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RESEARCH ARTICLE

Genome-wide analysis of salicylic acid and jasmonic acid signalling marker gene families in wheat

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ABSTRACT

- Jasmonic acid (JA) and salicylic acid (SA) phytohormone pathways are important regulators of stress tolerance. Knowledge regarding the diversity, phylogeny and functionality of wheat genes involved in JA and SA response is limited.
- Using *Arabidopsis*, rice and wheat genomic and wheat disease transcriptomic data, we deduced the size, phylogenetic diversity and pathogen-responsiveness of seven hormone-responsive gene families, and thus selected 14 candidates as potential hormone responsive gene markers. Gene-specific expression studies assessed the impact of exogenous JA and SA on their transcriptional activation in leaves of two distinct wheat cultivars. RNAseq data were interrogated to assess their disease responsiveness and tissue-specific expression.
- This study elucidated the number, phylogeny and pathogen-responsiveness of wheat genes from seven families, including 12 *TaAOS*, 6 *TaJAMyb*, 256 *TaWRKY* group III, 85 *TaPR1*, 205 *TaPR2*, 76 *TaPR3* and 124 *TaPR5*. This included the first description of the wheat *AOS*, *JAMyb*, *PR2*, *PR3* and *PR5* gene families. Gene expression studies delineated *TaAOS1-5B* and *TaJAMyb-4A* as JA-responsive in leaves, but not significantly responsive to SA treatment, while *TaWRKY45-B* was a SA- but not a JA-responsive marker. Other candidate genes were either unresponsive or non-specific to SA or JA.
- Our findings highlight that all seven gene families are greatly expanded in wheat as compared to other plants (up to 7.6-fold expansion), and demonstrate disparity in the response to biotic stress between some homoeologous and paralogous sequences within these families. The SA- and JA-responsive marker genes identified herein will prove useful tools to monitor these signalling pathways in wheat.

INTRODUCTION

The phytohormones salicylic acid (SA) and jasmonic acid (JA) have been widely studied for their roles in plant defence against pathogens (Aerts *et al.* 2021). Typically, SA is associated with systemic acquired resistance and resistance against pathogens that feed on living tissue (biotrophs and hemibiotrophs), while JA is associated with induced systemic resistance and resistance against pathogens that feed on dead tissue (necrotrophs). The perception of those phytohormones leads to the regulation of downstream signalling pathways and to the expression of genes that enhance disease resistance, including pathogenesis-related (PR) genes. Expression profiling of hormone-responsive marker genes provides information about the strength and timing of phytohormone signalling activation (Kouzai *et al.* 2016). Markers for testing the activation of JA and SA signalling pathways are well established in the eudicot model plant *Arabidopsis thaliana* (Table S1; Li *et al.* 2004; Schmitz *et al.* 2010; Singh *et al.* 2017; Wu *et al.* 2019). *Arabidopsis* exhibits all of the major defence responses described in other plants, including phytohormone signalling pathways, and thus *Arabidopsis*–pathogen interaction studies have led to a better understanding of plant immunity.

In *Arabidopsis*, *PR3*, *PR4* and *PDF1.2* genes are well-established as transcriptionally-responsive JA-responsive markers. However, the defence-associated plant defensin 1.2 (*PDF1.2*) and the defence-associated *PR4* were also used as markers of the ethylene pathway (Li *et al.* 2004; Poncini *et al.* 2017). PR3 proteins are chitinases from classes I, II, IV, VI and VII, belonging to the glycoside hydrolase family 19 (Tyler *et al.* 2010; Sarma *et al.* 2012). The JA biosynthetic gene allene oxide synthase (*AOS*) is a member of the cytochrome p450 CYP74 family and has been studied as a JA-responsive marker in *Arabidopsis* (Poncini *et al.* 2017; Singh *et al.* 2017), wheat and *Brachypodium* (Kouzai *et al.* 2016; Liu *et al.* 2016; Debona *et al.* 2017). The transcription factor *JaMyb* has been studied as a JA-responsive marker in rice (De Vleesschauwer *et al.* 2016; Ma *et al.* 2017).

Pathogen-responsive genes *PR1*, *PR2* and *PR5* are established transcriptionally-responsive SA-responsive markers. These have also been widely used in wheat, especially *PR1* (e.g. Niu *et al.* 2007; Debona *et al.* 2017; Reilly *et al.* 2021). PR1 and PR5, respectively, belong to the CAP (cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily and the thaumatin-like family (Gibbs *et al.* 2008; Petre *et al.* 2011). PR2 proteins are endo-1,3-β-glucanases, which

belong to the glycoside hydrolase family 17 (Higa-Nishiyama *et al.* 2006). The SA-responsive gene marker *AtWRKY70* falls into WRKY transcription factor subgroup IIIb (Kalde *et al.* 2003; Wu *et al.* 2005), and it functions as an activator of SA-responsive defences and a repressor of JA-responsive genes (Li *et al.* 2004; Li *et al.* 2006). It is closely related to the monocot SA-responsive marker gene *WRKY45* used in *Brachypodium* and rice (De Vleesschauwer *et al.* 2016; Kouzai *et al.* 2016; Ma *et al.* 2017).

Despite the global importance of wheat as a crop, information on gene expression during JA and SA signalling is fragmented, with only a few reports comparing the transcriptional response of several genes to more than one phytohormone (e.g., Ding *et al.* 2016; Qi *et al.* 2016). Many studies in wheat use homologues of *Arabidopsis* genes as hormone-responsive markers. The classic example is *PR1* which is used as a SA-responsive marker in wheat (Debona *et al.* 2017; Reilly *et al.* 2021). However, in wheat and other monocots species (*Oryza sativa*, *Brachypodium distachyon*), the expression of some *PR1* gene variants can also be induced by exogenous application of JA or methyl jasmonate (MJ) (Kouzai *et al.* 2016; Liu *et al.* 2016). In addition, increasing evidence suggests that JA and SA can activate common defence responses in rice and *Brachypodium* (De Vleesschauwer *et al.* 2013; Tamaoki *et al.* 2013; Kouzai *et al.* 2016). Such findings indicate that some knowledge about hormone activities gleaned from *Arabidopsis* cannot be directly transposed to other species.

Comparing the role of phytohormones across species is complicated due to variations in their biosynthesis, transport, perception and signalling. For example, in rice, levels of SA above 10 $\mu g \cdot g^{-1}$ fresh weight are often observed (Pál *et al.* 2014; Klessig *et al.* 2016), which is several orders of magnitude higher than the basal level in *Arabidopsis* which is estimated around 0.2 $\mu g \cdot g^{-1}$ fresh weight (Zhang & Li 2019). Even within a plant, tissues can vary in their hormone content and in their hormone responsiveness. In four wheat genotypes, more bound SA was found in leaves than in shoots (Kovács *et al.* 2014). In the wheat cv. Crusader, the gene expression of JA markers was found to be stronger in shoots than in roots, with more genes being responsive to MJ in shoots and stronger fold-changes (Liu *et al.* 2016). The response of wheat to MJ was also shown to be genotype-dependent, with application on spikes leading to a decrease in endogenous JA content in cultivar (cv.) NAUH117, but an increase in cv. Wangshuibai (Sun *et al.* 2016).

Wheat has a complex hexaploid genome, resulting in expansion within gene families as compared to in diploids such as *Arabidopsis*. Recent advances in sequencing and annotation of the wheat genome over the last decade opened the possibility of doing genome-wide analysis of gene families involved in phytohormone responsiveness in order to study their diversity and develop assays to determine their role and the level of subfunctionalisation, if any, of these genes in wheat hormonal pathways. In this study we investigated classic SA and JA signalling marker gene families within the wheat genome (IWGSC 2018), and studied their phylogeny and expression in response to biotic stresses in order to facilitate their use within future studies on wheat–pathogen interactions. We selected candidate gene families for JA- and SA-mediated signalling pathway markers based on a review of the literature: *AOS*, *JAMyb*, *WRKY70*, *PR1*, *PR2*, *PR3* and *PR5*. Phylogenetic analysis, coupled with *in silico* analysis of wheat disease transcriptomic data was used to delineate potential SA and JA responsive marker genes for disease studies. Thereafter, gene expression studies evaluated the response of candidate marker genes to SA and JA.

