Here we report creation of a unique and a very valuable resource for Plant Scientific community worldwide. In this era of post-genomics and modelling of multi-cellular systems using an integrative systems biology approach, better understanding of protein localization at sub-cellular, cellular and tissue levels is likely to result in better understanding of their function and role in cell and tissue dynamics, protein–protein interactions and protein regulatory networks. We have raised 94 antibodies against key Arabidopsis root proteins, using either small peptides or recombinant proteins. The success rate with the peptide antibodies was very low. We show that affinity purification of antibodies massively improved the detection rate. Of 70 protein antibodies, 38 (55%) antibodies could detect a signal with high confidence and 22 of these antibodies are of immunocytochemistry grade. The targets include key proteins involved in hormone synthesis, transport and perception, membrane trafficking related proteins and several sub cellular marker proteins. These antibodies are available from the Nottingham Arabidopsis Stock Centre.
Results and discussion

Antibody pipeline. The overall pipeline of antibody production is summarized in Fig. 1. It involved target selection, bioinformatic analysis of the target protein, identification of the antigenic regions within the protein, and probability analysis of chances of cross reactivity of the antigenic regions against non-target proteins. This was followed by cloning of the target region, antibody production and purification, quality control and validation.

Target selection. The key root–protein targets were selected based on their role in root development, as judged by either root related developmental phenotype(s) or the importance of a given pathway in root development. The emphasis has been on plant hormones due to their importance in regulating several aspects of plant growth and development, including primary and lateral root development. Thus, some targets included proteins involved in plant hormone biosynthesis, transport and signaling. In addition, we have also raised antibodies against key cell-wall and cytoskeleton-related proteins, and 4 popular subcellular marker proteins BIP (endoplasmic reticulum), γ-cop (golgi), PM-ATPase (plasma membrane), and MDH (plastid) to facilitate colocalization studies. A complete list of all the target proteins used for antibody production can be seen in Table 1 and Supplementary Tables S1 and S2.

Peptide v native or recombinant protein approach. There are two common approaches for antibody production, where animals are immunised against: (1) complete native protein or some parts thereof, or (2) small approximately 12–15 amino acid synthetic peptide (conjugated to an inert carrier protein)20. In addition, in a slight variation of the latter approach, short (3–5 amino acids) C-terminal peptides have also been used successfully21,22. Despite their small size, Edwards et al.23 discovered that antibodies raised against these peptides were highly specific to their target protein and did not cross react with the similar internal sequences, suggesting that C-terminal peptides may have a specific structure that can be exploited for antibody production.

The advantages of the peptide approach are that it is simple, convenient, and less likely to show non-specific cross reactivity. In comparison, the native or recombinant protein approach is more time consuming but increases the chances of a good immune response, due to the increased diversity and number of available epitopes.

Peptide antibodies. Because of its simplicity and less chance of non-specific cross reactivity, both standard peptide and short C terminal peptide approaches were tried initially. Surprisingly, this did not work very well in our hands with a very poor success rate. Even after affinity purification against the peptide, the detection rate remained very low and only one out of 24 antibodies worked satisfactorily (Supplementary Table S1). The only antiserum that worked well was affinity purified LAX2 (LIKE AUX1-2). It appears to be very specific as it detected a strong signal in the root apex in wild type Columbia roots but not in null lax2 mutants (Fig. 2).

For the 23 remaining antibodies, it is difficult to pinpoint why the success rate was so low, but one main reason could be the epitope prediction. The prediction methods identify individual stretches of amino acids (continuous epitopes), whereas epitopes are very often discontinuous, involving distant subsequences brought together by the protein’s tertiary structures24. However, prediction methods for the latter are not well developed and have met with little success. Also, a synthetic continuous (or even discontinuous epitope) peptide may still not fold correctly and hence not generate antibodies that recognize the native protein structure25.

Because of the low success rate of anti-peptide antibodies (from three different companies), this approach was abandoned, and efforts were turned to the recombinant–protein approach.

Recombinant-protein antibodies. 70 antibodies were raised against Arabidopsis root proteins using the recombinant protein approach (Supplementary Table S2). Bioinformatic analysis was used to identify potential antigenic regions and then the largest antigenic subsequence was checked for potential cross-reactivity by database searches using blastX24 (Fig. 3). A cut off of 40% similarity score (at amino acid level) was used as a guide to accept a given antigenic region for antibody production. In cases where blast results exceeded the cut off, we either chose another antigenic region or used a sliding window to obtain a smaller region that showed less than 40% sequence similarity. However, in cases of multi-gene families where it was not possible to obtain a reasonably large (~100 amino-acid) unique sequence, a more generic family-specific antibody was raised. Where possible, antibody cross reactivity was tested in the corresponding mutant backgrounds by either western immune detection and/or in situ localization.

