Received: 19 June 2020 | Accepted: 8 April 2021

DOI: 10.1111/ppa.13388

Plant Pathology WILEY

ORIGINAL ARTICLE

TaWRKY10 transcription factor is a novel jasmonic acid signalling regulator involved in immunity against Septoria tritici blotch disease in wheat

Alberto Campanaro[1] | Anjil K. Srivastava[1] | Cunjin Zhang[1] | Jack Lee[1] | Linda Millyard[1] | Angharad M. R. Gatehouse[2] | Ed Byrne[3] | Ari Sadanandom[1]

[1]Department of Biosciences, Durham University, Durham, UK

[2]School of Biology, Newcastle University, Newcastle Upon Tyne, UK

[3]KWS UK Ltd, Thriplow, Royston, UK

Correspondence
Ari Sadanandom, Department of Biosciences, Durham University, Durham, DH1 3LE, UK.
Email: ari.sadanandom@durham.ac.uk

Funding information
Biotechnology and Biological Sciences Research Council, Grant/Award Number: BB/P00038X/1

Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici*, is currently the main threat to worldwide wheat production in temperate climates. Understanding the genetic mechanisms that underpin the *Z. tritici*–wheat interaction will be crucial for generating new control strategies against STB. Plant hormones are essential regulators of growth and immunity. In particular, jasmonic acid (JA) plays a central role in defence against necrotrophic plant pathogens, but its role in mediating immunity against *Z. tritici* is largely unknown. Here we identify the transcription factor *TaWRKY10* that is taxonomically restricted to the grass subfamily Pooideae as a novel regulator of JA responses in wheat. We demonstrate by using virus-induced gene silencing that silencing *TaWRKY10* leads to increased resistance against *Z. tritici* with an earlier onset of necrotic symptoms, but with reduced pathogen sporulation. Wheat plants silenced for *TaWRKY10* show an up-regulated JA response. Transcriptional profiling of *TaWRKY10* knock-down plants indicates that it is a negative regulator of the JA receptor *TaCOI1* gene expression. Our data indicate that *TaWRKY10* down-regulates JA responses, contributing to the susceptibility of wheat to *Z. tritici*. We postulate that manipulating *TaWRKY10* may provide a strategy to boost STB resistance in wheat.

KEYWORDS
jasmonic acid, wheat hormone signalling, wheat immunity, wheat root elongation, WRKY, *Zymoseptoria tritici* (*Septoria*)

1 | INTRODUCTION

Zymoseptoria tritici is the causal pathogen of Septoria tritici blotch (STB), one of the most threatening wheat foliar diseases across temperate regions, causing up to 40% yield loss if untreated with fungicide (Orton et al., 2011). *Z. tritici* classification as a hemibiotrophic or a necrotrophic fungus is still a matter of debate; its life cycle is characterized by a long symptomless biotrophic phase, followed by a switch to a necrotrophic phase. During the biotrophic phase the *Z. tritici* spores germinate on the surface of leaves and hyphae invade the apoplastic space through the stomata. During this phase the fungus survives biotrophically, supposedly undetected by the host. The length of this period is variable, probably depending on environmental conditions, host recognition, or the properties of the pathogen strain (Dean et al., 2012). This latency period ends with *Z. tritici* rapidly switching to its necrotrophic feeding stage, where symptoms start to appear on foliar tissue in the form of chlorotic lesions that display characteristics of programmed cell death (Keon et al., 2007). The

appearance of leaf necrosis indicates the initiation of the pathogen reproductive stage. In this phase, pycnidia (the reproductive organ of the fungus) filled with pycnidiospores appear as black lesions on leaf surfaces. The mature pycnidia release spores that allow *Z. tritici* to colonize the plant canopy, affecting flag leaf productivity by reducing its photosynthetic potential, which ultimately impacts grain yield (Ponomarenko et al., 2011). Resistance to STB is one of the major targets in wheat breeding programmes. Currently 21 distinct genetic loci (*Stb* genes) as well as a large number of quantitative trait loci (QTLs) that confer STB resistance have been identified in various wheat germplasm screening programmes (Kettles & Kanyuka, 2016; Saintenac et al., 2018). However, the molecular mechanisms underlying wheat resistance to STB are largely unknown.

