Chapter 3

Materials and Methods
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Plant material

Plants of *Pisum sativum* (cv. Arkel) were raised from seeds (Pocha seeds Co. Pvt. Ltd. Pune, India). The pea seeds were soaked in water overnight and then surface sterilized with 0.2% (v/v) sodium hypochlorite solution. The seeds were kept covered in a moist black cloth at 25°C until germinated, usually for 3 d. The germinating seeds were then sown in plastic trays filled with soil and farmyard manure (3:1, v/v). The plants were grown in a greenhouse, average day/night temperature of about 30/20°C and photoperiod of 12 h and were watered twice daily. The second to fourth completely unfolded leaves were collected from 2 to 3 week-old plants for epidermal bioassays.

The seeds of *Arabidopsis thaliana* (wild type: Landsberg erecta or mutants abi1, abi2, aba2) were sterilized by using 80% ethanol in 0.1% Triton X-100 and 3% NaOCl. Seeds were then transferred in rows on ½ MS plates (Table 3.1), wrapped with Parafilm, incubated for 2 d at 4°C in dark to break dormancy. Then plates were transferred to culture room at 25°C in continuous light. One to two week old seedlings were planted in plastic trays containing 1:1:1 mixture of vermiculite, perlite and soilrite and transferring to growth chambers where the optimal conditions for growth was maintaining [light (125-150 µmol m⁻² s⁻¹), photoperiod 16/8 h (light/dark), temperature 25°C] and nutrient solution (Table 3.2) was supplied daily up to three weeks then once in every week.

Bioassays of stomatal closure in epidermal strips

The abaxial (lower) epidermis was peeled off from the leaves (pea or Arabidopsis) and cut into strips of ca. 0.16 cm². The epidermal strips were transferred to 3 cm diameter petri dishes containing 3 ml of “incubation medium” (10 mM MES-KOH pH 7.0 and 50 mM KCl) and the epidermal strips were exposed for 3 h to white light 250 and 150 µmol m⁻² s⁻¹ for pea and Arabidopsis respectively. A bank
Table 3.1  The composition of ½ MS Agar for rising the seedlings, germination and root growth assays.

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>(1 Litre)</th>
<th>Micronutrients</th>
<th>(1 Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄NO₃</td>
<td>1.65 g</td>
<td>CoCl₂ 6H₂O</td>
<td>0.025 mg</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1.9 g</td>
<td>KI</td>
<td>0.75 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.17 g</td>
<td>MnSO₄</td>
<td>10 mg</td>
</tr>
<tr>
<td>MgSO₄7H₂O</td>
<td>0.37 g</td>
<td>ZnSO₄7H₂O</td>
<td>2 mg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>0.44 g</td>
<td>CuSO₄</td>
<td>0.025 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₃BO₄</td>
<td>3 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FeSO₄ 7H₂O (in 0.1 mM Na₂EDTA)</td>
<td>27.8 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂MoO₄</td>
<td>0.25 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CaCl₂</td>
<td>0.025 mg</td>
</tr>
</tbody>
</table>

Sucrose 5g/L, MES 1g/L, Agar 10g/L, pH was maintained 5.8 with 1 M KOH and autoclaved.

Table 3.2  The composition of nutrient solution used for watering *Arabidopsis* plants.

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>(1 Litre)</th>
<th>Micronutrients</th>
<th>(1 Litre)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>0.505 g</td>
<td>FeSO₄ 7H₂O (in 50 mM Na₂EDTA)</td>
<td>27.8 mg</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.34 g</td>
<td>MnSO₄</td>
<td>10 mg</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.492 g</td>
<td>ZnSO₄</td>
<td>2 mg</td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>0.47 g</td>
<td>CuSO₄</td>
<td>0.025 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>H₃BO₄</td>
<td>3 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KI</td>
<td>0.75 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Na₂MoO₄</td>
<td>0.25 mg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CaCl₂</td>
<td>0.025 mg</td>
</tr>
</tbody>
</table>
of tungsten lamps, whose light was filtered through water jacket, provided the irradiation with white light. Photon flux was measured with a Li-Cor quantum sensor (Li-Cor Instruments Ltd, Lincoln, NE, USA). The temperature was maintained at 25 ± 1°C. Test compounds (scavengers or inhibitors) were added to the incubation medium 10 min before the addition of ABA, and the epidermal strips were kept under the same conditions for another 3 h.

