Chapter 6

Histological Staining and Hydrogen Peroxide Visualization of the Abscission Zone in *Setaria viridis*

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Abstract

The abscission zone (AZ) consists of specialized cell layers where cell separation or breakage occurs that result in organ detachment. Microscopic observation of the AZ is crucial for understanding its function. The AZ undergoes cellular and physiological changes prior to abscission, such as cell death, loss of chlorophyll, and the production of reactive oxygen species (ROS). These changes can be visualized using specific dyes and indicators under light or fluorescent microscopes. However, one challenge of using these dyes is their inefficient penetration into the tissue, especially when the epidermal layer has thick secondary cell walls. In this chapter, a detailed protocol to overcome this challenge is described. Using the fruit AZ of *Setaria viridis*, in which the epidermal cell wall is thick and lignified, we gently fix the dissected tissue, embed it in the Cryomatrix, and trim off the outer cell layers using a cryostat. The tissue with exposed inner cells can then be stained with fluorescent dyes to visualize organelles of interest, or 3,3′-diaminobenzidine (DAB) to visualize hydrogen peroxide accumulated in the tissue.

Key words Abscission Zone (AZ), Chlorophyll quantification, DAPI staining, Calcofluor white, 3,3′-Diaminobenzidine (DAB), Reactive oxygen species (ROS), Hydrogen peroxide, Cryosection, *Setaria Viridis*

1 Introduction

Abscission is a common process in which plants shed their unwanted organs, such as leaves, floral parts, fruits, and seeds. Abscission occurs at predefined cell layers at the abscission zone (AZ) and is regulated by developmental and environmental signals. In many species, the AZ exhibits particular anatomical characteristics, including small cell size with isodiametric shapes, dense cytoplasm, and differing cell wall compositions compared to the adjacent cells [1, 2]. However, recent studies in grass species revealed diverse anatomy of the AZ that may or may not be histologically distinct [3–6]. Microscopic observations immediately following abscission reveal that abscission occurs through either cell separation or cell breakage [7–10]. These observations

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demonstrate the importance of microscopy studies in understanding AZ differentiation and abscission function. High-resolution anatomical observations either use paraffin or resin-embedded plant sections under a light or transmission electron microscope, or whole-mount tissues under a scanning electron microscope.

Prior to abscission, the AZ undergoes a series of cellular and physiological changes. For example, cell death and loss of chlorophyll are observed right before abscission in some species [10, 12, 13]. Abscission is activated by hormonal regulation, including auxin, ethylene, and jasmonic acid [11]. Both the inhibitory effect of auxin and the accelerating effect of ethylene on abscission occur through the regulation of reactive oxygen species (ROS) at the AZ [14]. Exogenous application of H_2O_2 or induction of endogenous superoxide by mutating a manganese superoxide dismutase *MSD2* accelerates abscission, suggesting that ROS is involved in the timing of abscission downstream of hormonal signaling [14, 15].

ROS is a group of highly reactive molecules derived from oxygen (O_2), including hydrogen peroxide (H_2O_2), superoxide (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical (HO·), and other forms of peroxides [16]. ROS can be visualized using dyes such as 3,3′-diaminobenzidine (DAB), nitro blue tetrazolium (NBT), or 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). DAB is oxidized by H_2O_2 and peroxidases to generate a dark brown precipitate [17]. NBT specifically reacts to O_2^- and forms a blue precipitate. H_2DCFDA is ideal for detecting intracellular ROS in live cells, as it requires permeating into the cells, being cleaved by intracellular esterases, and being oxidized by ROS to convert to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) [18]. DAB, NBT, and H_2DCFDA have all been used for studying abscission, such as in *Arabidopsis* petal AZ, and tomato and *Capsicum* leaf AZ [12, 14, 15, 19]. Freshly prepared flowers or leaves are used for these experiments.

Despite the availability of various microscopy techniques and ROS dyes for studying the AZ, some are not suitable for certain species. In our study focusing on green millet (*Setaria viridis*), a model C_4 grass species and a relative of foxtail millet (*Setaria italica*), sorghum (*sorghum bicolor*), and maize (*Zea mays*), we encountered difficulties in visualizing chlorophyll loss at the cellular level and detecting ROS accumulation in the fruit AZ. For the visualization and quantification of chlorophyll lost, conventional microscopy methods require dehydration with alcohol before paraffin or resin embedding, a process that removes chlorophyll. Due to the small size of the fruit AZ in *S. viridis* (diameter 300–400 μm), measuring chlorophyll using other methods such as spectrophotometry or fluorometry is unlikely. Regarding ROS staining, the dyes fail to penetrate the AZ when fresh tissues are used due to the thick epidermal layer.