Plant hormones are essential regulators of growth and immunity (Balbi & Devoto, 2008). In particular, jasmonic acid (JA) plays a central role during defence against necrotrophic pathogens (Okada et al., 2015). JA-dependent defence has been widely studied in the model system *Arabidopsis thaliana* challenged with the necrotrophic pathogen *Botrytis cinerea* (Smirnova et al., 2017) and in *Oryza sativa* against *Magnaportha oryzae* (reviewed by Nasir et al., 2017). JA and its derivatives are fatty acid-derived hormones ubiquitously present in the plant kingdom (Wasternack, 2007). They act as regulatory molecules in many plant developmental processes that include fertility, sex determination, root elongation, and fruit ripening (Mandaokar et al., 2006). JA is also involved in activating plant defences against herbivory, wounding, and abiotic stress (Balbi & Devoto, 2008).

In recent years JA has been found to have a critical role in mechanisms that allow plants to switch to defence rather than growth, therefore transcriptionally reprogramming plant cells to activate defence mechanisms and arrest cell cycle and growth (Pauwels et al., 2008).

JA-Ile, the bioactive form of JA, is formed through a conjugation of JA and isoleucine (Ile) under the action of a GH3 family of amido synthetases. JA-Ile is subsequently recognized by CORONATINE INSENSITIVE 1 (COI1)-JASMONATE ZIM DOMAIN (JAZ) co-receptor complexes and activates a signalling cascade for the induction of a series of defence genes that are also expressed in response to wounding, insect herbivory, and necrotrophic pathogens (Balbi & Devoto, 2008).

In comparison to *Arabidopsis* and rice, knowledge on JA biosynthesis and signalling in wheat is limited. The PR genes *PR1.1* and *PR1.2* were highly induced in wheat 3 weeks after emergence by JA application (Liu et al., 2016), and the same pattern of expression was detected on wheat infected with the fungal pathogens *Tilletia tritici* and *Tilletia laevis* (Lu et al., 2006). Similarly, using a transcriptome-based method, it was revealed that JA biosynthesis genes such as *LOX*, *AOS*, *AOC*, and *OPR3* and JA signalling transduction genes, including *COI1*, *JAZ*, and *MYC2*, were induced in response to Fusarium head blight pathogen in a resistant wheat variety (Xiao et al., 2013). Recently, transcriptomic studies established that JA biosynthetic genes are up-regulated upon *Z. tritici* infection (Rudd et al., 2015). Nevertheless, the mechanism underpinning the signal perception and transduction remains unclear.

The WRKY transcription factor gene family is one of the largest and plant-specific transcriptional regulators (Bakshi & Oelmüller, 2014). WRKYs are part of an intricate plant signalling web and regulate multiple pathways from stress responses to growth (Bakshi & Oelmüller, 2014). It has been established through multiple studies that WRKY transcription factors are central regulators of innate plant immunity in plants (Bakshi & Oelmüller, 2014). The characteristic feature of the WRKY transcription factor family is its highly conserved 60 amino acid long WRKY domain at the N-terminus and zinc-finger-like motif at the C-terminus (Bakshi & Oelmüller, 2014). These motifs enable WRKY transcription factors to bind with high affinity to a specific DNA cis-acting element, (C/T)TGAC(T/C), named the W box, on target gene promoters. The regulatory networks controlled by WRKY genes allow plants to transduce environmental cues into differential expression of hundreds of genes, resulting in major physiological responses (Pandey & Somssich, 2009).

Although the role of JA in defence against necrotrophic plant pathogens is well established, its role in mediating immunity against *Z. tritici* is largely unknown. To reveal any role of JA in *Z. tritici*–wheat interaction, we studied the susceptibility of two wheat cultivars, KWS Lili and KWS Santiago, to STB, and investigated the role of the regulator *TaWRKY10* in defence against *Z. tritici* using a gene silencing approach.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Nicotiana benthamiana plants were used to propagate barley stripe mosaic virus (BSMV) to perform virus-induced gene silencing (VIGS), as previously described (Lee et al., 2015). These *N. benthamiana* plants were grown in environmentally controlled cabinets at 24 °C (day) and 22 °C (night) in long day conditions (16 hr light/8 hr dark). *Triticum aestivum* 'KWS Lili' and 'KWS Santiago' plants were grown in a temperature-controlled room at 24 °C in long day conditions (16 hr light/8 hr dark). KWS Lili derives from a cross between wheat cultivars Horizon and Timaru; KWS Santiago derives from a cross between Shereborne and Oakley.