The width of the stomatal aperture was measured under a research microscope (Nikon, Eclipse TE 200, Tokyo) with the help of a precalibrated ocular micrometer. Ten apertures were monitored at random in each of three different epidermal strips, from each treatment. The experiments were repeated for 3 different days, making each measurement of stomatal aperture an average of at least 90 stomata (Kolla et al., 2007; Suhita et al., 2004).

**Monitoring NO/pH/ROS**

The changes in NO/pH/ROS was monitored in guard cells of *Pisum sativum* by probing with 4,5-diaminofluorescein diacetate (DAF-2DA), 2',7'-bis(2-carboxy-ethyl)-5(6)-carboxy fluorescein-acetoxy methyl ester (BCECF-AM), 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA) (Irving et al., 1992; Murata et al., 2001; Neill et al., 2002a).

Paradermal sections of abaxial epidermis for fluorescence studies were prepared by mounting the epidermal sections on glass cover slips with the help of medical adhesive, Telesis V (Premiere Products Inc., Pacaima, California, USA) and sections were allowed to open under light for 3 h in incubation medium. The epidermal strips were loaded with 20 µM BCECF-AM (10 min) or 20 µM DAF-2DA (10 min) in incubation medium containing 0.05% Pluronic F-127 or 20 µM H$_2$DCFDA (10 min), in dark at 25 ± 1°C. The strips were rinsed quickly with three changes of incubation medium to wash off the excessive fluorophore. The dye-loaded strips were treated with test compounds as indicated, followed by ABA after 10 min. The strips were then monitored under confocal microscope (Leica,
TCS-SP-2, AOBS 4 channel UV and visible, Heidelberg, Germany) to observe the fluorescence of DAF-2DA or BCECF-AM or H$_2$DCFDA (Excitation 488 nm, emission 510-540 nm).

In experiments involving time-course monitoring of signalling components in guard cells, the epidermal strips were examined under an inverted fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan) fitted with a monochrome high-resolution digital cooled CD camera (CoolSNAP cf, Photometrics, Roper Scientific) that enabled to capture the images with DAF-2DA or BCECF-AM or H$_2$DCFDA fluorescence (filter: Nikon B-2E/C, excitation 465-495, emission 515-555). The captured images and the relative fluorescence emission of guard cells were analysed by using NIH Image for Windows (Murata et al., 2001; Suhita et al., 2004; Kolla et al., 2007).

**Image acquisition and analysis**

The levels of the fluorescence in the images acquired through either the epifluorescence microscope or inverted fluorescence microscope was determined by using NIH Image for windows. The images were imported to the NIH software and opened as TIFF files. A square box was drawn on the image window using the cursor and the intensity of fluorescence were calculated by analyzing the pixels of the square box in the fluorescent image. The mean values of square area box were obtained by taking the pixels within the given fluorescence image window. After taking “n” different pixel intensities of the square box of the same size in the non-fluorescent area was taken as the control (background).

The pixel intensity value of fluorescent guard cells was recorded as (X) and the background of the fluorescence images as (Y). The difference of the background and area of interest was calculated and Y-X gives the actual intensity of the fluorescent image. The intensity of fluorescence was obtained, as intensity of pixels in the control/beginning of the experiment and taken as 100%. Based on the % of control the experimental analysis was done with various treatments.
Preparation of mesophyll protoplast from Arabidopsis leaves

One gram of leaf material from Arabidopsis was digested for 3-4 h with 10 ml of “digestion medium” containing (1 % (w/v) Cellulase Onuzuka R-10, 0.2% (w/v) Macerozyme R-10, 400 mM Mannitol, 8 mM CaCl$_2$, 0.25% (w/v) BSA, 10 mM sodium ascorbate, 1 mM CaCl$_2$, 10 mM MES-KOH; pH 5.6. The digestion medium was filtered and the filtrate washed twice with 10 ml of “washing medium” containing 500 mM mannitol, 5 mM MES/TRIS, pH 5.8-6.0. Protoplasts were re-suspended in 1 ml of “suspension medium” containing 400 mM mannitol, 15 mM MgCl$_2$, 5 mM MES/KOH pH 5.8. The medium was adjusted to have 0.5 to 1.0 x 10$^6$ protoplasts ml$^{-1}$. The numbers of protoplasts were counted with a haemocytometer.

