Shockwave-induced DNA-free genome editing in tobacco: targeting the actin depolymerizing factor gene increases drought and salinity tolerance

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Abstract (150 words)

DNA-free genome editing involves the direct introduction of ribonucleoprotein (RNP) complexes into cells, but this strategy has rarely been successful in plants. Here we describe a new technique for the introduction of RNPs into plant cells involving the generation of cavitation bubbles using a pulsed laser. The resulting shockwave achieves the efficient transfection of walled cells in tissue explants by the creation of transient membrane pores. RNP-containing cells were rapidly identified by fluorescence microscopy, followed by regeneration and the screening of mutant plants by high-resolution melt analysis. We used this technique in tobacco to target the endogenous phytoene desaturase (pds) and actin depolymerizing factor (adf) genes. Genome-edited plants were produced with an efficiency of 5.6–8.7%. We also evaluated the effects of adf mutations in T2 mutant plants under drought and salinity stress, showing that adf acts as a key regulator of osmotic stress tolerance in plants.

Introduction

Genome editing in plants is widely used for the functional analysis of genes and the development of improved crop varieties. The CRISPR/Cas9 system is the most popular genome-editing tool because it is simple and adaptable, especially for the simultaneous mutation of multiple genes [1-3]. CRISPR/Cas9 has therefore been used to manipulate many traits of agricultural significance [4]. However, the major limitation of CRISPR/Cas9 technology in plant biology is the need for stable transformation, which involves the introduction of an expression cassette providing the Cas9 nuclease, the guide RNA (gRNA), and a selection marker [5]. The hostility towards genetically-modified plants in some jurisdictions calls for DNA-free genome editing methods in which RNPs containing the Cas9 nuclease and RNA components fulfilling the function of gRNA are first assembled in vitro and then introduced into plant cells using standard transfection procedures. The advantages of RNP-mediated genome editing include the prompt but transient activity of Cas9, reducing the frequency of off-target mutations, and the fact that the resulting genome-edited plants are considered transgene-free [6].

We have previously shown that RNP-mediated genome-editing experiments targeting the tobacco pds gene achieved a mutation efficiency of 19% when the RNP was delivered by particle bombardment, but the absence of a selection marker resulted in a laborious and time-consuming screening process [7]. Furthermore, particle bombardment causes severe cell damage and the efficiency of regeneration is...
PEG-mediated protoplast transfection is a gentler method for the delivery of CRISPR/Cas9 components (as DNA, RNA or RNPs), but the efficient regeneration of many plant species from protoplasts is not yet possible [11]. DNA-free genome editing therefore remains challenging in species recalcitrant to transformation, such as wheat, sorghum and woody plants [12]. A new method is required for the delivery of RNPs which is gentle and compatible with intact plant cells.

Shock waves induced by cavitation bubbles show great potential for the delivery of nucleic acids and proteins because they create transient pores in the plasma membrane, thus increasing its permeability [13, 14]. Cavitation bubbles are usually induced by acoustic stimuli such as ultra-sonication, and the resulting shock waves can promote the uptake of DNA by plant cells [15]. This non-invasive and chemical-free method has been used to transfet individual plant cells, protoplasts, plant cell suspension cultures, and intact tissue explants [16, 17]. Although the generation and oscillation of gas bubbles in liquid is usually achieved by acoustic cavitation [18, 19], shock waves can also be produced by a pulsed laser, an approach that has not yet been used for plants but was successful for the transfection of animal cells [20]. Based on these findings, we adapted the pulsed laser method for the transfection of intact plant cells with CRISPR/Cas9 RNPs.

The new pulsed laser technique has the potential to increase the efficiency of genome editing in a broad range of plant species. The rapid selection of RNP-containing cells was achieved by incorporating a labeled RNA that can be detected by fluorescence microscopy. This was followed by regeneration and the screening of mutant plants by high-resolution melt analysis (HRMA). We targeted the endogenous phytoene desaturase gene (pds) because mutations generate an easy detectable albino phenotype allowing the calculation of mutation efficiency. We also targeted the actin depolymerizing factor (adf) gene because mutations increase osmotic stress tolerance, allowing us to demonstrate the potential of our new method for the modification of agriculturally-relevant traits [21]. Accordingly, we also evaluated the phenotype of adf mutant T2 plants under drought and salinity stress, providing insight into the role of the actin cytoskeleton in abiotic stress tolerance.