Twenty-seven-day-old (GS 1, 4–5 leaves emerged) wheat plants were inoculated with the pathogen *Z. tritici* isolate IPO323 (see below). Inoculated wheat plants were grown at 24 °C and 90% humidity in an environmentally controlled cabinet. The samples were collected by excising leave tissue for transcriptional analysis 8 hr into the 16 hr light cycle of the growth cabinet.

2.2 | Preparation of pathogen inoculum and plant inoculation

Z. tritici isolate IPO323 was used for all plant assays, as described previously by Keon et al. (2007). Spores were grown on yeast extract peptone dextrose (YPD) agar (Sigma) for 7 days at 18 °C.

For plant inoculation, spores were suspended in water containing 0.1% (vol/vol) Tween 20 at a density of 7.5×10^6 spores/ml, then spray-inoculated on wheat leaves. Wheat plants were inoculated with spores 15 days after VIGS (GS 1; see below). Two replicates of five leaves each were used to determine spore production within pycnidia 28 days after inoculation for each experiment run. Spore counts were carried out using a light microscope and a haemocytometer after washing spores, as previously described by Lee et al. (2015). Area under the disease progress curve (AUDPC) was obtained from the percentage of leaf area covered by necrotic lesions and calculated using an AUDPC calculation algorithm in the R software package. Three independent experiments were performed to evaluate the phenotypes.

The time points considered for this experiment spanned from 11 to 17 days postinoculation (dpi).

2.3 | Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (RT-qPCR) was employed to analyse total gene expression in response to *Z. tritici* infection or after hormonal treatment. The total RNA was extracted using Direct-zol RNA prep Kit (Zymo Research) as per the manufacturer's instructions. The RNA was quantified and 1 μg RNA was used for cDNA synthesis. cDNA synthesis was done using SuperScript II reverse transcriptase (Invitrogen) following the manufacturer's guidelines. Quantitative PCR was performed using Brilliant III Ultra-Fast SYBR QPCR master mix (Agilent) by using ABI One Step Plus instrument and the analysis was done with the software provided using comparative quantification methods. *TaEF1* was used as internal control for normalization. The experiments were repeated three times. In each experiment, two pools of three leaves from three different plants were used as technical replicates. To ascertain gene expression changes in response to *Z. tritici*, leaves were collected immediately after inoculation of the pathogen for time point 0, and subsequently every 48 hr.

Gene expression analysis in response to JA treatment in roots was repeated three times. In each experiment, two pools of the whole root system from three different seedlings were used as a technical replicate. The $\Delta\Delta C_t$ method (Lee et al., 2015) was used to analyse the raw data. All the primers used in this study are listed in Table S2.

2.4 | Virus-induced gene silencing

Reverse genetics studies in *T. aestivum* were carried out using the VIGS platform (Baulcombe, 1999) based on BSMV, as previously described (Lee et al., 2015). Two independent silencing fragments were designed for each gene, in order to minimize the off-target effect. BSMV inoculations were performed on 12-day-old seedlings. Primers used to generate the BSMV construct are listed in Table S1. VIGS inoculation was performed as described by Lee et al. (2015).

2.5 | Sequence analysis

To retrieve genes and protein sequences used in this study, the publicly available databases GenBank, SwissProt, PLANTTFDB, NCBIBlast, Plant-Ensembl were used (Kersey et al., 2003). Domain sequences were aligned using Clustal-Omega. These sequence alignments were used to verify the presence of all the conserved domains that define a WRKY transcription factor.

2.6 | Hormone treatments

KWS Lili and KWS Santiago seeds were germinated on plates of Murashige and Skoog (MS) medium (Duchefa) and grown for 3 days at 24/22 °C (light/dark) in long-day conditions (16 hr light/8 hr dark). After germination the seedlings were moved individually into glass test tubes containing either MS or MS supplied with 10 μM JA (Sigma). Changes in marker gene expression (*TaLOX1*, *TaAOS*, *TaCOI1*, *TaMYC2*, *TaJAZ*, *TaTAT1*, *TaPDF1*) as well as root elongation were evaluated daily. Root growth inhibition was calculated using ImageJ software. Twenty-five plants of each treatment for each genotype were analysed, for a total of three biological replicates.

Gene expression in response to JA treatment analyses in roots were repeated three times. In each experiment, two pools of the whole root system from three different seedlings were used as a technical replicate. Sampling was performed by excising root tissue daily, at 8 hr into the 16 hr light cycle.

