STAR Protocols

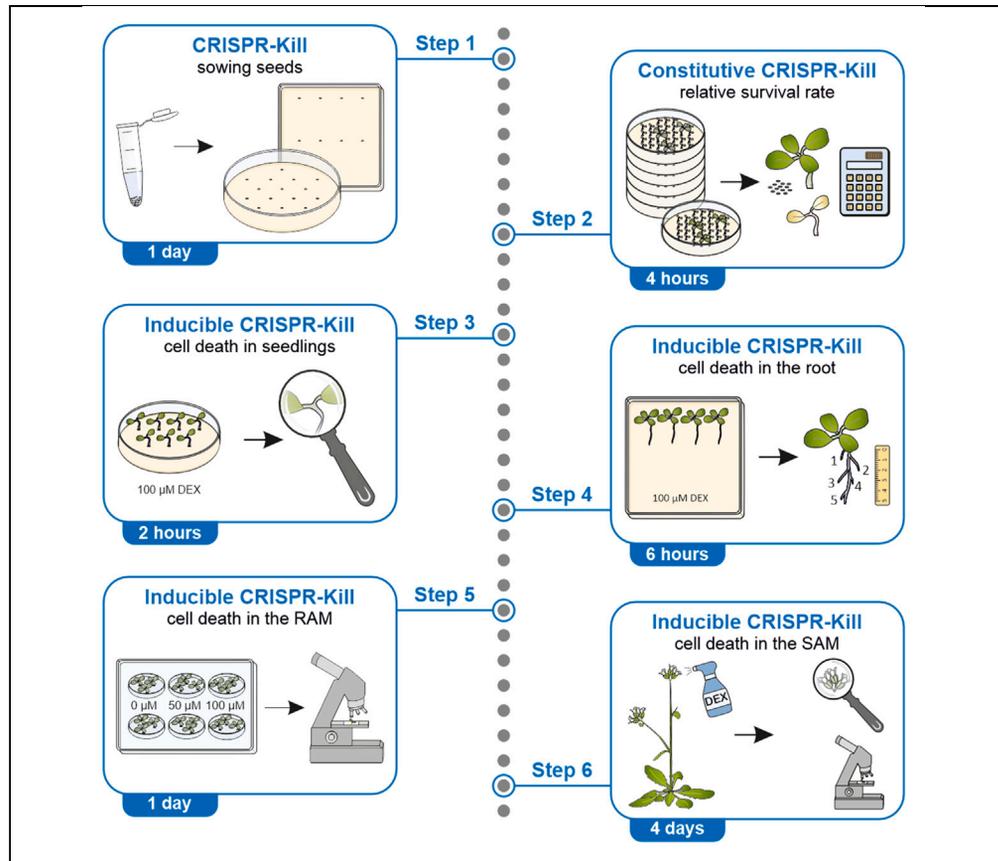**Protocol**

Protocol to efficiently induce CRISPR-Kill-mediated cell ablation in *Arabidopsis thaliana*

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Highlights

Application of the constitutive or chemically inducible CRISPR-Kill system

Independent induction of targeted cell death in different organs and developmental stages

Identifies macroscopic and microscopic effects of induced cell ablation

Concurrent detection of target cells through fluorescent markers

The CRISPR-Kill system enables targeted cell ablation by inducing multiple double-strand breaks in evolutionarily conserved repetitive genomic regions. Here, we present a protocol for the application of the CRISPR-Kill system to analyze the systemic and cellular effects of targeted cell death in Arabidopsis. We describe steps for generating constitutive and inducible CRISPR-Kill lines, chemically inducing CRISPR-Cas9-mediated genome elimination, and monitoring of cell death in shoot and root apical meristems. This enables the investigation of a wide range of questions in developmental plant biology.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Protocol

Protocol to efficiently induce CRISPR-Kill-mediated cell ablation in *Arabidopsis thaliana*

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SUMMARY

The CRISPR-Kill system enables targeted cell ablation by inducing multiple double-strand breaks in evolutionarily conserved repetitive genomic regions. Here, we present a protocol for the application of the CRISPR-Kill system to analyze the systemic and cellular effects of targeted cell death in *Arabidopsis*. We describe steps for generating constitutive and inducible CRISPR-Kill lines, chemically inducing CRISPR-Cas9-mediated genome elimination, and monitoring of cell death in shoot and root apical meristems. This enables the investigation of a wide range of questions in developmental plant biology.
For complete details on the use and execution of this protocol, please refer to Gehrke et al.[1]

BEFORE YOU BEGIN

This protocol was used in a recent publication (Gehrke et al., 2023[1]) to specifically induce CRISPR-Kill-mediated cell death in ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6)-positive cells. Therefore, the AHP6-promoter (pAHP6) was used to drive the constitutive expression of either the Cas9 nuclease from *Staphylococcus aureus* (SaCas9; uninducible approach) or the GR-LhG4 transcription factor (inducible approach) in protoxylem precursor and pericycle cells of the root apical meristem (RAM) as well as organ primordia in the shoot apical meristem (SAM). However, we have also used this protocol, or parts of it, with several other promoters before.[1,2]

Preparation of solid germination medium

⏲ Timing: 1 day

Before the experiment, prepare the medium to be used in subsequent steps according to the desired experimental setup one day in advance, unless stated otherwise.

1. Prepare herbicide-containing medium for the selection of plants carrying the respective transgene.
   a. Prepare herbicide-containing medium for the selection of primary transformants.
      i. Prepare germination medium with 0.8% (w/v) plant agar.
      ii. After the medium has been autoclaved and cooled below 50°C, add Cefotaxime (to kill Agrobacteria) and the respective herbicide.
      iii. Pour 75 mL each of the prepared medium into petri dishes (150 × 20 mm).
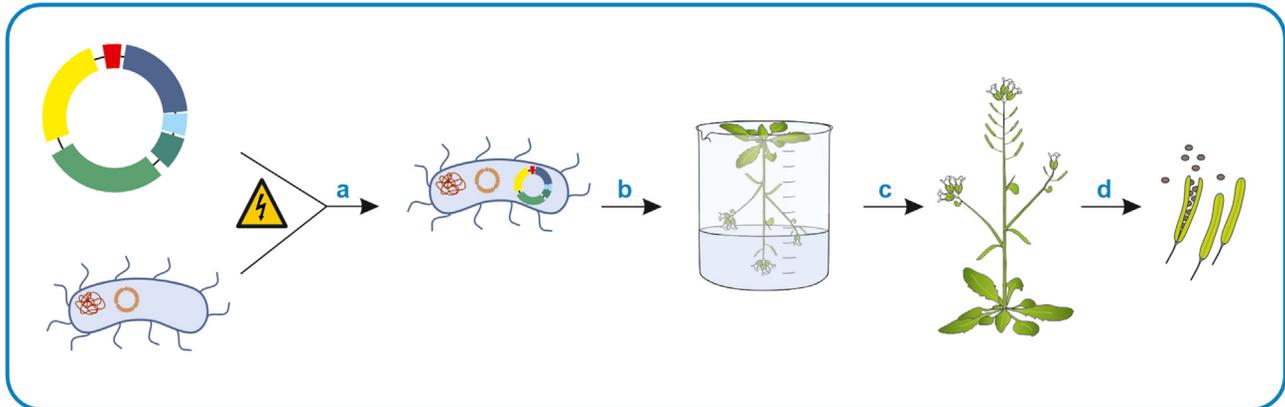
Figure 1. Workflow for generating constitutive, uninducible CRISPR-Kill lines
(A) Transformation of Agrobacteria with the respective expression vectors via electroporation.
(B) Agrobacteria-mediated T-DNA integration into *Arabidopsis* via the floral dip method.
(C) Cultivation of the transformed *Arabidopsis* plants.
(D) Harvesting the seeds of transformed *Arabidopsis* plants.