Results

Fluorescent protein delivery into plant cells using a pulsed laser-induced shockwave

Unlike acoustic wave cavitation, pulsed laser cavitation can target shockwaves to a specific region of the sample. To fine-tune the transient increase in membrane permeability, we induced shock waves to transfet intact plant cells with a fluorescent protein (Figure 1A). We placed 10 x 5 mm intact leaf explants from 3-month-old tobacco plants cultivated under sterile conditions into an imaging-grade Petri dish with a glass bottom (Figure 1B) and added 20 µL of 0.25 µg/µL DsRed (Figure 1B). We focused a high-powered multiphoton laser 2–5 µm below the leaf sample and irradiated for approximately 8 s at a laser power of ~2 W. This created a cavitation bubble-induced shockwave that successfully transfected the plant cells with DsRed (Supplementary Video S1). We analyzed leaf explant cells before and after the laser pulse and observed DsRed emission in individual transfected cells by confocal microscopy (Figure 1C and C'). A 3D-reconstruction of the transfected cells confirmed that DsRed was homogenously distributed in the cytoplasm (Figure 1C”) and not merely attached to the cell surface.
RNP delivery into plant cells using a pulsed laser-induced shockwave and visual selection

Having established a working procedure, we then used the pulsed laser-induced shockwave method for the direct delivery of RNPs into the intact cells of tobacco leaf discs, which are much easier to prepare and handle than protoplasts or zygotes. We introduced a preassembled RNP comprising the Cas9 protein, crRNA (crrNA) and ATTO-550-labeled trans-activating crRNA (tracrRNA) targeting either the tobacco pds or adf genes. The fluorescent tracrRNA allowed the direct screening of transfected cells so that a selectable marker gene was unnecessary (Figure 2A'). The sample size and experimental setup were the same as described above for the transfection with DsRed (Figure 1A and B). We screened for RNP-containing fluorescent cells 48 h after the laser pulse (Figure 2A and A'), identified and selected RNP-containing cells and excised small leaf tissue fragments of different sizes (0.1–0.3 cm radius) depending on the intensity and distribution of the RNP fluorescence signal (Figure 2A”). The selected leaf fragments were grown on MS medium to regenerate intact plantlets (Supplementary Figure S1).

Rapid and reliable identification of pds mutant tobacco plants

Loss-of-function mutations in the pds gene generate an albino phenotype, which allows the efficiency of genome editing to be determined by the visual inspection of regenerated plantlets [23, 24]. Following regeneration, we used a simple PCR-based HRMA procedure to identify the mutants. We analyzed a total of 1673 plants from the pds experiment and identified 147 homozygous/biallelic mutants and 442 heterozygous mutants. Representative images of homozygous pds mutant plants with the anticipated albino phenotypes (homozygous lines F19, I26, L35 and T10) are provided in Figures 2B and Supplementary Figure S2. The distinct melting curves of pds homozygous mutant T10 and the wild-type control are compared as an example in Figure 2C. The homozygous/biallelic mutants were characterized in more detail by Sanger sequencing across the target site, revealing 93 homozygous and 54 biallelic mutants, confirming the positive HRMA results (Figure 2E). A representative chromatogram is provided for line T10, showing a large deletion upstream of the protoscaler adjacent motif (PAM) (Figure 2D). Our new pulsed laser-induced shockwave method achieved an overall mutation efficiency of 8.7% in the pds gene (Supplementary Figure S3).

Generation of adf mutant tobacco plants and characterization under osmotic stress

We used the same HRMA method discussed above to screen 1011 plants from the adf experiment and identified 57 homozygous/biallelic mutants and 110 heterozygous mutants. In the former group, Sanger sequencing confirmed the positive HRMA results and resolved 48 homozygous mutants (e.g., lines A3, A57, A61, B2 and C60) and 9 biallelic mutants (e.g., lines C66 and C72). The distinct melting curves of adf homozygous mutant A57 and the wild-type control are compared as an example in Figure 3A along with the chromatogram of line A57 showing a single-base deletion at the PAM (Figure 3B). In most of the homozygous mutants we observed a single base pair deletion. Representative mutation patterns observed for the homozygous, biallelic and heterozygous mutants are shown in Figure 3C.

To visualize the actin filaments under normal irrigation conditions, we stained three homozygous mutant T0 adf plants (A57, A67 and B2) and wild-type controls with phalloidin. Confocal microscopy confirmed
the presence of more actin filaments in the leaves of the mutant plants (Supplementary Figure S4B) than wild-type controls (Supplementary Figure S4A). Furthermore, a standard test of cell membrane thermostability in 10 randomly-selected adf T0 mutants and wild-type controls under normal irrigation conditions revealed higher membrane thermostability among the mutants (Figure 3I).