To overcome these challenges, we developed a method involving gently fixed and cryo-trimmed AZ tissues. The method allows staining of tissues with various dyes and indicators, facilitating visualization under a light or fluorescent microscope. We stain our tissues with the nuclei indicator DAPI (4′,6-diamidino-2-phenylindole), the cellulose indicator Calcofluor White, and the H_2O_2 indicator DAB. Confocal microscopy is used to detect chlorophyll autofluorescence. With this method, differences in chlorophyll intensity and H_2O_2 accumulation between the wild-type and a nonfunctional AZ mutant of *S. viridis* can be detected [10]. This chapter provides a detailed description of the method.

2 Materials

2.1 Chemicals and Reagents

1. 0.2 M PIPES (1,4-Piperazinediethanesulfonic acid) stock buffer: Add 12.97 g of PIPES sodium salt (Na-PIPES) powder to ~160 mL H_2O. Under stirring conditions, adjust the pH using 10 N NaOH to pH 7.4. Add additional H_2O to reach a final volume of 200 mL (*see* **Note 1**).
2. 0.1 M PIPES buffer: Mix equal volumes of 0.2 M PIPES stock buffer and H_2O.
3. 4% PFA (paraformaldehyde) fixative: For 40 mL of fixative, combine 10 mL of 16% (w/v) PFA aqueous solution (EM grade) (Electron Microscopy Sciences), 40 μL Tween 20 (final concentration 0.1% (v/v)), 20 mL of 0.2 M PIPES buffer, and 10 mL of H_2O.
4. Sucrose solutions: To make a 2.3 M sucrose stock solution, dissolve 31.49 g of solid sucrose in H_2O to a final volume of 40 mL. Keep shaking or stirring until fully dissolved. To make 25%, 33%, 50%, 66%, and 75% (v/v) of 2.3 M sucrose solution, mix 2.3 M sucrose stock and H_2O with ratios of 1:3, 1:2, 1:1, 2:1, and 3:1 (v:v).
5. Thermo Scientific™ Shandon Cryomatrix™ Frozen Embedding Medium.
6. Dry ice.
7. DAPI and Calcofluor White solution: Add 4 μL of DAPI stock solution (1 mg/mL) (Thermo Fisher Scientific) and 2 μL of 5 mM Calcofluor White stain (Biotium) to 2 mL of H_2O. The final concentrations of DAPI and Calcofluor White are 2 μg/mL and 5 μM, respectively.
8. DAB solution: To make 10 mL of 0.1% (w/v) DAB solution, add 10 mg of solid DAB to 10 mL of water. Adjust the pH to 3.8 with 1 N HCl (*see* **Note 2**).

9. 95% (v/v) ethanol.
10. Ultrapure H_2O.

2.2 Consumables and Equipment

1. Feather™ Micro Scalpel, 45° (Electron Microscopy Sciences).
2. Fine forceps.
3. 2 mL microcentrifuge tubes.
4. Vacuum.
5. mPrep/s™ Capsules (Microscopy Innovations).
6. Tissue-Tek® Cryomold® 15 mm × 15 mm × 5 mm (Sakura Finetek).
7. Epredia™ Peel-A-Way™ Disposable Embedding Molds (Thermo Fisher Scientific) (*see* **Note 3**).
8. Coverslips, 24 × 40 mm and 22 × 22 mm.
9. Compartment Petri dishes (100 mm × 15 mm).
10. Benchtop centrifuge.
11. Benchtop open-air shaker.
12. Single-edge razor blades.
13. −80 °C laboratory freezer.
14. Common stereo microscope.
15. Leica CM1950 cryostat (Thermo Scientific).
16. TCS SP8-X confocal microscope (Leica Microsystems).
17. ZEISS Axio Zoom.V16 Fluorescence Stereo Zoom Microscope (Zeiss).

