

Characterization of selected members of clade A protein phosphatase 2Cs (PP2Cs) family in *Brassica juncea* var. *tumida*

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Abstract

Background and aims: Absciscic acid (ABA) is essential for plant stress adaptation and growth regulation, and clade A PP2Cs function as core negative regulators in ABA signal transduction. Nevertheless, the biological functions of clade A PP2C family genes remain largely uncharacterized in tuber mustard (*Brassica juncea* var. *tumida*). This study aimed to systematically characterize clade A *BjuPP2C* genes and explore their potential roles in abiotic stress response, stem swelling development, and resistance to *Plasmodiophora brassicae*.

Methods: We profiled the expression patterns of clade A *BjuPP2C* genes under multiple abiotic stresses and *P. brassicae* inoculation, as well as across successive stem swelling developmental stages. Four candidate genes (*BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*) with prominent expression changes were selected for CDS cloning. Subcellular localization assays were performed to determine their intracellular distribution, and yeast two-hybrid (Y2H) analysis was used to verify physical interactions between these PP2C proteins and PYR1-Like ABA receptors (BjuPYLs).

Results: Most clade A *BjuPP2C* genes were significantly induced by abiotic stresses and pathogen infection, and the transcript abundance of several clade A *BjuPP2C* members was upregulated during stem swelling development. In particular, *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were markedly upregulated both at the early stage of *P. brassicae* infection and the initial phase of stem swelling. Subcellular localization demonstrated that *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* localized to nucleus and *BjuPP2C56_B1* localized to nucleus and cytoplasm. Y2H results revealed that *BjuPP2C56_B1*, *BjuPP2C37_A1*, and *BjuPP2C37_B1* interact with BjuPYLs in tuber mustard, whereas no interaction was detected between *BjuPP2C24_O1* and BjuPYLs.

Conclusions: The clade A *BjuPP2C* genes participate in ABA-mediated stress response, clubroot resistance, and stem swelling development in tuber mustard. Four key PP2C members differ in subcellular distribution and their capacity to bind ABA receptors, laying a foundation for further dissecting the ABA regulatory network governing stem swelling development and pathogen defense in tuber mustard.

Keywords: abiotic stresses, *Brassica juncea* var. *tumida*, clade A *BjuPP2Cs*, *Plasmodiophora brassicae*, stem swelling.

Introduction

Reversible protein phosphorylation mediated by kinases and phosphatases governs plant growth and development (Cao et al., 2016; Ghanizadeh et al., 2025). Protein phosphatases (PPs) reverse phosphorylation by cleaving

phosphate moieties to regulate substrate activity. PPs fall into two primary groups: protein tyrosine phosphatases (PTPs) and serine/threonine phosphatases (STPs). STPs are subdivided into phosphoprotein phosphatases (PPPs) and metal-dependent protein phosphatases (PPMs). PP1, PP2A, and PP2B are PPP members, whereas protein

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Abbreviations: ABA - abscisic acid; BjuPYL - *Brassica juncea* var. *tumida* PYL protein; CDS - coding sequence; PP2C - Protein phosphatase 2C; PYL - PYR1-Like; Y2H - yeast two-hybrid.

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phosphatase 2C (PP2C) represents the major PPM subfamily (Cohen, 1989; Ghanizadeh *et al.*, 2025).

Abscissic acid (ABA) serves as a central hormone regulator mediating plant adaptation to diverse abiotic and biotic stresses, including salinity stress and pathogen infection (Kong *et al.*, 2015; Khan, 2025; Mahajan *et al.*, 2025). To date, the core regulatory cascade of the ABA signaling pathway consists of ABA receptors (PYR/PYL/RCAR), clade A PP2Cs, and SNF1-related protein kinases 2 (SnRK2s). Under unstressed conditions, PP2Cs physically interact with SnRK2s to dephosphorylate target sites and suppress SnRK2 kinase activity, thereby maintaining the ABA signaling pathway in a silent state. Upon ABA accumulation, ABA binds to PYR/PYL/RCAR receptors to form receptor-ligand complexes, which further associate with PP2Cs and suppress their phosphatase activity. The released SnRK2s are subsequently activated to initiate downstream ABA signaling cascades (Fujii *et al.*, 2009; Ma *et al.*, 2009; Park *et al.*, 2009).

In *Arabidopsis thaliana*, nine clade A PP2C members, namely AtABI1, AtABI2, AtAHG1, AtAHG3, AtHAB1, AtHAB2, AtHAI1, AtHAI2, and AtHAI3, act as key negative regulators of ABA signal transduction. Subcellular localization analyses have demonstrated that ABI1 and ABI2 are distributed in both the nucleus and cytoplasm, whereas AHG3 and HAI3 are predominantly localized in the nucleus (Park *et al.*, 2009). Dominant gain-of-function mutants *abi1-1* and *abi2-1* exhibit obvious ABA insensitivity during seed germination and cotyledon greening, confirming the negative regulatory roles of ABI1 and ABI2 in ABA responses (Merlot *et al.*, 2001; Zhang *et al.*, 2025). By contrast, *ahg1-1* and *ahg3-1* mutants display ABA hypersensitivity at the same developmental stages. Among these genes, *AHG1* displays the highest transcriptional abundance in seeds, implying its critical function in ABA-mediated seed developmental regulation (Nishimura *et al.*, 2007; Carrillo-Barral *et al.*, 2020). In addition, *HAB1* undergoes alternative splicing to generate two transcripts, *HAB1.1* and *HAB1.2*, which exert opposite regulatory effects on seed germination and cotyledon greening processes (Wang *et al.*, 2015).

Numerous studies have also characterized PP2C gene families across diverse *Brassica* crops. In *B. rapa*, sequence alignment, phylogenetic analysis, and conserved motif prediction revealed highly conserved structural features of BrPP2C proteins (Khan *et al.*, 2019). A total of 20 clade A PP2C homologs have been identified in *B. oleracea*; compared with their orthologs in *A. thaliana*, these genes generally exist as two to three copies, accompanied by distinct tissue-specific expression profiles (Ludwików *et al.*, 2013). In tuber mustard, genome-wide identification characterized 209 PP2C genes, and BjuPP2C52 was verified to interact with multiple flowering regulators such as BjuCOL5, BjuAP2, and BjuSVP-1, suggesting its potential roles in flowering time modulation (Zeng *et al.*, 2022). Furthermore, evolutionary analysis of 192 PP2CA genes from 15 *Brassicaceae* species revealed conserved evolutionary patterns and whole-genome duplication-driven gene expansion in *B. napus*. Functional divergence of BnaABI1 paralogs was mainly attributed to variations

in promoter regions rather than coding sequences (Babula-Skowrońska, 2021).

Tuber mustard (*B. juncea* var. *tumida*, AABB, 2n = 36) belongs to the *Brassicaceae* family (Yang *et al.*, 2016). Its swollen fleshy stem serves as the primary raw material for Fuling preserved mustard tuber, making it an economically important vegetable crop. Nevertheless, the molecular regulatory mechanisms underlying stem swelling development in tuber mustard remain largely unclear (Shi *et al.*, 2012; Du *et al.*, 2020). This crop frequently encounters multiple adverse environmental stresses, including salinity, low temperature, and infection by *P. brassicae*. These stresses severely hinder plant growth and development, induce clubroot disease, and restrict stem enlargement. As a devastating soil-borne biotic disease, clubroot markedly inhibits vegetative growth and tuber development of tuber mustard, thereby greatly reducing crop yield (Wang *et al.*, 2020a; Liao *et al.*, 2024; Wang *et al.*, 2025). Accordingly, exploring the expression characteristics related to stem swelling and stress responses is of great significance for high-yield breeding of tuber mustard. Clade A PP2Cs, core components in ABA signaling, are widely involved in plant stress adaptation and developmental regulation. However, there remains a lack of systematic expression profiling of clade A BjuPP2C genes in tuber mustard.

