Supporting Information for

Identification of cell populations necessary for leaf-to-leaf electrical signaling in a wounded plant

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**Plant Growth Conditions, Transformation and Chemicals.** *Arabidopsis thaliana* Columbia (Col) and T-DNA insertion lines in the Col background: *glr3.1* (SALK_063873), *glr3.2* (SALK_150710), *glr3.3* (SALK_099757), and *glr3.6* (SALK_091801) were from (3). Plants were sown individually in 7 cm diameter pots, stratified for 2 d at 4°C in the dark, and grown at 21°C under 150 μE m⁻² s⁻¹ light (10h light, 14h dark, 70% humidity) in an insect-free growth room. The transformation protocol was from (45). Chemicals were purchased from Sigma (Buchs, Switzerland) unless otherwise stated.

**Insect Bioassays.** Eleven pots of 4 week-old WT, and *glr* mutants were placed in Plexiglas boxes (28.5 x 19 x 19 cm). Four neonate *Spodoptera littoralis* larvae were placed on each plant and allowed to feed continuously over the duration of the assay. The caterpillars were collected and weighed on an MS104S balance (Mettler Toledo, Greifensee, Switzerland) after 12 days. The assays were replicated 3 times.

**Surface Potential Monitoring.** Surface potentials were monitored based on (3) with some modifications to permit automated data analyses. The electrophysiology setup consisted of a dual channel differential electrometer FD223a and LabTrax 4/16 acquisition system (both devices from World Precision Instruments, Sarasota, USA). FD223a probes (item FD223AP, World Precision Instruments) were fitted with Ag surface electrodes (chloridized silver wire, diameter 0.5 mm) and mounted on MM33 manual micromanipulators (Marzhauser Wetzlar GmbH & Co. KG, Wetzlar, Germany). Surface electrodes were placed on the petiole of leaf 8 and/or leaf 13. Low-resistance electrode–leaf interfaces were maintained with 10 μl drops of 10 mM KCl in 0.5% (w/v) agar. A reference electrode of the same type was placed in the soil. Surface potential recordings were acquired at 100 Hz using LabScribe3 (iWorx Systems, Inc., Dover, NH) software. Data was analyzed using custom-written scripts in MATLAB (MathWorks, Natick, MA). Two wounding protocols were employed. In order to obtain maximum reproducibility during the comparison of *glr* mutants (Fig. 1 A and B in the main text and for selection of complemented mutants), leaf 8 was wounded with an 808 nm laser at an energy density of 550 J/cm². Laser pulses (20s) produced a 2 mm diameter hole at the petiole-lamina junction. For experiments involving fluorescence microscopy approximately 50% of the leaf 8 lamina apical region was crushed with plastic forceps to induce a wound response. Note that crush-wounding is faster and more extensive than the laser wounding method, producing a stronger signal that causes surface potential changes in leaf 13 of *glr3.1 glr3.3* but not in *glr3.3 glr3.6*. The laser wounding protocol did not generate signals in leaf 13 of *glr3.1 glr3.3* or *glr3.3 glr3.6*.

For surface potential measurements, latency, duration and velocity were as defined in (3). In experimental series consisting of simultaneous electrical signals and GCaMP3 measurements, corresponding electrical signal parameters were calculated in MATLAB. Depolarization slopes were calculated in MATLAB based on data from between the depolarization initiation point and depolarization minimum. The depolarization inflection point was the minimum of the first derivative of the 5th order polynomial fitted to a selected data range. Maximal depolarization rate was a slope of linear function fitted to 41 experimental points (data collection rate 100 Hz) surrounding the depolarization inflection point.
Generation of Transcriptional GUS Fusions. GLR promoter-β-glucuronidase (GUSPlus) (46) transcriptional fusions were used to localize promoter activity at the tissue level. Promoters (approx. 3 kb) were first amplified from Arabidopsis genomic DNA. GLR3.1 primers: forward (5’ - 3’) cggggtacctccccctgctgatgtggtc, reverse (5’ - 3’) aagccgggggacaacagccagctgacact. GLR3.3 primers: forward (5’ - 3’) ggtaccacccaaaccgcttattcttttg, reverse (5’ - 3’) cccgggatctgataaagagatgaaagtaaattag. GLR3.6 primers: forward (5’ - 3’) cggggtaccttggtcattttgtcatctgcgac, reverse (5’ - 3’) aaggggcccgggcttctcaatttcaggagattcctg. Clones were inserted into pUC57-L4-Kpn1/Xma1/R1 using the XmaI, KpnI restriction enzymes and T4 ligase. Subsequent steps used recombination cloning (Gateway, Thermo Fisher Scientific/Invitrogen, Waltham, MA) to assemble constructs. Promoter fragments in pUC57, pEN-L1-GUSPlus-L2 and pEDO097pFR7m24GW carrying the fluorescence-accumulating seed technology (FAST) cassette (47) were combined using double Gateway cloning. WT plants were transformed and transgenics were first selected based on seed coat fluorescence using a MZ16 FA microscope (Leica, Wetzlar, Germany).

