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Molecular characterization of an AtPYL1-like protein, BrPYL1, as a putative ABA receptor in *Brassica rapa*

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ABSTRACT

Abscisic acid (ABA)-induced physiological changes are conserved in many land plants and underlie their responses to environmental stress and pathogens. The PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (PYLs)-type receptors perceive the ABA signal and initiate signal transduction. Here, we show that the genome of *Brassica rapa* encodes 24 putative AtPYL-like proteins. The AtPYL-like proteins in *Brassica rapa* (BrPYLs) can also be classified into 3 subclasses. We found that nearly all *BrPYLs* displayed high expression in at least one tissue. Overexpression of *BrPYL1* conferred ABA hypersensitivity to Arabidopsis. Further, ABA activated the expression of an ABA-responsive reporter in Arabidopsis protoplasts expressing BrPYL1. Overall, these results suggest that BrPYL1 is a putative functional ABA receptor in *Brassica rapa*.

1. Introduction

The phytohormone abscisic acid (ABA) regulates many physiological processes in plants, such as seed dormancy, seed germination, plant development and abiotic stress responses [1]. Our understanding of ABA perception and signal transduction in Arabidopsis and other organisms has improved substantially in recent years [2–6]. In terms of the molecular basis of the core ABA signalling network, the PYRABACTIN RESISTANCE1/PYR1-LIKE/REGULATORY COMPONENTS OF ABA RECEPTORS (hereafter referred as PYLs) receptors perceive the ABA signal and initiate the resulting physiological responses.

Several studies have shown that drought resistance can be acquired via molecular manipulation of ABA perception in Arabidopsis [7–16]. Genetic modifications, such as transgenic overexpression or conditional inducible expression of either wild-type, constitutively active or mutated versions of *PYL* receptors, conferred drought resistance to plants [9,10,12–16]. Chemical compounds that act as either ABA agonists/antagonists or ABA-mimicking ligands can also effectively control plant water use [7,8,11]. Similar manipulations are expected to be effective in regulating water dependency for many commercial crops.

The knowledge generated in *Arabidopsis* can now be utilized to identify ABA receptors in other organisms. A number of ABA receptors have been identified in other plants, such as 12 *PYL*-like genes in rice [17], 15 in tomato [16,18], 8 in grape [19], 6 in sweet orange [20], 21 in soybean [2], 13 in maize [6], and 14 in rubber tree [21]. *Brassica rapa* is one of the most popular vegetables in northern China, and shows a deep phylogenetic relationship to *Arabidopsis*. *Brassica rapa* contains two ABI5-like bZIP transcription factors, which are central downstream components of the early ABA signalling network [22]. However, the discovery of the PYR/PYL/RCAR-like ABA receptor family in *Brassica rapa* remains enigmatic.

In this work, we uncovered putative PYL-like ABA receptors genome-wide in *Brassica rapa*. Further, we validated one AtPYL1-like protein, BrPYL1, as a functional ABA receptor in plant cells.

2. Materials and methods

2.1. Protein properties and phylogenetic analysis of BrPYLs

The protein sequences of all AtPYLs were used as queries for searches against *Brassica rapa* database (BRAD, http://brassicadb.

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http://dx.doi.org/10.1016/j.bbrc.2017.04.116
0006-291X/

org/brad/index.php). To verify the conserved domains in putative BrPYLs, online tools such as CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), HMMER (Profile hidden Markov models for biological sequence analysis, http://www.ebi.ac.uk/Tools/hmmer/), Pfam (http://pfam.xfam.org/) and SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/) were used to perform PYR_PYL_RCAR_like domain prediction in BrPYLs respectively. Proteins which showed the presence of the conserved domains with confidence (E-value <1.0) were chosen for further analysis. All AtPYR/PYL/RCARs-BrPYLs were aligned with the MUSCLE tool and the maximum likelihood trees were generated using MEGA 6.0 as previously described. The molecular weight (kDa) and isoelectric point (pI) of BrPYLs were calculated by DNAstar.