MATERIAL AND METHODS

Bioinformatic analyses

The literature was analysed to select candidate marker families for JA- and SA-mediated signalling pathways (Table S1). Amino acid sequences of AtWRKY70 (At3g56400), AtAOS/AtCYP74A (At5g42650), AtPR1 (At2g14610), AtPR2 (AT3G57260), AtPR3 (AT3G12500) and, AtPR5 (AT1G75040) were retrieved from The Arabidopsis Information Resource (https://www.arabidopsis.org/). OsJAMyb (Os11g0684000) amino acid sequence was retrieved from EnsemblPlants (http://plants.ensembl.org/index.html). Those seven sequences were used as a query in BLASTp analyses performed against the wheat reference proteome (IWGSC RefSeq v1.1 High confidence database) using OmicsBox 2.0.10 (Götz *et al.* 2008). To ensure we selected all the genes from our family of interest, we determine our BLASTp cut-off based on the presence of canonical protein domains in combination with a reverse BLASTp using all the wheat hits as query against the *A. thaliana* proteome Araport11 (or the rice proteome IRGSP-1.0 for JAMyb). The presence of canonical domains was assessed by extracting the InterPro domain IDs and the Pfam domain IDs for every wheat protein sequence using BioMart in EnsemblPlants (Tables S2–S8; Kinsella *et al.* 2011). The reverse BLASTp ensured that the first *Arabidopsis* hit (or rice for JAMyb) was a protein belonging to the families/subgroups of interest. Comparative Blastp analysis between *Arabidopsis* and *Brachypodium* sequences was conducted using Emboss Needle (https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Phylogenetic analyses

Phylogenetic analyses were conducted to analyse the evolutionary relationship of the deduced proteins from our candidate families in *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica* and *Triticum aestivum*. For each selected family, the literature was screened to retrieve *A. thaliana*, *B. distachyon* and *O. sativa* subsp. *japonica* family members and their amino acid sequences were retrieved from EnsemblPlants (http://plants.ensembl.org/index.html) (Tables S2–S8). *Arabidopsis*, *Brachypodium*, rice and wheat amino acid sequences were aligned using "Multiple Alignment using Fast Fourier Transform" tool with default settings in Jalview version 2.10.5 (Waterhouse *et al.* 2009). Parameters used for alignment masking and phylogenetic tree construction are detailed in Table S9. Alignment completeness scores were determine using ALISTAT version 1.7 (Wong *et al.* 2020); sites with a low C_c score (C_c = number of unambiguous characters in the column/number of sequences) were masked (*i.e.*, excluded from further analysis) to only consider conserved positions. In addition, sequences with a C_r score below 0.5 (C_r = number of unambiguous characters in the sequence/alignment length) after masking were excluded

(because of their low completeness score). Alignments were analysed with Homo version 1.4 to test if all pairs of sequences met the phylogenetic assumption of evolution under globally stationary, reversible, and homogeneous conditions (Rouse *et al.* 2013). Within IQ-TREE web server (Trifinopoulos *et al.* 2016), the best-fitting model of sequence evolution was identified for each gene family using ModelFinder (Kalyaanamoorthy *et al.* 2017) and maximum-likelihood phylogenetic trees were constructed using IQ-TREE (Nguyen *et al.* 2015). Ultrafast bootstrapping was performed with 1000 replications (Hoang *et al.* 2018). The resulting phylogenetic trees were visualised using iTOL (Letunic & Bork 2021).

In silico expression analysis and candidate marker gene selection

In silico analysis of published RNA sequencing data was conducted to investigate the responsiveness of wheat genes to biotic stresses. Datasets were downloaded from the Expression Atlas database (https://www.ebi.ac.uk/gxa/home) and from BMC Genomics Journal (Pan *et al.* 2018) and included six disease studies. Three studies assessed the transcriptomic response of wheat seedling/leaves to *Fusarium pseudograminearum*, *Blumeria graminis* f. sp. *tritici*, *Puccinia striiformis* f. sp. *tritici* and *Xanthomonas translucens* (Ma *et al.* 2014; Zhang *et al.* 2014; Garcia-Seco *et al.* 2017). Three studies assessed the transcriptomic response of wheat spikelets to *Fusarium graminearum* (Kugler *et al.* 2013; Schweiger *et al.* 2016; Pan *et al.* 2018).

Wheat genes to be tested as markers for JA- and SA-mediated signalling were selected from the candidate gene families based on a combination of criteria including: responsiveness to fungal diseases, phylogenetic proximity to *A. thaliana* marker genes, available information from literature for monocots, the availability of published primers and the possibility of designing gene-specific primers. For candidate genes, in addition to assessing their pathogen responsiveness as described above, their expression profile in five different plant organs (leaf, stem, roots, spike, grain) was investigated using a developmental study dataset for cv. Chinese Spring (Pingault *et al.* 2015) downloaded from Expression Atlas database (https://www.ebi.ac.uk/gxa/home).

Plant material and growth conditions

Bread wheat (*Triticum aestivum*) cvs. Remus and CM82036 were used in this study. Seeds were germinated in the dark on moistened filter paper (Whatman No. 1) for 1 day at 4 °C followed by 2 days at 20 °C. Germinated seedlings were transferred into 2 l pots containing John Innes No. 2 compost (Westland Horticulture, UK) with two seedlings per pot, and were grown in a MD1400 Modular climate chamber (Snijders Labs) at 20 °C with 16-h light and 8-h dark per day.

Phytohormone treatment

Phytohormones were applied when three leaves were unfolded (growth stage 13; Zadoks *et al.* 1974). The first leaf was sprayed with 340 µl of a 0.1% ethanol solution supplemented or not (mock) with either 1 mM SA (Sigma: S7401) or 200 µM MJ (Sigma: 392707). As MJ is volatile, plants were transferred into sealed transparent bags after treatment until sampling was performed at either 10, 24 or 48 h post-treatment. Samples were frozen in liquid nitrogen and stored at −80 °C until processing. The experiment comprised three independent replica trials, each including two leaves from different plants per treatment per genotype. Prior to RNA extraction, the two leaves per treatment were pooled resulting in a total of three composite samples per treatment per genotype.

RNA extraction and cDNA synthesis

Samples were ground in liquid nitrogen using a mortar and pestle and RNA was extracted using the TRIzol™ Reagent (Invitrogen/Ambion: 15596018). DNase treatment of RNA was performed using the TURBO DNA-free™ Kit (Invitrogen/Ambion: AM1907), and cDNA was synthesised from 1 µg RNA using M-MLV Reverse Transcriptase (Invitrogen: 28025-013) according to the manufacturer's instructions, using oligo(dT) as a primer.

Gene expression analysis

The list of primers used in this study is detailed in Table S10. The wheat *T. aestivum* cell division control protein b (*TaCDCb*, NCBI Unigene: Ta54227) and *T. aestivum* translation elongation factor 1-alpha (*TaTEF1α*, TraesCS4A02G035500) were used as housekeeping genes. qPCR analyses were conducted using the Applied Biosystems QuantStudio™ 7 Flex system. Each reaction contained 1.25 µl of a 1:5 (v/v) dilution of cDNA sample, 0.2 µM of each primer (Table S10) and 1× Fast SYBR™ Green Master Mix (Thermo Fisher Scientific: 4385612) in a total volume of 12.5 µl. The following programme was used: 1 cycle of 20 s at 95 °C; 40 cycles of 1 s at 95°C and 20 s at 60 °C; and a final cycle of 15 s at 95 °C, 1 min at 60 °C and 1 s at 95 °C to generate the melting curve. All qRT-PCR analysis was done in technical triplicates (same sample measured three times) and the threshold cycle (Ct) values obtained were averaged. The Ct values obtained by qRT-PCR for both housekeeping genes (*TaCDCb* and *TaTEF1α*) were averaged and used with Ct obtained for target gene to calculate the relative gene expression using the equation: $2^{-(\text{Ct target gene}-\text{Average Ct housekeeping genes})}$ as described previously (Livak & Schmittgen 2001). The fold changes between hormone-treated and mock-treated samples were calculated following the $2^{-\Delta\Delta Ct}$ method (Schmittgen & Livak 2008). Log2(fold change) data were used to generate the heat map using Morpheus (https://software.broadinstitute.org/morpheus).

Statistical analysis

Means and standard errors were calculated from three biological replicates. Mann-Whitney *U* tests, performed in IBM SPSS Statistics 27.0 (IBM, Released 2020), were conducted to determine the difference in relative gene expression between hormone-treated and mock treated samples at a 5% significance (data not being normally distributed).

RESULTS

Selection and analysis of candidate gene families

We identified wheat members of seven gene families in the IWGSC RefSeq version 1.1 annotation (IWGSC 2018) based on

their homology with *Arabidopsis* hormone markers (or rice for JaMYB). As expected, the gene families were expanded in wheat due to the hexaploid nature of the genome and in some cases due to gene duplications (Tables S2–S8). These wheat sequences and *Arabidopsis*, rice and *Brachypodium* protein sequences previously described in the literature were used to generate maximum likelihood phylogenetic trees. Using publicly available RNA sequencing data, we determined the expression profile of the wheat genes during infection by five different pathogens: *F. graminearum*, *F. pseudograminearum*, *Blumeria graminis* f. sp. *tritici*, *Puccinia striiformis* f. sp. *tritici* and *Xanthomonas translucens*. The following two sections detailed the phylogenetic and transcriptomic results for candidate JA- and SA-responsive wheat genes.

Phylogeny and biotic stress responsiveness of candidate JA signalling marker genes

The phylogeny and biotic stress responsiveness of proteins deduced from *AOS*, *JAMyb* and *PR3* genes were examined, these being of interest as potential candidate JA-responsive genes. One *Arabidopsis*, four *Brachypodium* and four rice *AOS* genes were previously identified (Haga & Iino 2004; Kakei *et al.* 2015; Kouzai *et al.* 2016). However, *Brachypodium* gene Bradi3g01110 shares more homology with the *Arabidopsis AtCYP74B2/AtHPL1* than with *AtAOS/AtCYP74A* and was thus excluded from phylogenetic analysis (42% versus 39% homology, respectively). Analysis identified 12 putative *AOS* genes (constituting four triads of homoeologous genes) in bread wheat, with pathogen-responsive *AOS* genes present on all subgenomes (Fig. 1). Only one gene (TraesCS6B02G213300) was unresponsive to any of the diseases. The two triads on chromosome 4 contain paralogous genes, including the previously published *TaAOS* genes TraesCS4B02G237500 and TraesCS4D02G238800 (Chehab *et al.* 2007; Liu *et al.* 2016). Triad TraesCS4A02G061900/TraesCS4B02G237600/TraesCS4D02G238800 was previously found to be involved in *Fusarium* resistance and used as a JA signalling marker (Fan *et al.* 2019).