Generation of Translational GUS Fusions. GLR promoter::GLR-GUS translational fusions were used to localize GLR proteins at the cellular level. GLR3.1 and GLR3.3 genomic promoter and coding regions were amplified by PCR using InFusion kits (Takara Bio, Mountain View, CA) from the transformation-competent bacterial artificial chromosome library JAtY (48) with primer pairs containing Gateway recombination sites. GLR3.1: forward (5’ - 3’) gtatagaaaagttgggtaccttccccctgcgggatcgttggtc; reverse (5’ - 3’) ggtaccacccaaaccgcttattcttttg. GLR3.3: forward (5’ - 3’) cggggtaccttggtcattttgtcatctgcgac; reverse (5’ - 3’) aaggggcccgggcttctcaatttcaggagattcctg. Clones were inserted into pUC57-L4-Kpn1/Xma1-R1 using the XmaI, KpnI restriction enzymes and T4 ligase. Subsequent steps used recombination cloning (Gateway, Thermo Fisher Scientific/Invitrogen, Waltham, MA) to assemble constructs. Promoter fragments in pUC57, pEN-L1-GUSPlus-L2 and pEDO097pFR7m24GW carrying the fluorescence-accumulating seed technology (FAST) cassette (47) were combined using double Gateway cloning. WT plants were transformed and transgenic lines were selected with FAST technology. In the case of GLR3.6 the cDNA was used. This was cloned using primers: forward (5’ - 3’) caagtttgtacaaaaaagcaggcttaatgaagtggtttctgcttatgc; reverse (5’ - 3’) ccactttgtacaagaaagctgggttgttgcagcgacttgaaccatttg and recombined to produce pDONR/ZEO-L1-GLR3.6 cDNA-L2. Triple Gateway cloning with pUC57-L4-Kpn1-GLR3.6pro-Xma1-R1, pEN-R2-GUS-L3, pDONR/ZEO-L1-GLR3.6 cDNA-L2 and the destination vector pB7m34GW produced the desired GLR3.6pro::GLR3.6-GUS fusions which were transformed into the WT. Transgenics were then selected on half-strength MS medium (Duchefa Biochemie, Haarlem, Netherlands) with 40 µg/ml BASTA (PlantMedia, Dublin, OH).

Selection of Transgenic GUS Lines for Microscopy. For individual transcriptional and translational GUS fusions, at least 24 T1 plant lines were analyzed. The majority showed a similar expression pattern. From these, 12 T2 lines that showed similar promoter activity patterns were selected. From these, four T3 lines showing similar promoter activity patterns were used to generate T3 plants. Of these, one line was used to produce figures.