3 Methods

3.1 Tissue Dissection and Fixation

1. Dissect the AZ and the immediately surrounding tissues using a micro scalpel under a stereo microscope (*see* **Note 4**).
2. Immediately transfer the dissected tissues into the PFA fixative in a 2 mL microcentrifuge tube and keep them on ice (*see* **Note 5**).
3. Vacuum infiltrate for 10 min or until most tissues sink to the bottom (*see* **Note 6**).
4. Incubate samples at 4 °C overnight (*see* **Note 7**).
5. Rinse samples in 0.1 M PIPES buffer three times for 10 min each.

3.2 (Optional) Sucrose Infiltration (See Note 8)

1. Place samples in an mPrep/s™ Capsule using forceps. Add another capsule on top and place the pair of capsules into an empty 2 mL centrifuge tube.

2. Add 25% (v/v) of 2.3 M sucrose solution to the centrifuge tube, ensuring that samples are completely submerged in the solution.
3. Briefly centrifuge the tube using a benchtop centrifuge (*see* **Note 9**).
4. Gently shake samples on a benchtop shaker at about 40 rpm at room temperature for 1 h (*see* **Note 10**).
5. Take out the capsule pair from the centrifuge tube and remove excess sucrose solution by placing the bottom on a paper towel.
6. Replace the sucrose solution with 33% in the centrifuge tube. Place the capsule pair back into the tube.
7. Repeat **steps 3–6** for 50%, 66%, and 75% (v/v) of 2.3 M sucrose.
8. Replace the 75% sucrose solution with 100% and incubate overnight at 4 °C [20].

3.3 Sample Embedding

1. Remove samples from the 2.3 M sucrose or rinsing buffer and place them in a cryomold filled with Cryomatrix embedding medium.
2. Fill a clean cryomold with Cryomatrix embedding medium without introducing bubbles.
3. Place four to six samples longitudinally at the bottom of the Cryomatrix embedding medium. Avoid introducing bubbles around the samples.
4. Carefully transfer the cryomold to dry ice to freeze the samples and Cryomatrix embedding medium.
5. Proceed to the next step or seal the samples in a plastic bag and store at −80 °C (*see* **Note 11**).

3.4 Sample Sectioning

1. Place a new blade on the blade holder of the cryostat. Precool the cryostat at −20 °C before use (*see* **Note 12**).
2. Place samples in the cryostat and remove the cryomold (*see* **Note 13**). Leave samples in the cryostat for a few minutes to equilibrate the sample temperature to that of the cryostat.
3. Use a single-edge razor blade to cut and separate individual samples in the same cryomold.
4. Add a small amount of Cryomatrix embedding medium on a specimen chuck in the cryostat.
5. Quickly place one sample on the Cryomatrix embedding medium with the sample side facing up. Try to keep the block surface parallel to the specimen chuck. Allow the embedding medium to freeze.

6. Clamp the specimen chuck onto the cryostat. Adjust the orientation of the sample so that the front face of the block is parallel to the blade.
7. Start sectioning with a thickness of 20–30 μm and reduce to 10 μm when the sample is visible. Stop sectioning when the central vascular tissues in the pedicel are exposed (*see* **Note 14**).
8. Remove the sample from the specimen chuck with a single-edge razor blade and keep samples in the cryostat or on dry ice before staining.

3.5 Sample Staining and Image Acquisition

3.5.1 DAPI and Calcofluor White Staining, Imaging, and Chlorophyll Quantification

1. Prepare the DAPI and Calcofluor White solution in a disposable embedding mold.
2. Using forceps, transfer the trimmed frozen samples into the staining solution with samples facing down (*see* **Note 15**).
3. Incubate samples for 10 min in the dark.
4. Rinse samples for 5 min by transferring them into H_2O in a clean embedding mold.
5. Mount samples with 40 μL of H_2O on a 24 × 40 mm coverslip. Cover with a 22 × 22 mm coverslip (*see* **Note 16**). Gently press the edge of the coverslip to make samples flat (*see* **Note 17**).
6. Observe and image samples on a TCS SP8-X confocal microscope with an HC PL APO CS2 63×/1.20 water objective lens. DAPI and Calcofluor White are excited at a wavelength of 405 nm and imaged with an emission wavelength window of 415–630 nm. Chlorophyll autofluorescence is excited at a wavelength of 649 nm and imaged with an emission wavelength window of 660–780 nm. An example of the images is shown in Fig. 1.
7. To quantify the chlorophyll signal, download TIFF images from the Leica software. Open the image in FIJI. Select Image –> Color –> Split Channels. Keep the red chlorophyll channel. Then select Image –> Adjust –> Threshold (*see* **Note 18**). Adjust the maximum threshold to 255 and the minimum threshold to a value that the signal only overlaps with chloroplasts (*see* **Note 19**). Use the polygon tool to select the AZ area. Select Analyze –> Measure (Fig. 2). The average intensity of the chlorophyll in the selected AZ area was calculated as Mean × %Area/100.