> b. Prepare herbicide-containing medium for the cultivation of driver, effector and expression lines.
> > i. Prepare germination medium with 0.8% (w/v) plant agar.
> > ii. After the medium has been autoclaved and cooled below 50°C, add the respective herbicide(s).
> > iii. Pour 25 mL each of the prepared medium into petri dishes (92 × 16 mm).

Note: The plates can be used as soon as the medium is solid. Alternatively, the plates can be stored at 4°C for up to three months.

2. Prepare the dexamethasone (DEX)-containing medium for the induction of the CRISPR-Kill activity.
   a. Prepare the DEX-containing medium for the induction of the CRISPR-Kill activity in seedlings.
      i. Prepare germination medium with 0.8% (w/v) plant agar.
      ii. After the medium has been autoclaved and cooled below 50°C, add the required amount of DEX or, as a control, an equal amount of DMSO as well as the respective herbicide(s).
      iii. Pour 25 mL each of the prepared medium into petri dishes (92 × 16 mm).
   b. Prepare the DEX-containing medium for the induction of the CRISPR-Kill activity in the RAM.
      i. Prepare germination medium with 1% (w/v) plant agar.
      ii. After the medium has been autoclaved and cooled below 50°C, add the required amount of DEX or, as a control, an equal amount of DMSO.
      iii. Pour 50 mL each of the prepared medium into square petri dishes (100 × 100 × 20 mm).

Note: The plates can be used as soon as the medium is solid. We recommend using the plates on the same day. However, if the plates should be used later, they should be stored at 4°C in the dark up to three months.

Generation of the constitutive, uninducible CRISPR-Kill lines

⏱ Timing: 8 weeks

In order to analyze the effect of constitutive CRISPR-Kill-mediated cell elimination within a certain cell type, plant lines expressing the CRISPR-Kill system under the control of a corresponding tissue-specific promoter need to be generated (Figure 1). Depending on the target tissue, the

expression vectors pAHP6-SaCas9-ITS2 or pAHP6-SaCas9-ADH1 can be easily adapted by exchanging the pAHP6-promoter via classical cloning using the restriction enzyme EcoRI.

3. Transform *A. tumefaciens* with the CRISPR-Kill and control expression vectors.
   a. Gently thaw 50 µL of electrocompetent GV3101::pMP90 *A. tumefaciens* bacteria on ice and pre-cool one electroporation cuvette per construct at 4°C.
   b. Add 1 µL of either pAHP6-SaCas9-ITS2 or pAHP6-SaCas9-ADH1 expression vector (200 ng/µL) to 100 µL of the thawed Agrobacteria.
   c. Release the electrical pulse (U = 2500 V, K = 25 µf, R = 200 Ω, t = 5 s) and place the cuvette on ice immediately afterward.
   d. Add 500 µL of liquid YEB medium to the cells, pre-suspend and transfer the suspension to a new reaction tube and incubate the culture for 60 min at 28°C and 650 rpm.
   e. Plate 50 µL of the bacterial suspension on solid YEB medium with the respective antibiotics and incubate the plates for two days at 28°C.
   f. Prepare a preculture by transferring one Agrobacteria colony into 50 mL of liquid YEB medium with the corresponding antibiotics and incubate the culture for two days at 28°C.

   Note: To double-check the successful integration of the plasmid, verify the grown colonies via colony PCR.

   ⏸⏸ **Pause point:** For long-term storage of *A. tumefaciens* cultures, glycerol stocks can be prepared with 700 µL overnight culture and 300 µL 100% glycerol and stored at −80°C.

4. For the stable transformation of *A. thaliana*, perform the *A. tumefaciens* mediated floral-dip method.[3]
   a. Prepare the main culture by transferring 500 µL of the preculture into 400 mL of liquid YEB medium with the corresponding antibiotics and incubate the culture for 18–24 h at 28°C and 200 rpm to an OD600 of 1.7.
   b. Centrifuge the main culture for 15 min at 20°C and 6 000 *g* and resuspend the pellet in 800 mL of freshly prepared infiltration medium.
   c. Remove the pods as well as the already opened flowers from four to five week old *A. thaliana* plants and immerse the prepared plants in the bacterial suspension for 30 s.
   d. Incubate the plants for 18–24 h in a moist and dark place.
   e. Spray the plants with tap water and cultivate the transformed plants until seed maturity.

   ⏸⏸ **Pause point:** The generated T1 seeds can be stored at 20°C–23°C in the dark.
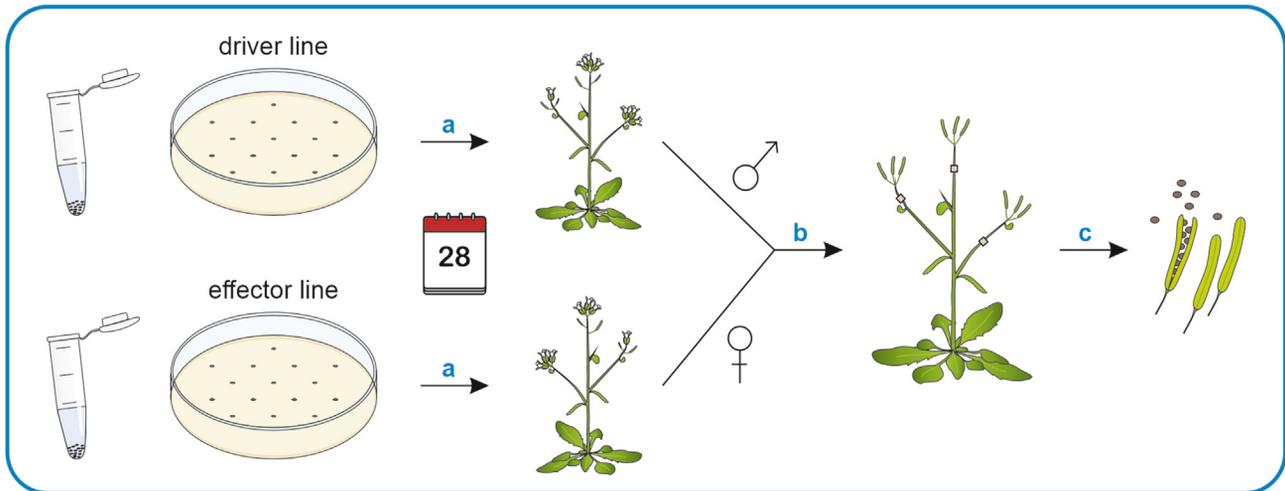
Generation of the DEX-inducible, tissue-specific CRISPR-Kill expression lines

⏱ Timing: 6 weeks

To enable the induction of CRISPR-Kill-mediated cell death at a precise time point, DEX-inducible expression lines must be generated. These are obtained by crossing inducible CRISPR-Kill effector lines with tissue-specific driver lines (Figure 2). Depending on the tissue to be eliminated, an appropriate driver line can be selected from a comprehensive set of established lines.[4] If the desired promoter is not available, the respective driver construct can simply be produced by golden gate cloning and used to transform *Arabidopsis* which are then propagated to homozygosity.