GUS Staining. 3.5 week-old plants expressing GUS reporter genes were collected and immediately prefixed in 90% acetone on ice for 1 h, washed twice with 50 mM sodium phosphate buffer (pH 7.2), placed in staining solution (10 mM Na2EDTA, 50 mM sodium phosphate buffer, 1 mM K4Fe(CN)6, 1 mM K3Fe(CN)6, 0.1% (v/v) Triton X-100, 0.5 mg ml⁻¹ X- Gluc (pH 7.2)), vacuum-infiltrated for 20 minutes and incubated at 37°C in the dark for 8 h. Rosettes were then washed with 50 mM sodium phosphate buffer and cleared with 70% ethanol.
Images of plants were taken with VHX-500F digital microscope (Keyence, Mechelen, Belgium). The petioles of leaves 6 or 7 were used for detailed GUS localisation. X-Gluc stained petioles were fixed in glutaraldehyde/formaldehyde/50 mM sodium phosphate (pH 7.2) 2:5:43 (v/v/v) for 30 min, dehydrated in an ethanol gradient (10%, 30%, 50%, 70%, 90% and twice absolute, 30 min in each concentration) and embedded in Technovit 7100 resin (Haslab GmbH, Ostermundigen, Switzerland) according to the manufacturer’s instructions. Transversal and longitudinal sections of the petiole (4 µm thick) were made on a RM2255 microtome (Leica, Wetzlar, Germany). The sections were mounted in 40% (v/v) glycerol and photographed with a Leica DM5500 microscope.

**Generation of Functional Translational VENUS Fusions.** Note that we did not succeed in generating functional GLRs that complemented the cognate glr mutants for GLR3.1 and GLR3.3. In all cases we used genomic DNA containing the coding regions as a basis for fusion protein production. GLRpromoter::GLR-yellow fluorescent protein (YFP) translational fusions were used to localize GLR proteins at the subcellular level. The YFP variant used was VENUS (49). For GLRs 3.1 and 3.3 the genomic promoter-coding region constructs used for GUS translational fusions were re-employed. Additionally, a genomic fragment with the GLR3.6 promoter and coding region was amplified from the transformation-competent bacterial artificial chromosome library JAtY (48) using the following primers: forward (5’-3’) gtatagaaaagttgggtacc ttcacgtacggtccat; reverse (5’-3’) tgtacaaacttgtcccggggttgcagcgacttgaaccat. These fragments were recombined with the pEN-L1-VENUS-L2 vector in a double Gateway reaction was used to produce GLRpro::GLR-VENUS fusions. All VENUS fusions were transformed into the cognate T-DNA insertion mutant backgrounds: glr3.1, glr3.3, and glr3.6 (3). Transgenic lines were selected with FAST technology and the restoration of WT electrical signals. To select functional GLR-VENUS protein fusions for microscopy only the complemented lines showing restoration of WT electrical signals were retained (Table S1). Plants were laser-wounded (see ‘Surface potential monitoring’) and restoration of WT-like wound-activated surface potentials on leaf 13 were sought. At least two independent transgenic lines for each construct were used to perform experiments. To determine subcellular GLR localizations, complemented lines were crossed with WAVE marker lines (27): WAVE 6R, endoplasmic reticulum; WAVE 9R vacuole membrane.

**Strategy for GLR Localization.** We found that GLR-VENUS fusions were visible in confocal microscopy in the roots and cotyledon petioles of seedlings. However, the laboratory’s previous work on wounding has focussed on the adult phase and chiefly on expanded leaves of 5 week-old plants on which electrodes were generally placed on the midvein. In order to conduct work relevant to this growth stage we therefore sought to visualize the GLR-VENUS fusions in the primary vasculature of expanded leaves of 4-5 week-old plants. This approach was unsuccessful and we were unable to gain optical access to core vascular cells either in fresh leaf tissue or in cleared tissues (i.e. using the ClearSee protocol) (23). We therefore developed a method that would allow us to isolate the midvein free from expanded leaves of adult-phase plants.

**Primary Vein Extraction and GLR-VENUS Localization.** To extract the primary vasculature, leaf 8 of a 5 week-old plant was severed at the leaf lamina-petiole junction. The entire lamina was then discarded. A transverse incision was then made with a razor blade or scalpel on the adaxial (upper) side of the basipetal petiole approximately 5 mm from the rosette center, to such a depth that the epidermis was cut but the midvein remained intact. The petiole envelope tissue was then gently pulled off so that the
primary vein was not torn off from the intact basal part of the petiole. The primary vein was then cut off from the petiole base with scissors for immediate use. In this way, vein segments of up to 1 cm long can be obtained routinely. Note that variations of the method can be used to allow observation of the vein while it is still attached to the petiole. The method also works for 6 week-old plants and in this case can yield veins segments of over 2 cm long.