3.5.2 H_2O_2 Staining with DAB

1. Pour the DAB solution into a compartment Petri dish.
2. Transfer trimmed frozen samples with forceps into the staining solution with samples facing down.
3. Incubate the samples for 2 h at room temperature in the light.

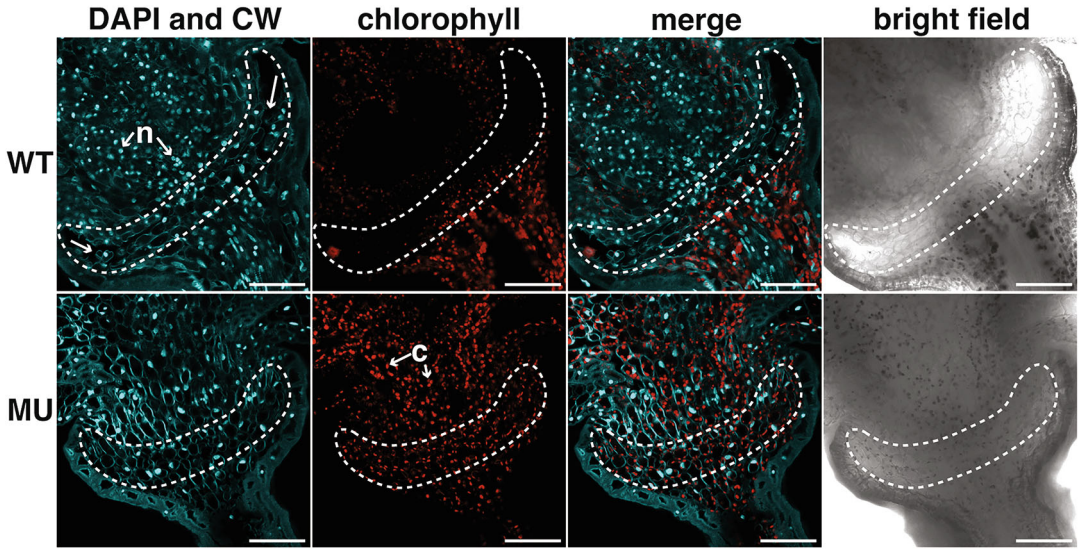

Fig. 1 Confocal microscopy reveals differences in cellular features between wild type (WT) and a non-shatter mutant (MU) in *Setaria viridis*. Nuclei and cellulose are stained with DAPI (4′,6-diamidino-2-phenylindole) and Calcofluor White (CW), respectively. The abscission zones (AZ) are marked with white dotted circles. Cell separation in WT is indicated by white arrows within the AZ. c, chloroplasts; n, nuclei. Bars, 50 μm

4. Transfer samples into 95% (v/v) ethanol in a 2 mL centrifuge tube and incubate for 1 h in the dark at room temperature on a benchtop shaker at about 40 rpm. Chlorophyll will be removed at this step (*see* **Note 20**).
5. Transfer samples into water in a compartment Petri dish (*see* **Note 21**). Place samples on a cover slide with water on the slide. Take images within an hour using a ZEISS Axio ZoomV16 fluorescence stereo zoom microscope (*see* **Note 22**). An example of the stained tissues is shown in Fig. 3 (*see* **Note 23**).

4 Notes

1. PIPES does not dissolve under low pH. It is necessary to use highly concentrated NaOH to increase pH. The solution gradually becomes clear when the pH is above 6. PIPES solution can be stored at 4 °C for 1 month without contamination. Sterilization may prolong the storage time.
2. DAB solution needs to be prepared freshly within an hour of the experiment. DAB is light-sensitive, so the solution needs to be kept in darkness before the experiment. Often, the DAB powder cannot be fully dissolved. It may be filtered before usage. However, we did not find any interference of the crystals in our experiments.

