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RESEARCH ARTICLE

Systematic expression analysis of cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 protein (CAP) superfamily in *Arabidopsis*

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Abstract

The Cysteine-rich secretory proteins (CRISPS), Antigen 5 (Ag5), and Pathogenesis-related 1 (PR-1) protein (CAP) superfamily members are found in multiple eukaryotic organisms, including yeasts, animals, and plants. Although one of the plant CAP family genes, *PR-1* is known to respond to pathogen infection in plants, the functions of other CAP family genes in *Arabidopsis* remain largely unknown. In this study, we conducted a comprehensive analysis of the similarities, loci, and expression patterns of 22 Arabidopsis CAP genes/proteins, providing a clue to elucidate their molecular functions. According to the promoter-β-glucuronidase (GUS) analysis, members of the *Arabidopsis* CAP family were expressed in various young tissues or organs, such as root and shoot meristems, reproductive tissues, and particularly at the lateral root initiation site before the formation of the lateral root primordium, with distinct expression specificity. In particular, *CAP51*, *CAP52*, and *CAP53* were specifically expressed in the cortical cells at the lateral root developing regions, suggesting that these genes may function in lateral root development. Thus, the expression patterns of Arabidopsis CAP family genes suggest that CAP family proteins may have certain function in the expressed organs or tissues in *Arabidopsis* plant.

KEYWORDS
antigen 5 (Ag5), *Arabidopsis*, cysteine-rich secretory proteins (CRISPS), gene expression pattern, pathogenesis-related 1 (PR-1)

1 | INTRODUCTION

The cysteine-rich secretory proteins (CRISPS), antigen 5 (Ag5), and pathogenesis-related 1 (PR-1) protein (CAP) superfamily members are widely distributed across the kingdoms of life, including bacteria, fungi, plants, and animals. The CAP superfamily proteins, comprising more than 3400 members from 1189 species, are defined by the presence of four conserved motifs of CAP1, CAP2, CAP3, and CAP4 domains, although the other sequences vary among species. Most members of CAP family proteins have the N-terminal secretion signal sequence (Gaikwad et al., 2020; Gibbs et al., 2008).

From the protein structure and the localization data of CAP family proteins, several functions of the family proteins have been suggested in some organisms. For example, the CAP proteins found as an excretory/secretory (ES) product of helminths and known as venom allergen-like proteins (VALs or VAPs) are specifically upregulated during their infection, suggesting they have a suppressor function of the host immune response (Wilbers et al., 2018). In mammals, CAP

proteins are expressed in the reproductive organs, in immune cells and tumors, or during embryogenesis, suggesting a function in autocrine or paracrine signaling in mammals (Choudhary & Schneiter, 2012; Gibbs et al., 2008).

In plants, the plant-type CAP family proteins, PR-1 have been reported. For example, overexpression of the PR-1 homolog enhanced plant resistance to heavy metals and pathogen stress in tobacco (Sarowar et al., 2005). The C-terminal 11-amino acid peptide fragment (CAPE) derived from tomato PR1b increases resistance to the bacterial pathogen *Pseudomonas syringae* pv. Tomato DC3000 and reduces larval growth of *Spodoptera litura* (Chen et al., 2014), and a C-terminal conserved 11-amino-acid peptide (AtCAPE1) derived from the salinity-upregulated gene PROAtCAPE1 (At4g33730) negatively regulates salt stress tolerance in *Arabidopsis* (Chien et al., 2015). In a model plant, *Arabidopsis thaliana*, a member of the CAP proteins is known as AtPR-1 (At2g14610.1), which is one of the pathogenesis-related proteins (PRs) classified into 22 family genes (Van Loon et al., 2006). AtPR-1 is highly expressed by pathogens, insects, and chemical treatments in pathogen-infected leaves and accounted for 10% of the total protein (Cornelissen et al., 1986) and was reported to have antifungal and antibacterial activities (Lincoln et al., 2018; Niderman et al., 1995; Rauscher et al., 1999). Thus, while the functions of a few members of CAP family proteins or genes have been reported in plants, the localization and functional analysis of most *Arabidopsis* CAP protein members remain poorly characterized.

In this study, as a first step toward understanding the molecular function of CAP family genes/proteins in *Arabidopsis*, we systematically renamed 22 *Arabidopsis* CAP genes/proteins based on their phylogenetic relationships and comprehensively investigated their expression patterns under normal growth conditions using a promoter: β-glucuronidase (GUS) reporter system.

2 | RESULTS

2.1 | Systematic nomenclature of 22 CAP family proteins of *A. thaliana*

Although the phylogenetic relationships and structural features of several predicted *Arabidopsis* PR-1 type CAP family proteins have been reported previously (Van Loon et al., 2006), they have not yet been systematically named. Therefore, based on the previously proposed phylogenetic relationship of *Arabidopsis* CAP family genes (Van Loon et al., 2006), we systematically named the Arabidopsis CAP family proteins as follows: CAP11 (At1g01310.1), CAP12 (At3g09590.1), CAP13 (At5g02730.1), CAP21 (At4g31470.1), CAP22/AtCAPE2 (At4g25780.1), CAP23/AtCAPE6 (At4g30320.1), CAP24/AtCAPE4 (At4g25790.1), CAP25/AtCAPE5 (At5g57625.1), CAP31 (At4g33710.1), CAP32/AtCAPE8 (At5g26130.1), CAP33 (At1g50050.2), CAP34 (At1g50060.1), CAP35/AtCAPE7/basic PR-1 (PRB1) (At2g14580.1), CAP36/AtCAPE9/PR1 (At2g14610.1), CAP37/AtCAPE3 (At4g33720.1), CAP38 (At3g19690.1), and CAP39 (At4g07820.1), CAP41/AtCAPE1 (At4g33730.1), CAP51/PR-1 homolog (PRH1) (At2g19990.1), CAP52 (At2g19970.1), CAP53 (At2g19980.1), and CAP61 (At5g66590.1) (Figure 1 and Table S1). We confirmed that all *Arabidopsis* CAP proteins possess 20–30 residues of the secretion signal sequence at the *N*-terminus and four conserved domains, CAP1; [G/D/E/R] [H/R] [F/Y/W] [T/V/S] [Q/A] [L/I/V/M] [L/I/V/M/A] W x x [S/T/N], CAP2; [L/I/V/M/F/Y/H] [L/I/V/M/F/Y] x C [N/Q/R/H/S] Y x [P/A/R/H] x [G/L] N [L/I/V/M/F/Y/W/D/N], CAP3; H N x x R, and CAP4; G [E/Q] N [I/L/V] (Van Loon et al., 2006) (Figure 1).

The CAP family genes are widely distributed across all *Arabidopsis* chromosomes and several CAP genes form gene clusters: *CAP33* (At1g50050) and *CAP34* (At1g50060) on chromosome 1; *CAP51* (At2g19990), *CAP52* (At2g19979), and *CAP53* (At2g19980) on chromosome 2; *CAP22* (At4g25780) and *CAP24* (At4g25790) on chromosome 4; and *CAP31* (At4g33710), *CAP37* (At4g33720), and *CAP41* (At4g33730) on chromosome 4 (Figure 2).

Cis-element analysis in the *Arabidopsis* CAP genes revealed that six different cis-element motifs, such as "Auxin responsive element (AuxRE)," "Myc transcription factors recognition motif (MYC)," "Myb transcription factors binding motif (MYB)," "GT-1 cis-acting element responsive to pathogen and salt stress (GT-1)," "low temperature responsive element (LTRE)," and "TGACG responsive motif responsive to methyl jasmonate (TGACG-motif)," exist within the 2.0 kbp upstream regions of CAP family genes (Figure 2). According to the comprehensive *cis*-element analysis of *Arabidopsis* promoter regions (Maruyama et al., 2012), there is no clear enrichment of the above *cis*-elements related to the various stress or phytohormone responses within the 2-kbp upstream region of the CAP family genes (Figure S1).