5. Surface-sterilize *Arabidopsis* seeds of CRISPR-Kill effector and tissue-specific driver lines.
   a. Fill ca. 50 seeds of the respective line into a sterile 1.5 mL reaction tube.
   b. Add 500 µL 4% (v/v) NaClO for surface sterilization.

   ⚠ CRITICAL: For optimal surface sterilization, use a freshly diluted 4% NaClO solution.

Figure 2. Workflow for generating DEX-inducible tissue-specific CRISPR-Kill expression lines
(A) Cultivation of driver and effector line seeds on selection medium until the primary inflorescence has formed.
(B) Pollination driver line plants with effector line plants.
(C) Harvesting of the generated expression line seeds.

    c. Shake the reaction tubes vigorously for 5 min in a laboratory orbital shaker.
    d. Centrifugate the reaction tubes for 1 min at 5 000 g at 20°C–23°C.

   Note: Work at a sterile bench for all further steps.

    e. Remove as much of the 4% NaClO as possible with a 1 mL pipette without aspirating any seeds in the tube.

   Note: A small remainder of the 4% NaClO in the tube is acceptable.

    f. To remove remaining NaClO, add 1 mL sterilized ddH$_2$O, shake the reaction tubes vigorously for 30 s, centrifuge the reaction tubes for 30 s at 5 000 g at 20°C–23°C and remove as much of the ddH$_2$O as possible with a 1 mL pipette without aspirating any seeds in the tube.
    g. Repeat step 5.f two more times.
    h. Add 1 mL sterilized ddH$_2$O.

   Note: Using 1 mL sterilized 0.1% agarose water instead of ddH$_2$O will simplify the sowing in step 6.a considerably.

    i. Stratify the seeds for 18–24 h at 4°C in the dark.
6. Cultivate CRISPR-Kill effector and tissue-specific driver lines.
    a. Sow the sterilized and stratified seeds by evenly distributing them on the prepared GM plates (92 × 16 mm) containing the required herbicide selection marker.

   ⚠ CRITICAL: Make sure you use the correct selection markers for the driver (Sulfadiazine) and effector lines (Gentamicin or DL-Phosphinotricin).

    b. Cultivate the sown seeds for two weeks in a growth chamber (22°C; 16 h light and 8 h dark).
    c. Transfer the seedlings to a soil culture (22°C; 16 h light and 8 h dark) and grow plants until the primary inflorescence is formed.
7. Cross the CRISPR-Kill effector line with the tissue-specific driver line.

a. Remove all the pods and already opened flowers from the plants of the CRISPR-Kill pOp6:Sa-Cas9-ITS2 or control pOp6:SaCas9-ADH1 effector line.

b. Remove sepals, petals and stamens from still fully closed buds of several inflorescences and expose the style.

c. Pollinate the prepared effector line plant with fully developed flowers of the pAHP6>>mTurquoise2 driver line plant by repeatedly brushing their stamens over the prepared stigma of the effector line plants.

d. Mark the pollinated stigma with colored tapes to facilitate subsequent identification of crossed inflorescences.

e. Trim newly formed shoots several times a week.

f. Harvest the ripe pods and dry the seeds for at least one day at 37°C.

⧗⧗ Pause point: The generated hybrid seeds can be stored at 20°C–23°C in the dark.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial strains		
Agrobacterium tumefaciens GV3101::pMP90	Koncz & Schell, 1986[5]	
Chemicals, peptides, and recombinant proteins		
Propidium iodide	Merck KGaA	Cat# P4170
Dexamethasone	Merck KGaA	Cat# D4903
Sulfadiazine	Merck KGaA	Cat# S8626
DL-Phosphinotricin (PPT)	Duchefa Biochemie B.V.	Cat# P0159
Gentamycin sulfate	Duchefa Biochemie B.V.	Cat# G0124
Spectinomycin HCL pentahydrate	Duchefa Biochemie B.V.	Cat# S0188
Rifampicin	Duchefa Biochemie B.V.	Cat# R0146
Dimethyl sulfoxide (DMSO)	Merck KGaA	Cat# 102952
Sodium hypochlorite solution, 12% Cl	Carl Roth GmbH + Co. KG	Cat# 9062.3
Cefotaxime sodium	Duchefa Biochemie B.V.	Cat# C0111
Acetosyringone	Merck KGaA	Cat# D134406
6-Benzylaminopurine (6-BAP)	Duchefa Biochemie B.V.	Cat# B0904
Silwet L-77	Hermann Meyer KG	
Murashige & Skoog medium including vitamins	Duchefa Biochemie B.V.	Cat# M0256
Plant agar	Duchefa Biochemie B.V.	Cat# P1001
Experimental models: Organisms/strains		
Arabidopsis thaliana: Col-0::pAHP6>>mTurquoise2	Schürholz et al. 2018[4]	
Arabidopsis thaliana: Col-0::pOp6:SaCas9-ITS2	Gehrke et al. 2023[1]	
Arabidopsis thaliana: Col-0::pOp6:SaCas9-ADH1	Gehrke et al. 2023[1]	
Recombinant DNA		
pAHP6-SaCas9-ITS2	Gehrke et al. 2023[1]	
pAHP6-SaCas9-ADH1	Gehrke et al. 2023[1]	
Software and algorithms		
ImageJ	Schneider et al.[6]	
ImageJ add-on SmartRoot	Lobet et al.[7]	
Fiji ImageJ	Schindelin et al.[8]	
Other		
Plant culture chamber	Percival Scientific	Cat# CLU-36L4
LSM 700 laser scanning microscope	Carl Zeiss AG	
Zeiss plan-apochromat x63/1.4 oil DIC M27	Carl Zeiss AG	Cat# 420782-9900-000
Petri dish 92 × 16 mm without cams	Sarstedt AG & Co. KG	Cat# 82.1472.001
Petri dish 92 × 16 mm with cams	Sarstedt AG & Co. KG	Cat# 82.1473.001
Petri dish 150 × 20 mm with cams	Sarstedt AG & Co. KG	Cat# 82.1184.500

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Petri dishes square 100 × 100 × 20 mm	Sarstedt AG & Co. KG	Cat# 82.9923.422
Cell culture plates CELLSTAR sterile, wells: 6	Carl Roth GmbH + Co. KG	Cat# CE54.1

MATERIALS AND EQUIPMENT
Germination medium (GM)

- Dissolve Murashige & Skoog medium [4.9 g/L] and sucrose [10 g/L] in ddH$_2$O and adjust the pH-value to pH = 5.7 with potassium hydroxide.
- For solid medium, add 8 g/L Plant agar for horizontally cultivated plates or 10 g/L Plant agar for vertically cultivated plates and autoclave the medium.
- For preparation of the selection medium, add the required herbicide(s) or antibiotics after the medium has been cooled below 50°C:
- Cefotaxim: 500 mg/L.
- Gentamicin: 60 mg/L.
- DL-Phosphinotricin: 6 mg/L.
- Sulfadiazine: 3.75 mg/L.

   Note: Store at 4°C for up to three months in the dark.

- For the preparation of induction medium, add the required amount of dissolved DEX after the medium has been cooled below 50°C:
- Dexamethasone D4903: 1–100 μM.

   Alternatives: In this study, DEX (D4903, Merck KGaA) was dissolved in DMSO. Alternatively, a water-soluble variant of DEX suitable for cell culture is also available (D2915, Merck KGaA).

   ⚠ CRITICAL: Dexamethasone is classified as a reproductive toxicant. It may damage fertility and may damage the unborn child.

   Note: Induction medium should be used within the same day or on the following day. Store at 4°C in the dark for 18–24 h.