Transient expression and reporter assays in mesophyll protoplasts of Arabidopsis

The transformation of protoplasts was based on the principles described by Himmelbach et al. (2002) and Yang et al. (2006), later modified by Moes et al. (2008). The following components were used: pRD29B::LUC (promoter of the desiccation-responsive gene RD29B (At5g52300) fused with luciferase, as reporter) and β-glucuronidase (GUS, fused with 35S promoter) for assessing transformation efficiency and normalization. Further, the protoplasts were transformed to express either normal (ABI1, ABI2) or mutant (abi1, abi2) forms of PP2Cs (fused with 35S promoter and GFP).

To 100 µl of protoplast suspension 10-20 µg/30 µl reporter DNA was added, followed by an equal volume of “PEG buffer” containing 40% PEG, 300 mM CaCl$_2$, 0.5 % MES-KOH, pH 5.8. The components were gently mixed by inverting 3-4 times and incubated for 3-5 min. The protoplasts were washed twice with 750 µl of washing media and re-suspended in 100-150 µl of washing media and incubated for expression at 22°C for 12 h.
To 50 µl of protoplast suspension, 100 µl of “cell lysis and GUS assay reagent” [25 mM Tris-phosphate, pH 7.8, 2 mM Dithiothreitol, 2 mM 1,2-diaminocyclohexane-N,N,N´,N´-tetraacetic acid, 10% (v/v) Glycerine, 1% Triton X100, 0.2 mM 4-Methylumbelliferyl-β-D-Glucuronid (MUG)] was added in black micro titer plate and measured GUS activity with micro plate reader (“HTS 7000 Plus Bioassay Reader”, Perkin Elmer, excitation-360 nm, emission-465 nm) for 7 min by using software program “HTSsoft”, then transferred 100 µl of the same lysate to luminometer tubes, measured the luciferase activity in luminometer (“flash’n glow”, Berthold) with the software "Berthold TubeMaster". Luminescence was measured for 10s as background and 20s for activity, after injection of “luciferase assay reagent” (20 mM Tricine/NaOH pH 7.8, 2.7 mM MgSO$_4$, 0.5 mM EDTA, 33.3 mM DTT, 0.53 mM ATP, (26 mg (MgCO$_3$)$_4$, 10 mg Coenzyme A, 7.5 mg Luciferin)/50 ml) (Moes et al., 2008).

**Seed germination and root elongation assays**

Under sterile conditions 100-150 seeds were plated on ½ MS Agar medium and incubated at 4°C for 2 d in dark to break dormancy. The plates were then transferred to culture room, with a continuous light (60 µE m$^{-2}$ s$^{-1}$) at 22°C. After 4 d, seeds were examined under a stereo microscope. Seeds were counted as germinated when the radicles emerged by 1 mm, and germination rate is calculated as percentage of the total number of seeds. For root elongation, five day old seedlings were transferred in a row to MS Agar containing different combinations of treatments and kept in a vertical position at 22°C in continuous light for 4 d. Root tip position was marked for every 24 h and root lengths were measured with the mm scale under a microscope (Moes et al., 2008).

**Replication and statistical analysis**

The data presented are the average values (± SE) of results from at least three experiments conducted on different days. Software from Sigma were used for
statistical analysis, student’s *t*-test (SigmaPlot for Windows Version 10.0) or one way ANOVA (SigmaStat for Windows Version 3.1).

**Solvents, chemicals and materials**

Abscisic acid was dissolved in 10 mM MES-KOH pH 7, while wortmannin, LY 294002, W-7, calmidazolium chloride, L-NAME, DPI, H$_2$DCFDA, BCECF-AM, DAF-2DA were dissolved in DMSO and all others in milli Q water. Most of the chemicals were from Sigma (Sigma Chemical Company, St Louis, MO, USA). Cellulase R-10, Macerozyme were from Sheishin Corporation (Tokyo, Japan), W-7 and other inhibitors were from BIOMOL (Plymouth, PA, USA). H$_2$DCFDA, DAF-2DA, L-NAME was from Calbiochem (La Jolla, CA, USA). Nylon filters were purchased from Sarayu Textiles, Mumbai. All other chemicals and materials were of analytical grade and were from following companies: Sisco Research Laboratories, E-Merk (India), Spectrochem, Loba Chemie, Himedia Laboratories and Qualigens: all from Mumbai.