2.2. Gene structure organization of BrPYL genes

The CDSs (coding sequences) of the *BrPYL* genes were downloaded from the *Brassica rapa* database (BRAD, http://brassicadb.org/brad/index.php). The CDS sequences of the *BrPYL* genes were used as the queries for local BLAST to search against the whole genome assembly of *Brassica rapa* (*B.rapa*_Chromosome_V1.5). The genomic sequences of *BrPYL* genes were then retrieved. The online Gene Structure Display Server (GSDS2.0, http://gsds.cbi.pku.edu.cn/) was used to decipher the architectures of *BrbZIP* genes as previously described [22,23].

2.3. RNA-seq data analysis of BrPYL genes

To determine the tissue-expression profile of *BrPYL* genes, we downloaded the RNA-seq data of different tissues (Callus, Flower, Leaf_1, Leaf_2, Root_1, Root_2, Silique and Stem) of *Brassica rapa* that was previously submitted into GEO database (https://www.ncbi.nlm.nih.gov/geo/, GEO accession number, GSE43245) [24]. Transcript abundance of *BrPYL* genes is calculated by fragments per kilobase of transcript per million fragments mapped (FPKM) values and the FPKM values were log2 transformed as previously described [24]. A heat map is generated via R (www.r-project.org).

2.4. qRT-PCR (real-time quantitative RT-PCR) analysis

Samples were treated with 0.1 mM ABA followed by total RNA extraction with TRIzol reagent (TaKaRa). Total RNA was used for reverse transcription with PrimeScript™ RT Master Mix (Perfect Real Time, TaKaRa). Real-time qRT-PCR was performed using a CFX96 real-time PCR machine (Bio-Rad, Hercules, CA, USA) and SYBR Premix Ex Taq kit (TaKaRa) to monitor double-stranded DNA products as previously described [22]. Data from real-time PCR was analyzed by Bio-Rad CFX Manager, and the standard curve method (delta-delta ct value) was used to calculate the relative expression of experimental genes normalized to the expression of cabbage *ACTIN2* (*BrACTIN2*/Bra037560) according to the manufacturer's instructions [22]. The following primers were used for qRT-PCR amplification: for *BrPYL1*, 5′- CGGATCTGGACCGTTGTTCTTG-3′ and 5′- CAGTGATCGACGCGAGCTTCTG-3'; for *BrPYR1c*, 5′- CGCAGACCTACAAGCACTTC-3′ and 5′- TCCTCTCGTCGTCCAGTATG-3'.

2.5. Construction of BrPYL1 transgenic plants and ABA inhibition of seed germination assay

To overexpress *BrPYL1*, the full-length CDS was amplified and cloned into *Sal*I and *Kpn*I sites of the binary vector p1307-3HA. The following primers were used: BrPYL1-SalF (5′-ACGCGTCGACATGGCAGATTCAGAGCCAATCAG-3′) and BrPYL1-KpnR (5′-GGGGTACCTCACATCACCGGAGGGTTTC-3′). The resulting p1307-3HA-BrPYL1 plasmid was transformed into the *Agrobacterium tumefaciens* strain GV3101 and then infiltrated into Col-0 plants with the floral dip method. Seeds (T0) from infiltrated plants were selected on MS medium containing 25 μg/L hygromycin (Roche). Homozygous T3 plants (derived from different T1 transformants) were used for ABA inhibition of seed germination as previously described [25–28]. Briefly, seeds were sterilized in a solution containing 20% sodium hypochlorite and 0.1% Triton X-100 for 10 min, washed five times with sterile water, and sown on MS

Table 1
Overall analysis of *PYL* genes in *Brassica rapa*.