2.2 | Analysis of promoter activity of CAP genes of *A. thaliana*

Next, we generated the *Arabidopsis* transcriptional reporter lines expressing a β-glucuronidase (GUS)-mNeonGreen fusion protein under the control of approximately 2.5-kbp 5′-upstream regions of 22 *Arabidopsis* CAP genes to study the promoter activities of 22 CAP genes in different organs or tissues. These *Arabidopsis* promoter-reporter lines allowed monitoring of the expression profiles of the CAP genes at the tissue level by GUS staining and at the cellular level spatiotemporally by fluorescence live imaging.

2.3 | Detection of the GUS activity in the root tip

In the root tip, the GUS activity of *CAP11*, *CAP21*, *CAP33*, *CAP34*, *CAP52*, and *CAP61* was detected in the cells of the meristematic zone; that of *CAP11*, *CAP24*, *CAP33*, *CAP34*, and *CAP52* was detected in the columella; and that of *CAP33*, *CAP34*, and *CAP52* was detected in the lateral root cap (Figure 3a,c,e). In the mature root region, the GUS activity of *CAP23*, *CAP24*, *CAP25*, and *CAP41* was detected in epidermis, especially in root hair, whereas the GUS activity of *CAP21*, *CAP22*, *CAP31*, and *CAP61* was detected in endodermis (Figure 3a,c,e).

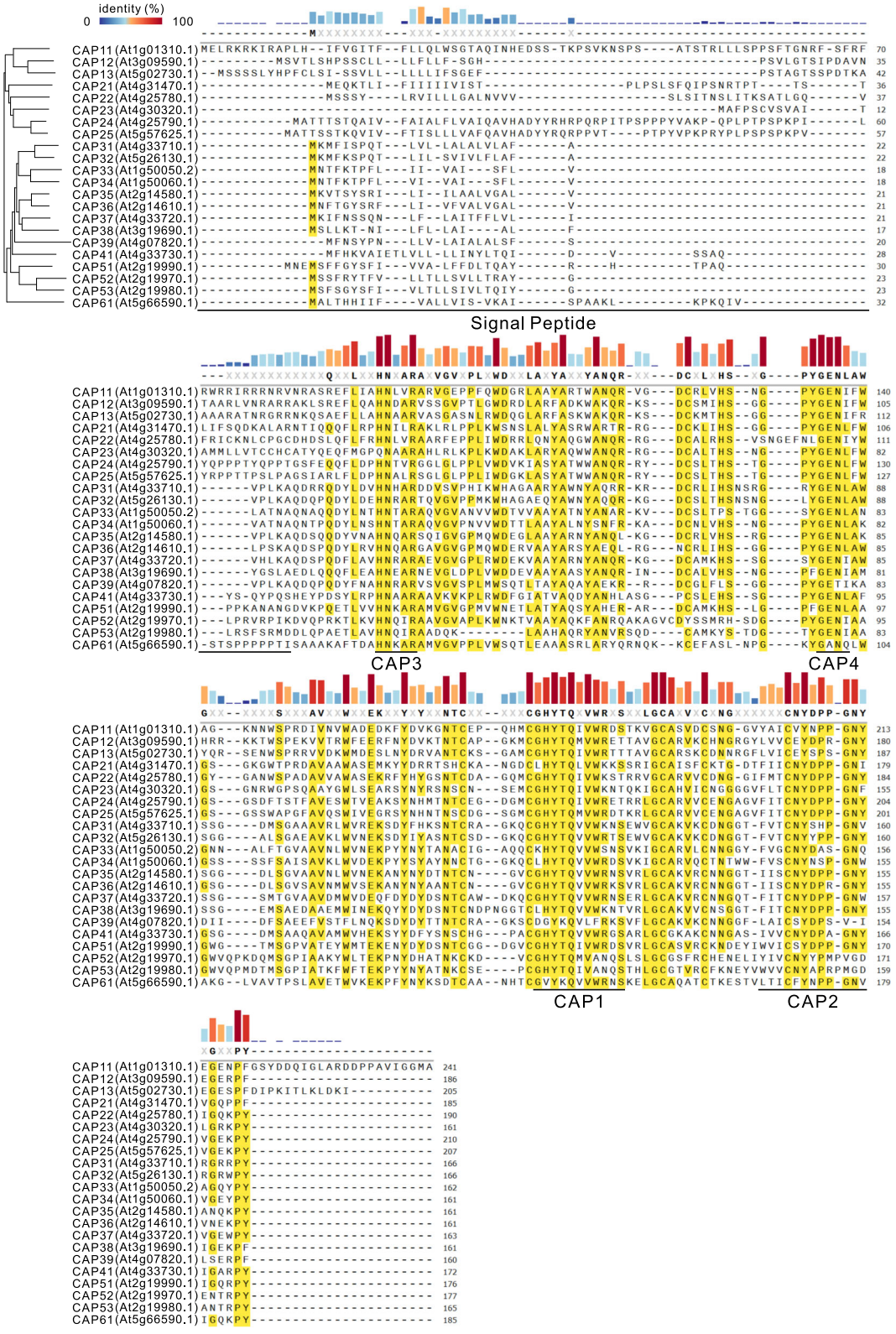


FIGURE 1 Phylogenetic tree and full-length alignment of the 22 *Arabidopsis* CAP protein sequences. The 50% more conserved residues are highlighted in a yellow box. The amino acid identity and conserved residues between the 22 CAP proteins are shown by the bar graph and the amino acid above the alignment. The signal peptide and conserved CAP motifs (CAP1–4) are underlined below the alignment.

