution buffer.
8. Centrifuge again and collect leftovers of supernatant with pipette.
9. Centrifuge combined eluates and transfer supernatant to new reaction tube. Repeat as long as there is matrix left (*see* **Note 26**).
10. Prepare filter device by washing with 500 µL water. Discard water with pipette.

11. Add up to 4 mL per sample on filter device. Centrifuge according to manufacturer's instructions at 15 °C. Stop centrifugation when a volume of 200 µL is achieved (*see* **Note 27**).
12. Use 2D-CleanUp Kit according to manufacturer's instructions for precipitation of proteins.
13. Dissolve pellet in appropriate amount of shotgun buffer (Fig. 1).
14. Determine protein concentration with 2D-Quant Kit according to manufacturer's instructions (*see* **Note 20**).

3.4 In-solution Digestion

1. Add 0.5 µL of reducing solution to 50 µg protein solution. Mix gently. Incubate for 60 min (*see* **Note 28**).
2. Add 2 µL of alkylating solution. Mix gently. Incubate for 60 min.
3. Add 2 µL of reducing solution. Mix gently. Incubate for 60 min (*see* **Note 29**).
4. Dilute sample to 0.6 M urea or less with diluting solution.
5. Add trypsin in ratio 1:50. With trypsin concentration of 200 ng/µL, 1 µL is able to digest 10 µg of protein.
6. Add TFA to adjust pH to 4. This quenches trypsin.
7. Desalt samples according to manufacturer's instructions with C18-columns, -tips or self-made tips. This will give you dried peptides to start with phosphopeptide enrichment.

3.5 Phosphopeptide Enrichment

1. Dissolve peptides in 100 µL equilibration buffer and centrifuge for 10 min at 14,000 × *g*.
2. 500 µg of (phospho)protein digest is the minimum recommended starting amount. For this amount of peptides weigh 12.5 mg of TiO_2 into a spin column with a 10 µm pore size polyethylene filter, screw cap and press-in bottom plug (*see* **Note 30**).
3. Equilibrate TiO_2 by adding 250 µL equilibration buffer to each column and incubate for 5 min.
4. Spin the column at 700 × *g* for 2 min.
5. Add dissolved peptides to the column and bind phosphopeptides to the TiO_2 chromatography medium for 15 min by closing the column and incubate it head-over-head.
6. Open the column and place it in a clean tube. Spin at 700 × *g* for 2 min, the flow-through fraction containing non-phosphorylated peptides can be desalted and stored at −20 °C for further analysis.
7. Wash the column twice with 250 µL equilibration buffer, wash buffer 1 and wash buffer 2 (spin at 700 × *g* for 2 min after each wash step to remove the respective solutions).

8. Elute phosphopeptides by applying 100 μL of elution buffer and incubate the closed column for 5 min head-over-head.
9. Collect the phosphopeptides in a clean 1.5 mL Eppendorf Protein LoBind® tube by centrifugation at 700 × *g* for 2 min.
10. Dry peptides completely in a concentrator.

4 Notes

1. The fine powder dissolves easier in solution. This shortens the addition time to solution and allows more time for stirring.
2. Other ammonium hydroxide might be used, but comparison with one different product showed higher efficiency of Sigma chemical.
3. First dissolve 8 M urea in 90 % of the final solution volume. This takes some time and can be expedited with mild heating of the solution. Let the solution cool down before adding the other chemicals. CHAPS should be added last. Storing at 4 °C can lead to crystallization. Stir in time for usage.
4. Storing at 4 °C can lead to crystallization.
5. Other precipitation could be used, but 2D Clean-up Kit showed high efficiency. For cost reasons precipitation and co-precipitation solution can also be used from 2D Quant Kit.
6. Other protein quantification method could be used. 2D Quant Kit gets rid of buffer ingredients which is particular important for protein quantification from incubation buffer.
7. For instance Thermo columns can be used up to 30 μg, Protea tips up to 1 mg. For cost reasons make your own tips.
8. 100–500 mg can be done in 2 mL reaction tube, up to 2 g in 15 mL reaction tube, everything up to 5 g should be done in 50 mL reaction tube. If more material than 5 g is used, please take multiple 50 mL reaction tubes and combine supernatants. For a good amount of phosphoproteins usage of at least 5 g of leaf material is recommended. 25 g of leaf material gives around 400 μg of phosphoproteins.
9. 5 g would need 15 mL of extraction buffer working solution. Produce an excess of ca. 2 mL solution in total to have an extra for pipetting errors.
10. Do not reuse extraction buffer working solution. β-mercaptoethanol, protease inhibitor and phosphatase inhibitor have to be added freshly each time.
11. This step should be done in 10 min. One minute accords to one round of mixing for every sample. If you have more than three samples (3 times 20 s) mix multiple samples at once (three 50 mL reaction tubes per hand are possible).