Initial quality control using dot blots against the recombinant protein revealed that most crude antisera could detect the target proteins in the picogram range, indicating a good titre (Supplementary Fig. S2). However, most of the crude antibodies did not show any signal when tested by in situ immunolocalization, the exceptions were PIN1, PIN2, PIN3, PIN4, PIN7 and PM-ATPase. Generic purification methods such as Caprylic acid precipitation26, Protein A or Protein G purification28 and signal amplification methods29 did not improve
the detection rate. Whereas affinity purification with the purified recombinant protein (Supplementary Fig. S3) resulted in significant improvement in detection rate: 38 (55%) antibodies could detect a signal with high confidence either by in situ immunolocalization (22 out of 38) or Westerns (20 out of 32 tested) or both (Figs. 4, 5, 6, 7).

A complete list of useful antibodies is given in Table 1. As can be seen, successful targets include several key proteins involved in hormone synthesis, transport and perception (LAX2, PIN proteins, AXR1, TIR1, GA3, GAI, RGA, GID, SLY1, ACO1), membrane trafficking related proteins (AXR4, GNOM, AtSYP21, AtSYP41) and other important root proteins including SHR, RSW1, RSW3 and WXR3. In several cases, the validity of the signal was checked against the respective mutant backgrounds by in situ immunolocalization (Fig. 5) or by detecting a single band, usually of the expected size, on Western blots (Fig. 6 and Supplementary Fig. S4). As evident in Fig. 5 (and also Fig. 2; Supplementary Fig. S3), all the antibodies that were checked against their mutant background for cross reactivity by in situ immunolocalization gave no detectable signal in the mutants except for anti-PIN3 where a faint signal was detected. This suggests that the bioinformatics approach that we used for our antibody pipeline is a robust approach and can be used as a guide for other antibody projects.

Figure 1. CPIB antibody pipeline. Targets for antibody production were identified and highly antigenic regions were determined by bioinformatics analysis. The proteins were then expressed in *E. coli*, purified by affinity chromatography and used for immunisation. Antibodies were then checked by dot blots, Westerns and in situ immuno-localisation and affinity purified if necessary.
Despite significant improvement in detection rates, several antisera still could not detect a signal by in situ immunolocalization. In some cases, this could be attributed to poor immune response in animals as the quality of the affinity purification was not very good, resulting in low levels of the IgG. But in other cases, despite good quality of affinity purification, no signal was detected. We cannot rule out the possibility that the target proteins are low abundant and hence are below the limits of our in-situ detection. Similarly, for westerns, in some cases, we did not detect a correct size band (Fig. 6; Supplementary Fig. S4). It is likely that some of this can be attributed to degradation or post-translational modifications or for some membrane proteins could be attributed to poor migration on the gel due to hydrophobic nature of these proteins. For most other proteins, we did detect a single correct size band (Fig. 6; Supplementary Fig. S4). Like in situ immunolocalisation (Figs. 2, 5, Supplementary Fig. S3), validation for a few antibodies (AXR4, ACO2, AtBAP31 and ARF19) have also been validated by westerns against their respective mutant backgrounds (data not shown). We envisage that with the help of the community, as more researchers use this resource, other antibodies will also be validated, and this information will be constantly reviewed and updated on Arabidopsis Stock Centre pages.

**Table 1.** List of successful antibodies raised in CPIB antibody project. *Single correct size bands are indicated as ‘OK’. Where bands do not match the correct size or there are multiple bands, approximate band sizes (kDa) are indicated. NT not tested. *Peptide antibody.
α-AtBIM1/AtbHLH046 (nucleus), α-CATALASE (peroxisome) and α-GNOM (endosome) because of their high expression in almost all cell files in the roots (Fig. 7).

In conclusion, we have raised 94 antibodies against key root-proteins using either small peptides (up to 15 amino acids) or recombinant proteins. Thirty-eight of these antibodies appear to be of good quality and 22 are of immunocytochemistry grade. CPIB antibodies form an extremely valuable communal resource for plant scientific community worldwide and will be available from Nottingham Arabidopsis Stock Centre (NASC).