In this study, we analyzed transcript patterns of clade A BjuPP2C genes under *P. brassicae* infection, NaCl, cold, ABA treatments, as well as successive stem swelling stages in tuber mustard. Most of these genes were induced by pathogen and abiotic stresses as well as stem swelling. Four highly induced genes (*BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*) were chosen for further assays. Subcellular localization showed that BjuPP2C37_A1, BjuPP2C37_B1, and BjuPP2C24_O1 localized exclusively to the nucleus, while BjuPP2C56_B1 was distributed in both nucleus and cytoplasm. Yeast two-hybrid (Y2H) assays confirmed physical interactions between BjuPYLs and BjuPP2C56_B1, BjuPP2C37_A1, and BjuPP2C37_B1, whereas no interaction was detected for BjuPP2C24_O1.

Materials and methods

Plant material and growth conditions: The seeds of *Brassica juncea* var. *tumida* cultivar Yong'an were used as plant material, and the permissions were obtained from Yangtze Normal University. The seeds were cultivated in light culture room following the methods previously used (Cheng *et al.*, 2019a). The details were as follows: the seeds were sterilized and sown in MS medium. The growth room was at 22°C and photosynthetic photon flux density of 90 $\mu\text{mol}/\text{m}^2\text{s}$ (long-day conditions; 16 h light/8 h dark). After 7 days, the seedlings were transplanted into soil and cultivated under controlled light conditions.

To explore the expression patterns of clade A BjuPP2C genes under biotic stress, swollen root tissues infected with *P. brassicae* were collected for dormant spore extraction. The diseased roots were thoroughly rinsed

with distilled water, and the epidermis was peeled off to isolate cortical tissues. Approximately 10 g of cortical samples were homogenized with 50 mL sterile water in a tissue homogenizer. The homogenate was subsequently filtered through eight layers of gauze into a clean beaker. The concentration of dormant *P. brassicae* spores was adjusted to OD₆₀₀ = 0.07 using sterile water. Uniform 14-day-old seedlings cultivated in the growth chamber were inoculated on the roots with an equal volume of spore suspension. Samples were harvested at a series of early infection time points (0, 6, 12, 24, and 72 h) post-inoculation with *P. brassicae*. Total RNA was isolated using the *RNA Extraction Kit* (Cat. No. B518631, Sangon Biotech, China) and reverse-transcribed into cDNA using the *RevertAid First Strand cDNA Synthesis Kit* (Cat. No. B300902, Sangon Biotech, China) for subsequent quantitative analysis. The time points of early *P. brassicae* infection were selected for the following reasons. Previous research has reported that the 6 - 24-h interval encompasses zoospore adhesion and preliminary root hair infection, a phase where initial host perception of pathogen signals occurs (Cai et al., 2023). The 72-h time point marks a critical node within this early infection window, during which *P. brassicae* completes penetration into root hair cells and induces widespread transcriptional reprogramming of hormone and defense-related pathways (Cai et al., 2023). Accordingly, this study selected the time points of 0, 6, 12, 24, and 72 h post-inoculation to analyze the transcriptional patterns of *BjuPP2Cs* during the early infection stage of *P. brassicae*.

To investigate the transcriptional expression profiles of clade A *BjuPP2C* genes under abiotic stress conditions, intact 5-day-old seedlings were directly exposed to 50 μ M ABA, 100 mM NaCl and low temperature (4°C) treatments for 3 h under continuous light conditions (Cheng et al., 2019a). To analyze the gene expression levels in different growth stages of stem swelling, tuber mustard stems at five key growth stages grown under field conditions were collected, including D1 (1-month-old seedlings, six-leaf stage), D2 (2-month-old seedlings, initial swollen stem stage), D3 (3-month-old seedlings, early swollen stem stage), D4 (4.5-month-old seedlings, rapid stem enlargement stage), and D5 (5-month-old seedlings, late swollen stem stage) (Cheng et al., 2019b).

Gene cloning and vector construction: For subcellular localization assay, total RNA was extracted from tuber mustard tissues and reverse-transcribed into first-strand cDNA as the amplification template using the *RNA Extraction Kit* (Cat. No. B518631, Sangon Biotech, China) and the *RevertAid First Strand cDNA Synthesis Kit* (Cat. No. B300902, Sangon Biotech, China). The full-length CDS sequences of target *BjuPP2C* genes were amplified using specific primers containing homologous arms matching the linearized vector ends. All primer pairs used in this study are listed in Table 1 Suppl. The backbone vector PTF101 was linearized by restriction digestion (Cai et al., 2025). Subsequently, the homologous recombination reaction was performed using a seamless cloning kit (Cat. No. B632219, Sangon Biotech, China),

in which inserts and linearized vector were mixed and incubated at 50°C for 20 min to achieve seamless recombination between the target fragments and vector backbone. The recombination products were transformed into *E. coli* DH5 α competent cells by heat shock. Positive single colonies were screened on selective solid medium supplemented with 50 mg/L spectinomycin, followed by colony PCR identification and sequencing verification. Plasmids extracted from correctly sequenced strains were transformed into *Agrobacterium* GV3101 competent cells for subsequent subcellular localization assay.

For Y2H assay, the full-length coding sequences (CDS) of target *BjuPP2C* and *BjuPYL* genes were amplified by PCR using *High Fidelity PCR Master Mix* (Cat. No. B639292, Sangon Biotech, China). The primers used in this study are detailed in Table 1 Suppl. The amplified fragments containing attB homologous recombination sites were subjected to the BP recombination reaction with the entry vector pDONR207 according to the Gateway cloning system protocol (*Gateway BP Clonase 2 Enzyme Mix*, Cat. No. 11789-020, Invitrogen, USA). The reaction mixture was incubated at 25°C for 3 h and then transformed into *E. coli* DH5 α competent cells. Positive colonies were screened on LB solid medium containing 50 mg/L gentamicin, and verified by colony PCR and sequencing to obtain correct entry clones. Subsequently, LR recombination reactions were performed between sequence-verified entry plasmids and destination vectors pGADT7 (AD vector) and pGBKT7 (BD vector), respectively. The LR reaction system was incubated at 25°C for 3 h to complete site-specific recombination (*Gateway LR Clonase 2 Enzyme Mix*, Cat. No. 11791-020, Invitrogen, USA). The recombinant products were transformed into *E. coli* DH5 α competent cells and cultured on LB medium supplemented with 100 mg/L ampicillin (for pGADT7) or 50 mg/L kanamycin (for pGBKT7). After PCR identification, the correct AD and BD recombinant plasmids were extracted using the *DiaSpin Plasmid Mini-Preps Kit* (Cat. No. B110091, Sangon Biotech, China) and preserved for the Y2H assays.

Subcellular localization analysis: The protein coding sequences of *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were cloned and inserted into the pTF101-GFP vector by seamless cloning. All successfully constructed recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, followed by transient infiltration into *Nicotiana benthamiana* leaves. At 2 days post-infiltration, GFP fluorescent signals were visualized under an Olympus BX53 upright fluorescence microscope (Olympus, Tokyo, Japan), equipped with an Olympus U-HGLGPS high-pressure mercury lamp light source (Olympus, Tokyo, Japan) and U-FGFP GFP-specific filter cube (excitation wavelength: 460 - 480 nm, emission wavelength: 500 - 540 nm) (Olympus, Tokyo, Japan).