To evaluate wheat response to *Z. tritici* infection under JA treatment, infected leaves were sprayed with 10 μM JA solution every 4 days starting at 2-week-old wheat plants. In order to maintain a standardized infection of plants, Lili and Santiago were inoculated at 27 days after germination even if not silenced. The pathotests were performed as described above.

2.7 | Root growth measurement

To evaluate the effect of *TaWRKY10* silencing on root growth, wheat seeds were germinated on plates as previously described, and then moved to pots of sand. The nutrients were supplied using 1/2 × Hoagland's solution. Seven weeks after germination the sand was removed by washing with water and pictures were taken and analysed using ImageJ software.

2.8 | Anthocyanin relative quantification

Anthocyanin extraction and quantification was done by collecting 3 g of leaf tissue for each sample, then extraction and quantification was done as previously described by Neff and Chory (1998). Three biological replicates were performed, with three technical replicates each.

3 | RESULTS

3.1 | Susceptibility of wheat cultivars to STB and *TaWRKY10* gene expression

As a first step to discover the role of WRKYs in *Z. tritici* defence, we defined the WRKY gene family in bread wheat using WRKY protein sequence information from the dicot model *A. thaliana*, and the monocot model rice (Figure 1a). Using WRKY protein sequence information from these model species (Table S1), we interrogated publicly available wheat sequence databases (PLANTTFDB, GenBank, SwissProt) and identified 71 WRKY genes in the wheat genome (TaWRKYs) and generated a phylogenetic tree based on predicted amino acid sequence similarity amongst wheat WRKY domain-containing proteins (Figures 1 and S1).

This evolutionary tree indicated that *TaWRKY10* (Figure 1b) is a Pooideae-specific WRKY transcription factor with no known homologs in other lineages except in *Hordeum vulgare* (*HvWRKY10*). To ascertain the role of *TaWRKY10* in STB, we investigated its transcription in two cultivars that are known to show different STB susceptibilities: KWS Lili and KWS Santiago.

Under control conditions, messenger RNA from 2-week-old seedlings was used as a template for RT-qPCR analysis. As indicated in Figure 1c, Lili showed lower *TaWRKY10* expression than Santiago, with the latter displaying 3-fold higher *TaWRKY10* transcription.

Using STB susceptibility scores developed by the Agriculture and Horticulture Development Board (AHDB), an industry levy board in the UK that provides annual information on the productivity of different cereal varieties under field conditions, yield losses and foliar damage were reflected at the adult plant stage. However, we