For microscopy, extracted primary veins were either treated with the ClearSee method (23, 24) or subjected to partial protoplasting to remove external vascular parenchyma cells in order to gain optical access to core vascular cells. For this, freshly isolated veins were placed in pH 5.5 digestion solution containing 500 mM D-Sorbitol (for GLR3.1 in Fig. 4A,B in the main text and Figs S3 and S5 (A-L) or 100 mM D-Sorbitol (for Fig. 4D), and 1 mM CaCl₂, 5 mM MES (4-Morpholine ethane sulfonic acid), containing 2 % (w/v) Cellulase Onozuka R10 (SERVA Electrophoresis GmbH, Tokyo, Japan), 0.3 % (w/v) Macerozyme R10 (SERVA Electrophoresis GmbH, Heidelberg, Germany). This solution was incubated at room temperature for up to 3 h with gently rocking. An additional co-localization experiment on plants expressing GLR3.6-VENUS was conducted in freshly isolated veins that were not subjected to protoplasting or to fixation (Fig. S5, M-O). Vein samples were observed with an SP8 microscope (Leica Microsystems CMS GmbH, Mannheim, Germany) using pre-set configurations for eYFP and/or RFP observation (with 10% maximum laser excitation intensity). Where mentioned, propidium iodide (PI) (40 µg/ml) and/or aniline blue (40 µg/ml) were used to facilitate localization. Staining was conducted for 10 min. PI and aniline blue were excited at 535 nm and 370 nm and detected in a 615–700, 490–520 nm windows, respectively. In some cases, extracted vascular strands or intact petiole segments were cleared for 48h using the ClearSee protocol (23), stained with PI and aniline blue (24) then observed under an LSM 700 microscope (Carl Zeiss MicroImaging GmbH, Jena, Germany. Whole-image contrast between pictures was equilibrated in Photoshop (Adobe Systems, California) or FIJI v1.48a (National Institutes of Health, USA). Co-localization coefficients were obtained using the ImageJ co-localization function.

**GCaMP3 Expressing Plants.** Wound-induced Ca²⁺ fluxes were detected with the single wavelength calcium biosensor GCaMP3 (32) which was cloned from plasmid #22692 (Addgene, Cambridge, MA) into pDONR/Zeo GCaMP3 was produced by fusion of: 1st position UBQ10pro and 2nd position GCaMP3 entry clones into double Gateway destination vector pEDO097 containing FAST cassette (47) and was transformed into the WT. Homozygous T3 generation plants were selected based on seed coat fluorescence. The GCaMP3 reporter was crossed into glr mutants from this background. The fluorescence output (ΔF/F₀) of GCaMP3 decreases approximately 2-fold at pH 6 compared to its maximum at pH 7 (50). Note that we did not detect decreases of GCaMP3 fluorescence to below base levels observed at the start of each experiment.

