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Genome-wide identification of trihelix transcription factor family genes in pear (*Pyrus bretschneideri*) and functional characterization of *PbrGT15* in black spot resistance

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Abstract

Pear (*Pyrus bretschneideri*), a valuable widely cultivated fruit, faces significant economic losses due to black spot disease caused by *Alternaria alternata* (Fr.) Keissl. Trihelix transcription factors (TFs) are crucial in regulating plant defense and autoimmunity. This study aimed to analyze the trihelix transcription factor (GT) genes within pear through genome-wide identification, phylogenetic, gene structure, synteny, and *cis*-acting elements analyses. Among the 31 trihelix genes, 28 were on 12 known chromosomes, while the remaining 3 were located on unknown chromosomes. These genes were categorized into five clades: SIP1, GT γ , GT1, GT2 and SH4, containing 7, 2, 9, 11 and 2 genes, respectively. Synteny analysis indicated eight duplicated gene pairs. Based on the expression pattern of *PbGT* genes in seven tissues from the database, the *PbGT* genes of the GT2 clade were selected for further investigation. The quantitative reverse transcriptase–polymerase chain reaction confirmed that *PbrGT5*, *PbrGT6*, *PbrGT15* and *PbrGT16* correlated with black spot disease resistance. Notably, the salicylic acid (SA) treatment significantly upregulated the expression levels of *PbrGT10*, *PbrGT13*, *PbrGT15* and *PbrGT23*. Among these, *PbrGT15* showed the highest induction to both SA and black spot infection. Subcellular localization demonstrated that *PbrGT15* functions as a nuclear protein. Virus-induced gene silencing of *PbrGT15* increased pear plants' susceptibility to black spot disease, indicating its pivotal role in enhancing resistance. These results indicated that *PbrGT15* positively regulated black spot disease resistance in pears.

Keywords Trihelix family, Disease, Resistant, Black spot, Salicylic acid

Introduction

Plants face various biotic and abiotic stresses during their growth. Biotic stress primarily arising from pathogenic sources (bacteria, fungi and viruses) adversely impacts yield and quality (Bhar et al. 2022). Signal transduction interacting with biotic stress has been shown to bolster stress tolerance (Peck et al. 2020). Plants integrate diverse signals using transcription factors (TFs) from various families, including v-myb avian myeloblastosis viral (MYBs) and dehydration responsive element binding proteins (DREBs) (Zandalinas et al. 2020). TFs play a crucial role in plant growth, development and

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stress responses (Osorio et al. 2012). Over 60 TF families have been classified based on their DNA-binding domains (Riechmann et al. 2000). The trihelix TFs derive their name from their helix-loop-helix-loop-helix structure in the DNA-binding domain and are also known as trihelix transcription factor (GT) due to their strong affinity for GT elements (Kaplan-Levy et al. 2012; Qin et al. 2014).

The GT family, relatively understudied since the 1990s, has recently regained interest. It encompasses genes from various plants, with *Arabidopsis* and *Oryza sativa* each containing 30 and 31 characterized GT genes, respectively. The GT genes have been divided into five clades: GT1, GT2, SH4, GT γ and SIP1 (Kaplan-Levy et al. 2012). The name of each branch is based on the name of the first gene that has been identified (Gao et al. 2009). Additionally, 63 GT genes are also found in soybean (Song et al. 2016), 20 in chrysanthemum (Song et al. 2016), 59 in maize (Du et al. 2016), 36 in tomato (Yu et al. 2015) and 94 in wheat (Xiao et al. 2019). These GT genes (Osorio et al. 2012) are involved in plant development, including flowers (Brewer et al. 2004), leaves (Li et al. 2008), stomata (Yoo et al. 2010), embryogenesis (Willmann et al. 2011) and seeds (Lin et al. 2007), respond to stresses. For instance, the GT2-like 1 (GTL1) factor in *Arabidopsis* regulates bacteria-triggered immunity (Völz et al. 2018). Paired GT factors ASR3/AITF1 in *Arabidopsis* regulate plant defense and autoimmunity (Wang et al. 2023). In *Arabidopsis*, the GT3a and GT3b of GT1 clade genes reportedly responded to salt and pathogen stress, mirroring findings in soybean (Ayadi et al. 2004; Park et al. 2004). Furthermore, *OsRML1*, a rice GT factor, displays increased expression in seedlings post-fungal blast infection (Wang et al. 2004). Notably, GhGT23 and GhGT26 in cotton improve salinity tolerance (Li et al. 2022; Li et al. 2023), while GhGT3b_A04 enhances resistance to *Verticillium dahliae* in cotton (Mao et al. 2023). The maize GT factor ZmGT3b is associated with the defense response against *Fusarium graminearum* (Zhang et al. 2021).

The pear, a *Rosaceae* family fruit, holds significant economic value with a rich cultivation history. Black spot, a devastating disease affecting pear cultivation and management globally, spreads *via* wind and rain, significantly impacting leaves, fruit and new shoots. This disease prompts premature leaf drop and fruit decay, thereby diminishing storage value, which consequently affects fruit yield and quality. However, limited studies have delved into the pear trihelix family, leaving the involvement of these genes in pear black spot uncertain. This study focuses on genome-wide identification, phylogenetic analysis, gene structure, synteny analysis, and

cis-acting elements of 31 GT genes in *Pyrus bretschneideri*. Transcriptome data was utilized to examine relative GT gene expression in tissues, particularly assessing the expression levels of GT2 clade genes in different resistant varieties (R-01 and S-01). Furthermore, this research elucidates the role of PbrGT15 in conferring black spot disease resistance using virus-induced gene silencing (VIGS).

Materials and methods

Plant materials and treatment

Leaves of *P. bretschneideri* ('Xiangmianli', R-01) and *P. sinkiangensis* ('Hongjuli', S-01) from an orchard (Baima, Nanjing, China) were pierced with sterile needles and inoculated with *Alternaria alternata* at the pinholes. RNA was extracted from these leaves after 0, 3, 12, 48 and 72 h. Subsequently, 45-day-old *P. betulaefolia* seedlings underwent salicylic acid (SA) treatment or VIGS at 25°C under a 16-h day/8-h night cycle. The plants were sprayed with 50 mL of 0.2 mM SA solution or water distilled. The true leaf of each plant was sampled with three biological replicates at 0, 6, 12, 24, 48 and 72 h, and stored at −80°C.

Identification of PbrGT genes in pear

A protein database of Chinese white pear was established (Wu et al. 2013). The trihelix protein sequences were downloaded from the TAIR database (<http://www.arabidopsis.org>) and the rice genome (<http://rice.plantbiology.msu.edu/>). A local BLASTP with an e-value of 10^{-5} and a 50% homology threshold was used to screen the pear local protein database. Subsequently, the Hidden Markov Model (HMM) was constructed using HMMER 3.0 (<http://hmmer.org/>) based on the trihelix protein sequence alignment results of *Arabidopsis* and rice, then searched in the local database. The hits containing the PF13837 (Myb/SANT-LIKE domain) were compared with the BLASTP results, and the overlapping genes were considered to be the PbrGT genes.

The ID, location, length, theoretical isoelectric point (pI) and molecular weight (MW) of PbrGT genes were determined using ExPASy (http://web.expasy.org/compute_pi/).

Phylogenetic analysis

Arabidopsis, rice and pear trihelix protein sequences were aligned with ClustalX 2.0 (Thompson et al. 1997). The unrooted trees were constructed with MEGA 7.0 using the neighbor-joining (NJ) method, repeated 1000 times with the bootstrap test.