Yeast Extract Beef (YEB) Agrobacterium growth medium		
Reagent	Final concentration	Amount
Beef extract	5 g/L	5 g
Peptone	5 g/L	5 g
Yeast extract	1 g/L	1 g
Sucrose	5 g/L	5 g
MgSO4 (x 7 H$_2$O)	493 mg/L	493 mg
ddH$_2$O	N/A	Fill up to 1000 mL
Total	N/A	1000 mL

- For solid medium, add 12 g/L plant agar and autoclave the medium.
- Add the following additives after the medium has been cooled below 50°C:
  ○ Rifampicin: 100 mg/L.
  ○ Gentamicin: 20 mg/L.
  ○ Spectinomycin: 100 mg/L.

   Note: Store at 4°C for up to three months.

- Adjust the pH value to pH = 5.7 with potassium hydroxide.
- Add 400 μL Silwet L-77.

Infiltration medium

Reagent	Final concentration	Amount
Murashige & Skoog medium	2.5 g/L	2 g
Sucrose	50 g/L	40 g
6-Benzylaminopurine (BAP)	5.25 µg/L	3.4 µL
Acetosyringon	100 mg/L	800 µL
ddH$_2$O	N/A	Up to 800 mL
Total	N/A	800 mL

⚠ CRITICAL: Use the prepared medium on the same day.

STEP-BY-STEP METHOD DETAILS

Below, we provide a detailed step-by-step protocol for the application of the constitutive and inducible CRISPR-Kill system as well as different analyses of the systemic and cellular effects in seedlings, the root and the shoot (Figures 3, 4, 5, 6 and 7.

Constitutive CRISPR-Kill: Macroscopic analysis of constitutive target cell ablation

⏲ Timing: 2 weeks

This section provides you with the protocol to visualize the morphological effects of the constitutively expressed CRISPR-Kill system in target cells on a macroscopic level (Figure 3).

1. Sow *Arabidopsis* T1 seeds on selection medium for primary transformant selection.
   a. Fill ca. 10 000 T1 seeds of the pAHP6-SaCas9-ITS2 CRISPR-Kill line and the pAHP6-SaCas9-ADH1 control line into one sterile 2 mL reaction tube each.
   b. Surface-sterilize the respective *Arabidopsis* seeds with 1 mL 4% NaClO, wash three times with sterilized ddH$_2$O and add 1 mL of 0.1% agarose water.

   Note: For a detailed protocol see Step 5 in the section "before you begin".

   c. Stratify the sterilized seeds for 18–24 h in the dark at 4°C.
   d. For each line, transfer the stratified seeds to a reaction tube containing 50 mL of 0.1% agarose water and mix the suspension gently.
   e. For each line, take 10 of the prepared GM plates (150 × 20 mm) containing cefotaxime and the respective herbicide, pour 5 mL of the seed suspension on each plate and distribute the seeds evenly on the medium by swirling gently.
   f. Cultivate the sown seeds for two weeks in a growth chamber (22°C; 16 h light and 8 h dark).
2. Determine the relative survival rate of the T1 primary transformants.
   a. For each line, determine the total amount of seeds as well as the total amount of viable primary transformants.

   Note: Plants are counted as primary transformants if they show a vital green-colored phenotype and, in addition to the cotyledons, developed first (true) leaves. Green-colored seedlings which only show cotyledons are considered dead.

   b. For each line, calculate the primary transformant efficiency by dividing the total number of viable primary transformants by the total number of seeds (troubleshooting 1).

   Note: Using the floral dip transformation method, the expected transformation efficiency can reach up to 3%.[1]
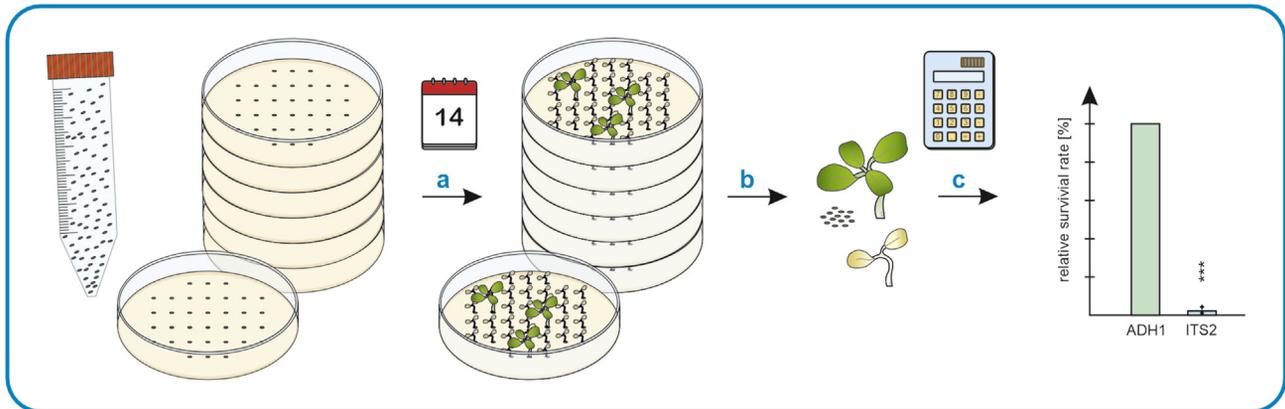
Figure 3. Workflow to analyze constitutive target cell ablation macroscopically
(A) T1 seed cultivation of the constitutive CRISPR-Kill line as well as the control line for two weeks.
(B) Determination of the total amount of seeds as well as the total amount of viable primary transformants.
(C) Determination of the relative survival rate of the CRISPR-Kill line.

c. Determine the relative survival rate by normalizing the primary transformant efficiency of the pAHP6-SaCas9-ITS2 CRISPR-Kill line to that of the pAHP6-SaCas9-ADH1 control line.

Note: If you use a different promoter for the constitutive uninducible CRISPR-Kill approach, which is not active in early developmental stages, you should not be able to detect a reduction in the relative survival rate in this experiment. For further characterization, you can continue to cultivate and phenotypically characterize the primary transformants.

Inducible CRISPR-Kill: Monitoring cell death in seedlings

⏱ Timing: 2 weeks

This section provides you with the protocol to visualize the morphological effects of DEX-induced CRISPR-Kill-mediated cell death in the target cells on a macroscopic level in seedlings (Figure 4).
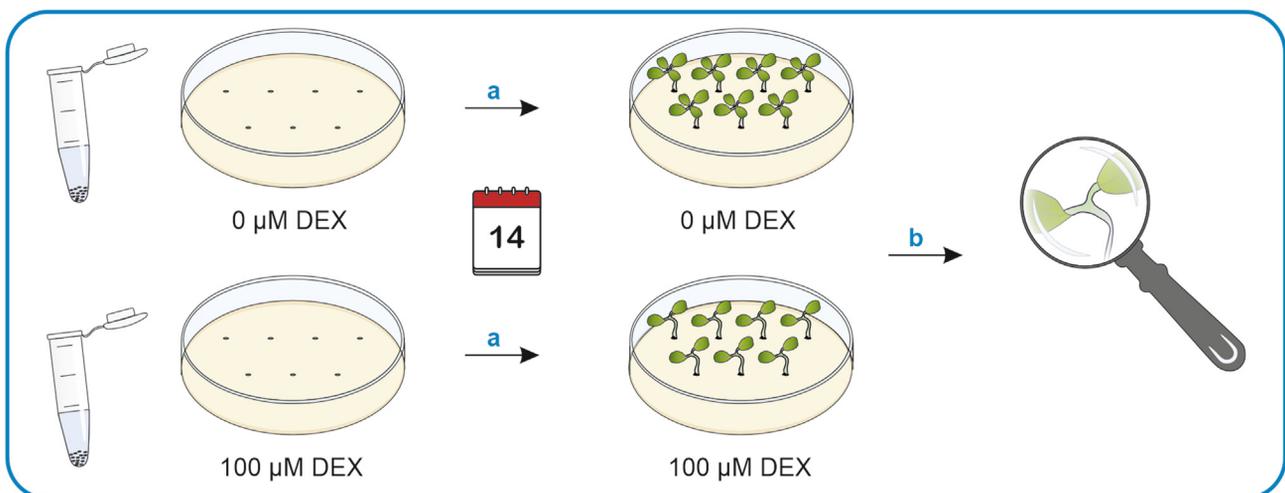
Figure 4. Workflow to analyze inducible target cell ablation in seedlings
(A) Cultivation of the inducible CRISPR-Kill expression line seeds on solid germination medium containing 100 μM DEX for inducing gene expression or 0 μM DEX as a control for two weeks.
(B) Characterization of the seedlings based on their phenotype.