Gene	Accession number	ORF (bp)	Chr	Protein				Subclass	Orthologs in Arabidopsis
				Length (aa)	MW (D)	pI	Position of PYL domain		
BrPYR1a	Bra021032	474	A08	157	17454.73	5.21	C-30~E-141	III	At4g17870,AtPYR1,RCAR11
BrPYR1b	Bra013262	576	A01	191	21658.53	6.7	C-30~E-176	III	At4g17870,AtPYR1,RCAR11
BrPYR1c	Bra012635	576	A03	191	21367.12	6.23	C-30~E-176	III	At4g17870,AtPYR1,RCAR11
BrPYL1	Bra024995	633	A06	210	23884.78	5.18	C-49~E-195	III	At5g46790,AtPYL1,RCAR12
BrPYL2	Bra007772	567	A09	188	20833.38	5.58	C-35~L-177	III	At2g26040,AtPYL2,RCAR14
BrPYL3	Bra008064	618	A02	205	22927.16	8.8	C-49~A-197	III	At1g73000,AtPYL3,RCAR13
BrPYL4a	Bra005115	450	A05	149	16173.32	8.36	C-52~L-148	II	At2g38310,AtPYL4,RCAR10
BrPYL4b	Bra000052	624	A03	207	22239.23	6.75	C-52~E-195	II	At2g38310,AtPYL4,RCAR10
BrPYL4c	Bra017125	615	A04	204	21987.82	6.52	C-49~E-192	II	At2g38310,AtPYL4,RCAR10
BrPYL5a	Bra028758	498	A02	165	18377.76	5.69	C-20~R-160	II	At5g05440,AtPYL5,RCAR8
BrPYL5b	Bra005853	612	A03	203	22726.57	5.96	C-58~R-198	II	At5g05440,AtPYL5,RCAR8
BrPYL5c	Bra009113	615	A10	204	22778.72	6.32	C-58~R-198	II	At5g05440,AtPYL5,RCAR8
BrPYL6a	Bra004967	639	A05	212	23445.3	6.88	S-62~E-208	II	At2g40330,AtPYL6,RCAR9
BrPYL6b	Bra000184	639	A03	212	23523.46	6.88	S-63~E-206	II	At2g40330,AtPYL6,RCAR9
BrPYL6c	Bra017012	618	A04	205	22764.53	6.38	S-56~E-199	II	At2g40330,AtPYL6,RCAR9
BrPYL7a	Bra000938	582	A03	193	21764.93	6.53	C-34~A-174	I	At4g01026,AtPYL7,RCAR2
BrPYL7b	Bra003463	564	A07	187	20767.83	6.33	C-35~L-174	I	At4g01026,AtPYL7,RCAR2
BrPYL8a	Bra022634	567	A02	188	21308.32	6.34	C-31~D-173	I	At5g53160,AtPYL8,RCAR3
BrPYL8b	Bra029093	555	A03	184	20906.88	6.76	C-28~D-170	I	At5g53160,AtPYL8,RCAR3
BrPYL8c	Bra003077	555	A10	184	21030.86	6.34	C-27~E-172	I	At5g53160,AtPYL8,RCAR3
BrPYL9	Bra033267	561	A10	186	20968.03	6.33	C-30~E-174	I	At1g01360,AtPYL9,RCAR1
BrPYL10	Bra026299	555	A01	184	20651.69	5.77	C-28~A-169	I	At4g27920,AtPYL10,RCAR4
BrPYL11	Bra025048	513	A06	170	18745.05	5.17	C-10~E-154	II	At5g45860,AtPYL11,RCAR5
BrPYL13	Bra013334	501	A01	166	18379.84	4.91	C-9~K-157	II	At4g18620,AtPYL13,RCAR7

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medium (Phytotech) with 0.3% Phytagel (Sigma-Aldrich) with different concentrations of ABA (Sigma-Aldrich). The plates were incubated at 4 °C for 4 days followed by incubation at 23 °C under continual illumination in growth chambers. Quantification of the percentage of seedlings with green cotyledons and radicle emergence was performed as previously described [22,27,28].

2.6. ABA responsive expression assay in protoplasts

The entire reconstitution of the ABA responsive expression assay *in planta* was performed as previously described [14,27,29]. Combinations of purified plasmids (via a Plasmid Maxiprep Kit, Vigorous Biotechnology) were introduced into Arabidopsis leaf mesophyll protoplasts according to the PEG-Ca^{2+} protocol. Transfected cells were then cultured for 4–8 h in the absence or presence of 5 μM ABA. Relative LUC activity was determined according to a Dual-Luciferase Reporter Assay Protocol provided by Promega as previously described [27]. The amount of purified plasmids used was as follows: *RD29B-LUC* (7 μg per transfection), *35S-rLUC* (2 μg per transfection), ABF2-HA, SnRK2.6-Flag, His-PYL1, 3HA-BrPYL1 (3 μg per transfection) and ABI1-HA (2 μg per transfection).