Syntenic analysis of PbrGT genes

The syntenic chains and duplication mode of the trihelix gene family were identified using MCScanX (Qiao

et al. 2015). The figure was generated using TBtools. The nonsynonymous (Ka) and synonymous (Ks) substitution ratio was calculated using the KaKs_Calculator 2.0 (Wang et al. 2010).

Gene structure construction, motif and *cis*-acting element identification

The exon/intron structure was obtained from GSDS 2.0 (<http://gsds.gao-lab.org/>) (Hu et al. 2015). The conserved protein motifs were analyzed using MEME (<http://meme-suite.org/tools/meme>) (Bailey et al. 2006).

The *cis*-acting elements in the 2 kbp upstream region of the genes were explored using the PlantCARE database (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>). The graphic was output using TBtools.

Tissue-specific expression of *PbrGT* genes based on transcriptome data

Using RNA-seq data of *P. bretschneideri* ‘Dangshansuli’ pear (Li et al. 2019), the expression of *PbrGT* genes was examined in seven pear tissues: leaf, fruit, petal, sepal, ovary, stem and bud, obtained from *P. bretschneideri* ‘Dangshansuli’ grown naturally. TBtools displayed the expression pattern using the value $\log_2$ (RPKM + 1) (Reads Per Kilobase of exon model per Million mapped reads) (Chen et al. 2020).

Expression pattern analysis by quantitative reverse transcriptase–polymerase chain reaction

The RNA samples were extracted using the Total RNA Kit (Foregene, Chengdu, China). Reverse transcription

Table 1 The *PbrGT* gene family information

Gene Name	Gene ID	Chromosomal localization	Gene length (bp)	Amino acid length	pI	MW (kDa)	Duplicate type
<i>PbrGT1</i>	Pbr018422.1	Chr1: 2630537–2632766	2230	244	9.16	39.94	Dispersed
<i>PbrGT2</i>	Pbr009547.1	Chr1: 7171343–7172086	744	173	9.27	27.90	WGD or segmental
<i>PbrGT3</i>	Pbr015653.1	Chr2: 5938554–5940352	1799	189	9.57	30.08	Dispersed
<i>PbrGT4</i>	Pbr010533.1	Chr5: 3191994–3194196	2203	299	6.06	51.38	WGD or segmental
<i>PbrGT5</i>	Pbr010534.1	Chr5: 3209966–3216320	6355	416	5.72	73.92	WGD or segmental
<i>PbrGT6</i>	Pbr010535.1	Chr5: 3233085–3235516	2432	377	6.60	64.69	Tandem
<i>PbrGT7</i>	Pbr017741.1	Chr6: 656360–663485	7126	624	7.86	102.97	WGD or segmental
<i>PbrGT8</i>	Pbr017747.1	Chr6: 730312–737437	7126	624	7.86	102.97	WGD or segmental
<i>PbrGT9</i>	Pbr038634.1	Chr6: 6812455–6817486	5032	498	5.65	82.46	WGD or segmental
<i>PbrGT10</i>	Pbr027610.1	Chr7: 3252473–3255479	3007	381	5.76	65.15	Dispersed
<i>PbrGT11</i>	Pbr024974.1	Chr8: 14388914–14389747	834	183	5.11	31.20	Dispersed
<i>PbrGT12</i>	Pbr021399.1	Chr10: 2390895–2392537	1643	319	9.18	50.92	WGD or segmental
<i>PbrGT13</i>	Pbr017590.1	Chr10: 7485551–7485967	417	92	9.30	16.06	Tandem
<i>PbrGT14</i>	Pbr036347.1	Chr10: 19162293–19164276	1984	298	6.10	51.60	WGD or segmental
<i>PbrGT15</i>	Pbr036348.1	Chr10: 19180580–19183637	3058	421	6.45	74.20	WGD or segmental
<i>PbrGT16</i>	Pbr036349.1	Chr10: 19196816–19199408	2593	347	8.73	61.13	Tandem
<i>PbrGT17</i>	Pbr004384.1	Chr12: 2197280–2198362	1083	246	8.79	40.64	Dispersed
<i>PbrGT18</i>	Pbr028877.1	Chr12: 2747789–2748785	997	148	9.18	26.53	WGD or segmental
<i>PbrGT19</i>	Pbr028880.1	Chr12: 2876022–2877018	997	148	9.18	26.53	Dispersed
<i>PbrGT20</i>	Pbr025445.1	Chr12: 11593544–11595698	2155	217	8.58	32.80	Dispersed
<i>PbrGT21</i>	Pbr010171.1	Chr14: 12521149–12524810	3662	405	5.57	71.99	WGD or segmental
<i>PbrGT22</i>	Pbr036910.1	Chr14: 13723668–13730972	7305	599	7.88	98.67	WGD or segmental
<i>PbrGT23</i>	Pbr019905.1	Chr15: 6109655–6111457	1803	345	5.77	63.83	WGD or segmental
<i>PbrGT24</i>	Pbr036250.1	Chr16: 800969–804447	3479	288	5.95	48.87	Dispersed
<i>PbrGT25</i>	Pbr037852.1	Chr16: 4904037–4906426	2390	224	5.62	38.36	WGD or segmental
<i>PbrGT26</i>	Pbr022283.1	Chr17: 14594021–14602779	8759	412	8.34	63.32	WGD or segmental
<i>PbrGT27</i>	Pbr012489.1	Chr17: 22968517–22971292	2776	293	5.54	46.54	WGD or segmental
<i>PbrGT28</i>	Pbr014466.2	Chr17: 25029705–25031870	2166	305	5.48	48.94	WGD or segmental
<i>PbrGT29</i>	Pbr027131.1	scaffold440.0: 7493–11225	3733	259	8.96	37.03	Dispersed
<i>PbrGT30</i>	Pbr027134.1	scaffold440.0: 20371–24103	3733	259	8.96	37.03	Dispersed
<i>PbrGT31</i>	Pbr041014.1	scaffold918.0: 139355–146119	6766	624	7.60	102.90	WGD or segmental

PbrGT pear trihelix transcription factor family gene, *WGD* whole-genome duplication, *pI* isoelectric point, *MW* molecular weight

utilized the HiScript III RT SuperMix (+gDNA wiper) (Vazyme, Nanjing, China). Primers, designed *via* Primer Premier 6.0 (Table S1), were employed. Quantitative reverse transcriptase–polymerase chain reaction (qRT–PCR) was conducted in a 20 μ L SYBR Green reaction (Synergy Brands) on a Roche LightCycler 480 instrument (Roche, Basel, Switzerland) with the following protocol: 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 20 s. The *TUB* gene was considered as the reference (Chen

et al. 2015), and the expression profiles were calculated *via* the $2^{-\Delta\Delta C_t}$ value (Livak and Schmittgen 2001). Each sample was repeated thrice.