3. Sow *Arabidopsis* seeds of the pAHP6>>SaCas9-ITS2 CRISPR-Kill as well as of the pAHP6>>Sa-Cas9-ADH1 control lines on selection medium containing the inductor DEX.
   a. Surface sterilize and stratify about 50 seeds of the respective expression lines.

   Note: For a detailed protocol see Step 5 in the section "before you begin".

   b. For each line, sow the sterilized and stratified seeds evenly distributed on the prepared GM plates (92 × 16 mm) containing:
      i. DEX [100 µM] and the respective herbicides.
      ii. DMSO and the respective herbicides.
   c. Cultivate the sown seeds for two weeks in a growth chamber (22°C; 16 h light and 8 h dark).
4. Characterize the seedlings based on their phenotype. Compare the influence of the inducer (DEX) concentration on plant growth and organ development and look for differences between the CRISPR-Kill and the control expression lines (troubleshooting 2).

Inducible CRISPR-Kill: Monitoring cell death in the root

⏱ **Timing: 2 weeks**

This section provides you with the protocol to visualize the morphological effects of DEX-induced CRISPR-Kill-mediated cell death in the target root cells on a macroscopic level (Figure 5).

5. Sow *Arabidopsis* seeds of the CRISPR-Kill expression lines as well as the control expression lines on selection medium.
   a. Surface sterilize and stratify ca. 50 seeds of the pAHP6>>SaCas9-ITS2 and pAHP6>>SaCas9-ADH1 expression lines.

   Note: For a detailed protocol see Step 5 in the section "before you begin".

   b. Sow the sterilized and stratified seeds evenly distributed in a row on the prepared GM square plates (100 × 100 × 20 mm) containing the required selection marker.
   c. Seal the plates with micropore and cultivate the plates in vertical orientation for five days in a growth chamber (22°C; 16 h light and 8 h dark).
6. Induce gene expression of the SaCas9-nuclease as well as the fluorescent reporter mTurquoise2.
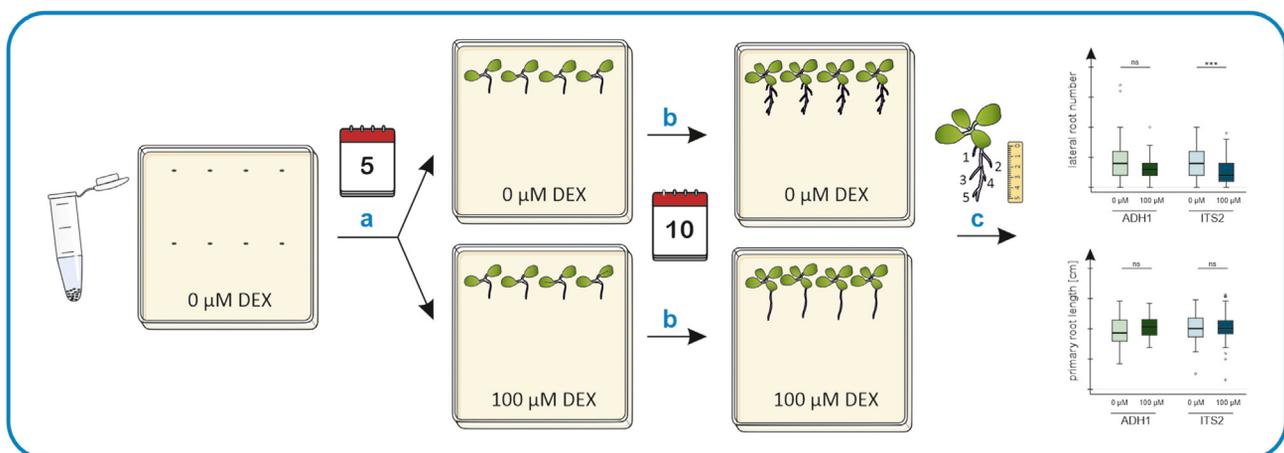
Figure 5. Workflow to analyze inducible target cell ablation in the root
(A) Cultivation of the inducible CRISPR-Kill expression line seeds on inductor-less germination medium for five days.
(B) Transferring seedlings to solid germination medium containing 100 µM DEX for inducing gene expression or 0 µM DEX as a control for 10 days.
(C) Characterization of root growth by determine the primary root length as well as the lateral root number.

a. To induce gene expression of the pOp6:SaCas9- and pOp6:mTurquoise2-constructs, transfer up to 6 seedlings per plate evenly distributed in a row on the prepared GM square plates (100 × 100 × 20 mm) containing 100 μM DEX or DMSO.
b. Seal the plates with micropore and cultivate the plates in vertical orientation for ten days in a growth chamber (22°C; 16 h light and 8 h dark).

⚠ CRITICAL: Make sure that the entire root is in contact with the medium.

7. Characterize the root growth (troubleshooting 2).
   a. Take a high-resolution photo of the seedlings on the cultivation plate.

   Note: Placing a scale next to the cultivation plate facilitates later root length analysis.

   b. To determine the number of lateral roots, count the number of lateral roots per plant.
   c. To determine the main root length, use the ImageJ plug-in SmartRoot.[7]

Inducible CRISPR-Kill: Monitoring cell death in the RAM

⏱ Timing: 1 week

This section provides you with the protocol to visualize the morphological effects of DEX-induced CRISPR-Kill-mediated cell death in the target cells of the RAM on a microscopic level (Figure 6).

8. Sow *Arabidopsis* seeds of the CRISPR-Kill expression lines as well as the control expression lines on selection medium.
   a. Surface sterilize and stratify ca. 50 seeds of the pAHP6>>SaCas9-ITS2 and pAHP6>>SaCas9-ADH1 expression lines.