Yeast two-hybrid analysis: The CDS sequences of *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were cloned into the pGBKT7 bait vector.

Similarly, the CDS sequences of *BjuPYL3*, *BjuPYL4-1*, *BjuPYL6-2*, *BjuPYL7-2*, *BjuPYL8-2*, *BjuPYL8-3*, *BjuPYL10-2*, and *BjuPYL11-1* were inserted into the pGADT7 prey vector. The resulting AD and BD constructs were co-transformed into yeast strain AH109. Yeast transformation was carried out per the Clontech Yeast Protocols Handbook. Transformed yeast colonies were cultured first on SD/-Leu/-Trp medium, then transferred to SD/-Leu/-Trp/-His/-Ade medium supplemented with or without 10 μ M ABA and incubated for 4 days (Wang *et al.*, 2020b). Parallel yeast two-hybrid assays with and without 10 μ M ABA were set up to discriminate ABA-dependent and ABA-independent interactions between PYL proteins and clade A BjuPP2Cs, according to Kanno *et al.* (2012). Yeast lacks endogenous ABA biosynthetic pathways and ABA transporters; thus, intracellular ABA originates solely from external supplementation, and 10 μ M ABA can passively diffuse into yeast cells to reach physiologically effective concentrations. ABA functions as a molecular glue that triggers conformational rearrangements in PYL receptors, consequently promoting assembly of stable PYL-PP2C complexes.

Gene expression analysis: Total RNA was isolated using the *RNA Extraction Kit* (Cat. No. B518631, Sangon Biotech, China) from tuber mustard seedlings subjected to *P. brassicae* infection and diverse abiotic stress treatments. After quality and integrity verification of RNA samples, residual genomic DNA was removed *via* DNase I digestion, and purified RNA was reversely transcribed into first-strand complementary DNA (cDNA) using the *RevertAid First Strand cDNA Synthesis Kit*

(Cat. No. B300902, Sangon Biotech, China). Subsequently, the synthesized cDNA templates were applied for quantitative real-time PCR (qPCR) detection. Relative gene expression levels were quantified following the comparative $2^{-\Delta\Delta C_t}$ method, with *BjuActin3* (*BjuA014894*) selected as the stable internal reference gene. All qPCR assays were performed with three independent biological replicates, and all primer pairs used in this study are detailed in Table 1 Suppl.

Statistical analysis: All data were analyzed using *SigmaPlot 10.0* (Systat Software, Inc., Chicago, IL, USA) and *SPSS 16.0* (SPSS Inc., Chicago, IL, USA). Means and standard deviations of all results were calculated, and for multiple groups of samples, the one-way ANOVA followed by the *Dunnnett's* test was used. The statistically significant treatments were marked with *** ($P < 0.001$), ** ($0.001 < P < 0.01$), and * ($0.01 < P < 0.05$) (Cheng *et al.*, 2019a).

Results

Correspondence of gene nomenclature systems used in this study: To clarify the relationships among different gene nomenclature systems adopted in previous studies on tuber mustard, a correspondence table (Table 1) was established to analyze the diverse gene naming conventions used in relevant research. Specifically, this table integrates three key nomenclature systems: the systematic locus names (gene ID) of tuber mustard genes (based on the latest genome annotation of *Brassica juncea* var. *tumida*),

Table 1. Correspondence of gene nomenclature systems used in this study. Tissue-specific expression pattern analysis of clade A BjuPP2Cs.

Group	Gene IDs	Gene name in Zeng <i>et al.</i> (2022)	Gene name in Babula-Skowrońska (2021)
ABI1	<i>BjuA004393</i>	<i>BjuPP2C56_A3</i>	<i>BjuA01.ABI1.a</i>
	<i>BjuA013512</i>	<i>BjuPP2C56_A2</i>	<i>BjuA03.ABI1.b</i>
	<i>BjuB040327</i>	<i>BjuPP2C56_B2</i>	<i>BjuB05.ABI1.a</i>
	<i>BjuA029591</i>	<i>BjuPP2C56_A1</i>	<i>BjuA08.ABI1.c</i>
	<i>BjuB043703</i>	<i>BjuPP2C56_B1</i>	<i>BjuB03.ABI1.b</i>
ABI2	<i>BjuB001428</i>	<i>BjuPP2C77_B1</i>	<i>BjuB02.ABI2</i>
	<i>BjuA044909</i>	<i>BjuPP2C77_A1</i>	<i>BjuA10.ABI2</i>
AHG1	<i>BjuA010184</i>	<i>BjuPP2C75_A1</i>	<i>BjuA03.AHG1</i>
	<i>BjuB041185</i>	<i>BjuPP2C75_B1</i>	<i>BjuB08.AHG1</i>
AHG3	<i>BjuA020514</i>	<i>BjuPP2C37_A1</i>	<i>BjuA05.AHG3</i>
	<i>BjuB006899</i>	<i>BjuPP2C37_B1</i>	<i>BjuB07.AHG3.b</i>
	<i>BjuB023632</i>	<i>BjuPP2C37_B2</i>	<i>BjuB01.AHG3.a</i>
<i>AtHAB1</i>	<i>BjuB030059</i>	<i>BjuPP2C16_B1</i>	<i>BjuB03.HAB1</i>
<i>AtHAB2</i>	<i>BjuB004338</i>	<i>BjuPP2C7_B2</i>	<i>BjuB03.HAB2</i>
<i>AtHAI1</i>	<i>BjuA038574</i>	<i>BjuPP2C78_A1</i>	<i>BjuA10.HAI1</i>
	<i>BjuB008108</i>	<i>BjuPP2C78_B1</i>	<i>BjuB02.HAI1</i>
<i>HAI2</i>	<i>BjuA019471</i>	<i>BjuPP2C3_A3</i>	<i>BjuA09.HAI2.b</i>
	<i>BjuB015401</i>	<i>BjuPP2C3_B1</i>	<i>BjuB06.HAI2</i>
	<i>BjuA037326</i>	<i>BjuPP2C3_A2</i>	<i>BjuA10.HAI2.a</i>
<i>HAI3</i>	<i>BjuO013253</i>	<i>BjuPP2C24_O1</i>	—

the gene names previously reported by Zeng et al. (2022) in their transcriptomic and functional study on tuber mustard stem swelling, and the clade A PP2C terminology proposed by Babula-Skowrońska (2021) for the classification of PP2C phosphatase genes in *Brassicaceae* species. In this study, we adopted the gene nomenclature reported by Zeng et al. (2022) for all subsequent analyses to ensure the consistency of gene naming and the accuracy of analysis results throughout the study.

To illustrate the tissue-specific expression patterns of clade A *BjuPP2Cs*, the qPCR assays to check the gene expression levels at different growth stages and tissues (root, stem, swollen stem, leaf, flower, and pod) were performed. According to the results, clade A *BjuPP2Cs* were expressed in multiple tissues. Interestingly, the expression levels of *BjuPP2C56_A3*, *BjuPP2C56_B1*, *BjuPP2C77_B1*, *BjuPP2C77_A1*, *BjuPP2C75_A1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, *BjuPP2C37_B2*, *BjuPP2C78_A1*, *BjuPP2C3_A3*, *BjuPP2C3_A2*, and *BjuPP2C24_O1* showed significant differences between stem and swollen stem (Fig. 1). The expression levels of *BjuPP2C56_A2*, *BjuPP2C56_B2*, *BjuPP2C56_A1*, *BjuPP2C37_B2*, *BjuPP2C16_B1*, *BjuPP2C7_B2*, *BjuPP2C78_A1*, and *BjuPP2C3_A3* were higher in root than those in other organs. Almost all the clade A *BjuPP2Cs* were expressed at very low levels in flower and pod, especially *BjuPP2C3_A3* and *BjuPP2C3_B1* (barely detectable expression) (Fig. 1).