Subcellular localization of PbrGT15

The coding sequence (CDS) without a stop codon of *PbrGT15* was cloned. The fusion plasmid 35S:*PbrGT15*:GFP was transfected into *Agrobacterium tumefaciens* GV3101 using freeze–thawing, then cultured

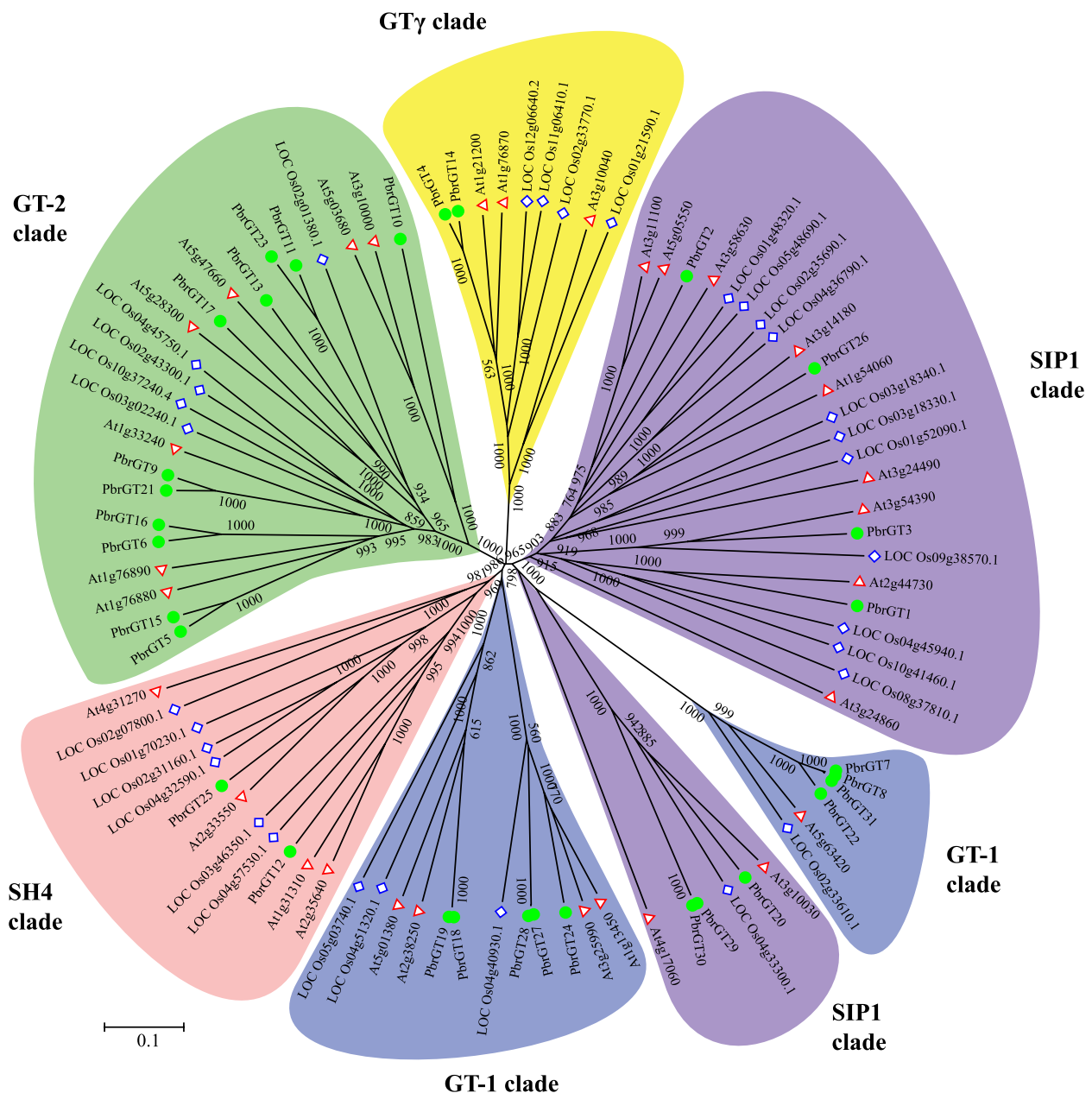


Fig. 1 Phylogenetic tree of trihelix transcription factor (GT) genes. The pear, *Arabidopsis* and rice GT genes are marked with green circles, red triangles and blue diamonds, respectively. The groups are represented by different colors: GT-1 (blue), GT-2 (green), GT γ (yellow), SIP1 (purple) and SH4 (pink). *PbrGT*, pear trihelix transcription factor family gene

for approximately 24 h at 28°C. The bacterial suspensions were pressure-injected into tobacco leaves and observed after 48–72 h incubation at 25°C using a confocal laser scanning microscope (LSM800, Zeiss, Jena, Germany), with subsequent image capture.

VIGS

PbrGT15 DNA fragment was cloned and inserted into the *pTRV2* vector for *PbrGT15* silencing in pear (Gao and Shan 2013). GV3101 cells harboring *pTRV1*, *pTRV2* and

PbrGT15-TRV were cultured overnight at 28°C, then harvested (5500 g, 5 min). The OD₆₀₀ value was then adjusted to 0.8 with infiltration buffer MMA (10 mM MgCl₂, 10 mM 2-(4 morpholino)-ethane sulfonic acid, 150 μM acetosyringone, pH 5.7). A 1:1 ratio mixture of *pTRV1* and *PbrGT15-TRV*, and *pTRV1* and *pTRV2*, was used for seedling infection. The infected seedlings were cultured under a 16 h light/8 h dark cycle for 20 days after a dark incubation period. Subsequently, isolated leaves were inoculated with *A. alternata* and cultured in the dark for six days,