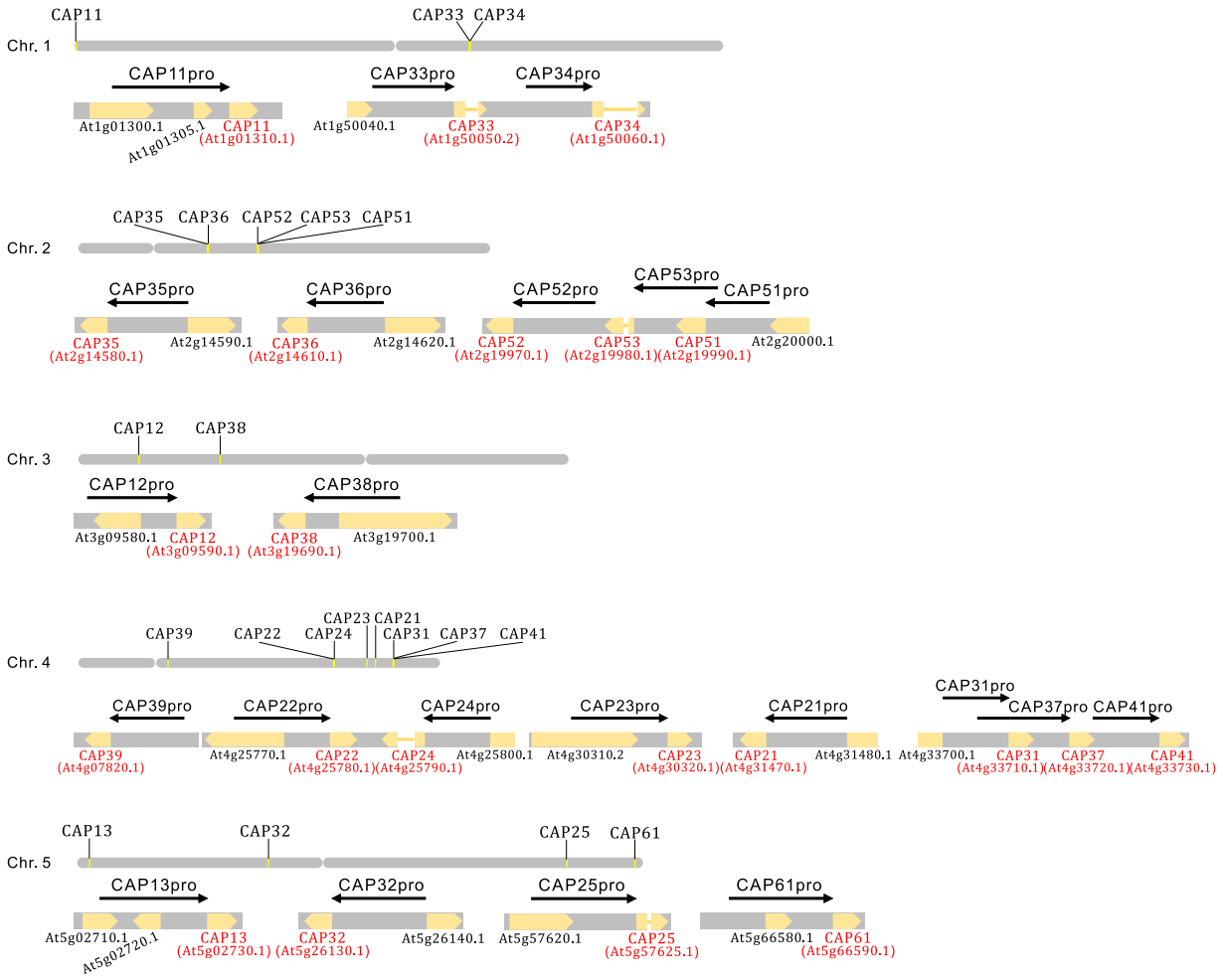


FIGURE 2 Chromosomal distribution of 22 Arabidopsis CAP genes (black; gene name) is indicated on the chromosome (gray horizontal bars). The genomic position and orientation of the CAP genes (red; gene name and AGI code) are indicated by the pale yellow arrow lines on the genome (gray horizontal bars), and their introns are indicated by pale yellow thin lines. The promoter region is indicated by the black arrows.

2.4 | Detection of the GUS activity in leaf and shoot

In the shoot apex during the vegetative growth phase, strong GUS activity of *CAP11*, *CAP31*, *CAP33*, *CAP34*, *CAP35*, *CAP36*, *CAP39*, and *CAP41* was detected at the position of the stipules, whereas the GUS activity of *CAP23*, *CAP24*, *CAP25*, *CAP51*, and *CAP52* was slightly detected in the same region (Figure 3a,c,e). The GUS activity of *CAP22*, *CAP31*, and *CAP41* was detected in the veins of young rosette leaves, while the GUS activity of *CAP61* was detected in the mesophyll of rosette leaves and cotyledons (Figure 3a,c,e). In contrast, the GUS activity of *CAP53* was observed only in the guard cells of rosette leaves and cotyledons (Figure 3e and Figure S2).

2.5 | Detection of the GUS activity in flower organs

In flower organs, *CAP11*, *CAP22*, *CAP38*, *CAP53*, and *CAP35* were slightly expressed in mature pollen and anthers, while *CAP21* and *CAP31* were slightly expressed only in the pollen grains. The expression of *CAP12* was observed in pollen tubes; *CAP31*, *CAP51*, and *CAP52* were expressed at the receptacle, while *CAP61* was expressed in the stamen filament, style, and sepal (Figure 3b,d,f).

2.6 | Expression profile of CAP genes in lateral roots

The lateral roots that emerge from the pericycle cell layer penetrate the endodermal, cortical, and epidermal tissues during emergence in *Arabidopsis* (Péret et al., 2009). *CAP51/PRH1* has been reported to be expressed in the cortical cells overlying the developing lateral root primordia at early stages of lateral root development, suggesting it is likely involved in the lateral root development under the control of AUXIN RESPONSE FACTOR7 (ARF7)-mediated auxin signaling (Zhang et al., 2020).

Like *CAP51/PRH1*, the GUS activity of *CAP52* and *CAP53* was observed at the lateral root initiation site (Figure 3e), although the GUS expression of *CAP52* was also observed in the ovules (Figure 3f).

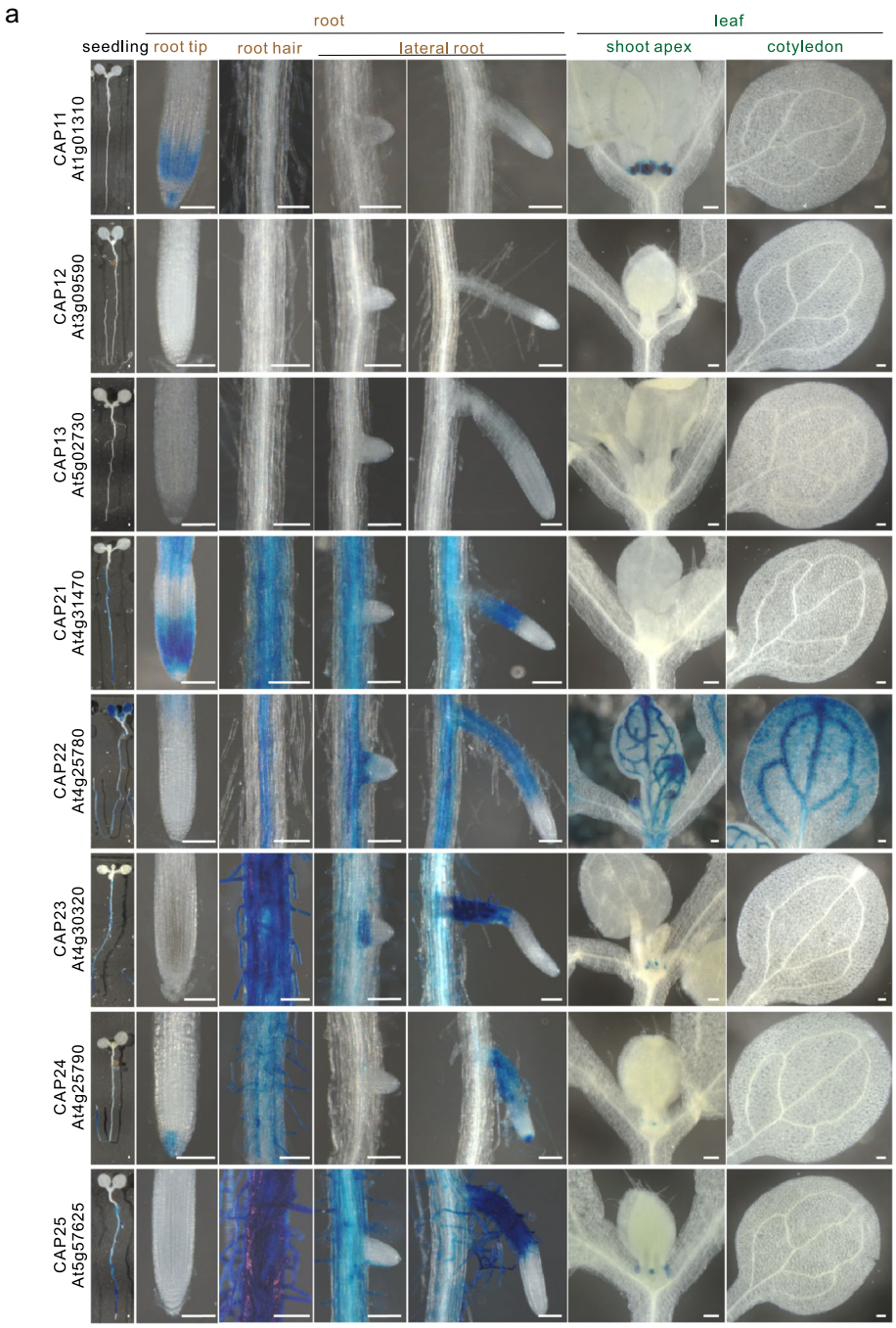


FIGURE 3 GUS staining of the transgenic lines expressing *GUS-mNeonGreen* under the promoter of each CAP gene (*pCAP::GUS-mNeonGreen*) in 9-day-old seedling, root tip, root hair, lateral root, shoot apex, and cotyledon (a,c,e), sepal, receptacle, petal, stamen, stigma, and silique (b,d,f). Bars = 100 μm.