To our knowledge, the rice *OsJAMyb* is the only *JAMyb* which has been characterised (the closest *Arabidopsis* homologue being an abscisic acid-inducible gene *AtMyb2* – 39% identity, which is thus not included in the tree) (Lee *et al.* 2001). Six orthologs of *OsJAMyb* were found in bread wheat, distributed across two triads on chromosomes 4 and 5 (Fig. 2). All were found to be responsive to at least one pathogen, and TraesCS4A02G172200 was the only wheat JAMyb that was unresponsive to *F. graminearum* in the three RNA sequencing studies analysed.

A total of 14 *Arabidopsis*, 8 *Brachypodium* and 16 rice *PR3* (glycoside hydrolase 19) genes were previously identified (Passarinho & de Vries 2002; Tyler *et al.* 2010; Sharma *et al.* 2013; Irigoyen *et al.* 2020). Our analysis identified 76 putative *PR3* genes in wheat, their deduced proteins distributed across all phylogenetic clades in Fig. 3; interestingly, protein AtPR3 (AT3G12500) was an outlier to the clades. The wheat *PR3* genes included 32 chitinases from class I, 24 from class II, 10 from class IV, 8 from class VI and 2 from class VII, with no obvious relationship between protein phylogenetic clade and class, as illustrated in Fig. 3. We found pathogen-responsive wheat proteins in all the classes. Interestingly, within one subclade, we found that the following six of the eight included wheat amino acid sequences encoded an additional kinase domain in their C-terminus: TraesCS1A02G383900, TraesCS1B02G410600, TraesCS1D02G392100, TraesCS5A0

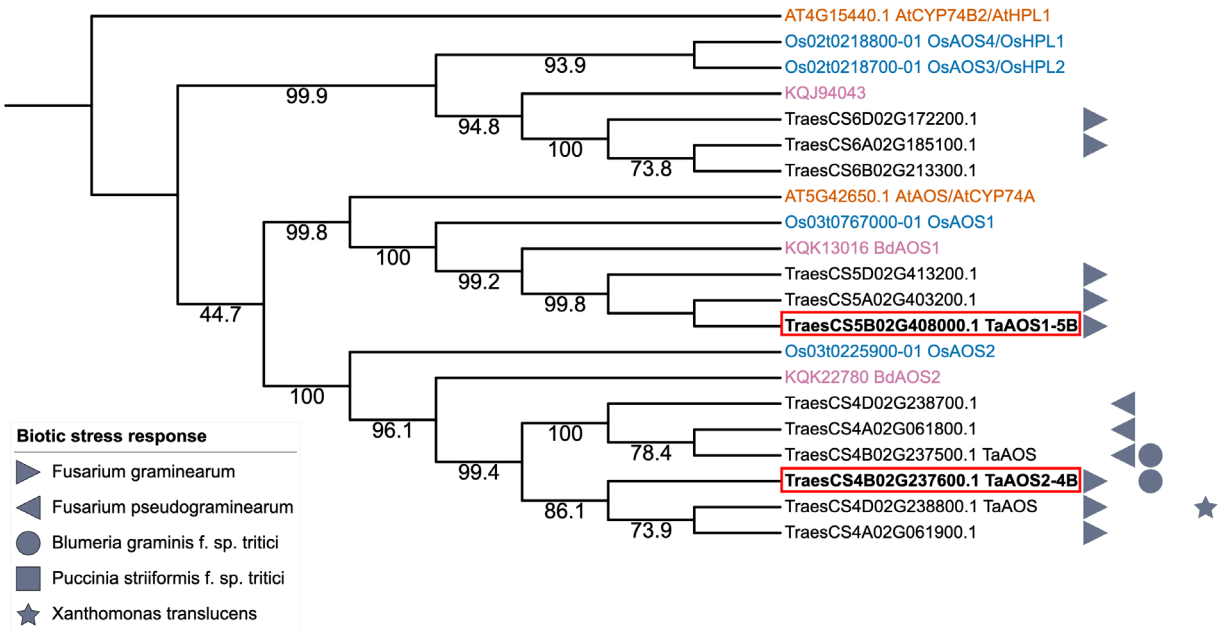

Fig. 1. Phylogeny and pathogen-responsiveness of AOS proteins from *Arabidopsis*, *Brachypodium*, rice and wheat. The maximum likelihood phylogenetic tree of AOS proteins from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al.* 2016) and visualised using iTOL (Letunic & Bork 2021). The tree was rooted using AtCYP74B2/AtHPL1 (AT4G15440.1) amino acid sequence. Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the AOS that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*).

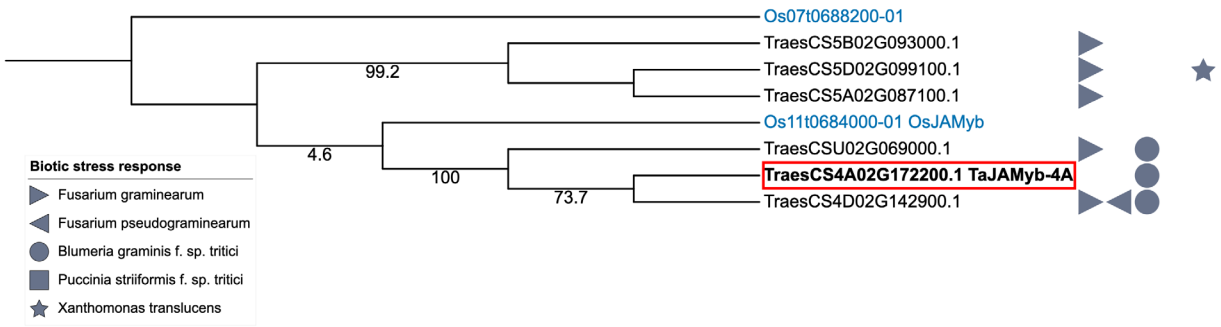

Fig. 2. Phylogeny and pathogen-responsiveness of JAMyb transcription factors from rice and wheat. The maximum likelihood phylogenetic tree of JAMyb proteins from *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al*. 2016) and visualised using iTOL (Letunic & Bork 2021). The tree was rooted using the Os07t0688200–01 amino acid sequence. Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the JAMyb that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*).