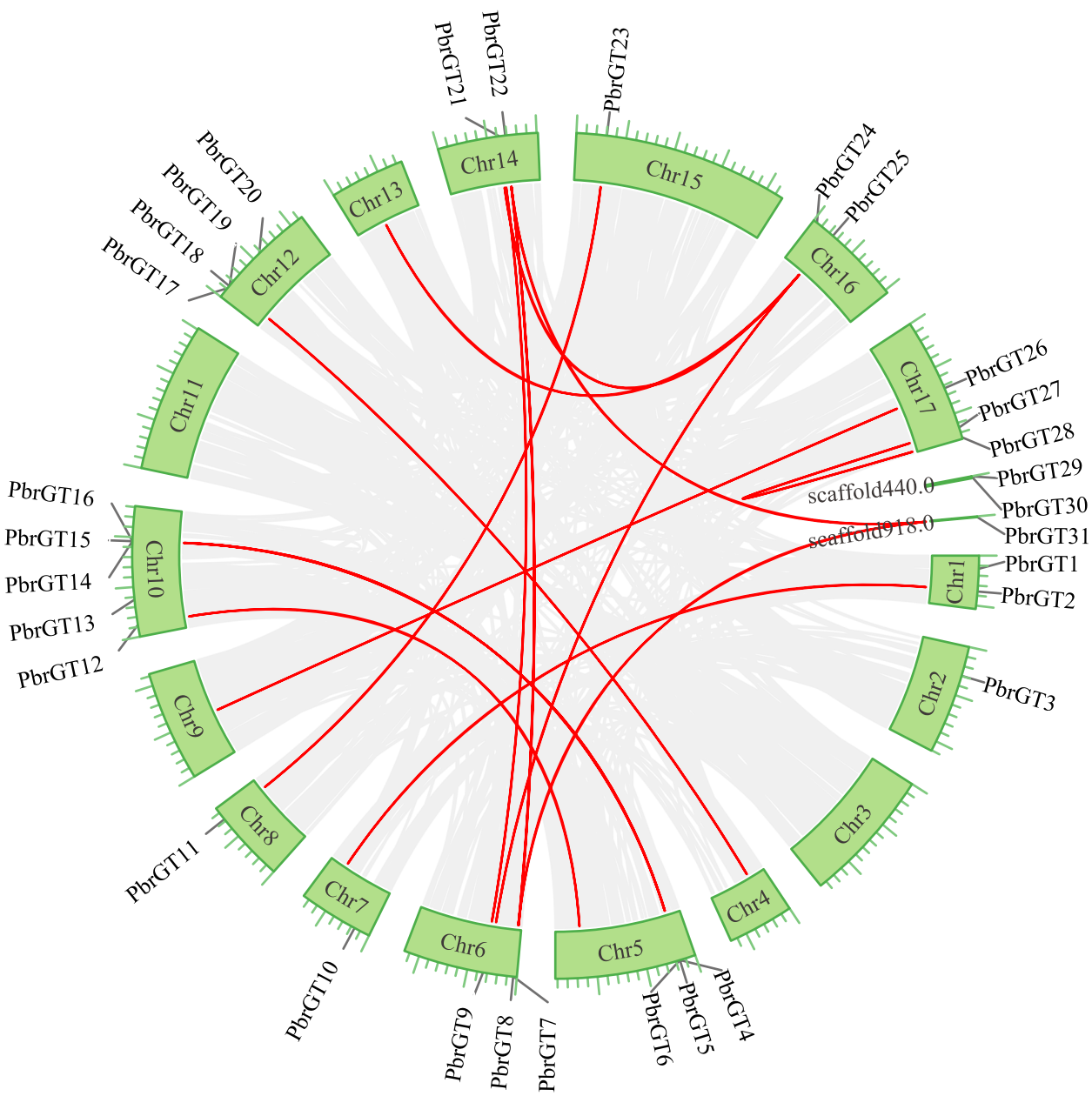


Fig. 2 Synteny of the *PbrGT* genes. *PbrGT* gene pairs are joined by a red line. The green box represents the chromosome, with the number being marked in the green box. *PbrGT*, pear trihelix transcription factor family gene; Chr, chromosome

followed by measuring the spot diameter. Electrolyte leakage (EL) was determined as described previously (Dahro et al. 2016). The enzyme activity, including phenylalanine ammonia-lyase (PAL), chitinase (CHI), catalase (CAT) and polyphenol oxidase (PPO) were measured using their respective test kits (Jiancheng, Nanjing, China).

Statistical analysis

Data were summarized using Microsoft Excel. Graph-Pad Prism 6 was used for data analysis and column chart creation.

Results

Identification and analysis of *PbrGT* genes in pear

From the pear protein databases, 31 *GT* genes were identified and labelled *PbrGT1* to *PbrGT31* based on their chromosomal locations (Table 1). Among these, 28 *PbrGT* genes are distributed across 12 distinct chromosomes, while the locations of other three *PbrGT* genes remain unknown. Notably, *PbrGT13* encoded the smallest protein (92 amino acids), whereas *PbrGT7/8/31* encoded the largest proteins, each comprising 624 amino acids. The pI of the *PbrGT* genes range from 5.11 to 9.57 and their MW ranged between 16.06 and 102.97 kDa.

Phylogenetic tree construction

To understand the phylogenetic relationships among *GT* proteins in *Arabidopsis*, rice and pear, we constructed an NJ phylogenetic tree using 30 amino acid sequences from *Arabidopsis*, 31 from rice (detailed in Table S2) and 30 from pear. Building upon prior research on *Arabidopsis* and rice (Kaplan-Levy et al. 2012), we categorized the 92 sequences into five distinct clades, encompassing 7, 2, 9, 11 and 2 pear *GT* proteins, respectively (Fig. 1).

Syntenic analysis

The pear genome exhibited diverse gene duplication events: 18 genes resulted from whole-genome duplication or segmental duplication (WGD or segmental), 10 from dispersed duplication and 3 from tandem duplication. Among these duplications, eight pairs were categorized as WGD or segmental genes (Table S3; Fig. 2). Notably, all duplicated genes demonstrated Ka/Ks values below 1, indicating strong purifying selection.

Gene structure, motif and *cis*-acting element analysis

The *PbrGT* proteins within the same branch showed analogous motif compositions and exon/intron structures, implying potential functional similarities. Using

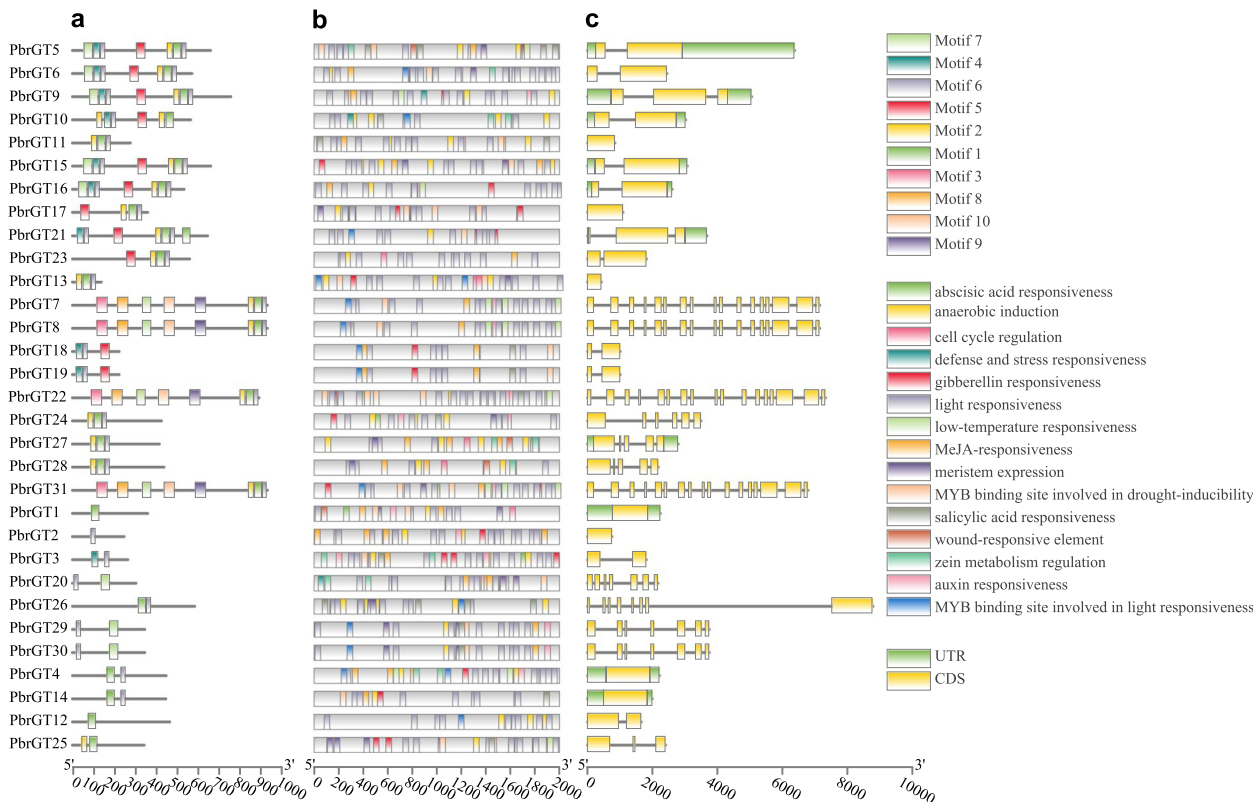


Fig. 3 *PbrGT* gene structures, *cis*-elements, and motif analysis. (a) Schematic diagram of the *PbrGT* gene motif. (b) *Cis*-acting elements of *PbrGT* genes. (c) CDS/UTR of *PbrGT* genes. *PbrGT*, pear trihelix transcription factor family gene; CDS, coding sequence; UTR, untranslated region