Stable inheritance of the adf mutation in generations T1 and T2

T0 adf homozygous mutant plants (48 events) were allowed to grow and set seed under controlled conditions in the greenhouse, but germination and seed setting were inefficient. After selfing, 25% of the T0 events did not set seeds, 31% set very few seeds, 16% set a few seeds but at a later time than wild-type plants, and 27% set seeds in a manner similar to wild-type plants. We grew T1 plants from 13 T0 events and confirmed the presence of the homozygous adf mutation by HRMA and Sanger sequencing (Supplementary Figure S5A and A'). We then generated T2 plants by selfing 10 T1 events for subsequent analysis under abiotic stress, and again confirmed the presence of the homozygous adf mutation by HRMA and Sanger sequencing (Supplementary Figure S5B and B').

Actin enrichment and membrane stability in adf homozygous mutant plants

T2 homozygous adf mutant plants (lines A57, A67 and B2) were stained with phalloidin to visualise actin enrichment and the formation of interlocking marginal lobes (IMLs). Figure 3D is a schematic representation of the apical cell membrane and cell–cell junction, which we used to determine the ratio of actin accumulation. Figure 3E and F show the Z projections of the apical cell membrane. The graph in Figure 3G shows the ratio of actin intensity in the apical cell membrane and cell–cell junction. Higher levels of actin were detected in the cell membrane of the mutant lines, along with the formation of IMLs (Figure 3E, E', F and F'). In the adf mutants, actin filaments were clustered at the cell–cell junctions, but this was not observed in wild-type controls (Figure 3E, E', F and F'). This resulted in a high ratio of actin accumulation favouring the cell–cell junctions in the adf mutant lines (Figure 3G) and an increase in the number of IMLs in the mutant cells (Figure 3H). These data, together with our previous results [21], suggest that the accumulation of actin facilitates the formation of IMLs in the adf mutant plants (Supplementary Figure S4C). A potential mechanism underlying the accumulation of cortical actin and IML formation in adf mutants is proposed in Supplementary Figure S4D.

The drought tolerance of the adf mutant plants was assessed by inducing soil moisture stress at the 4–6-leaf stage and measuring membrane injury using a conductivity meter, which is a widely accepted measure of drought tolerance based on membrane stability. Drought stress was applied for 5 days before the reintroduction of normal irrigation. We tested cell membrane stability on day 0 before the stress treatment, on day 5 of stress, and 5 days after the reinstatement of normal irrigation conditions (Figure 4A). Representative images of mutant and wild-type tobacco plants at the same time points are shown in Figure 4B. The adf mutant plants showed higher membrane stability under both normal irrigation and drought stress conditions. As the soil moisture level declined, the degree of cell membrane injury fell by 1–7% in the adf mutant plants but did not change in the wild-type controls, indicating that the mutant plants can adapt to soil moisture stress. The 10 mutant lines showed an injury rating ranging from 80% (lines A69 and C60) to 86% (lines A672 and C31) compared to 98% in the wild-type plants (Figure 4C).
Leaf water content under drought stress in the *adf* mutant plants

The relative water content (RWC) of the 10 T2 *adf* homozygous mutant plants lines was higher than that of wild-type plants under drought stress. On day 0 before soil moisture stress was applied, the RWC of the mutant and wild-type plants was similar at ~50%. After 5 days of drought, the RWC of wild-type plants had fallen to ~15% but remained much higher in the mutant plants, ranging from ~40% in lines A57, A67 and C60 to ~30% in line C6. Furthermore, the RWC of the mutant plants recovered quickly after normal irrigation was reinstated whereas the wild-type plants still showed a low RWC of ~10% after 5 days of normal irrigation (Figure 4E).

Stress-responsive genes are induced in the *adf* mutant plants

We selected the four mutant lines with the highest cell membrane stability (A57, A67, A69 and C60) as well as one with lower membrane stability (C31) for the comparative analysis of stress-responsive gene expression. The phenotype of C31 after 5 days of drought stress is shown in Supplementary Figure S6. We quantified the transcripts of *adf* and four stress-responsive genes encoding the proteins Hsp70, WRKY, ERF and DREB2 after 5 days of drought stress using the comparative CT method. In the four mutants with stable membranes, *adf* was induced minimally (0.93–1.5-fold), whereas the same gene was induced 4.5-fold in line C31 and 55.7-fold in wild-type plants under drought stress compared to normally irrigated wild-type plants (Figure 4D). We measured the expression of the four drought-inducible genes in the mutant plants relative to the wild-type controls. In the four mutants with the highest membrane stability, these genes were strongly induced by drought (50.2–132.3-fold for Hsp70, 58.7–108.3-fold for ERF, 54.19–138.85-fold for DREB2, and 55.33–77.17-fold for WRKY), with C60 showing the strongest responses and the most stable membranes (Figures 4F–I). The same four genes were only moderately induced in line C31 (8.63-fold for Hsp70, 10.33-fold for ERF, 18.25-fold for DREB2 and 15.88-fold for WRKY).