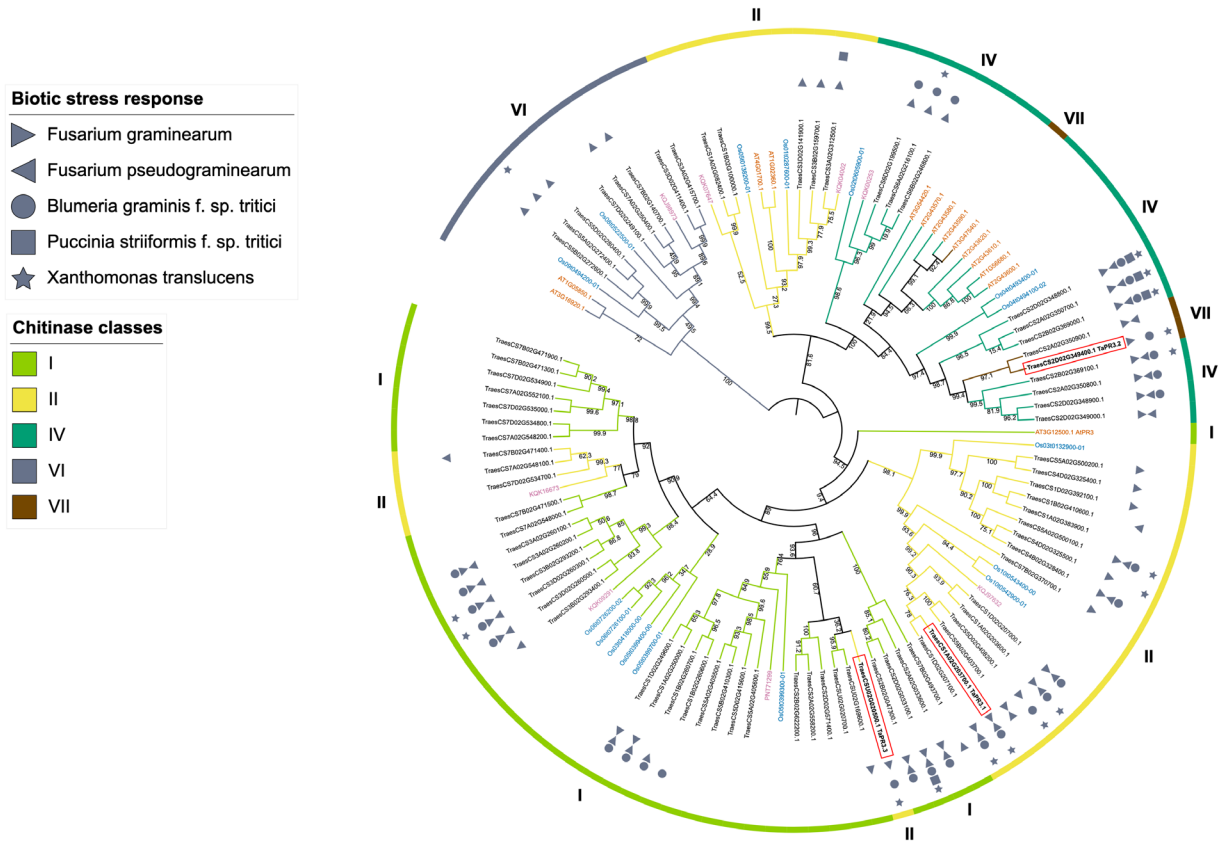

Fig. 3. Phylogeny and pathogen-responsiveness PR3 (glycoside hydrolase 19) proteins from *Arabidopsis*, *Brachypodium*, rice and wheat. The maximum likelihood phylogenetic tree of glycoside hydrolase 19 (PR3) proteins from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al*. 2016) and visualised using iTOL (Letunic & Bork 2021). The tree is not rooted. Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the AOS that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*). The outer coloured circle corresponds to the glycoside hydrolase 19 classes.

2G500100, TraesCS4B02G328400, and TraesCS4D02G325500. With the exception of TraesCS1A02G383900, these were disease-responsive. Those protein sequences cluster with the rice Os03t0132900-01 and the two wheat TraesCS5A02G500 200 and TraesCS4D02G325400, which do not possess a kinase domain.

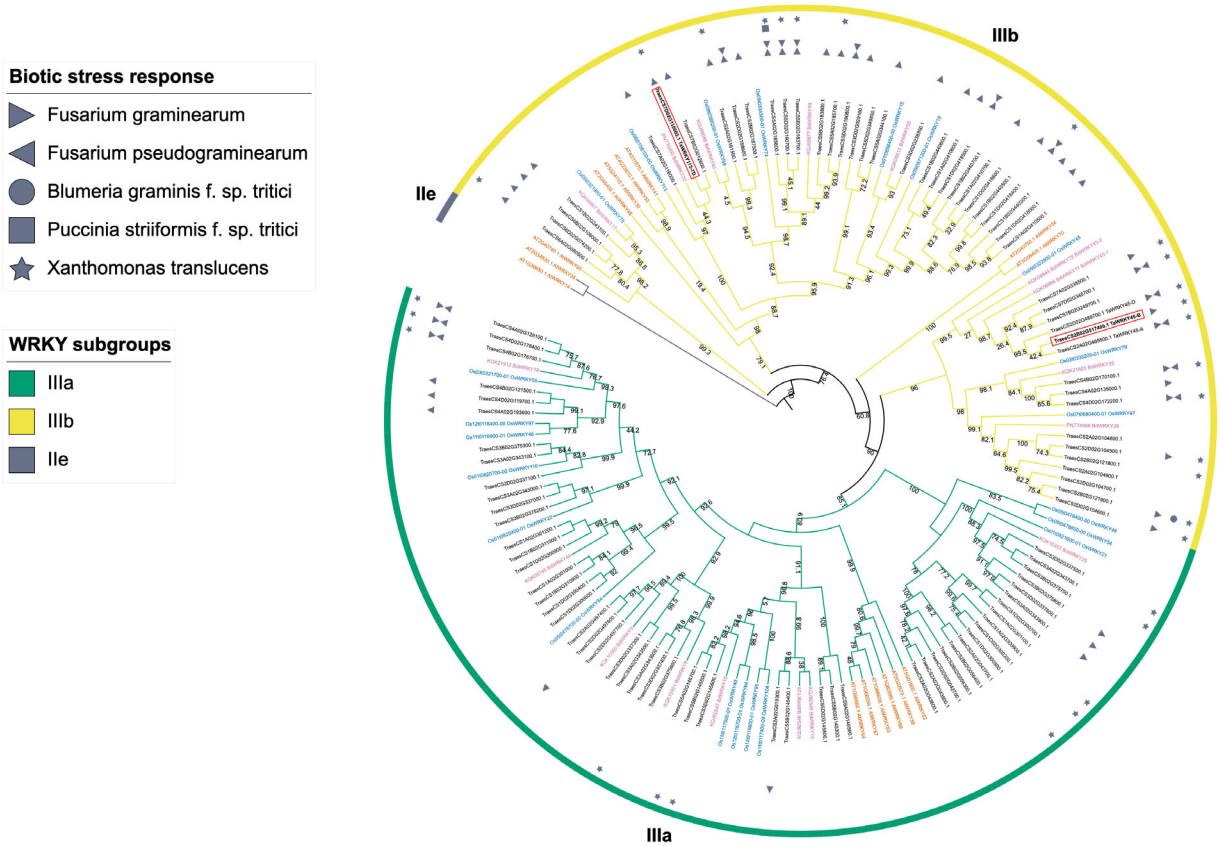

Fig. 4. Phylogeny and pathogen-responsiveness of WRKY transcription factor from subgroups IIIa and b in *Arabidopsis*, *Brachypodium*, rice and wheat. The maximum likelihood phylogenetic tree of WRKY group III proteins from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al*. 2016) and visualised using iTOL (Letunic & Bork 2021). The tree was rooted using the amino acid sequences of two WRKY proteins from group IIe (AT2G34830.1, AT1G30650.1). Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the AOS that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*). The outer coloured circle corresponds to the WRKY subgroups.

Phylogeny and biotic stress responsiveness of candidate SA signalling marker genes

The phylogeny and pathogen responsiveness of proteins encoded by *WRKY70*, *PR1*, *PR2* and *PR5* were investigated, being of interest because of their potential role as SA-responsive genes. 13 Arabidopsis, 17 Brachypodium and 22 rice genes were previously identified in the *WRKY* transcription factor group III (Rushton *et al*. 2010; Tripathi *et al*. 2012; Wen *et al*. 2014; Wang *et al*. 2015). Our analysis identified 99 putative *WRKY* wheat genes in the *WRKY* group III (52 and 47 in subgroups IIIa and IIIb, respectively) (Fig. 4). In subgroup IIIb, proteins AtWRKY70 and AtWRKY54 were in the same clade as the monocot WRKY45 sequences. Protein TaWRKY45-B (TraesCS2B02G517400) clustered with the SA-inducible BdWRKY45-1, BdWRKY45-2, OsWRKY45 (Chao *et al*. 2009; Kakei *et al*. 2015; Kouzai *et al*. 2016) and is encoded by the homoeolog of the SA-inducible and FHB resistance genes *TaWRKY45-A* and *TaWRKY45-D/TaWRKY70* (Bahraini *et al*. 2011; Bahrini *et al*. 2011; Kage *et al*. 2017; Wang *et al*. 2017). More wheat genes were found to be pathogen-responsive in the subgroup IIIb than in the subgroup IIIa, with 85% and 37% of the genes being responsive to at least one disease, respectively.

A total of 22 *Arabidopsis*, 11 *Brachypodium* and 23 rice *PR1* and *PR1*-like genes were previously described (van Loon *et al*. 2006; Kakei *et al*. 2015; Kouzai *et al*. 2016; Irigoyen *et al*. 2020). Our analysis identified 85 putative *PR1* wheat genes, and their encoded proteins were divided across the three clades previously described by Lu *et al*. (2011) and two additional clades (Fig. 5). Protein AtPR1 did not align with these clades, instead forming a cluster with 12 *Arabidopsis*, two wheat and three rice PR1-like proteins. Interestingly these two wheat PR1-like protein sequences (encoded by TraesCS3A02G477300 and TraesCS3D02G472000) possess an additional kinase domain in their C-terminus. The PR1 protein clades I, II and III contained monocot protein sequences but did not contain *Arabidopsis* sequences, which is in accordance with the findings of a previous study (Lu *et al*. 2011). Proteins encoded by previously characterised *TaPR1-1* (TraesCS5A02G183300) and *TaPR1-2* (TraesCS7D02G201400) belong to clades I and II, respectively (Fig. 5) and are both phytohormone-responsive in wheat (Ding *et al*. 2016; Liu *et al*. 2016). Also, protein TaPR1-1 clustered with that encoded by TraesCS7D02G161200, which was previously used as SA-responsive marker in wheat (Debona *et al*. 2017; Reilly *et al*. 2021). TaPR1 proteins from clades IV and V grouped with *Arabidopsis* PR1-like proteins and, unlike