the MEME search tool, ten motifs were identified. Figure 3a shows the organizational patterns of these motifs within the PbrGT proteins. Motifs 1 and 6 were conserved across most PbrGT proteins, while some comprised only a single motif and others incorporated up to eight motifs. Detailed motif representations are shown in Figure S1. From the PlantCARE database, 37 *cis*-acting elements were identified. Figure 3b shows the further analysis of 15 stress, hormone-related and plant growth and development-related elements. Except for *PbrGT12*, all *PbrGT* genes incorporated *cis*-acting elements associated with various hormones, including abscisic acid (ABA), gibberellin (GA), SA, and methyl jasmonate (MeJA). Regarding gene structure, seven *PbrGT* genes (*PbrGT1/2/4/11/13/14/17*)

lacked introns, while 10 genes (*PbrGT3/5/6/10/12/15/18/19/23/26*) had a single intron. Additionally, four genes (*PbrGT7/8/22/31*) featured 17 introns, while the remaining genes contained between 2 and 7 introns, as shown in Fig. 3c.

Expression of *PbrGTs* in different tissues

The expression data for *PbrGT* genes across seven tissues were obtained from a previous study (Li et al. 2019). Utilizing the log2-transformed RPKM values, a heatmap (Fig. 4, Table S4) was generated. The majority of *PbrGT* genes displayed tissue-specific patterns: 16 genes were predominantly expressed in petal, calyx, stem, ovary and fruit; 18 in the bud and 15 in the leaf. A subset of 12 genes exhibited high expression across all tissues, while

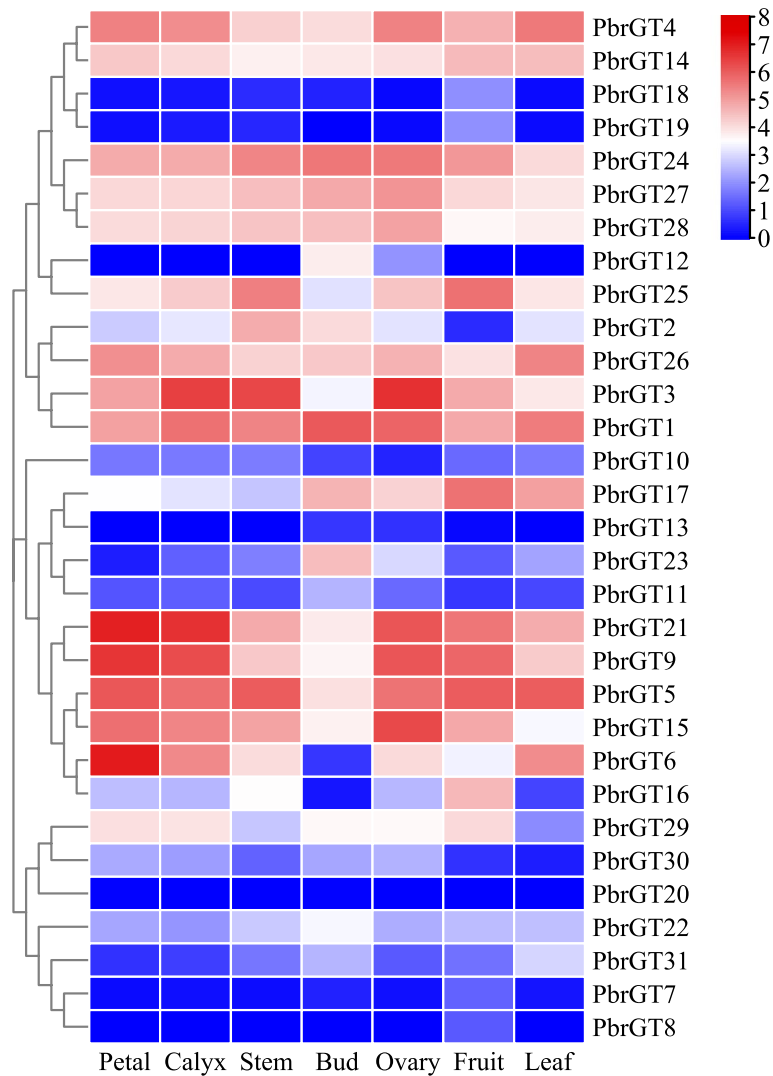


Fig. 4 Expression profiles of the *PbrGT* genes. Red indicates high expression levels, blue indicates relatively low expression levels. *PbrGT*, *pear trihelix transcription factor family gene*