Furthermore, we monitored the time course of the expression of *CAP51/PRH1*, *CAP52*, and *CAP53* using the mNeonGreen fluorescence during lateral root formation. The mNeonGreen fluorescence was observed using confocal laser microscopy every 2 h from 20 to 44 h after the induction of lateral root formation via the gravistimulation of roots. We confirmed that *CAP51*, *CAP52*, and *CAP53* were expressed in the cortex cells bordering the lateral root primordial cells. Notably, the fluorescence of *CAP51* and *CAP52* seemed to appear at the cortical tissues before the formation of the lateral root primordium (Figure 4a,b and Movies S1 and S2), whereas that of the *CAP53* line seemed to appear after the emergence of the lateral root primordium (Figure 4c and Movie S3).

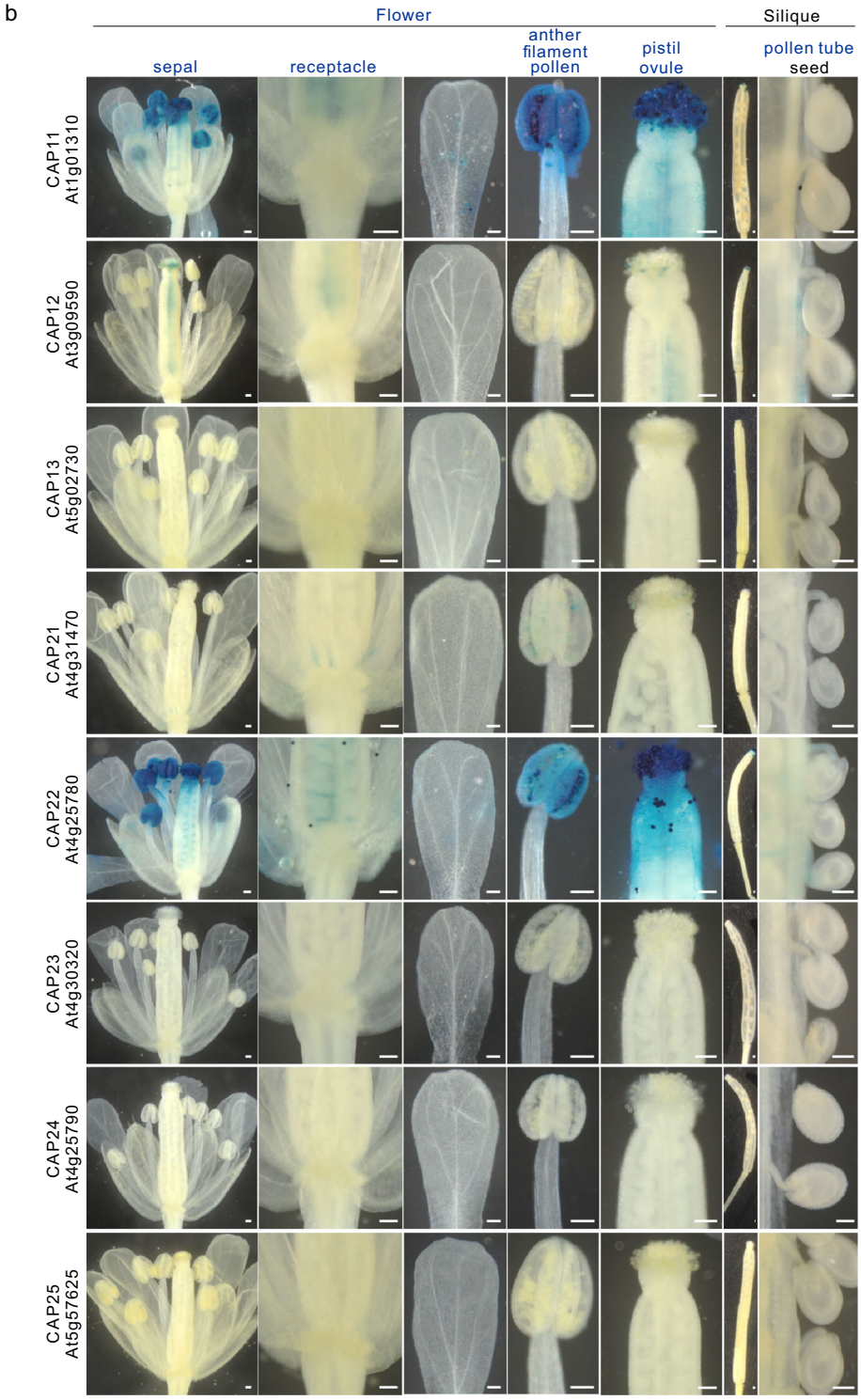


FIGURE 3 (Continued)

2.7 | Expression analysis of CAP genes from the publicly available data

We compared our promoter GUS analysis with the expression data from publicly available transcriptome data (Figure S3) or the e-Northerns Expression Browser database (Toufighi et al., 2005) (Figure S4). In root, the strong expression of *CAP34* and *CAP61* or *CAP34* and *CAP21* was shown based on publicly available transcriptome data (De Luis Balaguer et al., 2017; Krishnamurthy et al., 2018) or the e-Northerns Expression Browser database (Figure S4). In lateral