T2 generation *adf* mutants tolerate high salinity

Finally, we carried out seed germination tests in soil using different salt concentrations (100, 200 and 300 mM NaCl). The 13 mutants and the wild-type plants were morphologically similar when grown without NaCl (Figure 5A). The wild-type plants were unable to germinate in all three treated soils, indicating susceptibility to excess salinity (Figure 5B–D). In contrast, the mutant plants germinated and survived for the entire testing period (4 weeks) under all three salinity conditions, although there was an inverse relationship between shoot height and salt concentration. We measured the relative expression levels of the four stress-responsive genes at 300 mM NaCl in six mutants varying in germination efficiency and percentage (A67, C6, C60, A90, C31 and A69). We found that all four genes were upregulated in the mutant lines (4.43–19.6-fold for DREB2, 4.84–17.85-fold for WRKY, 3.65–7.51-fold for Hsp70 and 1.97–19.65-fold for ERF) compared to wild-type plants (Figure 5E–H). Lines C60, A90 and A69 showed the highest induction of stress-responsive gene expression, corresponding to their better germination and survival rates under salinity stress.
Discussion

CRISPR/Cas9 is the most versatile and widely-used genome-editing tool, facilitating the targeted mutagenesis of various loci in many different plant species [22]. We have described a new method for genome editing in plants that combines cavitation bubble-induced shockwaves for the transfection of plant cells with fluorescent RNPs, fluorescence microscopy to confirm transfection, regeneration based on visual selection, and HRMA to screen the mutant plants. These combined techniques allowed the rapid selection of tissues for regeneration following DNA-free genome editing. The efficient delivery of RNPs is required for effective DNA-free genome editing, and our cavitation bubble-induced shockwave overcomes the collective limitations of other methods such as particle bombardment, electroporation, PEG-mediated transfection and microinjection. Unlike PEG-mediated transfection, our method allows the direct introduction of RNPs into intact plant cells rather than protoplasts, thus reducing the time required for regeneration and making the method accessible in species that cannot yet regenerate from protoplasts. Unlike other physical delivery techniques, our method is gentle and therefore preserves a larger population of intact transfected cells, making it easier to isolate transfected cells and tissue segments for regeneration. A timeline for the new DNA-free genome editing method is provided in Supplementary Figure S3.

RNP-mediated genome editing has been laborious thus far because the absence of a selectable marker requires a time-consuming screening process in order to identify rare mutants in a large background of wild-type cells. In our experiments, the ATTO-550-labeled tracrRNA allowed the rapid identification of transfected cells by fluorescence microscopy. By selecting the region containing cells transfected with RNPs, we were able to avoid the inclusion of large numbers of wild-type cells, which facilitates the screening and regeneration of mutants. Following regeneration, a simple PCR-based HRMA procedure was sufficient to identify the mutants because this method can resolve sequences with single-nucleotide changes [23-25]. HRMA is also simpler, more sensitive, more specific, less expensive and quicker than other methods [26-30]. In this manner, we were able to screen 96 plants in 2 h, a much higher throughput than we previously achieved using the T7 assay for the detection and selection of mutants [7]. We showed that the combination of the new transfection technology combined with the improved selection process resulted in high mutation efficiencies of 5.6–8.7%.

The biological effect of the _pds_ gene mutation was easily verified by visual screening. To analyze how the mutation in the _adf_ gene affected cellular and molecular processes, we stained T0 _adf_ mutant plants with phalloidin to visualize the anticipated enhanced formation of actin filaments [21]. ADF proteins are key modulators of the actin cytoskeleton, and the downregulation of _adf_ promotes actin polymerization, which can be visualized using phalloidin [21, 31-32]. The principal components of the actin cytoskeleton include monomeric actin (G-actin) and filamentous actin (F-actin), with G-actin being the dominant form [33, 34]. ADF depolymerizes F-actin to G-actin. Loss-of-function mutations in the _adf_ gene should therefore promote the formation of more F-actin. In a previous study, we showed that the downregulation of _adf_ in transgenic sugarcane plants overexpressing Hsp70 increased cell membrane stability, actin accumulation, and the formation of IMLs, thus contributing to osmotic stress tolerance [21]. The _adf_ gene was induced 2500-fold in sugarcane under drought stress compared to irrigated control plants, which showed lower membrane stability, no actin accumulation and no IML formation under drought.
stress. We have also previously shown that IMLs in the abovementioned transgenic sugarcane plants and in native sweetcane (Erianthus arundinaceus) plants disappear when actin polymerization is inhibited using latrunculin A. This confirmed the role of filamentous actin in the maintenance and formation of IMLs and therefore in stress tolerance [21]. The transgenic sugarcane plants were able to survive 10 days in soil with only 8.1% moisture and were able to germinate in 300 mM NaCl [21]. Consistent with the above study, we also observed higher membrane stability followed by actin accumulation and the formation of IMLs under normal and drought stress conditions in adf mutant tobacco plants, resulting in higher drought and salinity stress tolerance.