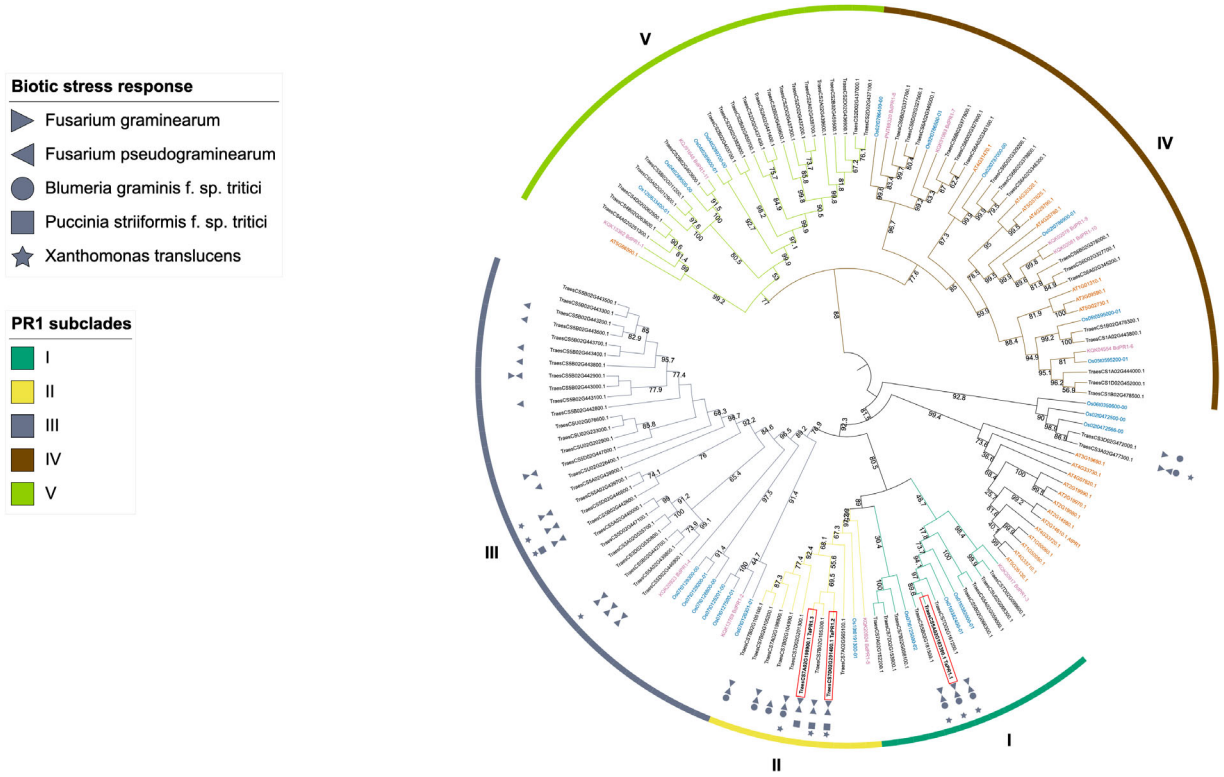

Fig. 5. Phylogeny and pathogen-responsiveness of PR1 gene families in *Arabidopsis*, *Brachypodium*, rice and wheat. The maximum likelihood phylogenetic tree of PR1 proteins from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al*. 2016) and visualised using iTOL (Letunic & Bork 2021). The tree is not rooted. Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the AOS that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*). The outer coloured circle corresponds to the PR1 subclades originally described by Lu *et al*. (2011) and expanded upon in this study.

the other clades, no encoding gene members were transcriptionally responsive to any of the pathogens examined herein.

A total of 51 *Arabidopsis*, 36 *Brachypodium* and 59 rice *PR2* (glycoside hydrolase 17) genes were previously identified (Hwang *et al*. 2007; Tyler *et al*. 2010; Gaudioso-Pedraza & Benitez-Alfonso 2014; Irigoyen *et al*. 2020). Our analysis identified 205 putative glycoside hydrolase 17 genes in wheat, separating into four protein clades, with *Arabidopsis*, *Brachypodium* and rice sequences in all of those clades (Fig. 6). About 60% of the wheat glycoside hydrolase 17 were found to be responsive to at least one pathogen, with the majority of these being within one clade that also included protein AtPR2 (AT3G57260).

A total of 24 *Arabidopsis*, 26 *Brachypodium* and 35 rice *PR5* thaumatin-like genes were previously identified (Liu *et al*. 2010; Petre *et al*. 2011; Iqbal *et al*. 2020; Irigoyen *et al*. 2020). Our analysis identified 124 putative thaumatin-like genes in wheat (Fig. 7), aligning within two major clades, with 18 genes being thaumatin-like protein kinases (*TLPK*). We identified two additional *TaTLPK*, as compared to Sharma *et al*. (2020) (Table S8). The 18 wheat TaTLPK proteins clustered with *Brachypodium* KQK02399 (Bradi2g01200) and rice Os01t0113350-00 and Os01t0113650-00 proteins, and about 46% of the wheat thaumatin-like sequences were found to be transcriptionally responsive to at least one of the pathogens examined.

Selection of candidate genes for hormonal profiling studies

For each of the JA- and SA-responsive gene families, we selected one to two hormone signalling marker candidate genes for further analysis, based on their homology to known hormone-responsive genes and/or their pathogen responsiveness (two for all families except *JAMyb* and *PR5*). The 14 selected candidate marker genes (Table 1) were tested for their transcriptional response to MJ and SA treatments in leaves of wheat cvs. Remus and CM82036 (10, 24 and 48 hpi). The response of each gene is summarised in Fig. 8 and Table S11. Table S12 also details the developmental and biotic stress response profile of these genes based on one publicly available developmental study (Pingault *et al*. 2015) and six diseases studies (Kugler *et al*. 2013; Ma *et al*. 2014; Zhang *et al*. 2014; Schweiger *et al*. 2016; Garcia-Seco *et al*. 2017; Pan *et al*. 2018).

Genes from the AOS and JaMYb families were responsive to MJ in wheat leaves

Two genes from the *AOS* family (*TaAOS2-4B* and *TaAOS1-5B*) and one gene from the *JAMyb* subfamily (*TaJAMyb-4A*) were expected to be MJ-inducible in wheat leaves based on the expression profile of their homologues. All three genes were significantly induced by MJ in both cultivars (Fig. 8, Table S11). *TaAOS2-4B* and *TaJAMyb-4A* were the most induced by MJ with a peak of

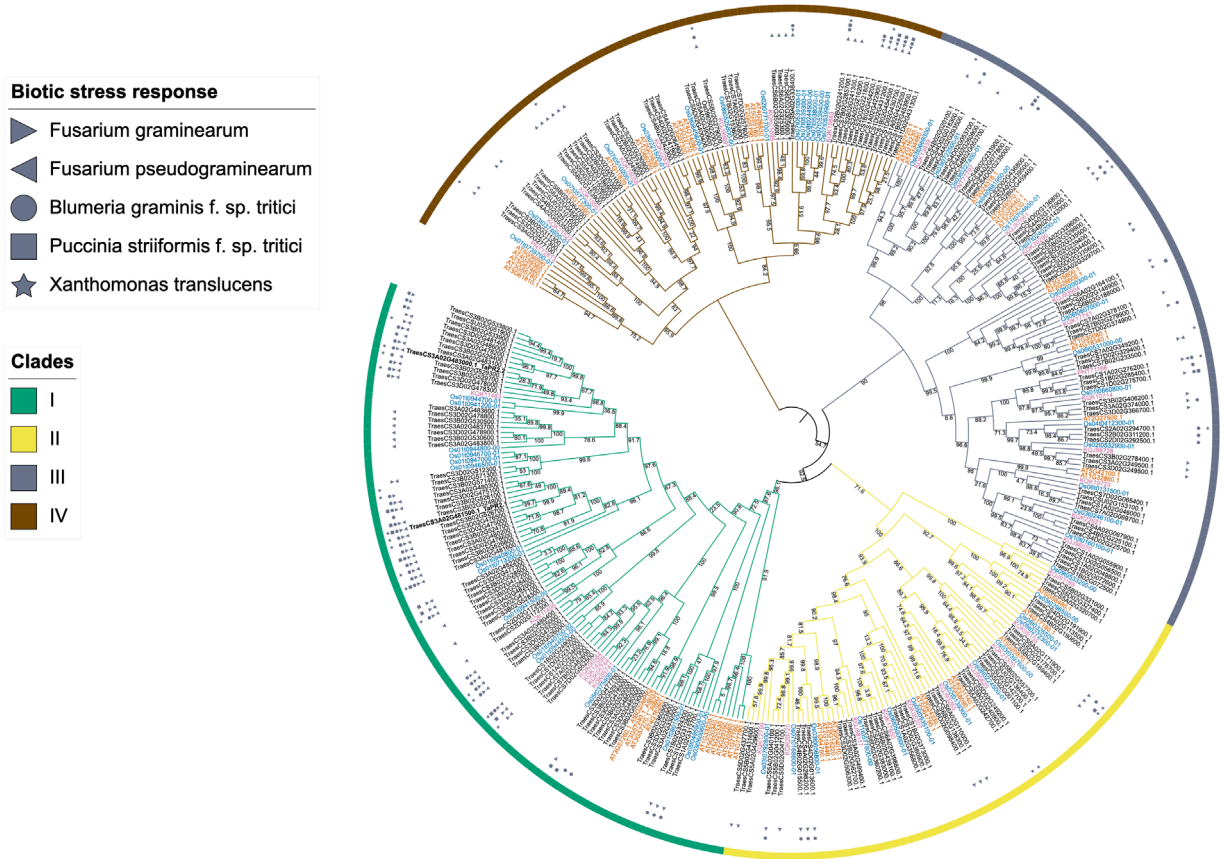

Fig. 6. Phylogeny and pathogen-responsiveness of PR2 (glycoside hydrolase 17) proteins from *Arabidopsis*, *Brachypodium*, rice and wheat. The maximum likelihood phylogenetic tree of PR2 proteins from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al.* 2016) and visualised using iTOL (Letunic & Bork 2021). The tree is not rooted. Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the AOS that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*).