**GCaMP3 Fluorescence Visualization and Quantification.** Calcium imaging used an SMZ18 stereomicroscope (Nikon Instruments Europe BV, Amsterdam, Netherlands) equipped with an ORCA-Flash4.0 (C11440) camera (Hamamatsu, Solothurn, Switzerland) and eGFP emission/excitation filter set (AHF analysentechnik AG, Tübingen, Germany). Light was supplied to the stereomicroscope using fibre optics. A 0.6x objective enabled visualisation of an area of 1225 mm² (35 x 35 mm) at the height of the rosette. This allowed simultaneous monitoring of GCaMP3 fluorescence in leaves 8 and 13. Signal in the intensity range interval of 90-300 from 16-bit camera output was converted to an 8-bit
intensity scale using Look Up Table (NIS-Elements; Nikon Instruments Europe BV, Amsterdam, Netherlands) and saved as the green component of a 24-bit RGB movie. Images with resolution of 512 x 512 pixels were acquired using NIS-Elements software with 1 frame s⁻¹ frequency. For data processing the 8-bit green component was extracted from the RGB movie using MATLAB. Wound-induced fluorescence changes were obtained by subtracting the first and consecutive movie frames recorded within 300 s after completion of wounding. To create maximal fluorescence intensity maps, maximal intensity values were calculated for each pixel from the movie. Integration of total fluorescence intensity from individual leaves or halves of the leaves was the sum of intensities of all selected pixels for 150 s after wounding. The optical system was tested for the presence of intrinsic optical distortions using the Camera Calibrator application in MATLAB. Tangential distortion was not detected and positive radial distortion minimally affected images only in the corners, therefore images were not corrected for intrinsic optical distortions. To account for uneven illumination a blank background image (mean of 10 background images after subtraction of its minimal intensity value) was subtracted pixel to pixel from the active image (i.e. images of rosettes). To compensate for loss of intensity in entire active images, the difference between mean intensities of the entire active images before and after correction was added to final image. Fluorescence in relation to depth of field of the stereomicroscope was tested using autofluorescent plastic slides (Chroma Technology Corp, Bellows Falls, USA) and in the range of height of Arabidopsis rosette (objective focal length ±10 mm) was stable. As a control for a non-wound calcium signal-inducing stimulus ice cold distilled water (30µl) was placed on leaf 8 at the blade-petiole transition zone. For analysis, the fluorescence signal was acquired and averaged from two ROIs (10x10 pixels, 0.68x0.68 mm) taken either side of the water droplet.

GCaMP3 fluorescence wounds is represented by the ratio ΔF/F calculated using the formula ΔF/F = (F-F₀)/F₀ where F is fluorescence from the moment of wounding to a given time-point and F₀ is the averaged baseline fluorescence in the ROIs in the first 10 s after wounding. Signal-to-noise ratio (SNR) analyses were performed by measuring fluorescence standard deviation (SD) in the first 10 s of recording following infliction of the wound. Average fluorescence per ROI throughout the movie was divided by the corresponding SD to give SNR ratios. Pairs of recordings from individual plants (surface potential and corresponding averaged fluorescence changes) were adjusted and averaged according to the surface potential depolarization minimum to allow the visual comparison of GCaMP3 fluorescence and electrical signals. Averaged fluorescence changes were quantitated in MATLAB using parameters (latency, amplitude, duration and velocity) analogous to those used for electrical signal quantitation (3). Latency was computed as the time between the wounding end point and the time at which the wound-induced increase of fluorescence exceeded the SD threshold calculated based on 10 preceding frames (i.e. 10 s). Fluorescence intensity amplitudes were calculated as differences between the maximal signal intensity after wounding and points corresponding to the initiation of fluorescence increase. Duration of the fluorescence changes was calculated with the peak analysis function available in the MATLAB Signal Processing Toolbox as the fluorescence peak width at half prominence. The apparent velocities of Ca²⁺ signals were calculated as the Euclidean distance between central pixels of two ROIs divided by the fluorescence wave transition time between the ROIs. Maximal slopes of fluorescence increase were calculated from a linear function fitted to 21 experimental points surrounding the fluorescence inflection point (inflection point +/-10 experimental points). As for membrane depolarization, the inflection point was specified.
as the minimum of the first derivative of the 5th order polynomial fitted to the data from the time of apparent increase of fluorescence to the point of maximal fluorescence. For simultaneous surface electrophysiology and calcium imaging, setups were placed in a custom-made Faraday cage. Acquisition of electrical signals was initiated manually with an approximately 1 s delay in relation to initiation of GCaMP3 image acquisition. Fluorescence changes were acquired from two ROIs (F1 and F2 in Fig. 5C) each of 10 x 10 pixels (0.68 x 0.68 mm) oriented along the axis of the leaf midvein and located either side of electrode. Signals from both ROIs were averaged. Each experiment was replicated a minimum of 7 times.