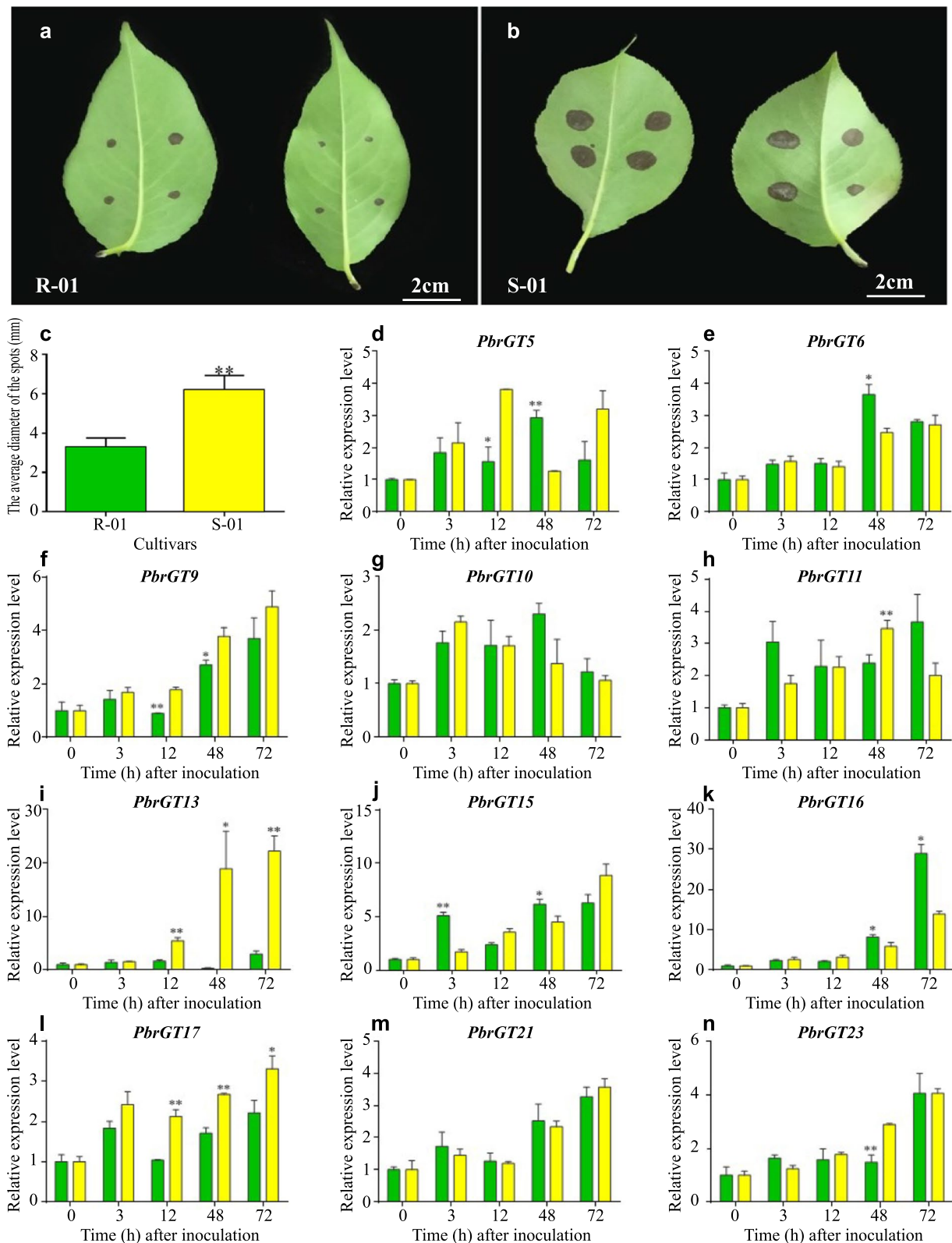


Fig. 5 Expression patterns of *PbrGT* genes in different cultivars. **(a)** Phenotype of R-01. Bars=2 cm. **(b)** Phenotype of S-01. Bars=2 cm. **(c)** Mean diameter of lesions (* $p < 0.05$; ** $p < 0.01$; $n = 12$). **(d)–(n)** Relative expression level: *PbrGT5*, *PbrGT6*, *PbrGT9*, *PbrGT10*, *PbrGT11*, *PbrGT13*, *PbrGT15*, *PbrGT16*, *PbrGT17*, *PbrGT21*, *PbrGT23*. (* $P < 0.05$; ** $P < 0.01$; $n = 3$). *PbrGT*, pear trihelix transcription factor family gene; R, resistant variety; S, susceptible variety

10 genes consistently exhibited low expression in all tissues. Interestingly, *PbrGT12*, *PbrGT22* and *PbrGT23* had limited expression outside the bud, thus suggesting their potential role in bud development.

Expression of *PbrGTs* in different cultivars

A marked difference was found in the leaf inoculation spot diameter between R-01 and S-01 (Fig. 5a-c). This discrepancy indicated differing resistance levels, with R-01 exhibiting superior resistance compared to S-01.

The GTL1, a GT2 clade member, enhances SA metabolism (Kaplan-Levy et al. 2012) and positively regulates

defense genes along with bacterial-triggered immunity (Völz et al. 2018). Therefore, the GT2 clade gene expression in R-01 and S-01 were analyzed using qRT-PCR (Fig. 5d-n).

In the resistant R-01 variety, significant expression level alterations were observed in 11 genes, all displaying bimodal patterns. An initial peak emerged after 3 h, with *PbrGT15* being notably higher in expression than in S-01. Subsequent peaks occurred at either after 48 or 72 h, wherein *PbrGT5*, *PbrGT6*, *PbrGT15* and *PbrGT16* displayed considerably elevated levels compared to S-01. These findings suggested that these four *PbrGT* genes were activated post-infection, crucial for

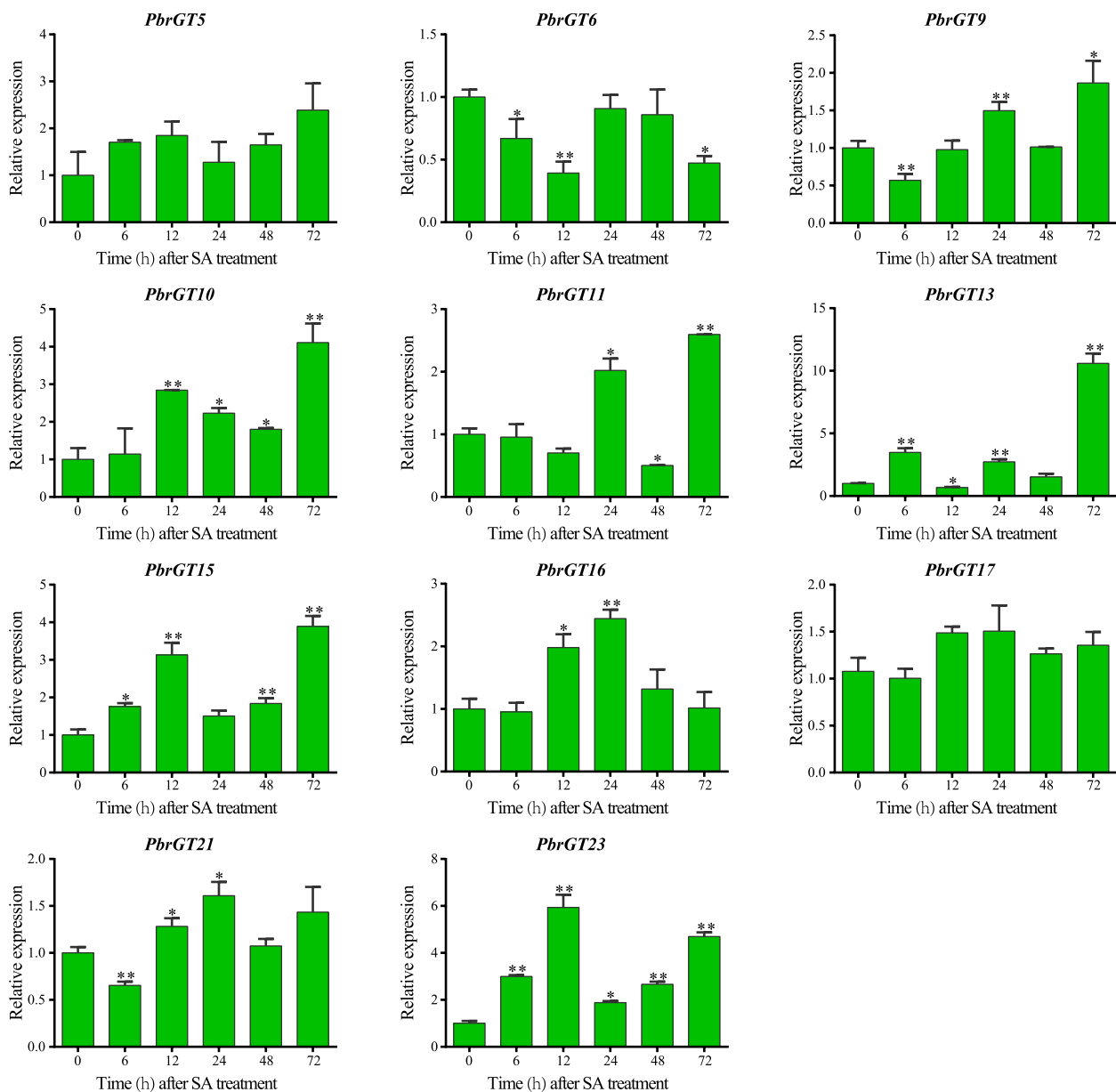


Fig. 6 Expression of *PbrGTs* under SA treatment. (* $P < 0.05$; ** $P < 0.01$; $n = 3$). *PbrGT*, pear trihelix transcription factor family gene; SA, salicylic acid

reinforcing disease resistance. Conversely, expression levels of *PbrGT9*, *PbrGT11*, *PbrGT13*, *PbrGT17* and *PbrGT23* were significantly subdued compared to S-01, indicating potential susceptibility correlations. The consistent expression patterns of *PbrGT10* and *PbrGT21* between R-01 and S-01 suggested that they might not directly impact resistance.