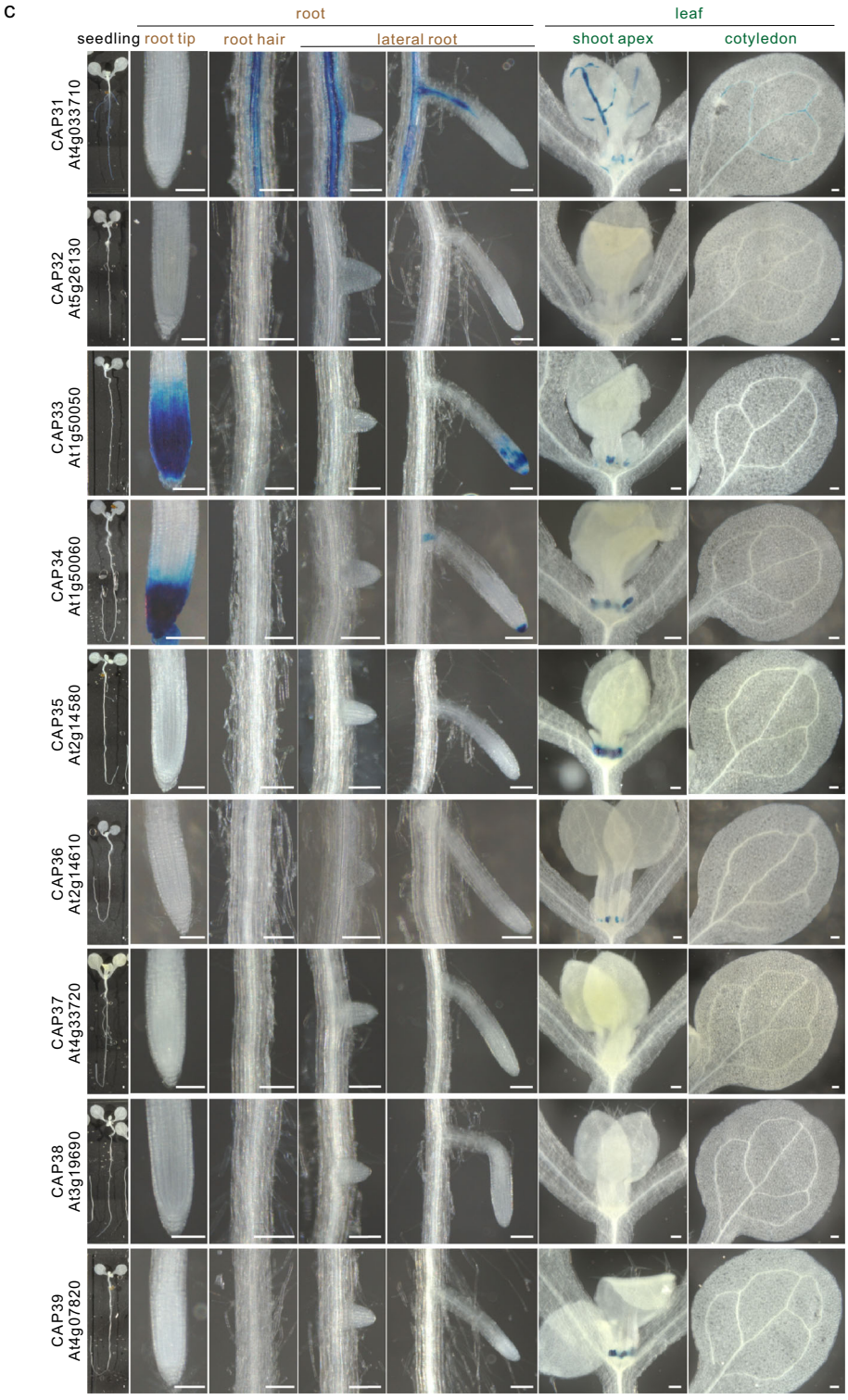
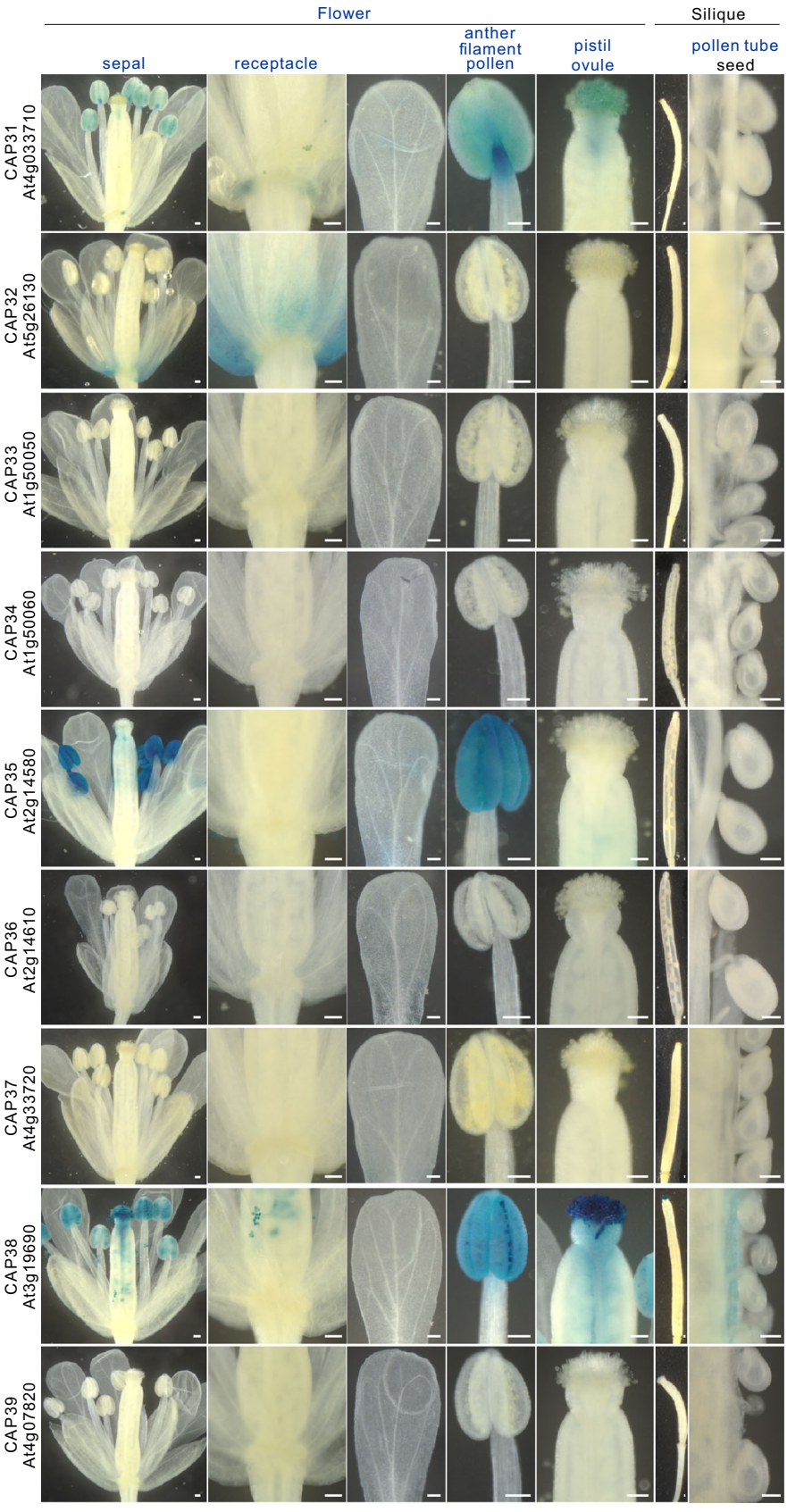


FIGURE 3 (Continued)

root, the strong expression of *CAP51* and *CAP52* was shown based on publicly available transcriptome data (Bellande et al., 2022; Hurný et al., 2020; Serrano-Ron et al., 2021). In shoot apical meristem, the strong expression of *CAP11*, *CAP12*, *CAP35*, and *CAP61* or *CAP22* was shown based on publicly available transcriptome data (Mandel et al., 2016) (Figure S3) or the e-Northerns Expression Browser (Figure S4). Collectively, our promoter GUS analysis results were consistent with these organ-level transcriptome data.



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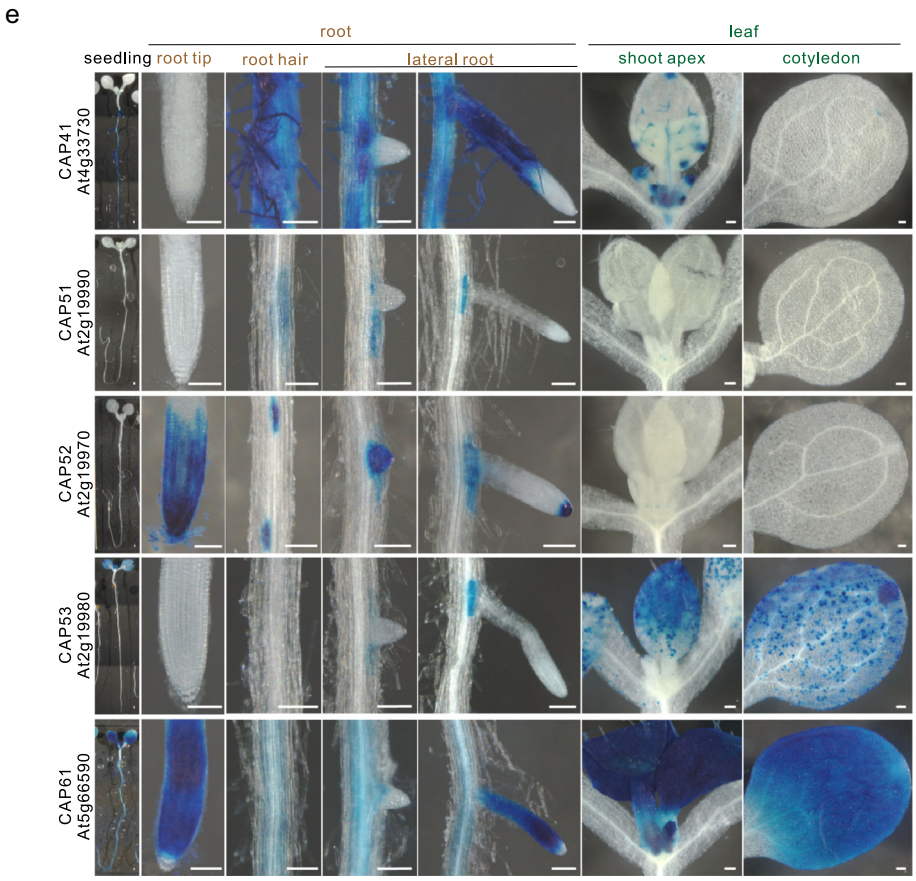


FIGURE 3 (Continued)

3 | DISCUSSION

We investigated the tissue- and cell-specific expression of all *Arabidopsis* CAP family genes using the promoter-reporter analysis using GUS-mNeonGreen fusion reporter protein and found that CAP family genes were expressed in various tissues and organs with different expression patterns, suggesting that *Arabidopsis* CAP genes have distinct functions in various tissues and organs (Figure 5). In addition, CAP genes were commonly expressed during the initial or early stages of tissue or organ development.