**Vein Exposure for Calcium Imaging.** Plants used were 5 weeks-old. To expose a region of the primary vasculature, two shallow incisions (approx. 2 mm apart) were made across the adaxial petiole such that only the epidermis and cortex were wounded. The two transversal incisions were then joined by cutting along the adaxial long axis of the petiole. Gentle pulling of leaf lamina brakes epidermal, mesophyll and bundle sheath cells while maintaining vascular tissue continuity. Fragments of the epidermis and cortex between incisions were detached with forceps in such a way as to create a movable ring of tissue on the vascular strand. In the last step, the movable ring was gently torn away using forceps. Blocks of agar (0.8%; AppliChem GmbH, Darmstadt, Germany) containing half-strength MS medium (Duchefa Biochemie, Haarlem, Netherlands) and pH adjusted to 5.7 were placed below the exposed vascular strand for 1 - 2 h to maintain sufficient humidity during tissue recovery prior to experimentation.
Fig. S1. Low abundance GLR3.1-VENUS pools in the phloem region.
Confocal images of GLR3.1-VENUS from a midvein. Partial protoplasting was used to remove peripheral vascular matrix. The image shows the sieve tube region. Yellow, GLR3.1-VENUS signal. Arrowhead, sieve plate(s) stained with aniline blue. Red, chloroplast autofluorescence. Bar = 10 μm.
**Fig. S2. GLR3.3-VENUS pools at the periphery of sieve plate.**

Face-on views of a sieve plate after full protoplasting of an extracted midvein in a digestion mix containing 500 mM sorbitol. (A) Plasmolysed sieve elements with sieve plates indicated by asterisks. GLR3.3-VENUS signal (yellow), scale bar = 10 µm. (B) Face-on view of sieve plate. Arrows indicate plate pores. (C) VENUS signal. (D), merge of (B) and (C). For B-D bars = 1 µm.
Fig. S3. GLR3.6-VENUS localizes to xylem contact cells.

Localization of GLR3.6-VENUS in contact cells. A petiole from a 3 week-old plant was fixed, cleared (using the ClearSee procedure: 23, 24) and stained with FM4-64. (A) The xylem region. V, xylem vessels. (B) FM4-64 signal from the same region. Asterisks indicate contact cells. (C) GLR3.6-VENUS signal (green) in contact cells. (D) Merged image. Scale bars = 50 µm. No protoplasting was used during sample preparation. Note that fixation/dehydration is likely to have caused deformation of GLR3.6-VENUS-tagged organelles; these images should be compared with those for fresh, non-dehydrated samples in Fig. 4D in the main text and Fig. S5 (M-O).
Fig. S4. Location of GLR pools in the primary leaf vein either side of the cambial region
Fig. S5. Functional GLR-VENUS fusion proteins co-localize with endomembrane markers.

(A, B, C) GLR3.1-VENUS in xylem contact cells localizes to the ER. (A) signal from VENUS. (B) signal from WAVE 6R ER marker. (C) overlay of signals in (A) and (B); scale bars = 10 µm. (D, E, F) GLR3.1-VENUS in the phloem sieve element region localizes to the ER. (D) signal from VENUS. (E) signal from WAVE 6R ER marker. (F) overlay of signals in (D) and (E); scale bars = 10 µm. (G, H, I) GLR3.3-VENUS localizes to the ER. (G) signal from VENUS. (H) signal from WAVE 6R ER marker. (I) overlay of signals in (G) and (H); scale bars = 5 µm. (J, K, L) GLR3.6-VENUS localizes to the vacuole membrane. (J) signal from VENUS. (K) signal from WAVE 9R tonoplast marker. (L) overlay of signals in (J) and (K). In all cases, freshly extracted primary veins were subject to treatment with a protoplasting mixture containing 500 mM sorbitol to remove outer layers of parenchyma cells. (M, N, O) GLR3.6-VENUS localization in an isolated primary vein that was imaged directly with no protoplasting and no incubation in sorbitol. (M) signal from VENUS. (N) signal from WAVE 9R tonoplast marker. (O) overlay of signals in (M) and (N). All scale bars = 10 µm. Note the difference in form of the vacuoles in J-L compared to M-O. This was likely due to vacuole volume reduction in the presence of sorbitol during preparation of samples shown in J-L.
Fig. S6. CGaMP3 expression does not have a major impact on wound-activated electrical activity.