The expression patterns of clade A *BjuPP2Cs* in *B. juncea* var. *tumida* under abiotic stresses:

Five-day-old tuber mustard seedlings were exposed to 50 μ M ABA, 100 mM NaCl and low temperature (4°C) for 3 h, followed by transcriptional expression analysis of clade A *BjuPP2C* genes. Compared with the untreated control (Ck), the transcript abundances of all clade A *BjuPP2C* genes were markedly upregulated under NaCl stress (Fig. 2). Under low-temperature conditions, the expression levels of *BjuPP2C56_A2*, *BjuPP2C56_B2*, *BjuPP2C56_A1*, *BjuPP2C77_A1*, *BjuPP2C75_B1*, *BjuPP2C7_B2*, *BjuPP2C3_A2*, and *BjuPP2C24_O1* were significantly induced, while the transcription of *BjuPP2C77_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, *BjuPP2C37_B2*, and *BjuPP2C3_A3* was notably suppressed (Fig. 2). Upon exogenous ABA treatment, *BjuPP2C56_A3*, *BjuPP2C56_A2*, *BjuPP2C56_B2*, *BjuPP2C56_A1*, *BjuPP2C77_A1*, *BjuPP2C75_B1*, *BjuPP2C37_B1*, *BjuPP2C37_B2*, *BjuPP2C16_B1*, *BjuPP2C78_A1*, *BjuPP2C78_B1*, and *BjuPP2C3_A2* were remarkably upregulated. In contrast, the expression of *BjuPP2C77_B1*, *BjuPP2C37_A1*, and *BjuPP2C7_B2* was distinctly inhibited by ABA application (Fig. 2).

The expression patterns of clade A *BjuPP2Cs* under *P. brassicae* stress:

To further investigate the gene expression patterns of clade A *BjuPP2Cs* during the early infection stage of *P. brassicae* in tuber mustard, qPCR assays were performed. Two-week-old tuber mustard seedlings were inoculated with *P. brassicae*. Root tissues were harvested at 0, 6, 12, 24 and 72 h post-inoculation,

and total RNA extracted from samples was reverse-transcribed into cDNA for subsequent qPCR detection. The results showed that compared with the untreated control (0 h), *BjuPP2C56_B1*, *BjuPP2C75_A1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* had relatively high expression levels and were highly induced at all time points during early *P. brassicae* infection (Fig. 3).

Gene expression levels of clade A *BjuPP2Cs* in *B. juncea* var. *tumida* during stem swelling stages:

The swollen stem is the raw material of “Fuling mustard”, yet the regulatory mechanism of stem swelling is still undiscovered. To further explore the regulation of clade A *BjuPP2Cs* in the formation and development of swollen stems in tuber mustard, we collected the stems in the field at different growth and developmental stages. The results showed that compared with the control (D1), the gene expression levels of *BjuPP2C56_A3*, *BjuPP2C75_A1*, *BjuPP2C3_B1*, and *BjuPP2C3_A2* were inhibited during stem swelling stages (D2 - D4 stages). By contrast, the expression levels of *BjuPP2C56_B1*, *BjuPP2C75_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* maintained relatively high expression levels and were significantly induced during stem swelling stages (D2 - D4 stages) (Fig. 4).

Subcellular localization analysis of *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*:

Stem swelling is a characteristic trait of tuber mustard, and the swollen stem constitutes its major economically valuable organ. Meanwhile, clubroot disease triggered by *P. brassicae* has become a critical limiting factor hampering the industrial production of tuber mustard. Accordingly, we selected four clade A *BjuPP2C* members (*BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*), whose transcripts were markedly upregulated both during the early phase of *P. brassicae* infection and stem swelling stages, for further functional investigation.

To investigate the subcellular localization of *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*, the protein coding sequences of these genes were inserted into the pTF101-GFP vector using the seamless cloning method. The results showed that *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were localized to the nucleus and *BjuPP2C56_B1* was localized to the nucleus and cytoplasm (Fig. 5). This localization pattern is consistent with that of *Arabidopsis* PP2C proteins AtABI1-5, AtAHG3-1, AtAHG3-2, and AtHAI3, implying that *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* exert their functions in the nucleus, while *BjuPP2C56_B1* functions in both the nucleus and cytoplasm.

Y2H analysis of interactions between *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, *BjuPP2C24_O1*, and *BjuPYLs*:

The clade A PP2Cs, acting as negative regulators in ABA signaling, could interact with PYLs in *Arabidopsis*. To further investigate the functions of *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* in *B. juncea* var. *tumida*, the Y2H assays