Expression profiles of *PbrGT* genes under SA treatment

The study employed qRT-PCR to assess GT2 clade gene expression post SA treatment. Significant expression variations were observed in 11 *PbrGT* genes (Fig. 6a–k), indicating a correlation between *PbrGT* genes and SA metabolism. *PbrGT5* and *PbrGT17* showed stable expression within 72 h post-SA spraying. Expression patterns for *PbrGT6*, *PbrGT9* and *PbrGT21* initially decreased after 6 or 12 h, then rose, and finally peaking values ~1–2 times higher than initial levels. In contrast, *PbrGT16* expression initially increased, peaking after 24 h before decreasing. The SA treatment notably induced the expression of *PbrGT10*, *PbrGT13*, *PbrGT15* and *PbrGT23*, displaying a bimodal pattern, with peak values 4–10 times their original levels.

Subcellular localization of PbrGT15 protein

The Plant-mPLOC tool deduced the subcellular localization of the PbrGT15 protein in the nucleus. Subsequently, the fusion protein PbrGT15::GFP was transiently expressed in tobacco leaves. As predicted, PbrGT15 was confirmed to be a nuclear-localized protein (Fig. 7).

Silencing *PbrGT15* by VIGS led to reduced disease resistance

The qRT-PCR analysis indicated sustained activation of *PbrGT15* post-infection, a key gene in pear black spot resistance. *PbrGT15* from the GT2 clade was further tested via VIGS. After 20 days, leaves from both VIGS-treated and control groups were collected. After six days post-inoculation, the experimental group displayed notably larger lesions than the control (Fig. 8a and b). The transcript abundance of *PbrGT15* in silenced plants (TRV-3/5/6) was reduced by 60–80% compared to the control group (TRV) (Fig. 8c and d). EL in TRV-3/5/6 leaves was significantly higher than in TRV leaves (Fig. 8e). Additionally, the PAL, CHI, CAT and PPO activities were notably reduced (Figs. 8f–i). These results emphasize *PbrGT15*'s role in enhancing black spot resistance. Moreover, the expression levels of defense-related genes, *PbrPR1* and *PbrNPR1*, in the VIGS-treated plants were significantly lower than those in the control plants (Fig. 8j and k).

Discussion

Biotic stress reduces yield or quality in plants, prompting the activation of defense systems, one of which involves integrating various signals through transcription networks using TFs (Zandalinas et al. 2020). The trihelix TFs are pivotal in regulating developmental processes and stress responses. However, the pear trihelix gene family remains understudied.

This study identified and systematically named 31 pear members based on chromosomal positions, following naming conventions similar to the *GT* family in *Arabidopsis* and

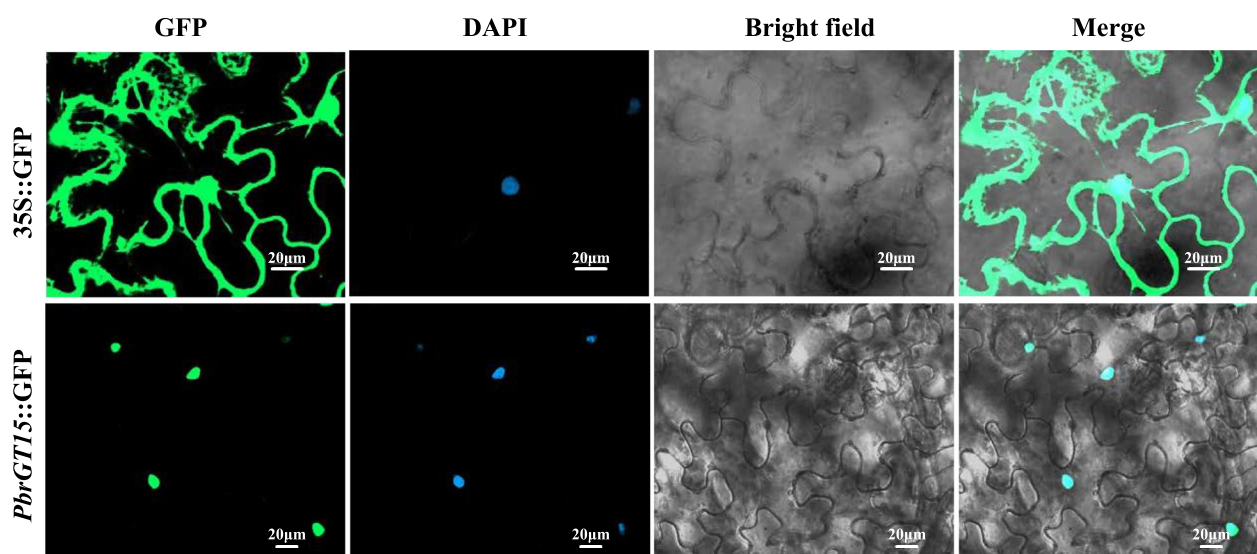


Fig. 7 Subcellular localization of PbrGT15. From left to right: GFP images, DAPI staining images, bright-field images and merged images; Bars = 20 μ m. *PbrGT*, pear trihelix transcription factor family gene; GFP, green fluorescence; DAPI, 4',6'-diamidino-2-phenylindole