The first discovery of plant CAP proteins, pathogenesis-related protein 1 (PR-1) was detected in tobacco leaves after infection with the tobacco mosaic virus (Cornelissen et al., 1986; Van Loon & Van Kammen, 1970) and C-terminal 11 amino acid peptide fragments derived from tomato or *Arabidopsis* function in defense against the bacterial pathogen and tolerance to salt stress (Chen et al., 2014; Chien et al., 2015). In this study, the expression of *PR-1/CAP36* (*At2g14610*) was low in almost all tissues and only in the stipules under normal growth conditions, suggesting that it may only function under pathogen infection conditions.

On the other hand, the *CAP51/PRH1* product participates in the development of lateral roots under the control of the ARF7 signaling pathway (Zhang et al., 2020). In this study, we found similar expression patterns and different expression timing of the cluster genes *CAP51/PRH1*, *CAP52*, and *CAP53* during lateral root development.

The functions of most CAP family genes are still largely unknown, except for a few CAP family genes that have been reported to be involved in abiotic and biotic stress responses and lateral root development in *Arabidopsis*. This study provides a clue to the elucidating of the molecular mechanisms of CAP family proteins in plants.

4 | MATERIAL AND METHODS

4.1 | Plant material and growth conditions

A. thaliana ecotype Columbia (Col-0) was used as the wild type (WT) in all experiments. *Arabidopsis* seeds were surface sterilized and germinated on 1/2 Murashige-Skoog (MS) 1.2% agar plates. The plants were grown on tilted plates at 45° in a growth chamber with a 16 h light/8 h dark photoperiod at 22°C. The 6-day-old seedlings were used for the time-course expression pattern analysis of the lateral roots. The 9-day-old seedlings were used for the GUS assay of the shoots and roots. The plants were transferred to soil, grown under white light with a 16 h light/8 h dark photoperiod at 22°C, and used for GUS assay of the flower at stage 15 and silique at stages 17 and 18.

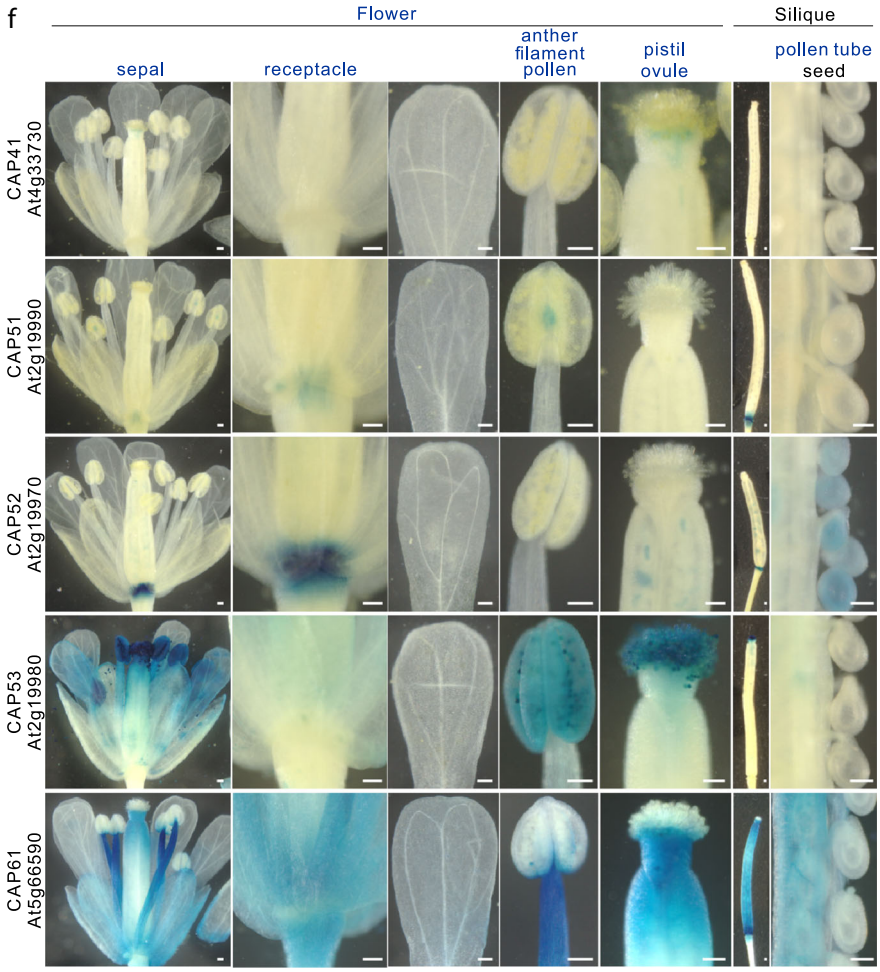


FIGURE 3 (Continued)

4.2 | Plasmid constructs and generation of the promoter-reporter transgenic plants

In each of the *Arabidopsis* CAP family genes, the promoter region comprises more than 1.5-kbp upstream region from the start codon of each CAP gene. Promoter sequences were generated by amplifying the *Arabidopsis* genome, and cloning into the pENTR entry vector was confirmed by DNA sequencing. Subsequently, the promoter sequences were subcloned into *Arabidopsis* expression vector pGWB533-GUS-mNeonGreen (Hirano et al., 2023) using the LR reaction to generate transgenic *Arabidopsis* plants expressing *GUS-mNeonGreen* under their promoters. The binary plasmids were transformed into Col-0 plants using the *Agrobacterium tumefaciens*-mediated floral dip method. The primers used are listed in Table S2.

4.3 | Histochemical GUS assays

For each transgenic plant, at least five independent lines were selected, and 9-day-old seedlings, flowers at stage 15, and siliques at stages 17 and 18 were used for the GUS assay. After samples were immersed in cold 90% acetone for 15 min at room temperature, the acetone was removed from the sample. After being washed twice with GUS buffer (50 mM sodium phosphate, pH 7.2, 0.5 mM $K_3Fe(CN)_6$, and 0.5 mM $K_4Fe(CN)_6$), the samples were vacuum infiltrated in GUS staining buffer (0.5 mg/ml 5-bromo-4-chloro-3-indoxyl-b-D-glucuronide, cyclohexylammonium salt (X-Gluc)/dimethylformamide and 0.1% (v/v) Triton X-100) for 2 min and incubated at 37°C overnight. After being rinsed twice with 70% ethanol and incubated in 70% ethanol for more than 2 h at room temperature, the samples were observed using a zoom microscope (ZEISS) (Koizumi et al., 2009).

4.4 | mNeonGreen fluorescence observations

Fluorescence and differential interference contrast (DIC) images were obtained using a Leica TCS SP8 laser scanning confocal microscope. For the detection of mNeonGreen, the excitation wavelength was set at 488 nm, and the emission wavelength was 493–550 nm. The captured images were processed using Leica LAS X software.