expression at 10 h. Across timepoints, *TaAOS2-4B* and *TaJAMyb-4A* response was stronger in cv. Remus than in cv. CM82036 with an average induction of 1346-fold and 103-fold, respectively, in cv. Remus, and an average induction of 51 and 9, respectively, in cv. CM82036. *TaAOS1-5B* was significantly induced by MJ in cv. Remus leaves at 24 h (2.1-fold) and 48 h (2.9-fold) and in cv. CM82036 leaves at 24 h (3.9-fold). In addition, *TaAOS2-4B* was also induced by SA in both cultivars at 10 and 24 h, but the induction was only significant in cv. Remus (12.1-fold and 3.4-fold at 10 and 24 h, respectively). Therefore, both *TaAOS1-5B* and *TaJAMyb-4A* were good JA markers in wheat leaves as they were both induced by MJ but not by SA in both tested cultivars. In terms of their disease-responsiveness, analysis of the RNAseq data indicated that *TaAOS1-5B* was responsive to *F. graminearum* and *TaJAMyb-4A* to *B. graminis* and neither to *F. pseudograminearum*, *Puccinia striiformis* or *X. translucens* (Figure S1); in a developmental context, both expressed at a low level (<17 TPM) in cv. Chinese Spring leaf, stem, root, spike and grain (Table S12).

A gene from the WRKY family was responsive to SA in wheat leaves

Two genes from the *WRKY* subgroup IIIb (*TaWRKY45-B* and *TaWRKY113-7D*) were tested for their response to MJ and SA in leaf tissue in order to verify their suitability as wheat gene markers to distinguish between the JA and SA response (Fig. 8, Table S11). Based on the SA-responsiveness of the rice and *Brachypodium* homologues (Chao *et al.* 2009; Kakei *et al.* 2015; Kouzai *et al.* 2016) and of homologues *TaWRKY45-A* and -*TaWRKY45-D/TaWRKY70* (Bahrini *et al.* 2011; Wang *et al.* 2017), *TaWRKY45-B* was expected to be SA-inducible. Indeed, *TaWRKY45-B* was found to be significantly induced by SA in both cvs. Remus and CM82036 and repressed by MJ in cv. Remus only (no significant MJ response in cv. CM92036; Fig. 8). The SA response was stronger in cv. CM82036 than in cv. Remus, with the upregulation trend being conserved across time points. In cv. Remus, the downregulation of *TaWRKY45-B* by MJ was significant only for 24 h (−2.2-fold) although the trend was conserved across time points. A non-significant upregulation of *TaWRKY45-B* by MJ was also observed in cv. CM82036 at 10 hpi but that seems to be due to a difference in behaviour in trial 1 in which MJ seems to be inducing *TaWRKY45-B*, in contrast with trial 2 and trial 3 (data not shown). In contrast, *TaWRKY113-7D* was not transcriptionally responsive to SA in either cultivar (Fig. 8) and is consequently not a good marker for the SA response in wheat leaves. Both *TaWRKY45-B* and *TaWRKY113-7D* were responsive to *F. graminearum and X. translucens*, but not to *B. graminis*, *P.*

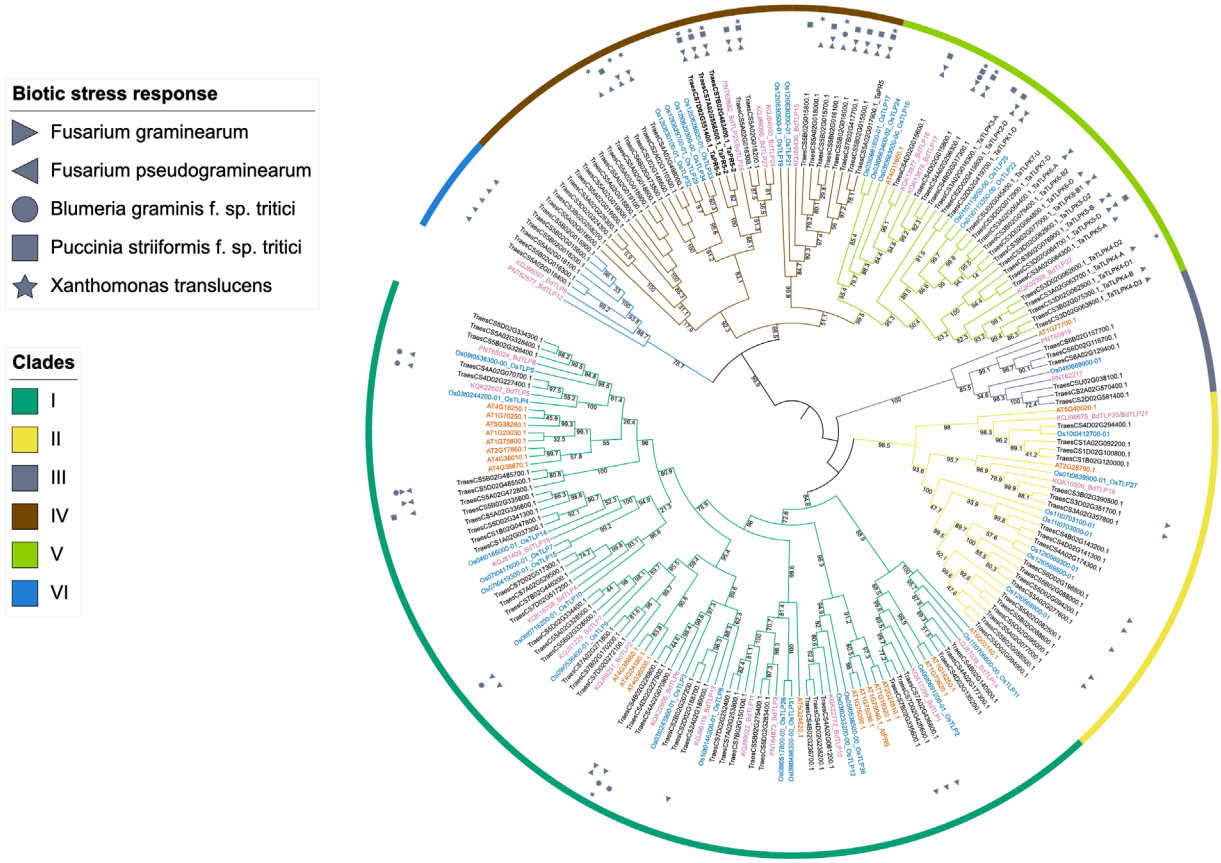

Fig. 7. Phylogeny and pathogen-responsiveness of PR5 (thaumatin-like) proteins from *Arabidopsis*, *Brachypodium*, rice and wheat. The maximum likelihood phylogenetic tree of PR5 proteins from *Arabidopsis thaliana*, *Brachypodium distachyon*, *Oryza sativa* subsp. *japonica*, and *Triticum aestivum* was inferred using IQ-TREE (Trifinopoulos *et al*. 2016) and visualised using iTOL (Letunic & Bork 2021). The tree is not rooted. Numbers on branches represent bootstrap values. Font colour represents plant species and bold font in a red rectangle represents the genes selected for further analysis as hormone-responsive markers. The symbols denote the AOS that are responsive (at the transcriptome level) to pathogens (*Fusarium graminearum*, *Fusarium pseudograminearum*, *Puccinia striiformis* f. sp. *tritici*, *Blumeria graminis* f. sp. *tritici*, *Xanthomonas translucens*).

Table 1. Candidate marker genes selected in this study for jasmonic acid (JA) and salicylic acid (SA) in bread wheat.

name	gene ID	Arabidopsis homologue	rice homologue	Brachypodium homologue
JA-related genes				
TaAOS2-4B	TraesCS4B02G237600	AtAOS: AT5G42650	OsAOS2: Os03g0225900	BdAOS2: Bradi1g69330
TaAOS1-5B	TraesCS5B02G408000	AtAOS: AT5G42650	OsAOS1: Os03g076700	BdAOS1: Bradi1g07480
TaJAMyb-4A	TraesCS4A02G172200	AtMYB78: AT5G49620	JAMb: Os11g0684000	Bradi4g04627
SA-related genes				
TaWRKY45-B	TraesCS2B02G517400	AtWRKY70: AT3G56400	OsWRKY45: Os05g0322900	BdWRKY45-2: Bradi2g44270
TaWRKY113-7D	TraesCS7D02G110600	AtWRKY53: AT4G23810	OsWRKY113: Os06g0158100	BdWRKY22: Bradi1g48770
Pathogenesis-related genes				
TaPR1-1	TraesCS5A02G183300	AtCAPE3: AT4G33720[a]	OsPR1-12: Os01g0382400	Bradi4g14920
TaPR1-2	TraesCS7D02G201400	AtCAPE3: AT4G33720[a]	OsPR1-101: Os10g0191300	BdPR1-3: Bradi1g57540
TaPR1-3	TraesCS7A02G198900	AtCAPE3: AT4G33720[a]	OsPR1-101: Os10g0191300	BdPR1-3: Bradi1g57540
TaPR2-1	TraesCS3A02G481500	AtPR2: AT3G57260	OsPR2: Os01g0944700	Bradi2g60541
TaPR2-2	TraesCS3A02G483000	AT4G16260	OsPR2: Os01g0944700	BdPR2: Bradi2g60490
TaPR3-1	TraesCS1A02G203700	AtPR3: AT3G12500	OsCht8: Os10g0542900	Bradi3g32340
TaPR3-2	TraesCS2D02G349400	AtEP3: AT3G54420	OsCht5: Os04g0494100	Bradi5g14430
TaPR3-3	TraesCSU02G020500	AT1G02360	OsCht9: Os05g0399400	Bradi1g29887
TaPR5-2	TraesCS7A02G558500, TraesCS7B02G483400, TraesCS7D02G551400	AtOSM34: AT4G11650	OsPR5: Os12g0628600	BdTLP25: Bradi4g09220

[a]Selected TaPR1 genes do not have AtPR1 (AT2G14610) as first hit but they have it as second hit.