Taken together, our results show that cavitation bubble-induced shockwaves generated by a pulsed laser can achieve the efficient transfection of walled plant cells with RNPs, even in the context of intact tissue explants. When used to deliver preassembled CRISPR/Cas9 components, targeted mutations were achieved with high efficiency. This method greatly simplifies the production of genome-edited plants, avoids the need to regenerate plants from protoplasts, and is potentially transferable to any plant species that can be regenerated in tissue culture, providing an opportunity to extend genome editing to include many currently recalcitrant plant species. In the longer term, this technique may accelerate the development of novel crops that address the needs of a growing population and the effects of climate change. We showed that the accumulation of actin filaments on the plant cell membrane plays a major role in stress tolerance by stabilizing the membrane, indicating a key role in osmotic stress tolerance. New crop varieties that produce more actin filaments could therefore be used to expand agricultural production to marginal soils.

Materials and methods

Plant material, resequencing of target genes and gRNA design

Genomic DNA was extracted from wild-type tobacco plants (Nicotiana tabacum cv Petit Havana SR1) (3–4 weeks old, when the plants had reached the 2–3 leaf stage) [35] using the NucleoSpin Plant II kit (Macherey-Nagel). The target regions of the selected pds and adf genes were amplified from genomic DNA using Q5 high-fidelity DNA polymerase (NEB). The PCR products were purified from agarose gels using the NucleoSpin gel and PCR clean-up kit (Macherey-Nagel) and sequenced using the Sanger method prior to gRNA design, using the primers listed in the Supplementary Table 1. The gRNA sequences were designed using the Crispr RGEN Tools, Cas-Designer and CRISPR-P v2.0 online. The gRNA targeting the pds gene was 5′-TTTTTTTGGAGATTACGTTTGG-3′ and the gRNA targeting the adf gene was 5′-CTTGGAGCTGAGGAGGAAGG-3′. BLAST analysis was used to identify any potential off targets in the crRNA sequences. To prepare RNP complexes, we used crRNA, tracrRNA labeled with ATTO-550 and Cas9 synthesized by Integrated DNA Technologies. We used crRNA XT for all experiments, which has additional chemical modifications to optimize stability and performance.

Transfection mediated by cavitation bubble-induced shockwaves

Shockwaves were generated using a Mai Tai DeepSee Multiphoton pulsed laser (Spectra-Physics) coupled to an inverted TCS SP8 confocal microscope (Leica Microsystems). In order to standardize the transfection conditions, leaf discs (−10 × 5 mm) were placed in a microscopy-grade 35-mm Petri dish.
with a glass bottom (ibidi µ-dish) containing 20 µL 0.25 µg/µL Discosoma striata red fluorescent protein (DsRed) R2G mutant, expressed from pGJ1425 (MPI, Cologne, Germany) [36]. For all experiments, the laser was set to 900 nm and the beam was focused 2–5 µm beneath the lowest epidermal cells of the leaf discs. The laser was used to irradiate a single focused region (six pulses over 1.29 s), creating a plasma that induced bubble formation. The resulting shockwaves transiently increased the permeability of the plasma membrane, allowing the uptake of DsRed into the leaf cells. The same laser setup was used for the delivery of RNPs, which were presented at the same concentration (0.25 µg/µL) as the DsRed protein.

**Selection of cells transfected with RNPs**

The leaf discs were visually inspected 48 h post-transfection under an Olympus X71 inverted fluorescence microscope equipped with appropriate filters for the detection of ATTO-550. Regions showing fluorescence were excised using pipette tips (1 mL and 200 µL capacity, shortened with scissors to achieve a radius of 0.1–0.3 cm) according to the area of RNP fluorescence. The selected regions containing the pds or adf RNPs were transferred to MS medium (4.4 g/L MS salts with vitamins (Duchefa), 20 g/L sucrose, 0.6 mg/L thiamine-HCl, 7 g/L agar, pH 5.8) with hormones (1 mg/L 6-BAP, 0.1 mg/L NAA) and incubated at 20–23°C. The regenerated tissue was subcultured onto plates with fresh medium every 2 weeks until shoots appeared [35]. The plantlets were then transferred onto MS medium without hormones and incubated at 20–25°C with a 16-h photoperiod (7000 lux) to induce root formation. The adf plants with roots were transferred to ED73 standard soil (Patzer) with 0–30% (v/v) sand and grown in the greenhouse with a 16-h photoperiod (10,000 lux, plus sunlight) at 70–90% humidity. All plants were regenerated without selection reagents. All pds plants were analyzed at the 2–3-leaf stage on MS medium. Only the heterozygous mutants and wild-type plantlets developed roots, but these plants were not transferred to the greenhouse.