   Note: For a detailed protocol see Step 5 in the section "before you begin".

   b. For each line, sow the sterilized and stratified seeds evenly distributed on the prepared GM square plates (100 × 100 × 20 mm) containing the required selection marker.
   c. Seal the plates with micropore and cultivate the plates in vertical orientation for ten days in a growth chamber (22°C; 16 h light and 8 h dark).
9. Induce gene expression of pOp6:SaCas9- and pOp6:mTurquoise2-constructs.
   a. Prepare sterile six-well plates and add 5 mL of sterile GM liquid medium containing 100 μM DEX, 50 μM DEX or DMSO to each well and transfer at least ten seedlings to each well.
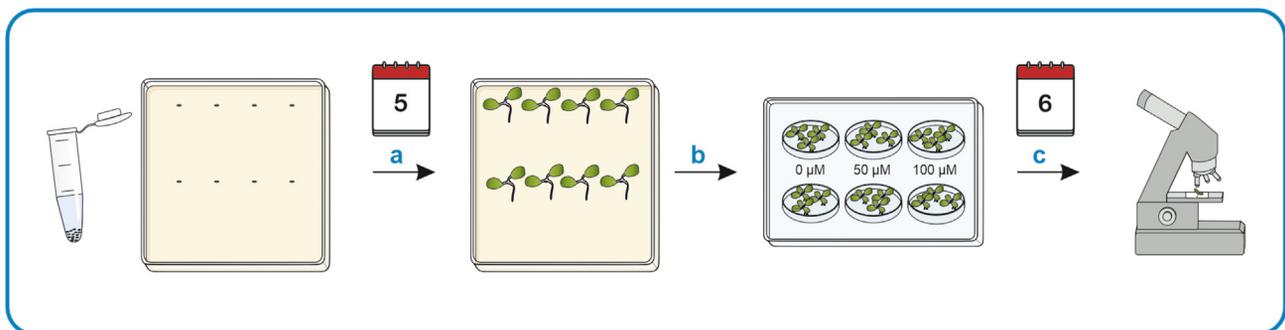
Figure 6. Workflow to analyze inducible target cell ablation in the root apical meristem (RAM)
(A) Cultivation of the inducible CRISPR-Kill expression line seeds on inductor-less germination medium for five days.
(B) Transferring seedlings to liquid germination medium containing 50 μM or 100 μM DEX for inducing gene expression or 0 μM DEX as a control for 24 h.
(C) Microscopic evaluation of CRISPR-Kill-induced cell ablation in target cells.

b. Cultivate the six-well plates for 24 h in a growth chamber (22°C; 16 h light and 8 h dark).

△ CRITICAL: Make sure that the entire root is immersed with the medium.

10. Visualize the induced cell death in the target tissue in the RAM.
    a. Carefully separate the primary root from the seedlings with tweezers, transfer the root tips to a slide with 150 µL 5 µg/mL Propidium iodide and place a cover slip on top.
    b. To visualize the target tissue, excite the fluorophore mTurquoise2 at 405 nm and detect the emission between 460 nm and 550 nm (troubleshooting 4).
    c. To visualize dead cells as well as the cell walls of living cells, excite the cell death marker Propidium iodide at 555 nm and detect the emission between 566 nm and 630 nm (troubleshooting 3, troubleshooting 4).
    d. For image processing, the Fiji ImageJ program can be used.[8]

**Inducible CRISPR-Kill: Monitoring cell death in the SAM**

⏱ Timing: 5 weeks

This section provides you with the protocol to visualize the morphological effects of DEX-induced CRISPR-Kill-mediated cell death in the target cells of the SAM on a macroscopic and microscopic level (Figure 7).

11. Sow *Arabidopsis* seeds of the CRISPR-Kill expression lines as well as the control expression lines on selection medium.
    a. Surface sterilize and stratify ca. 50 seeds of the pAHP6>>SaCas9-ITS2 and pAHP6>>SaCas9-ADH1 expression lines.

    Note: For a detailed protocol see Step 5 in the section "before you begin".

    b. For each line, sow the sterilized and stratified seeds evenly distributed on the prepared GM plates (92 × 16 mm) containing the respective selection marker.
    c. Cultivate the sown seeds for two weeks in a growth chamber (22°C; 16 h light and 8 h dark).
    d. Transfer the seedlings to a soil culture (22°C; 16 h light and 8 h dark) and let the plants grow until the primary inflorescence is formed.
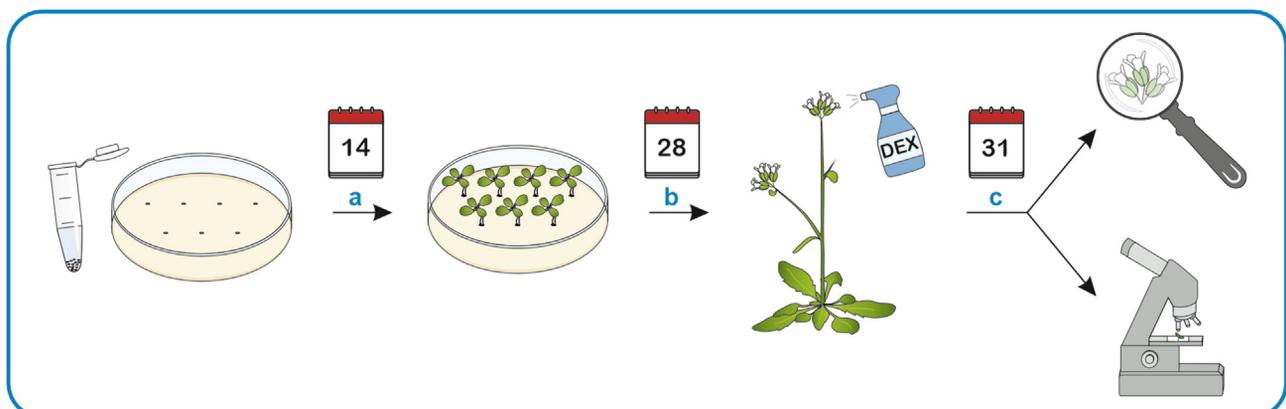
Figure 7. Workflow to analyze inducible target cell ablation in the shoot apical meristem (SAM)
(A) Cultivation of the inducible CRISPR-Kill expression line seeds on inductor-less germination medium for two weeks.
(B) Cultivation of CRISPR-Kill expression line plants in soil culture until the primary inflorescence has formed.
(C) Induction of gene expression by spraying the inflorescence with 10 µM DEX followed by macroscopic and microscopic characterization of CRISPR-Kill-induced cell ablation in SAM.

Note: Experiments on the shoot apical meristem are generally started when inflorescence stems have reached a height of 10–15 cm.

12. Induce gene expression of pOp6:SaCas9- and pOp6:mTurquoise2-constructs.
    a. Spray the inflorescence meristems with 10 μM DEX or, as a control, only with the solvent DMSO.
    b. Cultivate the treated plants for 72–96 h (22°C; 16 h light and 8 h dark).
13. Visualize the induced cell death in the target tissue in the SAM.
    a. 72–96 h after induction, dissect the apical meristem by removing flowers and lateral organs and place the terminal 2 cm of the apex upright into the agar medium of a small petri dish.
    b. Using binocular and fine forceps, remove all flower buds without disturbing the meristem dome.
    c. Add a drop of 10 mM propidium iodide to the meristem.
    d. Observe under a microscope, either by mounting the apex on a slide or, preferably place the petri dish on an upright microscope with dipping lense.
    e. To visualize the target tissue, excite the fluorophore mTurquoise2 at 405 nm and detect the emission between 460 nm and 550 nm (troubleshooting 4).
    f. To visualize dead cells as well as the cell walls of living cells, excite the cell death marker Propidium iodide at 555 nm and detect the emission between 566 nm and 630 nm (troubleshooting 3, troubleshooting 4).
    g. For image processing, the Fiji ImageJ program can be used.[8]