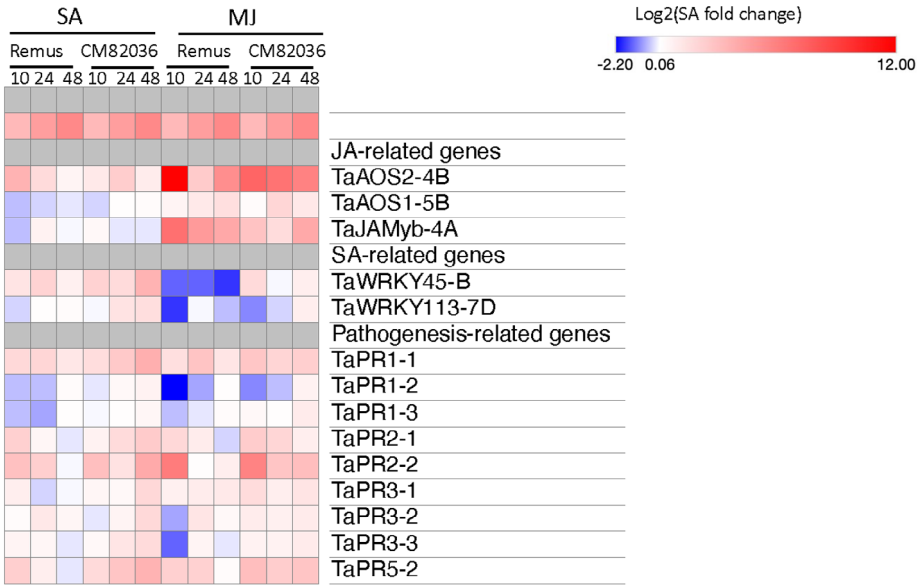

Fig. 8. Heat map indicating the change in expression between mock-treated and salicylic acid-treated or methyl jasmonate-treated leaves of wheat cvs. Remus and CM82036. Results presented are Log2 (fold change). Times of harvest were 10, 24 and 48 h post- treatment with either salicylic acid (SA), methy jasmonate (MJ) or mock treatment.

striiformis or *X. translucens* (Figure S1) and, in the absence of disease, were expressed at a low level (TPM < 9) in leaf, stem, root, spike and grain (Table S12).

PR genes are not good markers to distinguish between JA and SA pathway activation in wheat leaves

A total of nine *PR* genes from four different families were tested for their potential to distinguish between the JA and SA response in wheat leaves (Fig. 8, Table S11). As overviewed earlier, *PR1*, *PR2* and *PR5* are typically used as SA-responsive markers and *PR3* as a JA-responsive marker in *Arabidopsis*. The gene expression response of *TaPR1-1*, *TaPR1-2*, *TaPR1-3*, *TaPR2-1*, *TaPR2-2*, *TaPR3-1*, *TaPR3-2*, *TaPR3-3* and *TaPR5-2* in wheat to SA and JA is summarised in Figure 8. *TaPR2-1*, *TaPR2-2*, and *TaPR5-2* were found to be significantly induced by SA and MJ in both cvs. Remus and CM82036 for at least one time point. *TaPR3-1* and *TaPR3-2* were significantly responsive to SA and MJ in cv. CM82036, and in addition *TaPR3-2* was also found to be significantly SA responsive at 24 hpi. *TaPR1-1* was significantly upregulated by SA only in cv. CM82036 at 48 hpi; however, fold change values across timepoints suggests SA and MJ induction in both cvs. Remus and CM82036 (Fig. 8). *TaPR1-2* and *TaPR3-3* were found to be significantly downregulated by MJ in cv. Remus at 10 hpi, a tendency which seems to be conserved at 24 hpi as well, and in cv. CM82036 for *TaPR1-2* (−1.8-fold) but not *TaPR3-3* (1.6-fold). *TaPR1-3* was found to be significantly upregulated by MJ in cv. CM82036 at 48 hpi; this tendency does not seem conserved across time points in cv. CM82036 or in cv. Remus. Taken together, these results suggest that for the *PR* genes, phytohormone responsiveness in wheat leaves is variable in time, in strength and between cultivars. These results also show that some *PR* genes can be induced by both MJ and SA and therefore are not reliable markers to use in order to distinguish between the response to these two hormones. In terms of their disease-responsiveness, most of the *PR* genes were responsive to most diseases, with some sub-functionalisation observed between homoeologs. With the exception of *TaPR2-1*, none of the PR candidates were significantly upregulated in response to *P. striiformis*. Three genes were downregulated in response to the two biotrophs *P. striiformis* and *B. graminis* (*TaPr1-2*, *TaPR-1-3* and *TaPR5-2*) and the latter two were also downregulated in response to the bacterium *X. translucens*.

DISCUSSION

Based on an analysis of candidate genes from seven different gene families, this study identified two wheat genes as JA-responsive markers and one as a SA-responsive marker in leaves of two genetically distinct wheat cultivars. Wheat gene variants of both *AOS* (*TaAOS1-5B*; TraesCS5B02G408000) and *JaMYb* (*TaJAMyb-4A*; TraesCS4A02G172200) were identified as JA-responsive marker genes in leaves, being unresponsive to SA. Conversely, one WRKY variant (*TaWRKY45-B*; TraesCS2B02G517400) was shown to be a SA-responsive marker, and unresponsive to JA in wheat leaves. At the protein level, the MJ-responsive TaAOS1-5B clustered with AtAOS/At-CYP74A and OsAOS1, both of which are involved in JA biosynthesis and are JA-inducible (Laudert *et al.* 1996; Park *et al.* 2002; Mei *et al.* 2006; Zeng *et al.* 2021). The MJ-responsive *TaJAMyb-4A* is the first wheat *JAMyb* gene to be described; its induction by MJ and its lack of induction by SA suggest it plays a role in JA signalling similar to its ortholog *OsJAMyb*. *TaWRKY45-B* was significantly upregulated by SA in both cultivars and depending on the cultivar, either downregulated by or unresponsive to MJ, which is in accordance with the SA induction and MJ repression observed for its' homoeolog *TaWRKY45-D/TaWRKY70* in wheat cv. Xiaoyan 6 leaves (Wang *et al.* 2017). This SA inducibility is also shared by *TaWRKY45-B* orthologs *BdWRKY45-2* (Kouzai *et al.* 2016) and *OsWRKY45* (Chao *et al.* 2009). *TaJAMyb-4A* and *TaWRKY45-*

B were expressed at a low level (<10 TPM) in cv. Chinese Spring leaf, stem, root, spike and grain, while *TaAOS1-5B* was expressed at a medium level in cv. Chinese Spring stem and roots and at a low level in the other tissues (Pingault *et al.* 2015). Therefore, those genes have the potential to be used as hormone response markers in all wheat tissues. Interestingly, out of all pathogens studied, we found *TaAOS1-5B* was only responsive to *F. graminearum*, *TaWRKY45-B* to *F. graminearum* and *X. translucens*, and *TaJAMyb-4A* only to *B. graminis*. This is likely reflective of the experimental conditions, genotypes and time points assessed and it will be interesting to use the qRT-PCR assays developed herein to determine the response of these markers to these diseases in future experimentation. Herein, three datasets encompassing a range of genotypes and time points were analysed for the hemibiotrophic disease Fusarium head blight (FHB), as compared to the one dataset for the other diseases. And across these datasets it was found that both the JA marker genes were responsive to *F. graminearum*. This is as expected, since both JA and SA have been associated with defence against FHB (Qi *et al.* 2016; Wang *et al.* 2018), albeit the SA marker gene was not significantly activated by *F. graminearum* within these datasets. While the role of SA in defence against the biotrophic phase of FHB disease in wheat is not fully clarified, the pathogen has the capacity to metabolise this hormone (Qi *et al.* 2019b).