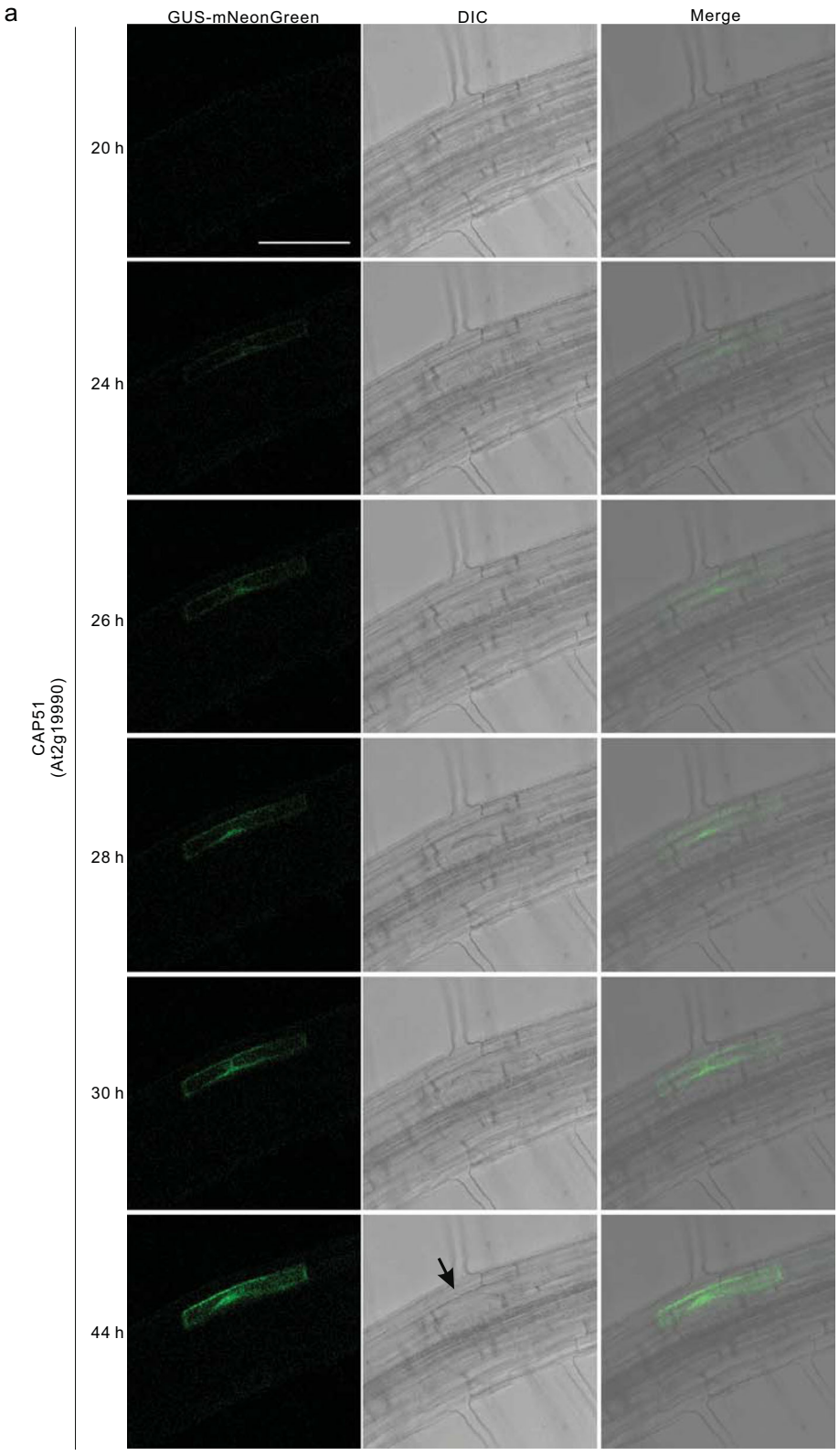
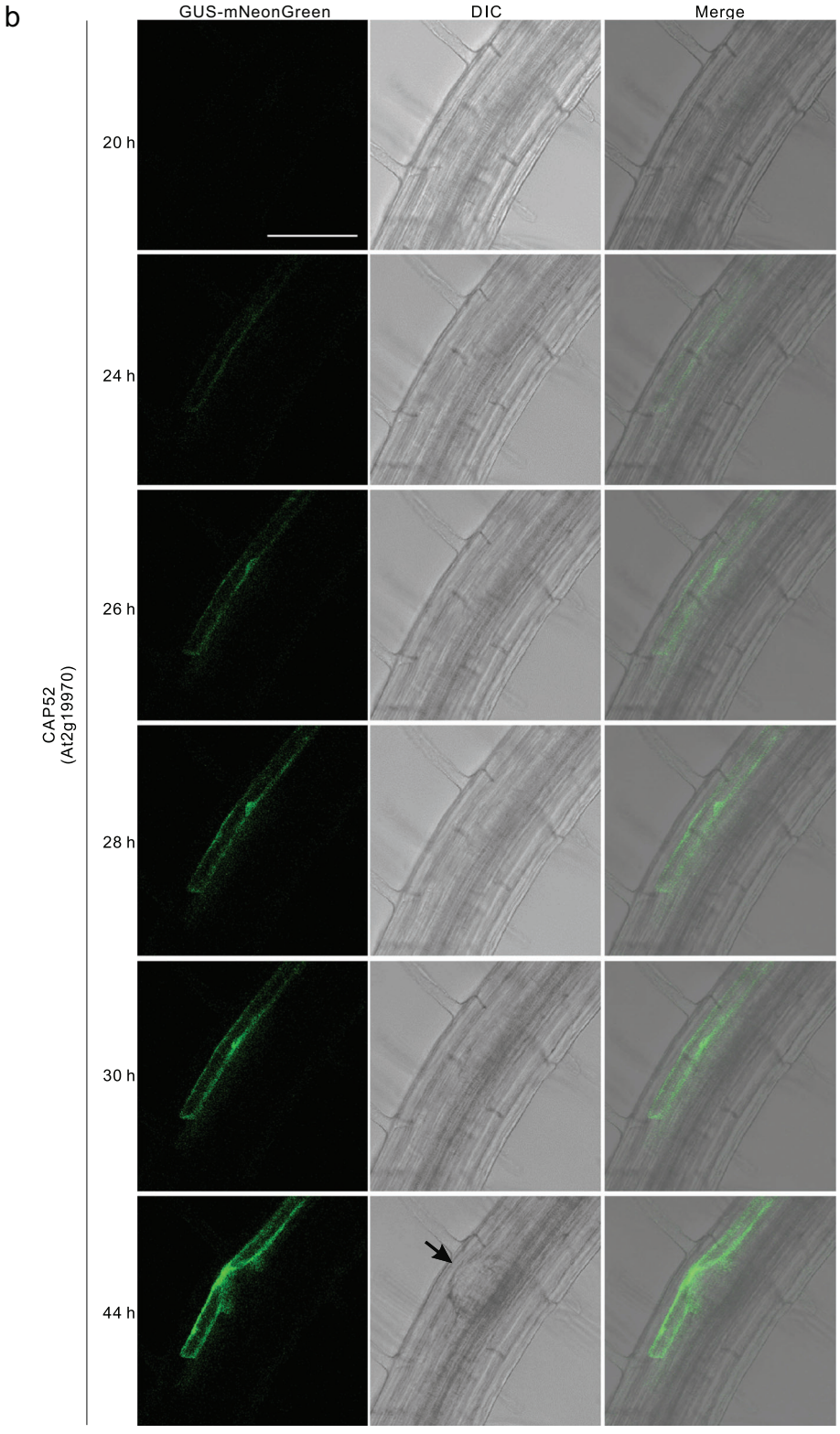


FIGURE 4 Confocal time-lapse images at 20, 24, 26, 28, 30, and 44 h time points of the root bending region after root gravistimulation for 20 h in the transgenic line expressing *GUS-mNeonGreen* driven by the *CAP51* promoter (a; *pCAP51*::*GUS-mNeonGreen*), *CAP52* promoter (b; *pCAP52*::*GUS-mNeonGreen*), or *CAP53* promoter (c; *pCAP53*::*GUS-mNeonGreen*). Black arrows indicate the lateral root primordia. Bars = 100 μm.