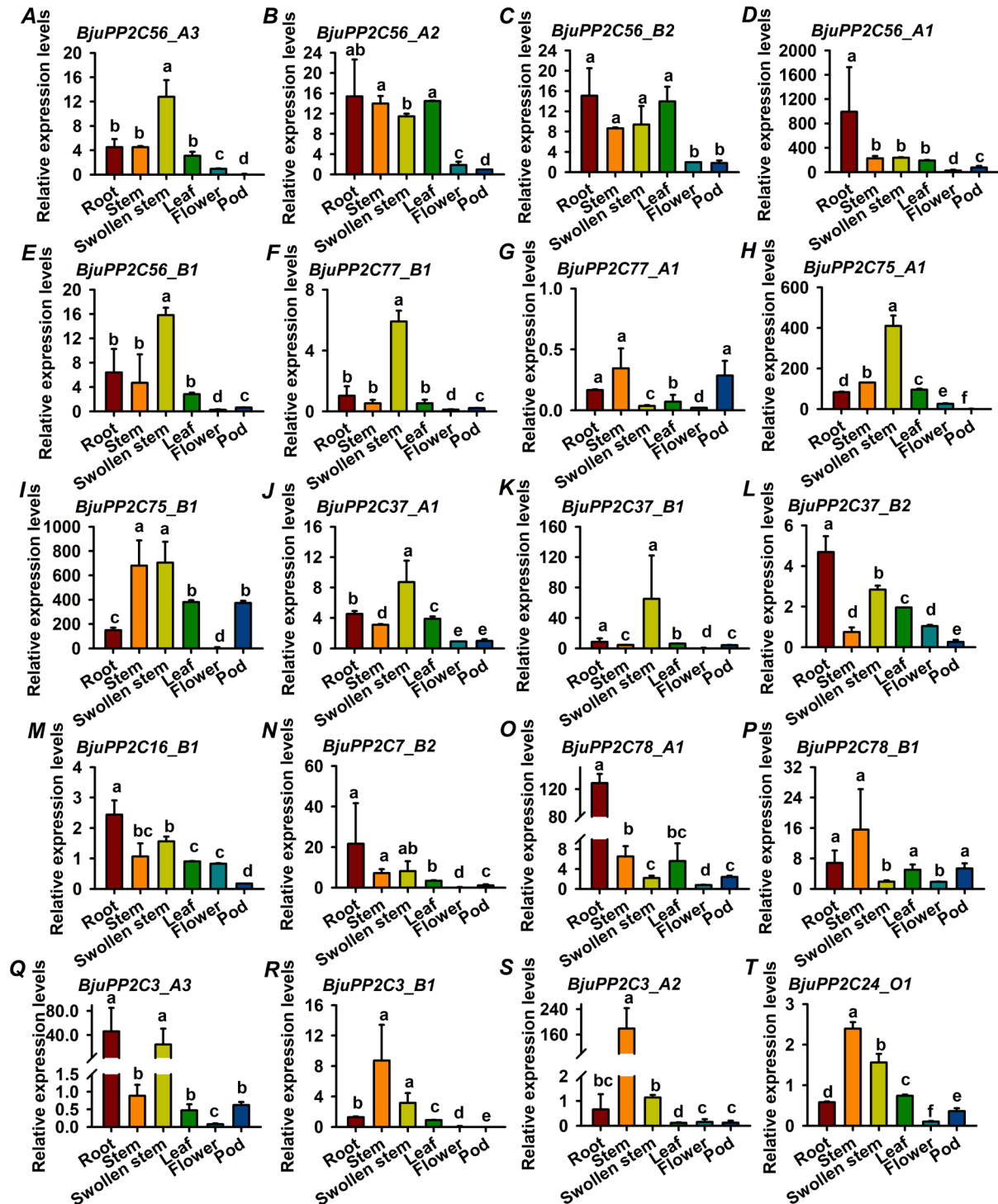


Fig. 1. Expression patterns of clade A *BjuPP2Cs* in different tissues. Gene expression levels were determined by qPCR and normalized to the reference gene *BjuActin3*. Data are presented as means $\pm$ SD ($n = 3$). Different letters indicate significant differences ($P < 0.05$).

were performed to test the interactions between some clade A *BjuPP2Cs* (*BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*) and ABA receptors (*BjuPYL3*, *BjuPYL4-1*, *BjuPYL6-2*, *BjuPYL7-2*, *BjuPYL8-2*, *BjuPYL8-3*, *BjuPYL10-2*, and *BjuPYL11-1*) with or without 10 μ M ABA. The results showed that

BjuPP2C56_B1 interacted with *BjuPYL3*, *BjuPYL6-2*, and *BjuPYL7-2*, and the interaction between *BjuPP2C56_B1* and *BjuPYL7-2* was induced by ABA. *BjuPP2C37_A1* interacted with *BjuPYL3* and *BjuPYL6-2* with or without ABA. *BjuPP2C37_B1* interacted with *BjuPYL3*, *BjuPYL6-2* and *BjuPYL7-2* with or without ABA (Fig. 6).

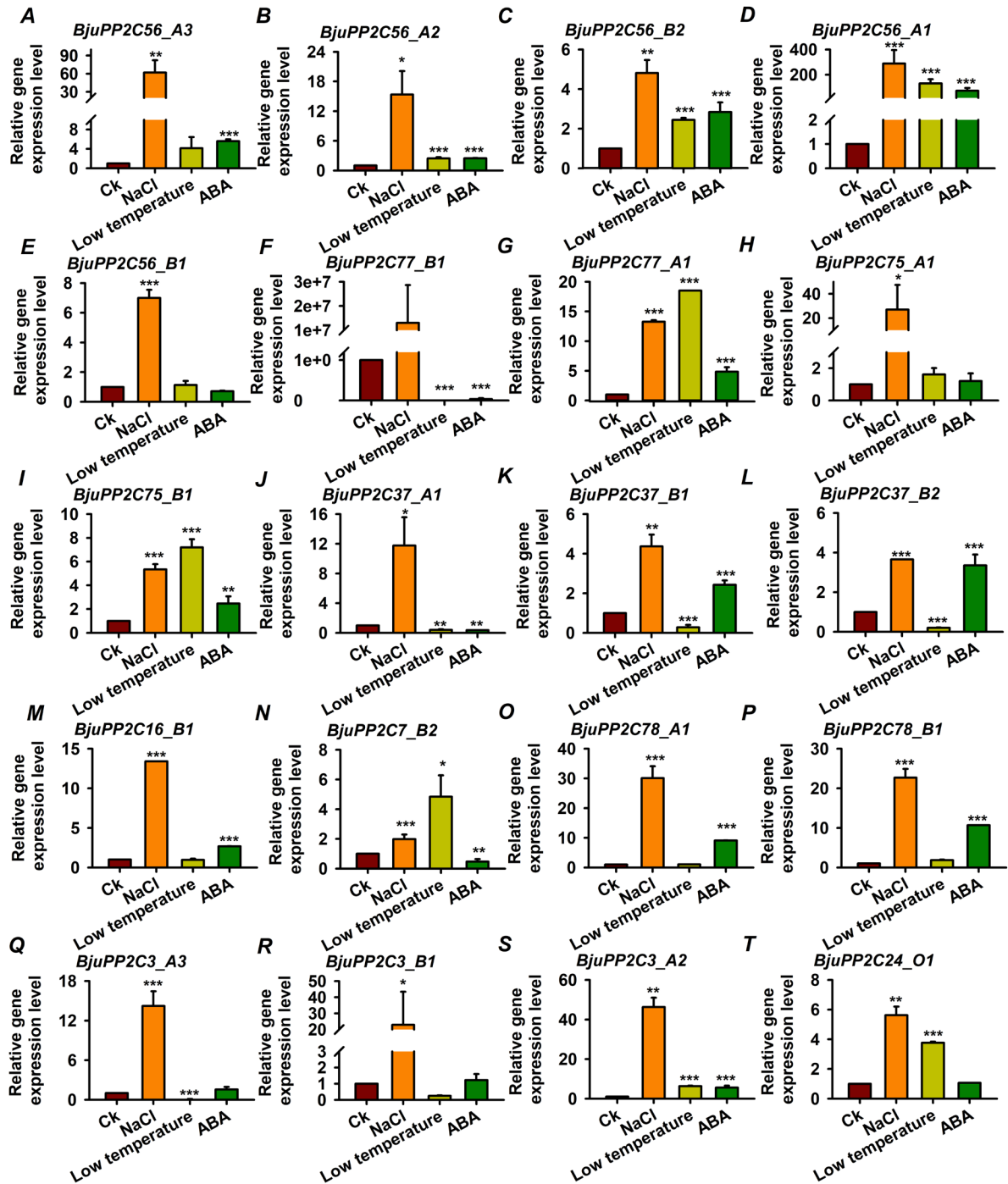


Fig. 2. Expression patterns of clade A *BjuPP2C* genes in tuber mustard seedlings under different abiotic stress treatments. Relative transcript levels of clade A *BjuPP2Cs* were detected under NaCl (100 mM), low temperature (4°C for 3 h), and exogenous ABA (50 μ M) treatments. The untreated seedlings were set as control (Ck). Error bars represent the standard deviation of three independent biological replicates. Asterisks indicate significant differences compared with Ck group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

ABA mediates abiotic stress tolerance, immune response and organ development in crucifers, with a conserved core signaling module consisting of PYR/PYL receptors, clade A PP2Cs and SnRK2 kinases (Cohen, 1989; Fujii et al., 2009). As major negative regulators of ABA signaling,

clade A PP2Cs bind SnRK2s and dephosphorylate them to shut down ABA cascades. Tuber mustard is an economically vital vegetable in China susceptible to clubroot disease caused by *P. brassicae*, which severely suppresses plant growth, stem swelling and crop yield (Shi et al., 2012; Cai et al., 2019; Wang et al., 2020a). Although clade A PP2Cs widely modulate plant growth