*UBQ10pro::GCaMP3* was crossed from the WT background into *glr* mutants. Leaf 8 of 5 week-old plants was crush-wounded and surface potentials were recorded on the petiole of distal leaf 13. *(A)* Duration of surface potential changes in wounded plants. *(B)* Amplitude of surface potential changes. *(C)* Maximum depolarization rates. All graphs show means +/- standard deviations (n = 4 - 13 plants per group). Statistical significance was calculated using an unpaired t test. n.s. not significant, *P < 0.05.*
Fig. S7. CGaMP3 signal intensities in response to cold water treatments.

(A) Averaged traces of GCaMP3 fluorescence after placing a 30 µL drop of ice-cold water on leaf 8. Solid lines and shaded areas between dotted lines represent averaged traces and standard deviation envelopes (3 traces averaged). Blue arrows indicate moment of cold water application.

(B) Peak amplitude of GCaMP3 fluorescence signal in glr mutants. Bar graphs show means +/- standard deviations (n = 3 plants). Statistical significance was calculated using an unpaired t-test, n.s., not significant.
Fig. S8. Differential GCaMP3 fluorescence in leaves 9 and 13 after wounding leaf 8.
The plants used were 5 weeks-old. Fluorescence for leaf 9 is indicated with black lines. GCaMP3 fluorescence for leaf 13 that shares a direct vascular connection with leaf 8 is shown in green. Leaf 8 was wounded. Solid lines and shaded areas between dotted lines represent averaged traces and standard deviation envelopes (4 - 6 traces were averaged for leaf 9, and 7 - 10 traces were averaged for leaf 13). Pairs of individual traces were adjusted and averaged according to maximal surface potential depolarizations that were recorded simultaneously on leaf 13 (not shown). Time zero represents the beginning of the time range used for averaging and corresponds to 20 s before the minimum of electrical signal depolarization.
Fig. S9. Maximal GCaMP3 fluorescence intensity after wounding.

(A) Maximal pixel intensity from wounded rosettes of 5 week-old plants. W, wound on leaf 8; D, distal leaf 13. (B) Integrated GCaMP3 fluorescence intensity per leaf after wounding leaf 8 (n = 7 - 10 plants per group). In (A) and (B) movies were recorded for 300 s after wounding. See Methods for integration procedure. Data are means +/- standard deviations. Statistical significance was calculated using one-way ANOVA and Tukey’s multiple comparisons test. Genotypes for which responses do not differ significantly are represented with the same letter.
Fig. S10. Signal-to-noise ratio analyses for GCaMP3 fluorescence in the WT and glr mutants.

In each case leaf 8 of a 5 week-old plant was wounded. The plots show signal-to noise (SNR) changes over time in distal leaf 13. Fluorescence was monitored in two ROIs (10 x 10 pixels) either side of a surface electrode as indicated in Fig. 5C.
Fig. S11. Asymmetrical Ca^{2+} signals in distal leaf 13 of the glr3.1 mutant.

(A) Schematic indicating the halves of leaf 13 that are proximal or distal to wounded leaf 8. (B) Integrated GCaMP3 fluorescence from each half of leaf 13 in the WT and glr mutants after wounding leaf 8. Integrations were from videos captured over a period of 300 s following wounding of leaf 8. Note significant asymmetry in leaf 13 in glr 3.1. (C) Ratio of integrated fluorescence in leaf 13 in the leaf half proximal to wounded leaf 8 over the leaf half distal to the wound. Bar graphs show means +/- standard deviations (n = 8 - 10 plants per group). Statistical significance was calculated using an unpaired t-test. n.s. not significant, *P < 0.05, **P < 0.005. (D to F) Schematic illustrating the differential response of leaves distal to the wounds in the WT and glr mutants. Leaves are numbered from oldest to youngest. The intensity of carmine represents the integrated GCaMP3 fluorescence in response to wounding leaf 8. Drawings show plants with clockwise growth spirals.
Table S1. Complementation of *glr3.1*, *glr3.3* and *glr3.6* single mutants with translational VENUS reporter fusions.