3. Results and discussion

3.1. The Brassica rapa genome encodes 24 putative PYL receptors

To identify PYL receptors in *Brassica rapa*, we used the 14 PYLs of Arabidopsis as queries to search the *Brassica rapa* database (BRAD, http://brassicadb.org/brad/index.php). To verify whether the isolated proteins harboured conserved domains, we analyzed the deduced amino acid sequences with online tools, such as CD-search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), Pfam (http://pfam.xfam.org/), HMMER (Profile hidden Markov models for biological sequence analysis, http://www.ebi.ac.uk/Tools/hmmer/) and SMART (Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de/). We ultimately identified 24 PYL-like proteins (Table 1). All the putative PYL proteins were named according to their similarity to Arabidopsis orthologues (Fig. 1 and Table 1).

To gain a more detailed outlook, we constructed phylogenetic trees of the PYLs between *Brassica rapa* and *Arabidopsis thaliana* (Fig. 1). The BrPYLs (PYLs in *Brassica rapa*) were categorized into 3 subclasses, similar to Arabidopsis. Nearly all AtPYLs had at least one orthologous gene in *Brassica rapa,* except AtPYL12. Although

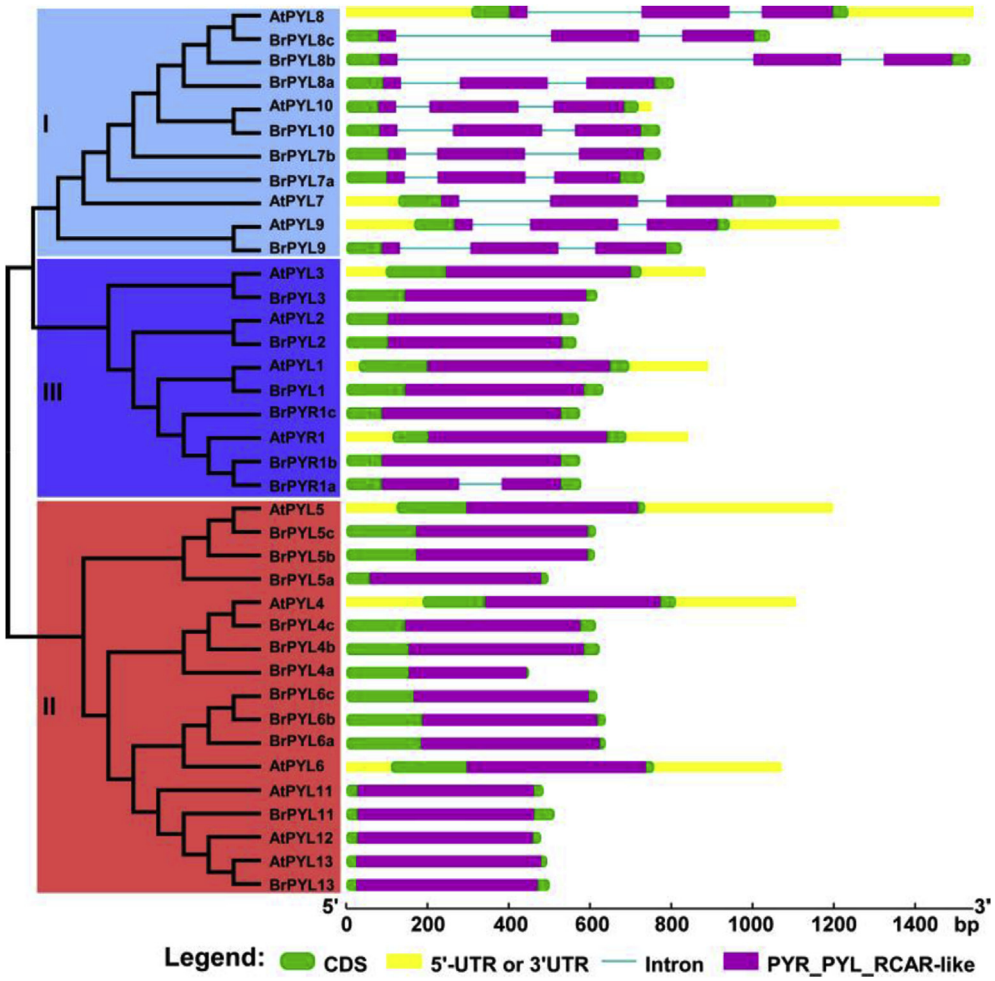

Fig. 1. The phylogenetic relationship and gene structure of BrPYLs and AtPYLs.
The protein sequences of 24 BrPYLs and 14 AtPYLs were aligned by the MUSCLE tool; the maximum likelihood tree was generated using MEGA 6.0. The three distinct subclasses were designated as I–III and labeled with different colored branches. Exon/intron organization of *BrPYL* and *AtPYL* genes was depicted with the online Gene Structure Display Server. The exons and introns are represented by green boxes and blue lines, respectively. The purple box denotes the PYR_PYL_RCAR-like domain region. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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AtPYL12 shared two orthologous genes in *Brassica rapa* with AtPYL13 and AtPYL11 (Bra013334 and Bra025048), their encoded proteins do not show high identity to AtPYL12. Bra013334 closely clustered with AtPYL13 and contained three key residues (Q-38 in CL1, F-71 in CL2 and T-135 in CL4) which affect ABA perception and distinguish AtPYL13 from other AtPYLs [14] (data not shown). Hence, Bra025048 was designated as BrPYL11, and Bra013334 as BrPYL13. Chromosomal distribution analysis revealed that Bra013334 (located on A01) and Bra025048 (located on A06) were located in different chromosomes (Table 1). Thus, the tandem gene duplication observed on Arabidopsis chromosome 5 between AtPYL11 (At5g45860) and AtPYL12 (At5g45870) is absent in

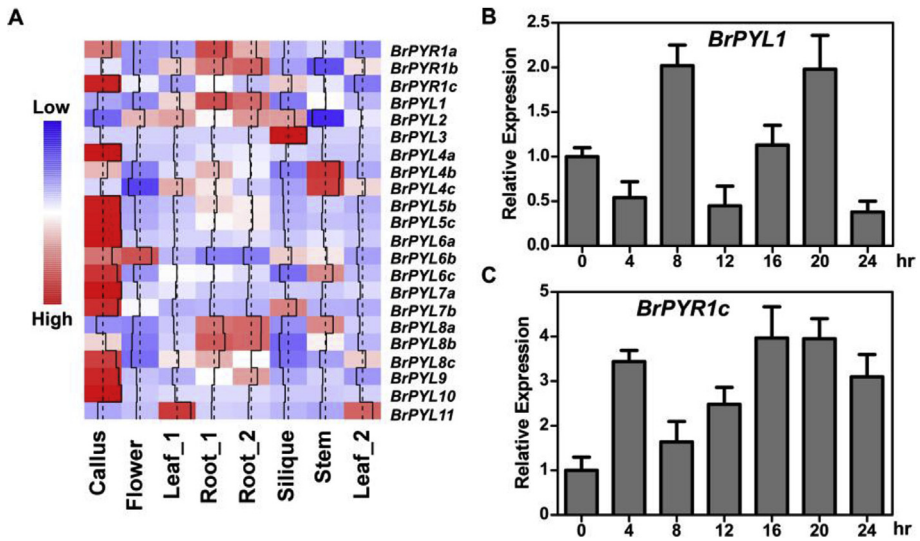

Fig. 2. Expression profile of *BrPYLs* genes in *Brassica rapa*.
(A) Tissue specific expression profile of *BrPYLs* genes. Expression levels are normalized according to the FPKM (fragments per kilobase of transcript per million fragments mapped reads) values and the FPKM values were log2 transformed as previously described [24]. A histogram displaying the transcript abundance of *BrPYL* genes was generated via R (www.r-project.org). Dashed vertical line indicates the average value of normalized expression levels of all *BrPYL* genes at each of the eight developmental stages, vertical trace line inside each column displays the expression level of individual *BrPYL* gene. (B) qRT-PCR analysis of the expression pattern of *BrPYL1* and *BrPYR1c* genes in response to ABA treatment. Eleven-day-old seedlings were treated with 0.1 mM ABA followed by sampling at 0, 4, 8, 12, 16, 20 and 24 h. The relative expression of *BrPYL1* and *BrPYR1c* genes was normalized to the expression of the cabbage *ACTIN2* gene (*BrACTIN2*/Bra037560) and expressed relative to the level in mock-treated seedlings.