**Genomic DNA extraction and HRMA**

Genomic DNA was extracted from tobacco plants regenerated from discs using the Quick extract plant DNA extraction solution (Lucigen) according to the manufacturer’s instructions. Leaf discs were collected from tobacco plants grown under sterile conditions at the 2–3-leaf stage. For initial screening, HRMA was carried out using a Quant studio3 Real-time PCR system with HRM software v3.1 (Thermo Fisher Scientific). Each 20-µL reaction contained 10 µL of MeltDoctor HRM master mix (Thermo Fisher Scientific), 0.25 µM of each primer and 50 ng genomic DNA. The melt curve analysis program included PCR amplification followed by melt analysis. For the pds gene, the following conditions were used: PCR stage, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 52°C for 1 min, and 72°C for 1 min; melt curve stage, 95°C for 15 s (1.6°C/s), 60°C for 1 min (1.6°C/s) and 95°C for 15 s (0.1°C/s). For the adf gene, the following conditions were used: PCR stage, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 57°C for 1 min, and 72°C for 1 min; melt curve stage, 95°C for 15 s (1.6°C/s), 60°C for 1 min (1.6°C/s), and 95°C for 15 s (0.1°C/s). All amplicons were 100–150 bp in length, and the site-specific primers listed in Supplementary Table 2 were designed using Primer 3 software.
Sanger sequencing and analysis

The target sites for genome editing were amplified by PCR using 50 ng genomic DNA, 1.5 mM dNTPs, 0.25 µM of each primer, and one unit of Q5 high fidelity DNA polymerase for each 20-µL reaction. For the *pds* gene, the reaction was heated to 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 56°C for 30 s, and 72°C for 30 s, then a final extension step at 72°C for 7 min before cooling to 4°C. For the *adf* gene, the reaction was heated to 98°C for 30 s, followed by 30 cycles of 98°C for 10 s, 53°C for 30 s, and 72°C for 30 s, then a final extension step at 72°C for 7 min before cooling to 4°C. The PCR products were purified from agarose gels using the NucleoSpin gel and PCR clean-up kit and sequenced using the Sanger method on a 3730 DNA analyzer (Applied Biosystems). The primers used for Sanger sequencing are listed in Supplementary Table 3.

The sequencing data were analyzed using Clone Manager v9 Professional (Scientific & Educational Software, Denver) by alignment with the wild-type reference sequence followed by analysis of the chromatograms. For samples with heterozygous or biallelic mutations, the amplified PCR product was subcloned into the pTOPO vector from the TOPO TA Cloning Kit (Thermo Fisher Scientific) and plasmids from three or more independent clones were sequenced for confirmation.

Phalloidin staining and ImageJ analysis of *adf* mutant plants

Tobacco leaf discs (~10 × 5 mm) were stained with AlexaFluor 488 phalloidin as previously described [37] with slight modifications [38]. The discs were fixed in 3.5% (v/v) formaldehyde in phosphate-buffered saline (PBS, pH 7.4), at room temperature overnight. After washing in PBS, the discs were immersed in 0.5% (v/v) Triton X-100 in PBS (pH 7.4) at room temperature overnight. The discs were then washed three times in PBS and stained with 0.66 mM AlexaFluor 488 phalloidin in PBS (Thermo Fisher Scientific) at room temperature for 1 h in the dark before rinsing in PBS and mounting in PBS on glass slides. Actin microfilaments were observed under a Leica inverted TCS SP8 confocal microscope. Actin intensity in the Z projection of the apical membrane of the cells and the average intensity of the cell–cell junction were measured with the help of ImageJ software [39] for at least three independent plants of each mutant and wild-type line.

Cell membrane thermostability analysis

The cell membrane thermostability test estimated the percentage of cell membrane injury as previously described [40] and is an indicator of drought tolerance [41]. This parameter was studied in T0 and T2 mutant plants as well as wild-type plants. In the T0 plants, we analyzed the membrane stability under normal irrigation. In the T2 generation, the third fully-opened leaf was collected from mutant and wild-type plants on day 0 and 5 after the application of drought stress and 5 days after the reinstatement of normal irrigation. Leaf discs (0.5 cm diameter) weighing 200 mg were washed 3 x 2 min times with 20 mL distilled water. The leaf discs were then immersed in 20 mL distilled water in 2.5 cm x 15 cm tubes covered with aluminum foil and incubated at 60°C in a thermostatically controlled water bath for 20 min before cooling to 10°C for 12 h to allow the diffusion of electrolytes. An initial conductance reading was taken at 30°C using a conductivity meter, then the tubes were heated to 100°C for 20 min and a second conductance reading was taken after cooling to 30°C. Membrane injury % = 1 – [(1 – T1/T2)/(1 – C1/C2)]
× 100, where T and C refer to the values for treatment and control samples, and the subscripts 1 and 2 denote the initial and final conductance readings, respectively.