Materials and methods
Cloning, expression and purification. The target sequences for antibody production were chosen based on antigenicity plots (DNASTAR) and blastX24. The target sequences were PCR amplified from a 5-day-old root-cDNA library, using gene specific primers and cloned into linearized pENTR/Directional-TOPO vector (Thermofisher Scientific) as per manufacturer’s instructions. Positive clones were identified by colony PCR and further confirmed by sequencing. These entry vectors were then recombined into the gateway destination vector pDEST17 to create N terminal translation fusions with a 6×Histidine tag (6xHis) as per manufacturer’s instructions. Positive colonies were identified by colony PCR and plasmid DNA was further validated by PCR and restriction digestion. Finally, these recombinant plasmids were transformed into E. coli expression strains Rosetta or BL21-AI (Thermofisher Scientific).

Three or four colonies were initially tested in a small-scale expression trial. For BL21-AI, small-scale induction trials were run as per manufacturer’s instructions. In short, overnight cultures were used to inoculate (1:100 dilution) fresh growth media (Luria broth) containing appropriate antibiotics and allowed them to grow to an OD_{600} of about 0.4. The expression of the target protein was induced by arabinose (0.2%) and 0.5 ml samples were withdrawn at 0, 2, 4 and 24 h after induction. Samples were centrifuged and the pellet was heated in sample buffer as above and subjected to SDS–PAGE. For Rosetta, the auto-induction method was used and plated cells were heated in sample buffer as above and subjected to SDS–PAGE.

For large scale protein production, single colonies were grown for 16 h at 37 °C in 3 ml LB. 1 ml of this culture was then used to inoculate 400 ml of auto induction medium and grown for 16 h at 37 °C. The cells were harvested at 6000 rpm for 5 min and were resuspended in 10 ml of Binding Buffer (8 M Urea/40 mM Sodium Phosphate Buffer, pH 7.4/0.3 M NaCl/0 mM Imidazole), then solubilized for 1 h at room temperature with gentle shaking. The lysate was sonicated for 30 min in ice using a water-bath sonicator and centrifuged at 8000 rpm for 45 min. The supernatant was then passed through 0.45 μm sterile filter and purified straightaway using the AKTAexpress protein-purification system (GE Healthcare, UK).

Figure 2. C-terminal antipeptide LAX2 antibody detects strong signal in wildtype Columbia (but not lax2 mutant) roots upon immunolocalization. Affinity purified LAX2 anti peptide antibody was used for in situ immunodetection of LAX2 (green) in 4-day old wild type Columbia or lax2 mutant roots. Primary and Alexafluor 488 coupled secondary antibodies were used at 1:200 dilutions. Seedlings were counter stained using propidium iodide (red). Scale bar 20 μm.
A two-column purification strategy was used that combined affinity purification (HisTrap nickel columns-GE Healthcare, UK) with desalting. A typical purification protocol involved equilibration of the HisTrap column with 2 column volumes of binding buffer; passage of the cleared lysate through the equilibrated column; washing of the column with five column volumes of binding solution; followed by elution of the recombinant His-tagged protein using a 20–500 mM continuous imidazole gradient. The machine was programmed to pass the largest peak on to a 2 × Sephadex G25 column assembly and 2 ml fractions were collected through a built-in fraction collector. Peak protein fractions were then used for protein assay by the Bradford method, and the extent of purification was subsequently checked by SDS-PAGE.

**Antibody production.** One milligram of protein was sent to Scottish National Blood Transfusion Services (Edinburgh, UK) for immunization, which consisted of a primary injection and two booster injections 4 weeks apart, with test bleeds taken at 4, 8 and 12 weeks after the initial injection.

**Affinity purification of antibody.** One ml of Sulfo-Link Coupling Resin (Thermo Scientific, UK) was placed into a disposable 5 ml polypropylene column (Thermo Scientific, UK) and equilibrated with 5 ml of coupling buffer (50 mM Tris–HCl, pH 8.5/5 mM EDTA). Five hundred micrograms of the recombinant protein were added to the column and incubated with rotation for 30 min at room temperature. The column was placed upright for 30 min without mixing, followed by washing with 3 ml of coupling buffer. Two mls of 50 mM of Cysteine in coupling buffer was added to the column and rotated for 15 min at room temperature, followed by washing...
washing with 6 ml of 1 M NaCl. The column was washed with 20 ml of 10 mM Tris–HCl, pH7.5/0.5 M NaCl, 10 ml of 100 mM Glycine (pH2.5), and 20 ml of 10 mM Tris–HCl, pH7.5. Fifty ml of 1 M Tris–HCl, pH7.5 were added to 5 ml of crude antiserum and filtered through with 0.45 µm filter. The buffered antiserum was added to the column and incubated with rotation overnight at 4 °C. On the next day, the antiserum was allowed to drain out, and flow-through was passed over the column twice at room temperature. The column was washed with 20 ml Tris–HCl, pH7.5 and 10 ml of 10 mM Tris–HCl (pH7.5)/0.5 M NaCl. Two-hundred and fifty ml of antibody were collected into 1.5 ml Eppendorf tubes containing 50 µl of 1 M Tris–HCl, pH8.0 with 2 ml of 100 mM Glycine, pH2.5. Each fraction was used for measurement of protein concentration and SDS-PAGE. Fractions were stored at − 80 °C.