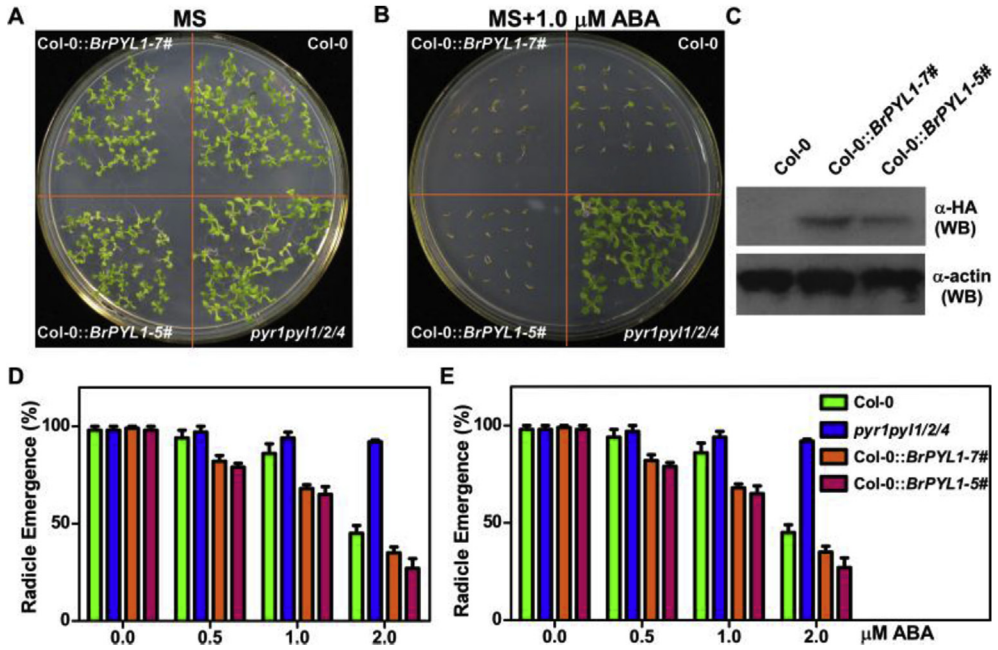

Fig. 3. Overexpression of *BrPYL1* confers ABA hypersensitivity to Arabidopsis during seed germination.
(A, B) Sensitivity of seed germination to ABA. The seeds of Col-0, *pyr1pyl1/2/4*, and transgenic lines carrying HA-tagged *BrPYL1* (Col-0:: *BrPYL1*) were germinated on MS medium (A) and MS medium supplemented with 1.0 μM ABA (B). (C) Immunoblots of HA-BrPYL1 protein levels in the transgenic lines (Col-0:: *BrPYL1*). The anti-actin based immunoblot served as a loading control. The emergence rate of radicle (D) and green cotyledons (E) from Col-0, *pyr1pyl1/2/4*, and Col-0:: *BrPYL1* transgenic seeds plated on MS supplemented with ABA. Approximately 100 seeds were used in each experiment. Error bars represent SD (seed number > 100). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Brassica rapa.

3.2. Gene structure analysis of BrPYLs

To gain a more detailed insight into the phylogenetic relationship between BrPYLs and AtPYLs, we investigated their overall intron/exon profile. Nearly all the BrPYLs displayed a similar intron/exon structure as their Arabidopsis orthologues, except BrPYR1a (Bra021032) which has an intron insertion in the PYR_PYL_RCAR-like region compared with AtPYR1 (Fig. 1).

3.3. Expression profiles of BrPYLs

To determine the expression profile of *BrPYLs*, we compared their transcript abundance in different tissues (callus, flower, leaf, stem, root and silique) by analysing the RNA-seq data from the GEO database [24]. Nearly all the *BrPYLs* are highly expressed in at least one tissue, except *BrPYL5a* (Bra028758) and *BrPYL13* (Bra013334) (Fig. 2A). Previous study revealed that ABA inhibited or attenuated the gene expression of many *AtPYLs* of subclass III [30]. To examine whether similar repressing effect occurred, we determined the expression profile of two subclass III members, *BrPYL1* (Bra024995) and *BrPYR1c* (Bra012635), in response to ABA stimulation. We did not observe either repression or stimulation of *BrPYL1* by ABA (no more than a 2-fold increase or decrease, Fig. 2B). In contrast, ABA induced *BrPYR1c* expression (more than 2-fold) after 4 h, which subsequently decreased at 8 h and persisted up to 24 h (Fig. 2C).