EXPECTED OUTCOMES

This protocol describes the procedure for characterizing the morphological consequences of CRISPR-Kill-mediated cell death in pAHP6-positive cells in the RAM and the SAM by macroscopic and microscopic analyses. The expected phenotypes resulting from the constitutive and inducible CRISPR-Kill activity were described in Gehrke et al. 2023[1,2] in detail. In this study, the CRISPR-Kill system was used to induce cell death in the root as well as in the shoot at precisely defined time points, so that the local effects in the RAM and SAM could be analyzed independently of each other. While this protocol is specific to the use of the pAHP6 promoter, the protocol described here can also be used or adapted to characterize the consequences of CRISPR-Kill activity in other cell types when using corresponding tissue-specific promoters. In this case, the tissue-specific promoter in the established constitutive CRISPR-Kill constructs can simply be exchanged using a classical cloning approach, thus redefining the target tissue.[1,2] Also, in the inducible CRISPR-Kill system, the tissue to be eliminated can be flexibly selected by combining the inducible CRISPR-Kill effector lines established in Gehrke et al. 2023[1] with a comprehensive repertoire of existing driver lines.[4] These lines, established by Schürholz et al. 2018,[4] enable tissue-specific induction in the root and shoot with a strong focus on the indeterminate meristems.[4] Should the desired tissue-specific line not yet be included in this repertoire, the promoter of the driver line constructs can simply be adapted using Golden Gate cloning, followed by the establishment of the respective driver line. This opens up a broad field of possible analyses to answer a wide variety of questions in basic research.

With the protocol presented here, we have addressed different cell types at different developmental stages in seedlings, the root and the shoot. Therefore, the procedures described can be applied directly or modified slightly for the cell structures to be investigated. In some approaches, it might be worth considering the extension of the system with a second constitutively and tissue-specifically expressed reporter. This would, for example, allow to characterize re-differentiation after cell death-induced ectopic cell divisions. In addition, some approaches may require de-induction instead of continuous induction. In this case, washing out of the inductor DEX or transferring the plants to inductor-free medium could be a valid option, although the effect would not be immediately apparent.

By using the inducible CRISPR-Kill system, valuable insights into the developmental plasticity of certain cell types could be gained, but also could the cellular and molecular responses to cell

elimination in combination with positional signaling and intercellular communication be investigated. Thus, microscopic analysis of CRISPR-Kill lines could be a promising method to elucidate wound response mechanisms in order to understand the underlying signaling cascades for restorative pattern formation. The analysis of the cellular consequences with regard to the maintenance of stem cell character after tissue-specific cell ablation in the SAM could be of particular interest, as these cells are often difficult to reach with the laser. For this application, the inducible CRISPR-Kill system offers a precise and easily controllable alternative to previous mechanical ablation approaches. In addition, as the inducible CRISPR-Kill system is based on multiple double-strand break (DSB) induction in repetitive DNA regions, the characterization of DNA damage-mediated cell death per se can also be specifically investigated. This could be of particular interest for target cells that exhibit high resistance to genotoxic damage, as has been described in case of the quiescent center in the RAM.[9–11]

Besides using the tissue-specific CRISPR-Kill system to answer a wide range of basic research-related questions, it could also be used to manipulate plant secondary metabolism. The accumulation of secondary metabolites in a particular tissue requires the spatio-temporal coordination of a variety of cellular processes, which could allow the identification of suitable tissue-specific promoters for the expression of the CRISPR-Kill system in secondary metabolite-producing cells. The precise elimination of these cells could lead to an altered metabolite composition of a plant, which could be of particular interest for the food or pharmaceutical industry. Another potential application of the tissue-specific CRISPR-Kill system could be to combat plant diseases in agriculture. The spread of plant-specific pathogens and the physiological consequences could be prevented by early controlled cell ablation of infected, transgenic host cells.

As the target regions of the CRISPR-Kill system are not only highly conserved in plants, but also in other multicellular eukaryotes, an application of the CRISPR-Kill system might also be feasible in animals and fungi. This hypothesis is supported by recently published data on the elimination of cancer cells through the induction of multiple DSBs in non-coding repetitive sequences.[12]

The broad spectrum of potential applications is the greatest advantage of the CRISPR-Kill system, as it has, in addition to tissue engineering-based applications, considerable potential in genome engineering approaches. In contrast to other genetic cell ablation techniques, the CRISPR-Kill system acts at the DNA level and targets specific genomic regions. This creates the possibility of reprogramming the CRISPR-Kill system, so that multiple DSB induction in the repetitive centromere regions occurs in a chromosome-specific context, which could result in the elimination of individual chromosomes.

LIMITATIONS

We used the protocol described here to successfully ablate pAHP6-positive cells in the model plant *A. thaliana*. However, we performed similar approaches with a variety of different promoters to efficiently induce CRISPR-Kill-mediated cell death in several cell types.[1,2] When choosing the tissue-specific promoter, particular attention must be paid to achieving a high expression level of the SaCas9 nuclease to ensure simultaneous DSB induction in the repetitive elements and thus efficient induction of cell death. The use of a weak promoter can lead to an insufficient concentration of the SaCas9 nuclease, inducing only a small amount of DSBs in the repetitive elements, which can be repaired by the cell's own repair machinery before cell death is induced. A weak promoter limits not only the success of the constitutive, direct CRISPR-Kill approach, but also the efficiency of the inducible system, as the concentration of the GR-LhG4 transcription factor determines the expression level of the pOp6-controlled SaCas9 nuclease. We therefore recommend using only strong promoters for the expression of the SaCas9 nuclease or the GR-LhG4 transcription factor. If, despite its low strength, a specific promoter has to be used, a fusion of the respective promoter with enhancers might be worth considering, as was done with the egg cell-specific EC1.1 promoter which was fused to the EC1.2 enhancer.[13] Furthermore, the selected promoter should only be active within

the targeted cell type, although multi-tissue promoters can also be used with the inducible CRISPR-Kill system. However, such leaky expression patterns need to be considered during the evaluation of the morphological effects.

Since the CRISPR-Kill system is based on the induction of multiple DSBs in highly conserved genomic regions, it should be possible to adapt the system for applications in other plant species relatively easily. However, a limiting factor could be the cutting efficiency of the employed Cas nuclease. While Cas9 from *S. aureus* has been shown to provide the highest mutagenesis efficiencies in *Arabidopsis*,[14,15] other Cas9 orthologs or even nucleases from different CRISPR/Cas-systems, such as type V Cas12a nucleases, could be more suitable in other organisms. In the case of Cas12a, the use of engineered Cas12a variants with optimized cleavage efficiencies is highly recommended. Especially in plants, in which the cutting efficiency of the wild-type enzymes is limited by low cultivation temperatures, the optimization of enzyme activity at low temperatures through protein engineering is particularly attractive. In *Arabidopsis*, for example, the temperature-tolerant Cas12a variant of *Lachnobacterium spiraceae* (ttLbCas12a) and the improved Cas12a variant of *Eubacterium rectale* show a several-fold increase in editing efficiency at 22°C compared to their wildtype enzyme.[16,17] Furthermore, it was recently shown that the insertion of introns into the Cas9 but also into the ttLbCas12a coding sequence drastically improves the efficiency of editing in plants.[18,19] To identify the most suitable nuclease for the CRISPR-Kill system, the cutting efficiency of these nucleases in repetitive elements can be evaluated by ubiquitous expression of the construct, resulting in a plant survival rate that directly correlates negatively with the efficiency of DSB induction. Survival rates of under 10% indicate a sufficient cutting efficiency, although rates up to 20% might still indicate sufficient cutting. Besides the high impact of the cutting frequency, the transformation efficiency could also be a limiting factor when adapting the CRISPR-Kill system to other plants. While *Arabidopsis* can be easily transformed via Agrobacteria-mediated T-DNA integration, most crops require regeneration procedures or are not susceptible to transgene integration.[20]

TROUBLESHOOTING

Problem 1
All T1 plants are alive (related to Step 2).