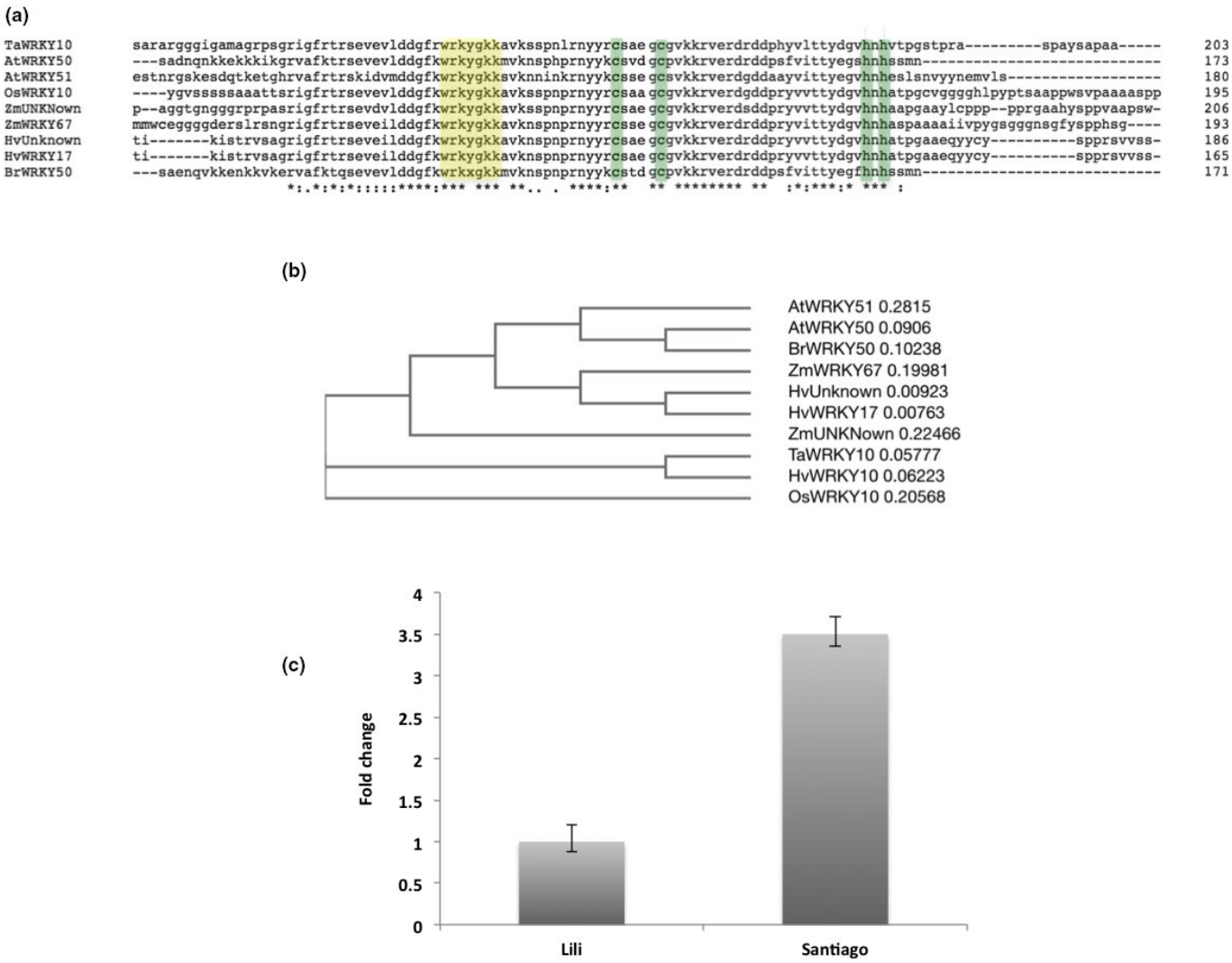

FIGURE 1 Characterization of *TaWRKY10*. (a) Multiple sequence alignment of *TaWRKY10* amino acid sequence with other known WRKY domain proteins (*Arabidopsis*, rice, maize, barley, and brassica). Alignment was generated using Clustal Omega. WRKY consensus sequence and zinc finger motifs are in yellow and green, respectively. *TaWRKY10* belongs to the subfamily of type II WRKY transcription factors. (b) Phylogenetic tree generated using Clustal Omega. *TaWRKY10* displays only one homolog in *Hordeum vulgare*. (c) Quantitative reverse transcription-PCR performed on leaves of 2-week-old wheat seedlings of cultivars Lili and Santiago. Error bars, ± *SEM* of raw data for three biological replicates [Colour figure can be viewed at wileyonlinelibrary.com]

observed differential *TaWRKY10* gene expression at the 2-week-old growth stage, and therefore to reconcile these differences and to ascertain if we could observe the same susceptibility phenotypes in 4- to 6-week-old plants as observed in field conditions, we performed *Z. tritici* infection assays in wheat varieties under controlled conditions in the laboratory.

Both varieties were infected with virulent *Z. tritici* isolate IPO323 (Arraiano & Brown, 2006). White light pictures were taken daily, with pycnidia and spore counts performed at 30 dpi, as previously described (Lee et al., 2015).

Lili leaves started to show the first symptoms of STB necrotic lesions between 14 and 15 dpi, while for Santiago these necrotic lesions were observed earlier, at 12 dpi (Figure 2a). To provide a more accurate quantification of STB symptoms, the percentage of blotched area in the leaves was determined from white light pictures using ImageJ software, with the data analysed by exploiting the AUDPC method (Madden et al., 2007). The AUDPC quantification indicated a significant difference in disease severity and progression between Lili and Santiago at 11 to 17 dpi, with a value of 251.84 for Lili and 413.25 for Santiago (Figure 2b,c). These data indicated that the spread of the lesions was slower in Lili compared to Santiago. *Z. tritici* sporulates within necrotic lesions containing pycnidia on the surface of leaves (Keon et al., 2007). Therefore, we quantified sporulation by performing pycnidia and spore counts via the spore wash technique (Lee et al., 2015). *Z. tritici* in Santiago produced on average 43 pycnidia per leaf and 250 spores/µl, while on Lili it only produced on average 33 pycnidia per leaf and 180 spores/µl (Figure 2d,e). The data demonstrate that Lili produced significantly fewer pycnidia and spores per leaf, indicating that *Z. tritici* is less successful in causing STB in Lili compared to Santiago (Figure 2d,e).