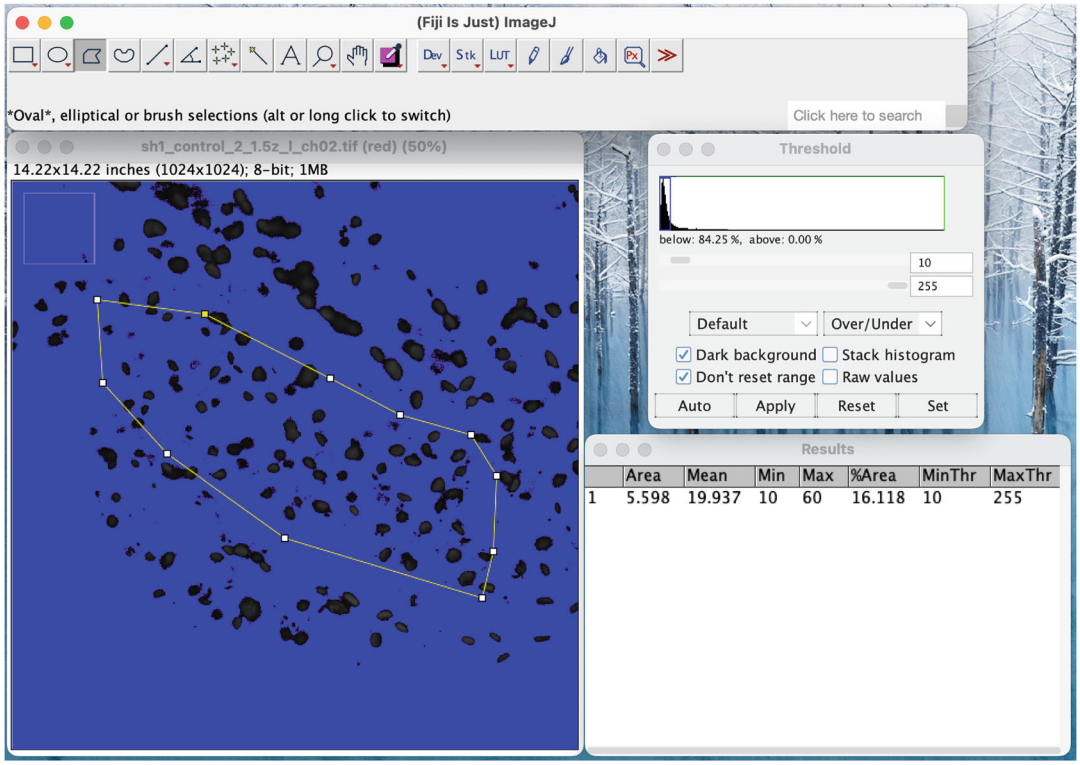

Fig. 2 Screenshot of chlorophyll quantification using FIJI. The image threshold has been set between 10 and 255. In other words, signal intensity below 10 is considered as background (shown in blue); signal intensity between 10 and 255 is considered as true signals (shown in black). The polygon tool was used to select the region of interest, which is the left half of the AZ in the example image. The results show selected measurement parameters, including Area (area within the polygon that has signals between 10 and 255), Mean (the sum of the gray values of all pixels between 10 and 255 in the selected polygon divided by the number of pixels), Min (minimum gray value within the polygon), Max (maximum gray value within the polygon), %Area (the ratio between area with pixels between 10 and 255 and the total selected area in the polygon), MinThr (minimum threshold), and MaxThr (maximum threshold)

3. The holder can be replaced by any similar products. We selected this product based on its small size, availability in the lab, and convenience in manipulating samples.
4. This step helps with fixative infiltration. Smaller samples with more uniform hardness are essential for obtaining intact samples in the later sectioning step.
5. A 2 mL centrifuge tube was chosen due to the small AZ size (~1 mm) of *S. viridis*. Use larger containers for larger samples. The volume of the fixative should be at least ten times the sample size.
6. Multiple vacuum infiltrations with short durations are better than one long infiltration step. We keep the cap open during infiltration. In between the infiltration steps, gently tapping the