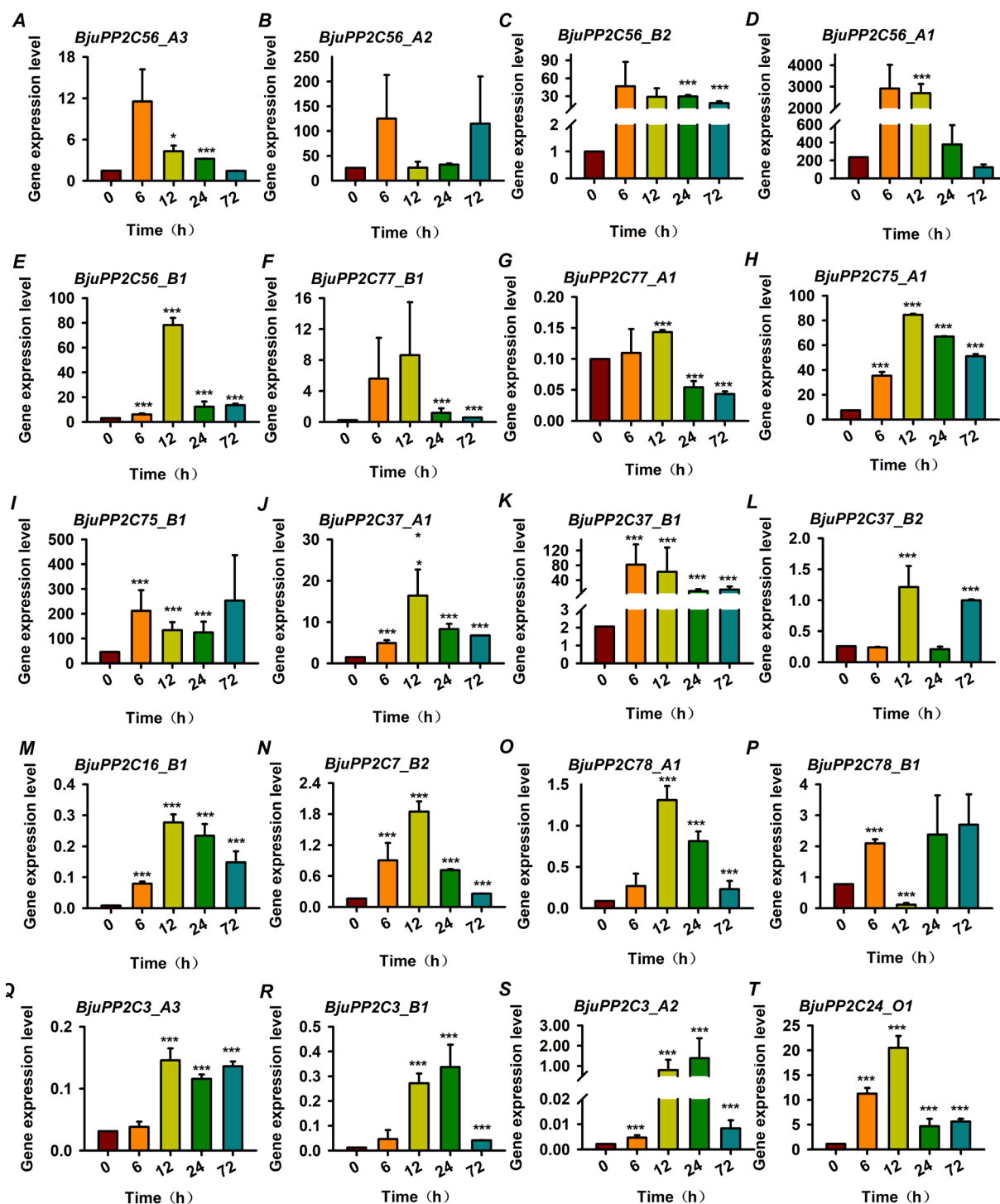


Fig. 3. Temporal expression profiles of clade A *BjuPP2C* genes in tuber mustard responding to *P. brassicae* infection. The relative transcript abundance of clade A *BjuPP2C* genes was quantified at 0, 6, 12, 24, and 72 h post-inoculation. Seedlings at 0 h post-inoculation were used as the control. Error bars represent the standard deviations of three independent biological replicates. Asterisks denote statistically significant differences relative to the control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

and stress resistance, their stress-responsive expression patterns and molecular features remain uncharacterized in tuber mustard. Here, we systematically characterized clade A *BjuPP2C* members, covering their subcellular localization, physical interactions with *BjuPYLs*,

transcriptional responses to biotic and abiotic stresses, and expression patterns during different stem swelling stages.

In this study, all clade A *BjuPP2C* genes were significantly upregulated under NaCl stress (Fig. 2), which was consistent with previous findings that clade A *PP2Cs*