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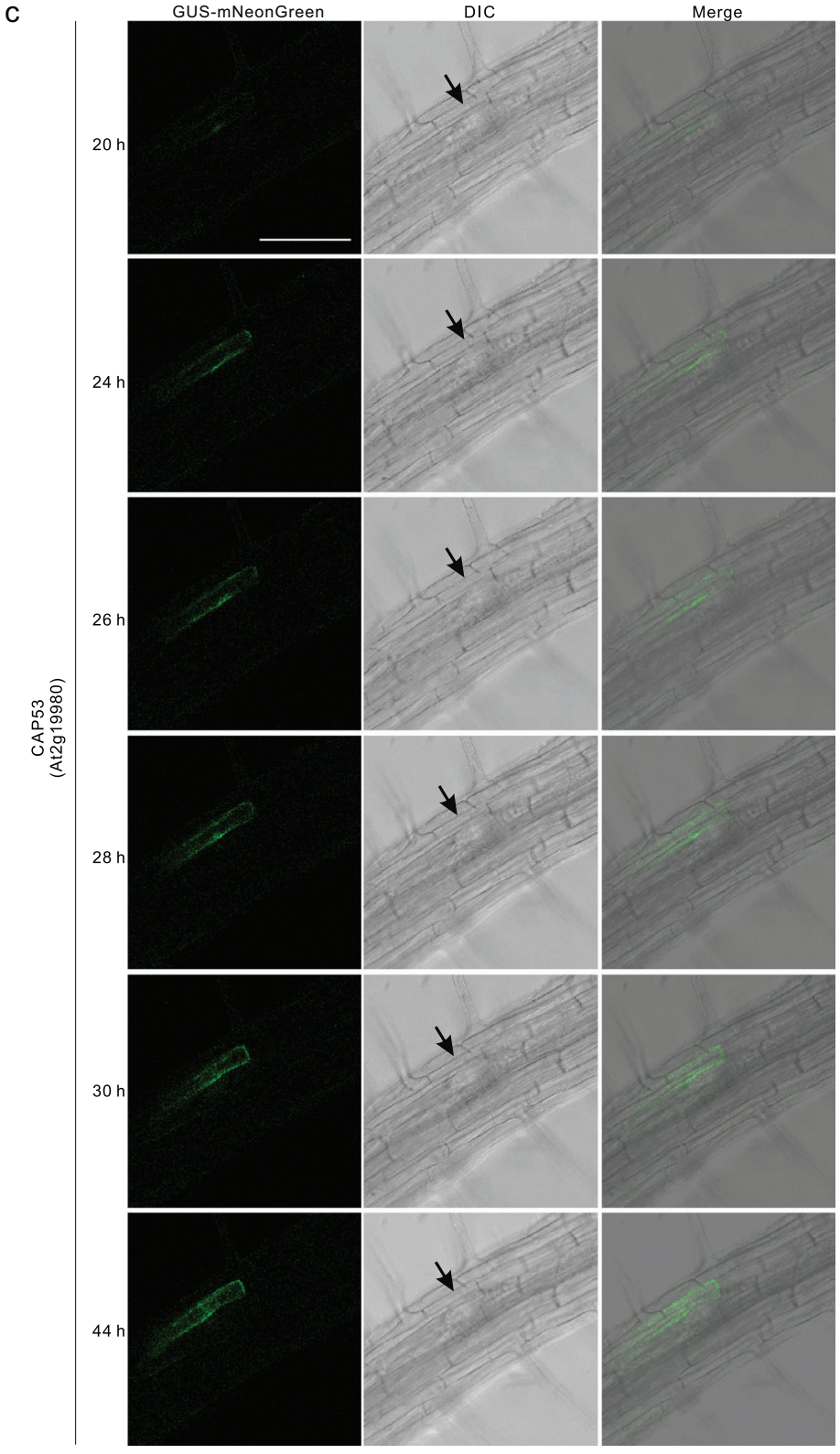

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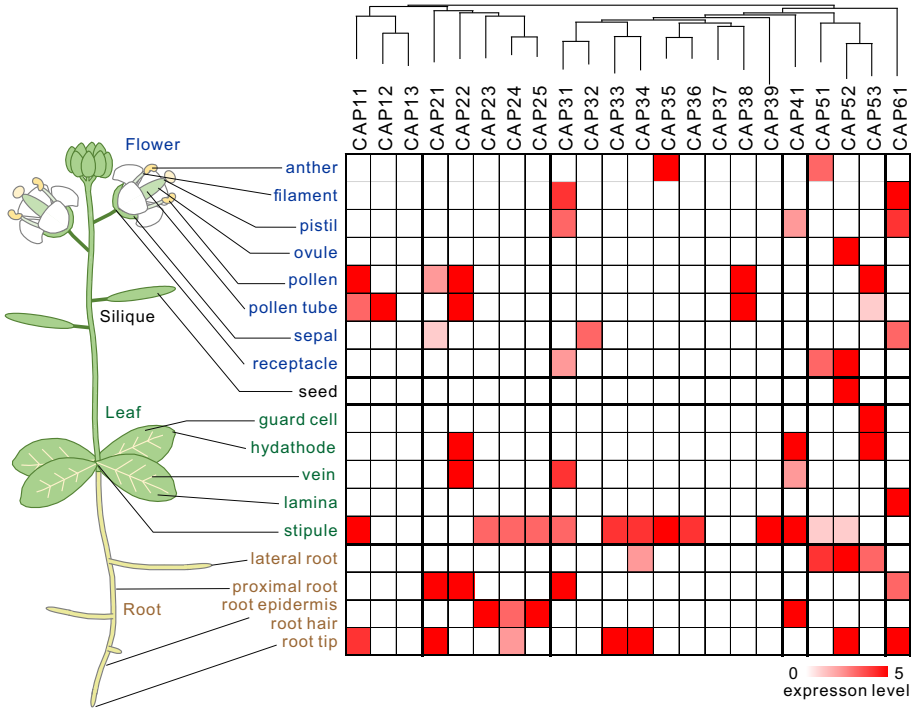

FIGURE 5 Summary of the tissue-specific expression pattern of CAP genes based on GUS staining in this study. Expression levels are indicated by red gradients (bottom).

4.5 | Time-course expression pattern analysis during the LRP formation

Lateral root initiation is induced mechanically by gravitropic curvature at the site of bending (Ditengou et al., 2008). Then, after rotating the 6- or 7-day-old seedlings by 90° on 1/2 MS medium with 0.8% agar in a glass bottom dish (AS ONE) for 20 h, the root bending region was observed at 20, 24, 26, 28, 30, and 44 h using a Leica TCS SP8 laser scanning confocal microscope.

4.6 | Identification of promoter sequences and alignment of amino acid sequences for 22 *Arabidopsis* CAP genes/proteins

The promoter regions, genome sequences, amino acid sequences, and five prime untranslated regions (5′UTR) of 22 *Arabidopsis* CAP genes/proteins were obtained from The Arabidopsis Information Resource (https://www.arabidopsis.org/). Full-length amino acid sequences were aligned using the SnapGene software and ClustalOmega method. Signal peptide sequences of CAP proteins were also identified using SignalP 3.0 (https://services.healthtech.dtu.dk/services/SignalP-3.0/).

4.7 | Promoter *cis*-regulatory elements analysis of *Arabidopsis* CAP genes

The 2-kbp upstream region from the start codon of each CAP gene was analyzed by online analysis software PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) (Higo et al., 1999) and PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002).

4.8 | Expression analysis of *Arabidopsis* CAP genes by bioinformatics databases

The expression patterns of 21 *Arabidopsis* CAP genes were predicted using e-Northerns w. Expression Browser (https://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi) (Toufighi et al., 2005) in the Bio-Analytic Resource for Plant Biology (BAR) (https://bar.utoronto.ca/).

AUTHOR CONTRIBUTIONS

Tomoko Hirano and Masa H. Sato conceived and designed this study. Megumi Matsuzawa performed almost all study experiments. Megumi Matsuzawa and Takumi Nakayama generated the transgenic lines. Megumi Matsuzawa, Tomoko Hirano, and Masa H. Sato analyzed the data. Tomoko Hirano and Masa H. Sato wrote the manuscript and supervised the study.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

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SUPPORTING INFORMATION

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