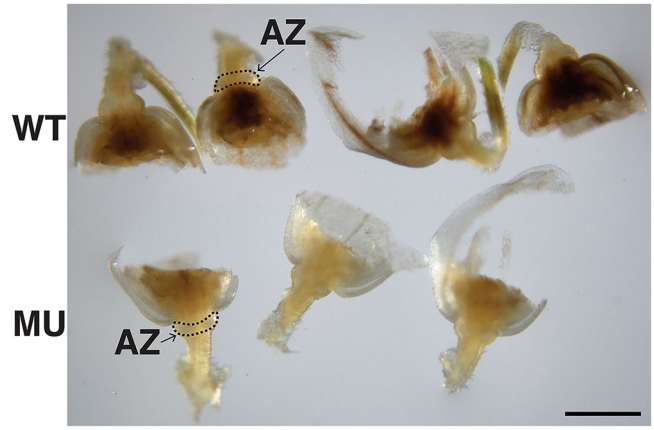

Fig. 3 DAB staining reveals differences in H_2O_2 accumulation next to the AZ between wild-type (WT) and a non-shatter mutant (MU). Brown color indicates H_2O_2 accumulation. The AZ is marked by black dotted circles. Bar, 500 μm

tube helps remove the bubbles attached to the samples and facilitates sample sinking.

7. Shorter fixing times, such as 30 min to 2 h at room temperature, may be used for small samples.
8. High concentrations of sucrose preserve cell morphology and protect the cells from the formation of ice crystals during freezing. We found that omitting this step resulted in cell collapse and irregularly shaped nuclei. If cellular and subcellular morphology is crucial for downstream experiments, then this step is essential. However, for visualization at the tissue level as demonstrated in DAB staining, this step can be omitted.
9. This step removes bubbles in the lower capsule.
10. The incubation time can be adjusted. Samples sink to the bottom when infiltration is complete, which is a sign to move on to the next step.
11. We stored samples for up to 6 months without affecting downstream experiments.
12. The specific temperature of the cryostat and the sample holder should be tested. In general, when the temperature is too cold, sections are brittle and tend to shatter; when the temperature is too warm, sections tend to stick on the knife and compressed folds occur. Since our goal is not to obtain individual sections, but to trim off the outer layers of cells, the requirement for sectioning temperature is less stringent. We tested a range from −18 °C to −22 °C, and they all worked.
13. Samples should be kept on dry ice during the transfer between −80 °C and the cryostat.

14. The vascular tissue may not be visible due to small sample size. Since the AZ of *S. viridis* is in a sphere shape, our trick is to section until we observe the largest diameter of the AZ. Therefore, it is essential to cut thin sections to avoid over-trimming. We also section multiple samples to identify ideal ones for imaging.
15. There is no need to remove the Cryomatrix embedding medium as it will melt during incubation.
16. We use two coverslips instead of a coverslip and a cover slide because the trimmed face of samples may either face up or down. We image the samples with the correct orientation and then flip the coverslip to image the rest of the samples.
17. It is important to only press the edge of coverslips gently, as pressure may damage samples.
18. After setting the threshold, do not click "Apply," otherwise, the signal values become binary, either 0 or 255. Directly go to the measurement step. The results will show the minimum gray value as the set minimum threshold.
19. The autofluorescence may vary among samples even with the same acquisition setting. We normally test a few images and select a minimum threshold that removes background noise in most images. Because of the background noise, it is essential to repeat the experiments with enough replicates. We used 6–10 replicates in our experiments.
20. The incubation time depends on the size of the tissue. Larger samples may need larger containers and longer incubation time.
21. Tissues are softer and easier to manipulate in water compared to those in ethanol.
22. Keep incubation time at each step the same among samples and replicates, including the last water incubation. This is because the brown precipitate keeps developing over time.
23. We failed to detect O_2^- using NBT staining with the same sample preparation, probably due to the instability of O_2^-. This sample preparation is also not suitable for DCF staining, which requires living cells.

References

1. Valdovinos JG, Jensen TE (1968) Fine structure of abscission zones: II. Cell-wall changes in abscising pedicels of tobacco and tomato flowers. Planta 83:295–302. https://doi.org/10.1007/BF00385339
2. Sexton R, Roberts JA (1982) Cell biology of abscission. Ann Rev Plant Phys 33:133–162. https://doi.org/10.1146/annurev.pp.33.060182.001025
3. Hodge JG, Kellogg EA (2016) Abscission zone development in *Setaria viridis* and its domesticated relative, *Setaria italica*. Am J Bot 103:998–1005. https://doi.org/10.3732/ajb.1500499