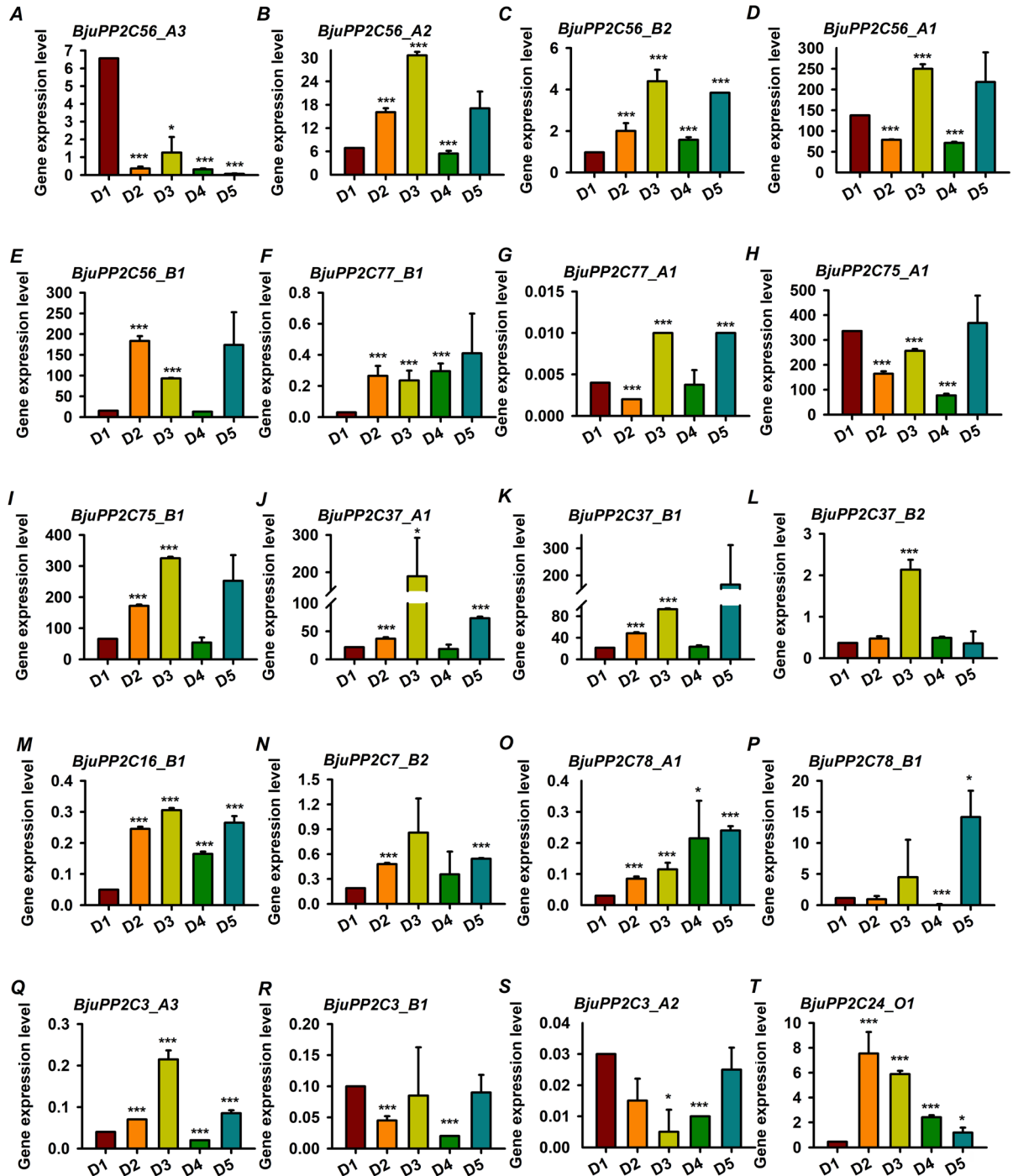


Fig. 4. Expression patterns of clade A *BjuPP2Cs* at different developmental stages of tuber mustard swollen stems. Relative expression levels of clade A *BjuPP2C* genes were detected across five stem growth stages: D1 (six-leaf seedling stage), D2 (initial stem swelling), D3 (early stem swelling), D4 (rapid stem enlargement), and D5 (late mature swelling stage). D1 was set as the control. Error bars represent standard deviations of three independent biological replicates. Asterisks indicate significant differences relative to the D1 control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

act as core regulators of ABA-mediated salt stress tolerance in *Brassica* crops. Consistently, *PP2C* genes in *B. rapa* are strongly induced by salt treatment and contribute to the modulation of osmotic stress adaptation (Khan et al., 2019). Unlike the uniform response to salinity, *BjuPP2C* paralogs exhibited obvious functional divergence under

low-temperature and exogenous ABA treatments: certain members were markedly upregulated, whereas others were significantly repressed (Fig. 2). Such divergent expression patterns among duplicated *PP2C* homologs have also been documented in *B. oleracea*, in which clade A *PP2C* duplicates display distinct expression specificities under

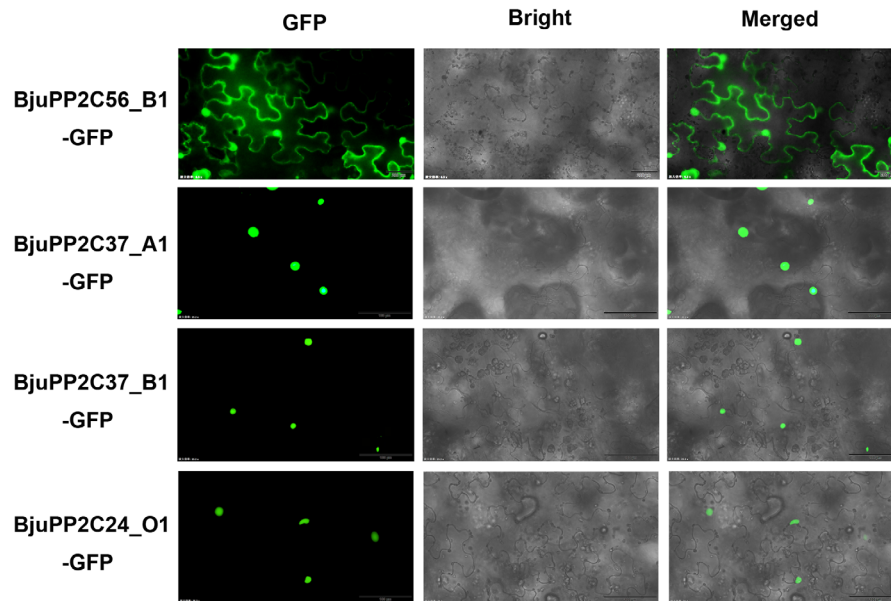


Fig. 5. Subcellular localization of BjuPP2C56_B1, BjuPP2C37_A1, BjuPP2C37_B1, and BjuPP2C24_O1. GFP fluorescence was detected in tobacco leaves at 2 days post-infiltration. GFP: GFP laser channel. Bright: bright-field channel. Merged: merge of GFP and bright-field channels. Bars = 100 μ m.

cold and ABA stimuli and execute non-redundant functions in cold adaptation (Ludwików *et al.*, 2013). These collective results indicate that the whole-genome triplication event in *Brassica* species has driven the functional differentiation of clade A PP2C genes, enabling duplicated paralogs to evolve specialized regulatory pathways for responding to diverse abiotic stresses. Gene duplication generally leads to three evolutionary fates: subfunctionalization, neofunctionalization, and nonfunctionalization (Khan *et al.*, 2019; Lynch and Conery, 2000). In agreement with this evolutionary theory, clade A BjuPP2C genes in tuber mustard have 1 - 5 duplicated copies with highly differentiated expression profiles. Notably, the *BjuABII* subfamily exhibited prominent expression divergence under different treatments: all *BjuABII* members except *BjuPP2C56_B1* were ABA-inducible, while only *BjuPP2C56_A2*, *BjuPP2C56_B2*, and *BjuPP2C56_A1* responded positively to low-temperature stress (Fig. 2). These distinct stress-responsive expression signatures further verify the subfunctionalization of duplicated *BjuPP2C* genes in allotetraploid tuber mustard.

As a typical biotrophic pathogen, *P. brassicae* severely threatens the sustainable production of tuber mustard, one of the most economically important vegetable crops in southwest China. Early infection is the critical and decisive stage of clubroot disease occurrence, in which the pathogen invades root epidermal cells, induces dramatic physiological reprogramming, and initiates root gall formation (Wang *et al.*, 2020a). Plants rapidly activate a series of stress-responsive signaling cascades to resist pathogen colonization at the early infection stage. ABA signaling acts as a core regulatory pathway that coordinates plant immune responses and abiotic stress adaptation, in which clade A PP2C proteins serve as key negative regulators. In this study, we analyzed

the expression pattern of clade A *BjuPP2Cs* at the early stage of *P. brassicae* infection. The results showed that multiple clade A *BjuPP2C* genes, including *BjuPP2C56_B1*, *BjuPP2C75_A1*, *BjuPP2C75_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1*, were significantly induced during the early stage of *P. brassicae* infection (Fig. 3). This early and strong transcriptional activation indicates that clade A *BjuPP2Cs* are highly sensitive to clubroot pathogen stimulation and may participate in the early immune response of tuber mustard against *P. brassicae* infection. These candidate PP2C members may mediate the crosstalk between ABA signaling and clubroot resistance, thereby regulating the disease adaptation of tuber mustard at the initial infection stage.

Stem swelling is the core economic trait of tuber mustard, and swollen stems serve as the raw material for Fuling preserved mustard. Nevertheless, the molecular regulatory network governing stem swelling development remains largely unclear. In this study, tuber mustard stem development was classified into five sequential stages (D1 - D5) based on seedling age, morphological phenotype and stem transverse diameter: D1: 1-month-old seedlings at the six-leaf stage, pre-swelling stage without visible stem bulging; D2: 2-month-old seedlings, initial swelling stage with slight protuberance at the stem base; D3: 3-month-old seedlings, early rapid-swelling stage with obvious enlargement in stem volume and diameter; D4: 4.5-month-old seedlings, rapid-growth peak stage with sustained fast stem expansion; D5: 5-month-old seedlings, late swelling stage with slowly decelerated growth and basically stabilized stem size for harvest (Cheng *et al.*, 2019b).