Potential solution
The primary transformant selection did not work. Make sure your germination medium contains the appropriate amount of the required selection marker.

Problem 2
After induction, no phenotypic differences are visible between the CRISPR-Kill and control lines (related to Steps 4, 7).

Potential solution
Make sure that both T-DNAs are integrated by cultivating the plants on selection medium. Two selection markers must be added to the medium: Sulfadiacine for the driver construct and gentamycin or DL-Phosphinotricin for the effector construct. An additional PCR-based verification can also be helpful. Verify that the concentration of the inductor DEX is correct.

Problem 3
In the target tissue, the signal of the fluorescent marker but not of the cell death marker PI is visible (related to Steps 10, 13).

Potential solution
Test various different DEX concentrations with different induction periods.

Problem 4
No signal of either the fluorescence marker or the cell death marker PI is visible in the target tissue (related to Steps 10, 13).

Potential solution
Make sure that both T-DNAs are integrated by cultivating the plants on selection medium. Two selection markers must be added to the medium: Sulfadiacine for the driver construct and gentamycin or DL-Phosphinotricin for the effector construct. An additional PCR-based verification can also be helpful. Verify that the concentration of the inductor DEX is correct.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Holger Puchta (holger.puchta@kit.edu).

Technical contact
Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Fabienne Gehrke (fabienne.gehrke@kit.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
This study did not generate datasets.

AUTHOR CONTRIBUTIONS
F.G.: writing – original draft, visualization, writing – review and editing. S.W.: writing – review and editing, funding acquisition. H.P.: writing – review and editing, funding acquisition.

DECLARATION OF INTERESTS
The authors declare no competing interests.

REFERENCES

1. Gehrke, F., Ruiz-Duarte, P., Schindele, A., Wolf, S., and Puchta, H. (2023). An inducible CRISPR-Kill system for temporally controlled cell type-specific cell ablation in Arabidopsis thaliana. New Phytol. 239, 2041–2052. https://doi.org/10.1111/nph.19102.

2. Schindele, A., Gehrke, F., Schmidt, C., Röhrig, S., Dorn, A., and Puchta, H. (2022). Using CRISPR-Kill for organ specific cell elimination by cleavage of tandem repeats. Nat. Commun. 13, 1502. https://doi.org/10.1038/s41467-022-29130-w.

3. Clough, S.J., and Bent, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735–743. https://doi.org/10.1046/j.1365-313x.1998.00343.x.

4. Schürholz, A.-K., López-Salmerón, V., Li, Z., Forner, J., Wenzl, C., Gaillochet, C., Augustin, S., Barro, A.V., Fuchs, M., Gebert, M., et al. (2018). A Comprehensive Toolkit for Inducible, Cell Type-Specific Gene Expression in Arabidopsis. Plant Physiol. 178, 40–53. https://doi.org/10.1104/pp.18.00463.

5. Koncz, C., and Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of Agrobacterium binary vector. Molec. Gen. Genet. 204, 383–396. https://doi.org/10.1007/BF00331014.

6. Schneider, C.A., Rasband, W.S., and Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

7. Lobet, G., Pagès, L., and Draye, X. (2011). A novel image-analysis toolbox enabling quantitative analysis of root system architecture. Plant Physiol. 157, 29–39. https://doi.org/10.1104/pp.111.179895.

8. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

9. Fulcher, N., and Sablowski, R. (2009). Hypersensitivity to DNA damage in plant stem cell niches. Proc. Natl. Acad. Sci. USA 106, 20984–20988. https://doi.org/10.1073/pnas.0909218106.

10. Furukawa, T., Curtis, M.J., Tominey, C.M., Duong, Y.H., Wilcox, B.W.L., Aggoune, D., Hays, J.B., and Britt, A.B. (2010). A shared DNA-damage-response pathway for induction of

stem-cell death by UVB and by gamma irradiation. DNA Repair 9, 940–948. https://doi.org/10.1016/j.dnarep.2010.06.006.

11. Timilsina, R., Kim, J.H., Nam, H.G., and Woo, H.R. (2019). Temporal changes in cell division rate and genotoxic stress tolerance in quiescent center cells of Arabidopsis primary root apical meristem. Sci. Rep. 9, 3599. https://doi.org/10.1038/s41598-019-40383-2.

12. Tan, I.-L., Perez, A.R., Lew, R.J., Sun, X., Baldwin, A., Zhu, Y.K., Shah, M.M., Berger, M.S., Doudna, J.A., and Fellmann, C. (2023). Targeting the non-coding genome and temozolomide signature enables CRISPR-mediated glioma oncolysis. Cell Rep. 42, 113339. https://doi.org/10.1016/j.celrep.2023.113339.

13. Wang, Z.-P., Xing, H.-L., Dong, L., Zhang, H.-Y., Han, C.-Y., Wang, X.-C., and Chen, Q.-J. (2015). Egg cell-specific promoter-controlled CRISPR/Cas9 efficiently generates homozygous mutants for multiple target genes in Arabidopsis in a single generation. Genome Biol. 16, 144. https://doi.org/10.1186/s13059-015-0715-0.

14. Steinert, J., Schiml, S., Fauser, F., and Puchta, H. (2015). Highly efficient heritable plant genome engineering using Cas9 orthologues from Streptococcus thermophilus and Staphylococcus aureus. Plant J. 84, 1295–1305. https://doi.org/10.1111/tpj.13078.

15. Fauser, F., Schiml, S., and Puchta, H. (2014). Both CRISPR/Cas-based nucleases and nickases can be used efficiently for genome engineering in Arabidopsis thaliana. Plant J. 79, 348–359. https://doi.org/10.1111/tpj.12554.

16. Schindele, P., and Puchta, H. (2020). Engineering CRISPR/LbCas12a for highly efficient, temperature-tolerant plant gene editing. Plant Biotechnol. J. 18, 1118–1120. https://doi.org/10.1111/pbi.13275.

17. Pietralla, J., Capdeville, N., Schindele, P., and Puchta, H. (2024). Optimizing ErCas12a for efficient gene editing in Arabidopsis thaliana. Plant Biotechnol. J. 22, 401–412. https://doi.org/10.1111/pbi.14194.

18. Grützner, R., Martin, P., Horn, C., Mortensen, S., Cram, E.J., Lee-Parsons, C.W.T., Stuttmann, J., and Marillonnet, S. (2021). High-efficiency genome editing in plants mediated by a Cas9 gene containing multiple introns. Plant Commun. 2, 100135. https://doi.org/10.1016/j.xplc.2020.100135.

19. Schindele, P., Merker, L., Schreiber, T., Prange, A., Tissier, A., and Puchta, H. (2023). Enhancing gene editing and gene targeting efficiencies in Arabidopsis thaliana by using an intron-containing version of ttLbCas12a. Plant Biotechnol. J. 21, 457–459. https://doi.org/10.1111/pbi.13964.

20. Altpeter, F., Springer, N.M., Bartley, L.E., Blechl, A.E., Brutnell, T.P., Citovsky, V., Conrad, L.J., Gelvin, S.B., Jackson, D.P., Kausch, A.P., et al. (2016). Advancing Crop Transformation in the Era of Genome Editing. Plant Cell 28, 1510–1520. https://doi.org/10.1105/tpc.16.00196.