3.4. Overexpression of BrPYL1 in Col-0 confers hypersensitivity to ABA

To determine whether BrPYLs also participate in the ABA response, we further investigated BrPYL1, which is closely clustered with AtPYL1 (Fig. 1A). We generated Col-0 transgenic lines containing 35S-3HA-BrPYL1 which ubiquitously express HA-tagged BrPYL1. We examined how ABA treatment affected seed germination in these transgenic lines. The transgenic lines expressing 3HA-BrPYL1 were hypersensitive to ABA treatment compared to the quadruple mutant *pyr1pyl124* and the parental Col-0, and displayed delayed seed germination (Fig. 3A–B). We also determined the germination frequencies (green cotyledon and radicle emergence ratios) of these plant lines exposed to different concentrations of ABA. Again, 3HA-BrPYL1 transgenic plants displayed a similar ABA hypersensitivity compared to the quadruple mutant and parental lines (Fig. 3D–E). These findings indicate that BrPYL1, like AtPYL1, positively regulates the inhibition of seed germination by ABA.

3.5. BrPYL1 promotes ABA-induced RD29B-LUC expression in protoplasts

The ABA signalling pathways of Arabidopsis and rice have been successfully reconstituted *in vitro* via transient expression of core components in protoplasts [29,31]. The core signalling pathway includes PYL, the PP2C phosphatase ABI1, the kinase SnRK2.6 and the transcription factor ABF2. Because only a few ABA signalling components are known in *Brassica rapa* [22], we attempted to reconstitute the ABA signalling pathway via substituting AtPYL1 with BrPYL1. As previously reported [14,29], protoplasts expressing AtABF2 and AtSnRK2.6, but not AtABF2 alone, displayed ABA-dependent induction of *RD29B-LUC* (Fig. 4, plasmid combinations 1 and 2). In contrast, ABA did not induce *RD29B-LUC* in protoplasts expressing AtABI1, AtABF2 and AtSnRK2.6 (Fig. 4, plasmid combination 3). Importantly, protoplasts expressing BrPYL1 or AtPYL1 together with AtABI1, AtSnRK2.6 and AtABF2 displayed ABA-dependent induction of *RD29B-LUC* (Fig. 4, plasmid combinations 4 and 5). Thus, BrPYL1 is as sufficient as AtPYL1 to reconstitute the ABA signalling pathway and ABA-dependent induction of *RD29B-LUC* by ABF2.

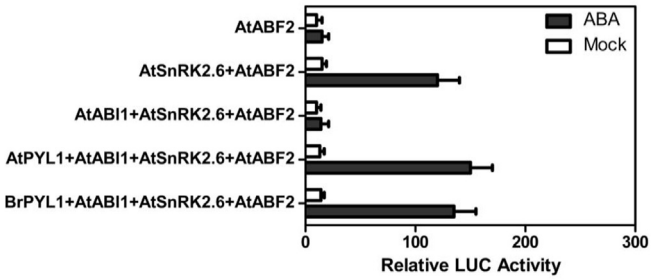

Fig. 4. BrPYL1 can induce ABA-dependent expression of *RD29B-LUC* by AtABF2 in protoplasts. Protoplasts from Col-0 wild-type plants were used for transfection. *RD29B::LUC* and *35S::rLUC* (*LUC* gene from *Renilla reniformis*, also known as sea pansy) were used as the ABA responsive reporter and internal control, respectively. Protoplasts were incubated for 5 h in the absence (open column) or presence (fixed column) of 5 μM ABA after transfection. Error bars indicate SEM (n = 3).

In the current study, we identified 24 BrPYLs in *Brassica rapa* and verified BrPYL1 as a putative ABA receptor. We observed gene copy number variations between BrPYLs and AtPYLs (24 versus 14). Taken together, our data support a co-linear relationship between *Brassica rapa* and *Arabidopsis thaliana* with respect to early ABA perception. Further investigation will illuminate the biological significance of BrPYLs in both ABA-dependent and -independent physiological responses.

Conflict of interest

The authors declare no conflict of interest.

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