To explore the functions of clade A *BjuPP2C* genes in stem swelling, we detected their expression levels from D1 to D4. Compared with D1, *BjuPP2C56_A3*,

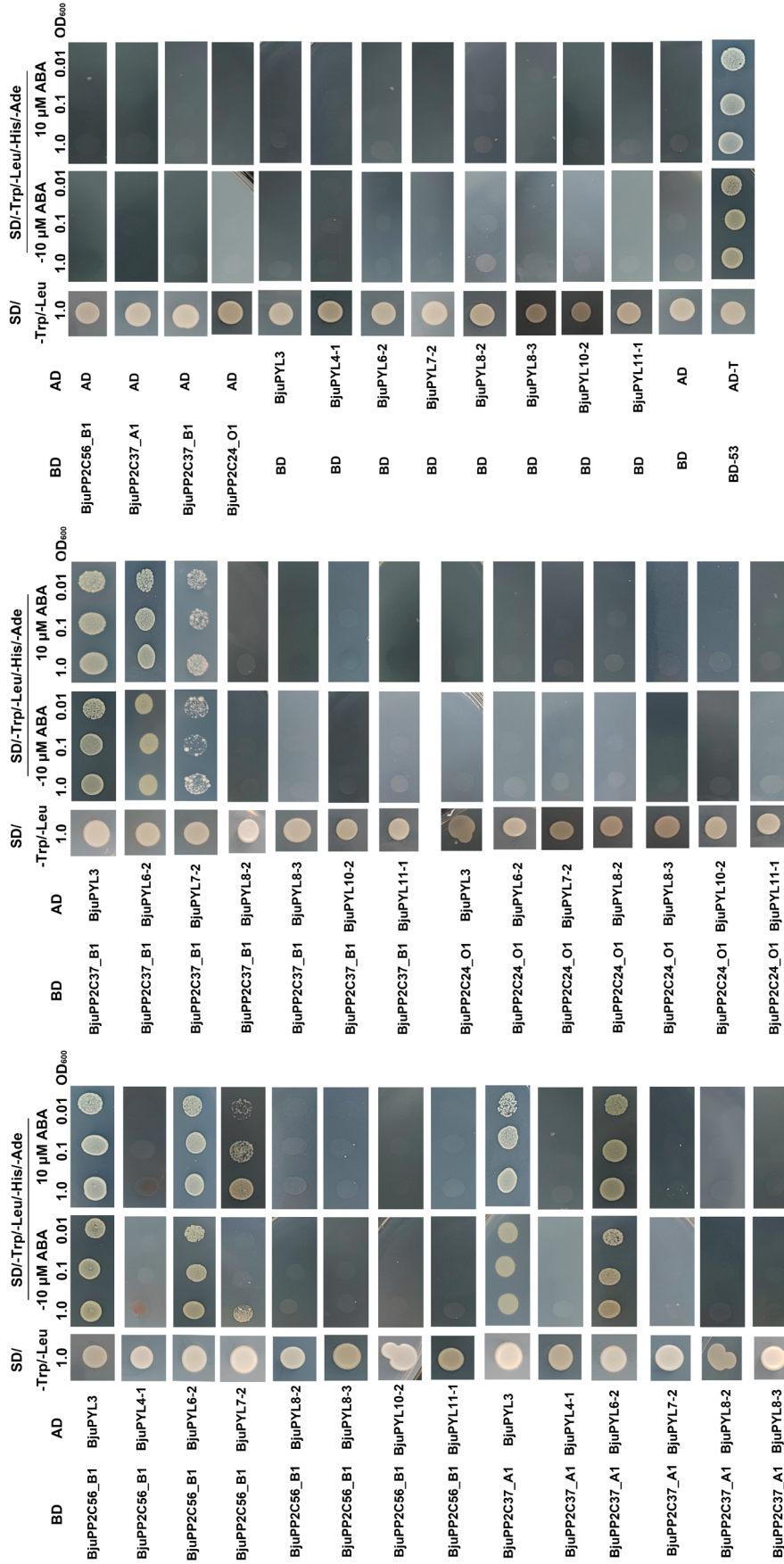


Fig. 6. Y2H verification of protein interactions between BjuPP2C56_B1, BjuPP2C37_A1, BjuPP2C24_O1, and BjuPYLs. Y2H assays were conducted to examine physical interactions between the four BjuPP2C proteins and eight BjuPYL members (BjuPYL3, BjuPYL4-1, BjuPYL6-2, BjuPYL7-2, BjuPYL8-2, BjuPYL8-3, BjuPYL10-2, and BjuPYL11-1) in the presence or absence of 10 μM ABA. SD/-Trp/-Leu: selective medium deficient in tryptophan and leucine. SD/-Trp/-Leu-His-Ade: selective medium deficient in tryptophan, leucine, histidine, and adenine. Co-transformation of AD-T and BD-53 served as positive controls, while empty AD and BD vectors were used as negative controls.

BjuPP2C75_A1, *BjuPP2C3_B1*, and *BjuPP2C3_A2* were downregulated from D2 - D4. Since clade A PP2Cs act as core negative regulators in ABA signaling, decreased transcription of these genes may release repression on SnRK2 kinases, triggering ABA cascades to promote cell division and expansion for stem growth (Fig. 4). In contrast, *BjuPP2C56_B1*, *BjuPP2C75_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were significantly induced in D2 - D4. This opposite expression trend indicates subfunctionalization among clade A BjuPP2C paralogs in allotetraploid tuber mustard. Different PP2C copies antagonistically modulate ABA signaling strength to coordinate cell proliferation, elongation, and organ formation (Fig. 4).

Yeast two-hybrid assays verified the interaction between some clade A BjuPP2Cs (*BjuPP2C56_B1*, *BjuPP2C37_A1*, and *BjuPP2C37_B1*) and ABA receptors (*BjuPYL3*, *BjuPYL6-2*, and *BjuPYL7-2*), and subcellular localization confirmed that *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were localized to the nucleus and *BjuPP2C56_B1* to the nucleus and cytoplasm, which were highly consistent with the subcellular localization and interaction pattern of PP2C-PYL modules in *A. thaliana* and *B. napus* (Figs. 5 and 6) (Park *et al.*, 2009; Babula-Skowrońska, 2021). The fluorescence distribution pattern of *BjuPP2C56_B1* was highly similar to that of the empty pTF101-GFP control. Two possible interpretations are proposed for this observation. On one hand, this dual nucleocytoplasmic pattern may represent the authentic native subcellular localization of *BjuPP2C56_B1*. On the other hand, partial proteolytic cleavage of the *BjuPP2C56_B1*-GFP fusion protein may occur in plant cells. Degradation of the fusion protein would release free GFP peptides, which diffuse freely across the nucleus and cytoplasm and produce a signal pattern indistinguishable from empty GFP.

Conclusion

Taken together, 20 clade A *BjuPP2Cs* were analyzed in tuber mustard. Among them, *BjuPP2C56_B1*, *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were significantly induced both at the early stage of *P. brassicae* infection and throughout the D2-D4 stem swelling stages. Subcellular localization assays revealed that *BjuPP2C37_A1*, *BjuPP2C37_B1*, and *BjuPP2C24_O1* were localized to the nucleus and *BjuPP2C56_B1* was localized to the nucleus and cytoplasm. Y2H assays further confirmed physical interactions between selected BjuPYL proteins (*BjuPYL3*, *BjuPYL6-2*, *BjuPYL7-2*) and *BjuPP2C56_B1*, *BjuPP2C37_A1*, and *BjuPP2C37_B1*, yet no detectable interaction was observed between *BjuPP2C24_O1* and these three BjuPYL members.

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