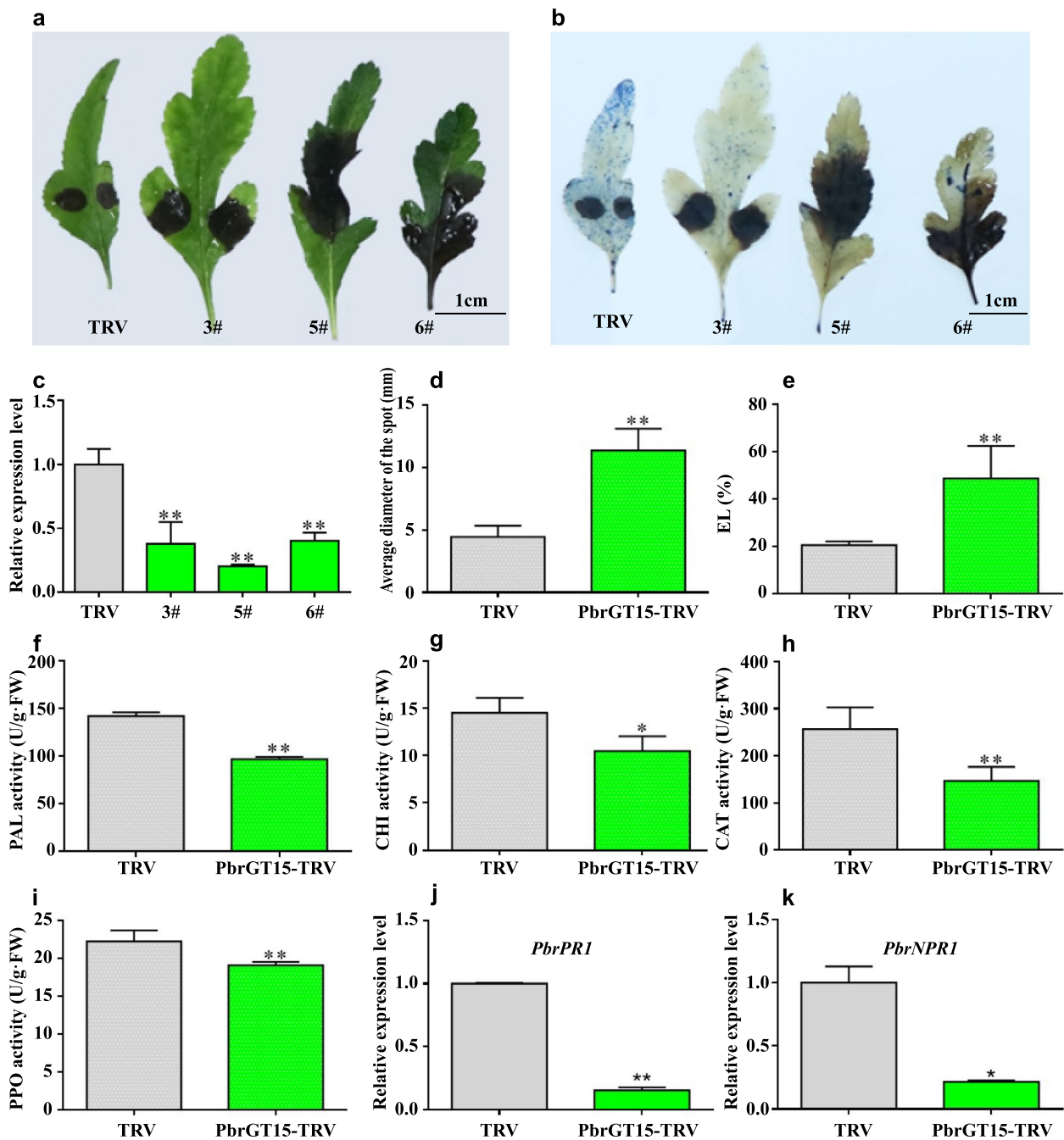


Fig. 8 Silencing of *PbrGT15*. (a) Inoculated leaf phenotype. (b) Trypan blue-dyed leaves. (c) Expression of *PbrGT15*. (d) Mean diameter of lesions. (e) EL (%) value. (f) PAL enzyme activity. (g) CHI enzyme activity. (h) CAT enzyme activity. (i) PPO enzyme activity. (j) Relative expression of *PbrPR1*. (k) Relative expression of *PbrNPR1*. (*, $P < 0.05$; **, $P < 0.01$; $n = 3-12$). TRV, tobacco rattle virus; EL, electrolyte leakage; PAL, phenylalanine ammonia-lyase; CHI, chitinase; CAT, catalase; PPO, polyphenol oxidase; *PbrPR1*, pear pathogenesis-related protein 1; *PbrNPR1*, nonexpressor of pathogenesis-related protein 1

rice. The count of pear *GT* genes closely resembled that of *Arabidopsis* and rice. Phylogenetic analysis categorized *PbrGTs* into five subfamilies: GT1, GT2, SH4, SIP1 and GT γ (Gao et al. 2009). Gene duplications in pear involved whole-genome duplication, segmental duplication, dispersed duplication and

tandem duplication. WGD or segmental duplication primarily contributed to *PbrGT* gene amplification. Eight pairs of duplicated genes were identified, all originating from WGD or segmental genes. Analysis of Ka/Ks values indicated that the *GT* genes underwent rigorous purifying selection.

Phylogenetically clustered genes often share similar motifs, implying the conservation of these motifs' importance in specific group functionalities. Promoter *cis*-elements are pivotal in stress responses (Fujita et al. 2006). The analysis revealed numerous *cis*-acting elements associated with hormones like ABA, auxin, GA, MeJA and SA within the *GT* gene family. Following SA treatment, several genes showed significant expression changes, as confirmed by qRT-PCR.

From the RNA-seq dataset analysis unveiled diverse expression patterns among *GT* genes across different tissues. A total of 12 genes displayed consistently high ubiquitous expression levels across all tissues, while 10 genes were consistently low-expressed. Moreover, a subset of 15 genes demonstrated increased expression in leaves, thereby suggesting a potential association with leaf functionality.

Multiple trihelix TFs, such as ASR3/AITF1 (Wang et al. 2023), GT3a, GT3b in *Arabidopsis* (Ayadi et al. 2004; Park et al. 2004), OsRML1 in rice (Wang et al. 2004), GhGT3b_A04 in cotton, and ZmGT3b in maize (Zhang et al. 2021), play roles in defense responses against biotic stress. GTL1, a member of the GT2 subfamily in *Arabidopsis*, is integral to the MPK4 signaling cascade, essential for plant immunity and SA homeostasis (Völz et al. 2018). SA, a pivotal plant hormone for pathogen resistance (Wang et al. 2020; Malamy et al. 1990; Gaffney et al. 1993), induces systemic acquired resistance (SAR) associated with upregulated pathogenesis-related (PR) genes (Zhou and Zhang 2020). Genes encoding PR proteins and enzymes are upregulated during pathogen invasion (Kachroo et al. 2020; Jia et al. 2023). Endogenous SA acts as a signaling molecule inducing SAR, while externally applied SA enhances plant resistance to various biotic and abiotic stresses (Vlot et al. 2009). Based on the constructed phylogenetic tree, 11 *GT* genes were identified within the GT2 subfamily. *PbrGT5*, *PbrGT6*, *PbrGT15* and *PbrGT16* showed markedly elevated expression compared to the susceptible cultivar S-01, indicating their rapid activation post-infection and crucial role in disease resistance. Furthermore, SA treatment significantly induced the expression of *PbrGT10*, *PbrGT13*, *PbrGT15* and *PbrGT23*, suggesting their swift activation following SA exposure.

The qRT-PCR findings indicated a swift response of the *PbrGT15* gene in disease-resistant pear varieties after SA treatment. Furthermore, its membership in the pear GT2 subfamily suggests its potential role in regulating black spot resistance. This prompted a detailed analysis of *PbrGT15*. The VIGS results further confirmed its proactive role in enhancing pear resistance to black spot disease. Subsequent qRT-PCR analyses demonstrated

higher *PbrGT15* expression in resistant varieties compared to susceptible ones. Moreover, since disease resistance reduced post-gene silencing, it highlighted a positive correlation between *PbrGT15* and black spot resistance.

Conclusion

This study analyzed the expression of pear *GT* genes across different cultivars post-SA treatment, and utilized VIGS to knock out specific genes, confirming that *PbrGT15* was pivotal in enhancing pear black spot resistance.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-023-00018-z>.

Additional file 1: Figure S1. The ten motifs of pear trihelix transcription factor family gene (*PbrGT*) detected by the online tool MEME.

Additional file 2: Table S1. Primer sequences designed using Primer Premier 6.

Additional file 3: Table S2. Overview of the *GT* genes in *Arabidopsis* and rice.

Additional file 4: Table S3. Ka/Ks and duplicate type analysis for the paralogous gene pairs in *PbrGT* genes.

Additional file 5: Table S4. Transcriptome data of *PbrGT* genes in various tissues.

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Not applicable.

Authors' contributions

SZ and XH designed experiments. ZZ and HX performed all bioinformatics analysis. ZZ performed all molecular biology experiments. ZZ, XX, XC and TL analyzed all data. SZ, XH and ZZ wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

Date will be shared upon request by the readers.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests, no financial or nonfinancial interests.

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