Dot blot. As a measure of the antibody titre, antisera were tested on dot blots. These were prepared by spotting 10 ng to 100 pg of expressed recombinant proteins on nitrocellulose or PVDF membranes which were used for western immunodetection. In short: blocking (5% Non-fat milk-1 h); primary antibody (1:200 dilution-1 h); washing (3 × 5 min in TBST (Tris buffered saline; 0.1% Tween20); secondary antibody (alkaline phosphatase conjugate—1:5000-1 h); washing ((3 × 5 min in TBST) and detection using NBT/BCIP substrate solution in 0.1 M Tris–HCl buffer pH 9.5 containing 0.1 M NaCl and 0.05 M MgCl₂.

Plant protein extraction. Five to 7 day old Arabidopsis seedlings or 4 week old root cultures were ground using liquid nitrogen and homogenized in homogenization buffer (50 mM HEPES, pH7.5/0.5 M sucrose/0.1% sodium ascorbate/1 mM DTT/0.5% polyvinyl polpyrolidone, insoluble/protease inhibitor), centrifuged and the crude extracts were used for protein estimation by the Bradford method²⁹. For membrane proteins, microsomal fractions were prepared as described previously³⁰.
Western immunodetection. Proteins (25 µg) were separated by SDS-PAGE and transferred to PVDF membrane using Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad). These membranes were probed as described above for dot blots with some modification. Primary antibodies were normally used at a dilution of 1:200–1:5000 (37°C 12–16 h) whereas secondary antibodies were routinely used at a dilution of 1:5000 (37°C 2–3 h).

In situ immunolocalization. This was carried out on three to 4-day old Arabidopsis roots as described previously. Crude or affinity-purified antisera were used at 1:50–1:400 dilutions (37°C 5 h) whereas second-
ary antibodies were used normally at 1:200 dilutions (37°C 5 h). The images were captured using Leica SP2 confocal laser scanning microscope (Leica Microsystems UK Ltd).

Ethical statements. As the animals were involved in the antibody production through several companies, we have enquired with the companies and we can confirm that (1) all experimental protocols were approved by a named institutional and/or licensing committee/s. (IACUC committee; PTU/BS has a project license under the Animals (Scientific Procedures) Act 1986, which permits contract immunisation of animals and (2) all methods were carried out in accordance with relevant guidelines and regulations (USDA guidelines; PTU/BS work is carried out in compliance with the requirements of ISO9001).

Figure 6. Typically, CPIB antibodies show single correct size band upon western immunodetection. Twenty-five microgram of Arabidopsis total proteins were separated by SDS-PAGE and transferred to PVDF membranes. The blots were then used for Western immunodetection of target proteins using affinity-purified primary antibodies and HRP conjugated secondary antibodies. Marker sizes are indicated on the left of the bands whereas expected band sizes are indicated below the images.
Figure 7. CPIB antibody project has raised antibodies against several popular sub-cellular markers: BiP (ER), γ-cop (golgi), PM-ATPase (PM) and malate dehydrogenase (plastid). Antibodies were raised against popular sub cellular marker proteins (A–H) and other root proteins (AXR4 (ER), BIM1 (nucleus), catalase (peroxisome) and GNOM (endosome) that potentially can be used as subcellular markers (I–P). The antibodies were used for immunodetection of the targets (green) in 4-day old wild type Columbia roots. Primary and Alexafluor 488 coupled secondary antibodies were used at 1:200 dilutions. Seedlings were counter stained using propidium iodide (red). Scale bar 20 μm.
Data availability

All the antibodies will be available from Nottingham Arabidopsis Stock Centre (NASC).

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Author contributions
J.O., M.W., N.L. and R.S. performed experiments and contributed experimental data; J.O., M.J.B. and R.S. designed experiments; J.O., C.H. and R.S. wrote and edited the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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