**Plant water status**

The RWC of excised third leaves from mutant and wild-type plants at the 4–6-leaf stage was determined on day 0 and 5 after the induction of drought stress and 5 days after the reinstatement of normal irrigation. The RWC was calculated based on the fresh weight (FW), turgid weight (TW) and dry weight (DW) of 200-mg leaf samples. The FW was determined on a mass balance immediately after sample collection. The TW was determined after soaking the leaf discs in deionized water for 4 h at room temperature in a closed Petri dish and then blotting off any surface drops. The DW was determined after oven drying at 90°C for 72 h. The RWC was determined as previously described [42] using the following calculation:

\[ \text{RWC} = \left( \frac{FW - DW}{TW - DW} \right) \times 100 \]

**Gene expression analysis using the comparative CT method**

Total RNA was isolated from leaf samples using Trizol reagent [43] followed by treatment with DNase (Thermo Fisher Scientific). First-strand cDNAs were synthesized from total RNA using the Revert Aid first-strand cDNA synthesis kit and oligo (dT) primers (Thermo Fisher Scientific) with β-actin as an internal control because its expression was not modified by drought stress. Gene-specific primers designed using Primer Express v3 (Applied Biosystems) were then used for 40 cycles of specific amplification (Supplementary Table 4). Each reaction comprised 12 µL Powerup SYBR green master mix (Thermo Fisher Scientific), 1 U Taq DNA polymerase and 10 pmol of each gene-specific primer and was carried out on a Step One real-time PCR system (Applied Biosystems). The CT values for both the target and internal control genes were used to quantify the transcripts by comparative CT normalization. All reactions were performed in triplicate, and the expression of the target gene was calculated using the formula \(2^{-\Delta\Delta\text{CT}}} [[(\text{Ct gene of interest} – \text{Ct internal control}) \text{ sample} – (\text{Ct gene of interest} – \text{Ct internal control}) \text{ control}] [44]. The \(\Delta\Delta\text{CT}\) values reflect the relative expression of the target gene following exposure to osmotic stress.

**Seed germination assay**

Ten T2 seeds from each mutant line and wild-type controls were grown under identical controlled conditions in a greenhouse. To evaluate germination under salt stress conditions, the trays were irrigated daily with 0, 100, 200 or 300 mM NaCl for a period of 6 weeks. The number of seeds that germinated and the number of plants that survived were recorded at the end of 6 weeks. The experiment was carried out three times.

**Statistical analysis**

For statistical analysis of the data, three replicates from each of the mutant events and wild-type plants were used. Mean value and standard deviation were evaluated using the XLSTAT 2013.5 program to analyze all the data to compare the mutant events and wild-type plants under normal and stress conditions.
References