4. Fu Z, Song J, Zhao J, Jameson PE (2018) Identification and expression of genes associated with the abscission layer controlling seed shattering in *Lolium perenne*. AoB Plants 11:ply076. https://doi.org/10.1093/aobpla/ply076
5. Yu Y, Leyva P, Tavares RL, Kellogg EA (2020) The anatomy of abscission zones is diverse among grass species. Am J Bot 107:549–561. https://doi.org/10.1002/ajb2.1454
6. Zeng X, Tagiri A, Kikuchi S, Sassa H, Komatsuda T (2020) The ectopic expression of *Btr2* in *Aegilops tauschii* switches the disarticulation layer from above to below the rachis node. Front Plant Sci 11:582622. https://doi.org/10.3389/fpls.2020.582622
7. Roberts JA, Elliott KA, Gonzalez-Carranza ZH (2002) Abscission, dehiscence, and other cell separation processes. Annu Rev Plant Biol 53:131–158. https://doi.org/10.1146/annurev.arplant.53.092701.180236
8. Ogawa M, Kay P, Wilson S, Swain SM (2009) Arabidopsis dehiscence zone polygalacturonase1 (ADPG1), ADPG2, and QUARTET2 are Polygalacturonases required for cell separation during reproductive development in *Arabidopsis*. Plant Cell 21:216–233. https://doi.org/10.1105/tpc.108.063768
9. Yu Y, Beyene G, Villmer J, Duncan KE, Hu H, Johnson T, Doust AN, Taylor NJ, Kellogg EA (2023) Grain shattering by cell death and fracture in *Eragrostis tef*. Plant Physiol 192:222–239. https://doi.org/10.1093/plphys/kiad079
10. Yu Y, Hu H, Voytas DF, Doust AN, Kellogg EA (2023) The *YABBY* gene *SHATTERING1* controls activation rather than patterning of the abscission zone in *Setaria viridis*. New Phytol 240:846–862. https://doi.org/10.1111/nph.19157
11. Patharkar OR, Walker JC (2017) Advances in abscission signaling. J Exp Bot 69:733–740. https://doi.org/10.1093/jxb/erx256
12. Bar-Dror T, Dermastia M, Kladnik A, Znidaric MT, Novak MP, Meir S, Burd S, Philosoph-Hadas S, Ori N, Sonego L et al (2011) Programmed cell death occurs asymmetrically during abscission in tomato. Plant Cell 23:4146–4163. https://doi.org/10.1105/tpc.111.092494
13. Patharkar OR, Walker JC (2016) Core mechanisms regulating developmentally timed and environmentally triggered abscission. Plant Physiol 172:510–520. https://doi.org/10.1104/pp.16.01004
14. Sakamoto M, Munemura I, Tomita R, Kobayashi K (2008) Involvement of hydrogen peroxide in leaf abscission signaling, revealed by analysis with an in vitro abscission system in *Capsicum* plants. Plant J 56:13–27. https://doi.org/10.1111/j.1365-313X.2008.03577.x
15. Lee J, Chen H, Lee G, Emonet A, Kim S, Shim D, Lee Y (2022) MSD2-mediated ROS metabolism fine-tunes the timing of floral organ abscission in Arabidopsis. New Phytol 235:2466–2480. https://doi.org/10.1111/nph.18303
16. Mittler R, Zandalinas SI, Fichman Y, Breusegem FV (2022) Reactive oxygen species signalling in plant stress responses. Nat Rev Mol Cell Biol 23:663–679. https://doi.org/10.1038/s41580-022-00499-2
17. Šnyrychová I, Ayaydin F, Hideg É (2009) Detecting hydrogen peroxide in leaves in vivo—a comparison of methods. Physiol Plant 135:1–18. https://doi.org/10.1111/j.1399-3054.2008.01176.x
18. Jambunathan N (2010) Determination and detection of reactive oxygen species (ROS), lipid peroxidation, and electrolyte leakage in plants. Methods Mol Biol 639:292–298. https://doi.org/10.1007/978-1-60761-702-0_18
19. Lee Y, Yoon TH, Lee J, Jeon SY, Lee JH, Lee MK, Chen H, Yun J, Oh SY, Wen X et al (2018) A lignin molecular brace controls precision processing of cell walls critical for surface integrity in arabidopsis. Cell 173:1468–1480.e9. https://doi.org/10.1016/j.cell.2018.03.060
20. Knapp E, Flores R, Scheiblin D, Scheiblin D, Modla S, Czymmek K, Czymmek K, Yusibov V (2012) A cryohistological protocol for preparation of large plant tissue sections for screening intracellular fluorescent protein expression. BioTechniques 52:31–37. https://doi.org/10.2144/000113778