Several PR genes were induced by both SA and MJ. *PR1*, *PR2* and *PR5* genes, which are typically SA-responsive markers in *Arabidopsis* (Cao *et al.* 1994; Li *et al.* 2004; Yang *et al.* 2008; Schmitz *et al.* 2010; Wu *et al.* 2019), were also induced by MJ in wheat leaves. This is not surprising as some studies have shown that wheat genes from those families were induced by exogenous application of MJ (Ding *et al.* 2016; Liu *et al.* 2016). *PR3* genes, which are typically JA markers in *Arabidopsis* (Yang *et al.* 2008; Schmitz *et al.* 2010), were also induced by SA. Those results suggest that SA and JA signalling pathways could converge to induce defence genes in wheat. This hypothesis is supported by the fact that in *Brachypodium* and rice, some *PR1* genes were found to be induced by both SA and JA (Kouzai *et al.* 2016). De Vleesschauwer *et al.* (2013) suggested a crosstalk model for rice in which SA and JA signalling pathways converge through the transcription factor OsWRKY45 to induce common defence PR genes. All those findings suggest that SA and JA can activate a common pathway in monocot species. Therefore, *PR* genes should not be used as hormone-specific markers in wheat, at least in leaves. This convergence of pathways between SA and JA in wheat, as well as the interconnectivity observed between other phytohormone pathways in wheat (Qi *et al.* 2019a) highlight the importance of having a transcriptional reference map of phytohormone responses in wheat. Qi *et al.* (2019a) started to pave the way by studying the response of wheat spikes to seven phytohormones using microarray data. However, no such study exists for wheat leaves and roots.

In the course of identifying JA- and SA-responsive marker genes for wheat, we identified 12 *TaAOS*, 6 *TaJAMyb*, 256 *TaWRKY* (belonging to WRKY group III), 85 *TaPR1*, 205 *TaPR2* (glycoside hydrolase 17), 76 *TaPR3* (glycoside hydrolase 19) and 124 *TaPR5* (thaumatin-like) putative genes from the wheat reference IWGSC RefSeq v1.1 high confidence database (IWGSC 2018). To our knowledge, the wheat *AOS*, *JAMyb*, *PR2*, *PR3* and *PR5* gene families have not been previously described in detail. For the gene families *WRKY* and *PR1* we expanded on the work of others, our work benefiting from the whole wheat genome annotation that is now available. We identified nearly five times more putative *WRKY* group III genes as compared to recent studies (e.g. Ning *et al.* 2017). We found 85 putative *TaPR1* genes as compared to the 23 previously identified (Lu *et al.* 2011). In our phylogeny, the PR1 groups I, II and III (described by Lu *et al.* (2011)) comprised proteins encoded by disease-responsive wheat genes, clustering with *Brachypodium* and rice proteins, while groups IV and V included proteins encoded by non-disease-responsive wheat genes and clustering with *Arabidopsis* PR1-like, *Brachypodium* and rice proteins. As an aside it was interesting to note that, in clade V, all 20 wheat sequences possess the canonical CAP domain (IPR014044) but only three of them (TraesCS2B02G403600, TraesCS5A02G012900, TraesCS5B02G011200) possess the Allergen V5/Tpx-1-related site (IPR018244, Table S2).

We identified two *TaPR1*, six *TaPR3* and 18 *TaPR5* genes with integrated kinase domains, which has also been reported for other PR proteins. Two *PR-1* receptor kinases, *TcPR-1f* and *TcPR-1g*, have been described in *Theobroma cacao* (Teixeira *et al.* 2013; Tosarini *et al.* 2018), and 28 thaumatin-like protein kinases (*TLPK*) were identified in five cereal crops (Sharma *et al.* 2020). We identified two additional wheat *TLPK* (*TaTLPK4-B*, *TaTLPK5-D2*), which (at the protein level) clustered with the 16 *TLPK* previously identified by Sharma *et al.* (2020). The presence of a kinase domain in PR proteins suggests that they have evolved to function in signalling pathways.

Our analyses revealed the phylogenetic relationships between *Arabidopsis*, *Brachypodium*, rice and bread wheat protein sequences for AOS, JAMyb, WRKY group III, PR1, PR2 (glycoside hydrolase 17), PR3 (glycoside hydrolase 19), and PR5 (thaumatin-like). Our analyses revealed that monocot species have more AOS genes than *Arabidopsis*, with one AOS gene in *Arabidopsis* and three, four and 12 in *Brachypodium*, rice and wheat, respectively. The wheat triad TraesCS4A02G061900/-TraesCS4B02G237600/TraesCS4D02G238800, which was previously found to be involved in *Fusarium* resistance (Fan *et al.* 2019), was found in the same cluster as OsAOS2 which is involved in herbivore-induced JA biosynthesis (Zeng *et al.* 2021), suggesting an importance of this cluster for pest/disease resistance. In the JAMyb subfamily, two wheat triads (six genes) clustered with OsJAMyb, which is a rice MYB transcription factor involved in general defences (Lee *et al.* 2001; Yokotani *et al.* 2013; Cao *et al.* 2015; Yang *et al.* 2020). In the WRKY transcription factor subgroup IIIb, monocot *WRKY45* sequences are known to be SA-responsive in *Brachypodium*, rice and wheat (Chao *et al.* 2009; Bahrini *et al.* 2011; Kakei *et al.* 2015; Kouzai *et al.* 2016). In the *PR1* family, the previously published *TaPR1-1* and *TaPR1-2* (clades I and II, respectively) were part of pathogen-responsive gene triads in our study. Interestingly genes from clades IV and V were not pathogen-responsive, suggesting that those *PR1* genes may not participate in disease response. In the PR2 family, proteins encoded by wheat pathogen-responsive genes were found in all clades of the trees, most clustering together in clade I (including TaPR2-1 and TaPR2-2). In the PR3 family, pathogen-responsive genes were found in all five chitinase classes suggesting that the pathogen responsiveness of PR3 genes is not linked to the presence/absence of the N-terminus chitin-binding domain as chitinases from classes II and IV are lacking in this domain (Tyler *et al.* 2010; Sarma *et al.* 2012). In the PR5 family, proteins encoded by wheat pathogen-responsive genes

were found in all clades with more pathogen-responsive genes in clade V containing the TLPK and clade VI containing TaPR5-1 and TaPR5-2.

Our study provided evidence of specialisation within wheat gene families and between homoeologs in terms of their hormone responsiveness. For example, of the three genes *TaPR1-1*, *TaPR1-2* and *TaPR1-3*, only *TaPR1-1* was responsive to SA. Two *AOS* genes were induced by JA as expected, but one (-*TaAOS2-4B*; TraesCS4B02G237600) was also induced by SA. In accordance with our results, it was shown in *Brachypodium* and rice that *PR1* genes from the same cluster can respond differently to SA, JA and ethylene, with different *PR1* genes being responsive to all three hormones, to only one or two, or to none (Kouzai *et al.* 2016). This result highlights the fact that genes from the same family may have a different function. Indeed, in the *in silico* analysis we found that members of the same gene families can respond differently to diseases, even within the same subclade. For example, *TaJAMyb-4A* was not found to be *F. graminearum*-responsive in wheat spikes, while the expression of its homoeolog TraesCS4D02G142900 was found to be promoted by *Fusarium* treatment in the spikes of five wheat cultivars (Kugler *et al.* 2013; Pan *et al.* 2018).

In conclusion, we identified wheat members of seven phytohormone-responsive gene families and facilitated future research on those families involved in defence. Our findings elucidated the phylogeny and disease responsiveness of genes within those seven families and highlighted that there is disparity in the response to biotic stress between some homoeologous and some paralogous sequences within families. The SA- and JA-responsive marker genes identified will prove useful in future studies that aim to elucidate the role of these hormones in wheat stress responses.

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AUTHOR CONTRIBUTIONS

A.H., A.P. and F.D. designed the research; F.D. supervised the experiments; A.H. performed the experiments and analysed the data. A.H. wrote the article, edited and amended by A.P. and F.D.; F.D. won the funding to conduct the research.

CONFLICT OF INTEREST

None.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Maximal fold change in the expression of *TaAOS1-5B*, *TaJAMyB-4A*, *TaWRKY450B* and *TaWRKY113-7D* gene expression in response to the pathogens.

Table S1. InterPro and Pfam domains for wheat proteins identified in this study and used for the phylogeny analyses.

Table S2. AOS protein IDs for Arabidopsis, Brachypodium, rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S3. JAMyb protein IDs for rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S4. PR3 (glycoside hydrolase 19) protein IDs for Arabidopsis, Brachypodium, rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S5. WRKY (subgroups IIIa and b) protein IDs for Arabidopsis, Brachypodium, rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S6. PR1 protein IDs for Arabidopsis, Brachypodium, rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S7. PR2 (glycoside hydrolase 17) protein IDs for Arabidopsis, Brachypodium, rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S8. PR5 (thaumatin-like) protein IDs for Arabidopsis, Brachypodium, rice and wheat amino-acid sequences used to construct the phylogenetic tree.

Table S9. Parameters used for alignment masking and phylogenetic tree construction[1].

Table S10. Primers used in this study.

Table S11. Fold change in expression between mock-treated and salicylic acid-treated or methyl jasmonate-treated leaves of wheat cvs. Remus and CM82036[1].

Table S12. Developmental and stress response profile of candidate markers[1].

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