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Figure 1: Cavitation bubble-induced shockwaves promote the transfer of DsRed/RNPs into intact tobacco cells. A) Schematic representation of the laser setup. B) Representative tobacco leaf sample used for DsRed/RNP transfection on a microscopic-grade Petri dish. Scale bar = 1 cm. C) Confocal microscope image of tobacco leaf sections before laser-assisted transfection. White arrow points to the region chosen for laser targeting. Scale bar = 50 µm. C'). The same region 10 min after the shockwave-mediated uptake of 20 µL DsRed solution (0.25 µg/µL). DsRed fluorescence was detected by confocal microscopy. C'') 3D reconstruction of DsRed-positive cells 10 min after laser treatment. White arrow points to the region chosen for laser targeting. Scale bar = 50 µm.
Figure 2: Analysis of RNP-mediated pds mutations in T0 plants. A) Non-transfected tobacco leaf section does not show any fluorescence. Scale bar = 50 µm. A’) Detection of ATTO-550-labeled fluorescent RNPs by fluorescence microscopy in cells 10 min after laser-assisted transfection. Scale bar = 50 µm. A”) Representative sampling of RNP-containing regions of different sizes (0.1–0.3 cm in radius) depending on the intensity of the RNP signal. Scale bar = 1 cm. B) Albino pds homozygous mutant lines F19, I26, L35 and T10 compared to a wild-type (WT) plant. C) Representative image showing the high-resolution melt analysis of a pds mutant (line T10, red line) compared to wild-type (WT) (blue line). D) Representative DNA sequence chromatogram of WT and pds mutant plant (line T10). The red line shows the PAM, the blue arrow in the mutant shows the start of the deletion, and the yellow line shows the nucleotides just after the PAM in the WT and the starting region of the PAM in the mutant. E) Alignments of representative genome-edited mutants. The mutants marked in red correspond to the albino mutant lines in (B).
Figure 3: Analysis of RNP-mediated adf mutations in T0 plants. A) Representative image showing the high-resolution melt analysis of an adf mutant (A57, red line) compared to wild-type (WT, blue line). B) Representative DNA sequence chromatogram showing WT and mutant line A57. The red line shows the PAM, and the yellow line show the first bp of the PAM. C) Sequence alignment of genome-edited mutants. D) Schematic representation of the apical cell membrane and cell–cell junction. E) Representative image of phalloidin-stained apical cell membrane Z projection in WT cells. E’) Representative image of phalloidin-stained cell-cell junction in WT cells. Scale bar = 20 µm. F) Representative image of phalloidin-stained apical cell membrane Z projection in adf mutant A67. F’) Representative image of phalloidin stained cell–cell junction in adf mutant A67. Scale bar = 20 µm. G) The ratio of actin intensity between the apical cell membrane and cell–cell junction in WT and adf mutant line A67 determined using ImageJ. Data are means ± SD (n = 3). The dots represent individual cells. H) The number of IMLs from each cell. Data are means ± SD (n = 3). The dots represent individual
18 cells. I) Cell membrane thermostability measured using a conductivity meter under irrigated conditions in WT and *adf* mutant T0 plants. Data are means ± SD (n = 3).

Figure 4: Analysis of drought stress tolerance in *adf* mutant T2 plants. A) Schematic representation of the experimental timeline. B) Representative images of wild-type (WT) and *adf* mutant plants on days 0 and 5 of drought stress and 5 days after the restoration of normal irrigation. C) Membrane thermostability of the *adf* mutant and WT plants on days 0 and 5 of drought stress and 5 days after the restoration of normal irrigation.
restoration of normal irrigation. D) Relative expression of adf in the adf mutants compared to WT plants under drought stress calculated using the comparative CT method. E) Relative water content of the adf mutant and WT plants on days 0 and 5 of drought stress and 5 days after the restoration of normal irrigation. F–I) Relative expression of stress-responsive genes in adf mutant plants (A57, A67, A69, C31 and C60) compared to WT controls under drought stress calculated using the comparative CT method: F) Hsp70; G) ERF; H) DREB2; I) WRKY. Data are means ± SD (n = 3).

Figure 5: Analysis of salinity stress tolerance in adf mutant T2 plants. A) Wild-type (WT) (white dotted square) and adf mutant plants at 0 mM NaCl. B) WT (white arrow) and adf mutant plants at 100 mM NaCl. C) WT (white arrow) and adf mutant plants at 200 mM NaCl. D) WT (white arrow) and adf mutant plants at 300 mM NaCl. E-H) Relative expression of stress-responsive genes in adf mutant plants (A67, C6, C60, A90, C31 and A69) compared to WT controls at 300 mM NaCl calculated using the comparative CT method: E) DREB2; F) WRKY; G) Hsp70; H) ERF. Data are means ± SD (n = 3).
Figure S1. Regenerated *adf* mutant plantlets in MS medium after selective RNP-positive tissue sampling by fluorescence microscopy.
Figure S2. Regenerated *pds* mutant plantlets (homozygous mutations) showing the typical albino phenotype.
**Figure S3.** Schematic representation of the genome-editing workflow showing the timeline for direct delivery of RNPs and regeneration.

**Figure S4.** A) Representative microscopic image of wild-type (WT) cells after staining with phalloidin. B) Representative image of T0 *adf* mutant plant line A67 with corresponding phalloidin-stained cells. Scale bar = 50 µm. C) Schematic representation of actin-mediated IML formation in the cell membrane. D) Schematic representation of the putative mechanism of actin-mediated membrane stability and IML formation.
Figure S5. A) Representative image showing the high-resolution melt analysis of a T1-generation *adf* mutant (line C60). A′) Chromatogram of wild-type (WT) and T1-generation mutant (line C60) plants. B) Representative image showing the high-resolution melt analysis of a T2-generation *adf* mutant (line C60). B′) Chromatogram of wild-type (WT) and T2-generation mutant (line C60) plants.

Figure S6. Representative images of wild-type (WT) and *adf* mutant (C31) plants on day 5 of drought stress.

Video S1: The laser-mediated cavitation bubble-induced shockwave that facilitated the introduction of RNPs into plant cells.