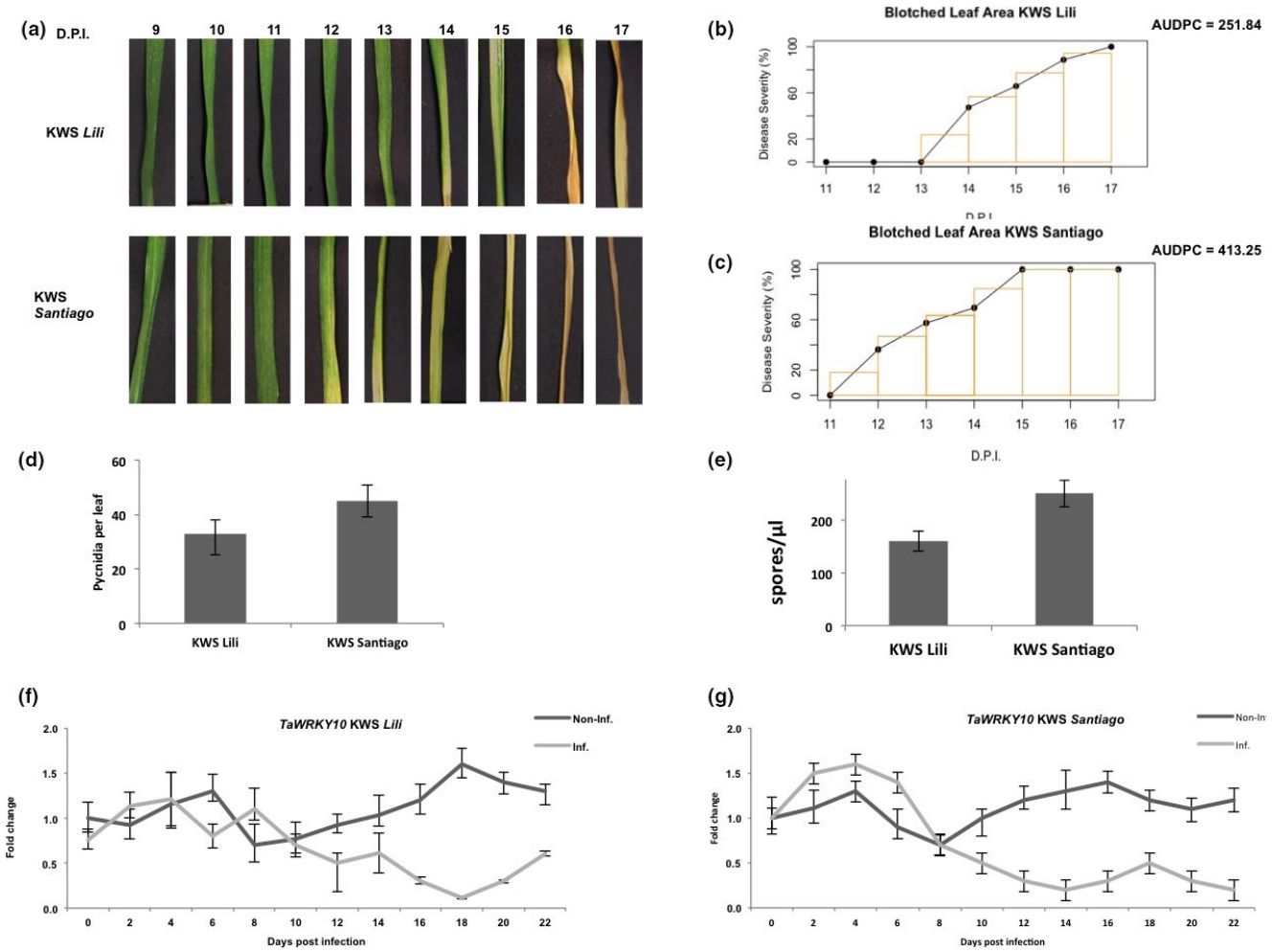

FIGURE 2 Susceptibility to Septoria tritici blotch (STB) at the seedling stage in wheat cultivars Lili and Santiago. (a) White light picture of a single leaf of Lili and Santiago infected with *Zymoseptoria tritici* over a time-course from day 9 to day 17. Area under the disease progress curve (AUDPC) corresponding to Lili (b) and Santiago (c) (average of three biological replicates), calculated by evaluating the percentage of blotched leaf area of each cultivar. The mathematical calculation was performed using the appropriate R package. (d) Average number of pycnidia per leaf in Lili and Santiago; Lili was significantly more resistant to STB than Santiago (Student's *t* test, $p = 3.3 \times 10^{-4}$). (e) Spore wash was performed 32 days after initial *Z. tritici* inoculation. Student's *t* test showed significance difference between Lili and Santiago sporulation $p = 1.2 \times 10^{-9}$. Level of expression of *TaWRKY10* gene over 22 days of STB time-course, in cultivars Lili (f) and Santiago (g). Error bars, ± *SEM* of raw data for three biological replicates [Colour figure can be viewed at wileyonlinelibrary.com]

A time-course experiment indicated that *TaWRKY10* gene expression was down-regulated in both cultivars in response to the pathogen, 2 days prior to the switch to the necrotrophic phase (Figure 2f,g). Taken together with the differential susceptibility to STB in Lili and Santiago, our data suggest a potential involvement of *TaWRKY10* in wheat defence against STB.

3.2 | Effect of silencing *TaWRKY10* gene expression on resistance to STB

Because Santiago showed the greatest expression of *TaWRKY10*, and was the most susceptible variety to STB, we wanted to ascertain if *TaWRKY10* gene activity could contribute to the differences in susceptibilities between Lili and Santiago.

Due to the large hexaploid wheat genome, isolating single gene knockouts is currently not feasible. Therefore, VIGS (Baulcombe, 1999) was employed to silence *TaWRKY10*. BSMV has previously been modified to effectively silence the expression of many wheat genes (Matthew, 2004). Gene silencing vectors based on BSMV (Lee et al., 2012), carrying the 5′ untranslated region (UTR) and 3′ UTR of *TaWRKY10* mRNA, were used to induce sequence-specific degradation of the