Data are means +/- standard deviations. Letter a shows statistically significant difference regarding to WT. Student *t*-test with *P*-value < 0.05. All complemented lines are used for confocal microscope experiments.

| Leaf 8 | Construct | Leaf 13 |
|--------|-----------|---------|
|        |           | Amplitude | Duration | Amplitude | Duration | n |
|        |           | (mV) | (s) | (mV) | (s) | |
| WT     |           | -85±13 | 35±5 | -54±9 | 72±9 | 12 |
| glr3.1 |           | -87±7  | 27±7<sup>a</sup> | -47±10 | 4±1<sup>a</sup> | 8 |
| glr3.1 | *GLR3.1pro:GLR3.1-VENUS #5.1* | -88±13 | 25±9 | -46±10 | 70±12 | 6 |
| glr3.1 | *GLR3.1pro:GLR3.1-VENUS #6.1* | -91±10 | 30±14 | -49±9 | 74±8 | 6 |
| glr3.3 |           | -67±21 | 21±6<sup>a</sup> | -45±15 | 19±5<sup>a</sup> | 10 |
| glr3.3 | *GLR3.3pro:GLR3.3-VENUS #2.3* | -90±8  | 34±12 | -48±13 | 72±17 | 6 |
| glr3.3 | *GLR3.3pro:GLR3.3-VENUS #5.2* | -86±8  | 28±9  | -50±3  | 74±5  | 6 |
| glr3.6 |           | -91±7  | 28±5<sup>a</sup> | -56±10 | 30±7<sup>a</sup> | 9 |
| glr3.6 | *GLR3.6pro:GLR3.6-VENUS #18.11* | -85±18 | 31±8  | -58±13 | 69±34 | 6 |
| glr3.6 | *GLR3.6pro:GLR3.6-VENUS #28.10* | -85±9  | 36±5  | -63±13 | 85±11 | 6 |
Captions for Movies S1 to S3

Movie S1. Caterpillar feeding induces distal Ca\(^{2+}\) fluxes.
The movie shows a *Pieris brassicae* larva feeding on a 2 week-old WT plant expressing the UBQ10pro:GCaMP3 reporter. Note induced fluorescence distal to the feeding site. Speed is real time. Scale bar = 0.5 cm.

Movie S2. Wound activated surface potential changes and GCaMP3 fluorescence traverse an exposed vein.
(A) Experimental setup. The midvein of leaf 13 of a 5 week-old plant was exposed over a length of approximately 2 mm. e1, position of a surface electrode. Equidistant red squares indicate regions used for estimating apparent velocities of GCaMP3 fluorescence changes. F1 shows the frame used to compare fluorescence changes with electrical activity recorded at e1. (B) Movie of the experiment. W, position of wound; C, clamp to prevent leaf movement during wounding. The exposed vein is encircled in red. The movie is in real time. (C) Apparent velocities of Ca\(^{2+}\) fluxes estimated from points A, B, C and D in (A). Data represent average values +/- standard deviation from at least three repetitions of velocity estimates from movie presented in (B). (D) Simultaneous display of GCaMP3 fluorescence (red trace) and electrical activity (blue trace) monitored at F1 and e1 respectively synchronized with the video presented in (B).

Movie S3. GCaMP3 fluorescence after wounding leaf 8 of 5 week-old plants.
In each case the frame includes distal leaf 13 (this leaf carries an electrode which is visible in the image). Note that these videos are 10x accelerated.

Supporting references

45. Clough SJ & Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16(6):735-743.

46. Broothaerts W, et al. (2005) Gene transfer to plants by diverse species of bacteria. *Nature* 433(7026):629-633.

47. Shimada TL, Shimada T, & Hara-Nishimura I (2010) A rapid and non-destructive screenable marker, FAST, for identifying transformed seeds of *Arabidopsis thaliana*. Plant J 61(3):519-528.

48. Zhou R, Benavente LM, Stepanova AN, & Alonso JM (2011) A recombineering-based gene tagging system for *Arabidopsis*. Plant J 66(4):712-723.

49. Nagai T, et al. (2002) A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat Biotechnol 20(1):87-90.

50. Helassa N, et al. (2015) Fast-response calmodulin-based fluorescent indicators reveal rapid intracellular calcium dynamics. Sci Rep 5:15978.