(50 mg/mL in 100% dimethylformamide) (Life Technologies, Immunoselect NBT and BCIP Combo cat. no. 8280SA): Mix the following: 10 mL Tp3, 44 μL NBT, 33 μL BCIP.

10. Bead extraction buffer: 0.2*M* Tris-HCl, pH 8.0, 10 m*M* EDTA, 40 m*M* β-mercaptoethanol, 1% (w/v) PVP-40, 0.1 m*M* PMSF (store as a 100X stock at –20°C in isopropanol), 1X proteinase inhibitors, 20 m*M* ascorbate.

3. Methods

1. Prepare samples by crushing leaf material (*see* **Notes 1–4**) in 2 vol (2 mL/g tissue) of extraction buffer. The extract is transferred to a 1.5-mL Eppendorf tube and clarified by centrifugation in a microcentrifuge for 2-5 min. The supernatant (80 μL) is mixed with 20 μL 5X SDS-PAGE sample buffer, boiled for 3–5 min, and then clarified by centrifugation to remove insoluble material. Samples are electrophoretically separated by SDS-PAGE ***(2)***. Prestained mol-wt markers (we use Amersham Rainbow Markers, cat. no. RPN 755) are used to monitor the electrophoresis and the subsequent Western transfer of proteins.
2. The transfer procedure described by Towbin et al. ***(3)*** is used. The gel is equilibrated in transfer buffer while the sandwich for tank blotting is prepared (*see* **Note 5**). The transfer sandwich (we use the Bio-Rad [Hercules, CA] Mini-Protean II electrophoresis cell and Western transfer apparatus) is made in the following order, submerged under buffer: (cathode-negative electrode) plastic support sandwich holder, coarse foam pad, two layers of Whatman 3MM, protein gel, nitrocellulose or PVDF (*see* **Note 6**), two layers of Whatman 3MM, coarse foam pad, plastic support sandwich holder (anode-positive electrode). Mark the membrane with a pencil for orientation, as required. Use a glass rod or pipet to roll out air bubbles trapped in the sandwich. Take care not to allow the gel and membrane to slide out of alignment. Transfer is carried out for 0.5 h at 90 V (*see* **Note 7**).
3. Rinse membrane in 1X PBS to remove traces of transfer buffer and methanol.
4. Block the membrane in blocking solution (0.3 mL/cm^2) for 1–2 h at room temperature in a small plastic container.
5. Incubate membrane with primary antibody diluted in antibody dilution buffer (*see* **Notes 8** and **9**) for 1–2 h at room temperature.
6. Wash membrane 3 × 5 min in PBS-T.
7. Incubate membrane with secondary antibody diluted in antibody dilution buffer at 1:1000 dilution for 1 h at room temperature. We use Life Technologies goat antirabbit (cat. no. 19815-018) or goat antimouse (cat. no. 9715SA) antibodies conjugated to alkaline phosphatase.
8. Wash membrane 2 × 5 min in PBS-T.
9. Wash membrane for 2–5 min in Tp3.
10. Develop blot in 10 mL NBT/BCIP until bands appear. Wash immediately in water once desired balance of signal and background is attained. Blot membrane between Whatman 3MM filters to dry (*see* **Note 10**). The membrane is conveniently stored and can be scanned or photographed while taped between sheets of cellulose acetate overhead transparency (*see* **Note 11**).