12. Put the reaction tubes in appropriate rack and fasten with tape. Place horizontally on plate shaker, fasten with tape and shake with 300 motions/min in 4 °C room.
13. Second centrifugation is necessary since a lot of leaf material does not centrifuge to a pellet and would interfere with filtration.
14. Up to 15 mL can be stirred in a 50 mL reaction tube. For more volume use appropriate beaker. The solution should not be too high to allow for maximum stirring efficiency with minimum stirring speed. Take identical reaction tubes and magnetic stirrer for same volumes.
15. This procedure gives seven steps to add ammonium sulfate. Roughly estimate the amount you have to add in every step to have an equal distribution.
16. If you use a beaker transfer the solution before incubation to reaction tube which can be used in centrifuge to avoid remnants of precipitation process in beaker.
17. Be careful, pellet is rather loose.
18. Pellet might not be broken up during washing steps. For proper washing use pipette by pulling up and down again. Attention, pellet might stick to pipette tip.
19. Do not dry pellet too long since solubility decreases. Pellet should not smell like acetone when dry.
20. With starting material of 25 g 80–100 μL would be sufficient. If pellet dissolves poorly use sonication intervals of 15 min or leave at 4 °C overnight.
21. Be aware that no more than 15 samples can be handled at once. If necessary due to comparison reasons get helping hands.
22. This is the basic scale. Up-scaling to 15 mL reaction tubes (6× scale) is recommended due to efficiency reasons.
23. Solution can solidify at 4 °C. Have a look every 10 min. Hand warming helps to liquefy again.
24. Supernatant can be dumped simply. $Al(OH)_3$ pellets very well.
25. Remove all of the incubation buffer since it can interfere with the elution.
26. Matrix is visible as white remnants. $Al(OH)_3$ can interfere with filter device and 2D Clean-Up Kit.
27. If more than 4 mL eluate were obtained repeat centrifugation with same filter device to obtain around 200 μL in total.
28. For 50 μg or less take stated amount of solution. Above 50 μg adapt to proper volume.
29. This will consume any unreacted IAA.
30. The peptide-to-beads ratio is critical for achieving optimal phosphopeptide enrichment. *See* ref. 19 for details.

References

1. Guo M, Huang BX (2013) Integration of phosphoproteomic chemical and biological strategies for the functional analysis of targeted protein phosphorylation. Proteomics 13:424–437
2. Manning G, Plowman GD, Hunter T et al (2002) Evolution of protein kinase signaling from yeast to man. Trends Biochem Sci 27:514–520
3. Wang D, Harper JF, Gribskov M (2003) Systematic trans-genomic comparison of protein kinases between *Arabidopsis* and *Saccharomyces cerevisiae*. Plant Physiol 132:2152–2165
4. De la Fuente van Bentem S, Roitinger E, Anrather D et al (2006) Phosphoproteomics as a tool to unravel plant regulatory mechanisms. Physiol Plant 126:110–119
5. Champion A, Kreis M, Mockaitis K et al (2004) *Arabidopsis* kinome: after the casting. Funct Integr Genomics 4:163–187
6. Hunter T (2000) Signaling—2000 and beyond. Cell 100:113–127
7. Herskowitz I (1995) MAP kinase pathways in yeast: for mating and more. Cell 80:187–197
8. Hamel LP, Nicole MC, Sritubtim S et al (2006) Ancient signals: comparative genomics of plant MAPK and MAPKK gene families. Trends Plant Sci 11:192–198
9. Meng X, Zhang S (2013) MAPK cascades in plant disease resistance signaling. Annu Rev Phytopathol 51:245–266
10. Olsen JV, Blagoev B, Gnad F et al (2006) Global in vivo and site-specific phosphorylation dynamics in signaling networks. Cell 127:635–648
11. Olsen JV, Vermeulen M, Santamaria A et al (2010) Quantitative phosphoproteomics reveals widespread full phosphorylation site occupancy during mitosis. Sci Signal 3:1–15
12. Choudhary C, Olsen JV, Brandts C et al (2009) Mislocalized activation of oncogenic RTKs switches downstream signaling outcomes. Mol Cell 36:326–339
13. Nakagami H, Sugiyama N, Ishihama Y et al (2012) Shotguns in the front line: phosphoproteomics in plants. Plant Cell Physiol 53:118–124
14. Lassowskat I (2008) Optimierung Erweiterung und Anwendung der Metalloxid-Affinitätschromatographie zur Phosphoproteomanalyse in *Arabidopsis thaliana*. Diploma thesis Martin-Luther-University Halle-Wittenberg, Halle/Saale Germany
15. Wolschin F, Wienkoop S, Weckwerth W (2005) Enrichment of phosphorylated proteins and peptides from complex mixtures using metal oxide/hydroxide affinity chromatography (MOAC). Proteomics 5:4389–4397
16. Lassowskat I, Naumann K, Lee J et al (2013) PAPE (Prefractionation-Assisted Phosphoprotein Enrichment): a novel approach for phosphoproteomic analysis of green tissues from plants. Proteomics 1(3):254–274
17. Hoehenwarter W, Thomas M, Nukarinen E et al (2013) Identification of novel in vivo MAP kinase substrates in *Arabidopsis thaliana* through use of tandem metal oxide affinity chromatography. Mol Cell Proteomics 12:369–380
18. Agrawal GK, Thelen JJ (2005) Development of a simplified economical polyacrylamide gel staining protocol for phosphoproteins. Proteomics 5:4684–4688
19. Li QR, Ning ZB, Tang JS et al (2009) Effect of peptide-to-TiO_2 beads ratio on phosphopeptide enrichment selectivity. J Proteome Res 8:5375–5381

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