endogenous *TaWRKY10* mRNA and therefore a knock down in its gene expression in Lili and Santiago. BLAST analyses confirmed that both DNA fragments (*BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2*) were unique to *TaWRKY10*. The analysis was carried out using two databases: NCBI-BLAST (*T. aestivum* nucleotide collection) and PLANT ENSEMBL *T. aestivum* DNA database. The siRNA finder software si-fi predicted *TaWRKY10*-specific silencing (Lee et al., 2015). The effectiveness of gene silencing was confirmed by RT-qPCR on mRNA from emerging leaves of plants 14 days after *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2* inoculation (Figure 3a,b). *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2* yielded 70% and 65% silencing of *TaWRKY10* in Lili and 68% and 60% silencing in Santiago, respectively, compared to empty vector controls.

Fourteen days after silencing treatment, the third to fifth wheat leaves were subjected to *Z. tritici* inoculation (7.5×10^6 spores/ml) and the symptom development was recorded for 28 days (Figure 3c), before pycnidia and spore counts were performed to measure fungal development (Figure 3d,e). White light pictures of disease progression were taken daily. As shown in Figure 3c, silencing *TaWRKY10* led to an earlier onset of disease symptoms compared to the controls for both varieties. In Lili no difference in symptom development was observed until 14–15 dpi, after which the *TaWRKY10*-silenced lines began to show necrotic lesions, whereas lesions appeared 2 days later on the empty vector-inoculated control plants. For Santiago, the same pattern of earlier onset of symptoms was observed. The empty vector control leaves started to show blotching phenotype at 14 dpi, while in the *TaWRKY10*-silenced plants, STB symptoms appeared at 11 dpi. After 30 days, *Z. tritici* in the inoculated leaves started to sporulate through pycnidia production. The pycnidia production across multiple plants over 2 cm leaf sections was quantified. Pycnidia formation was significantly reduced on *TaWRKY10*-silenced leaves, with 45% reduction on *BSMV:TaWRKY10_1*- and 43% reduction on *BSMV:TaWRKY10_2*-infected plants compared to empty vector BSMV:00 control (Figure 3d). As expected from the reduced number of fruiting bodies, spore production was also reduced in the *TaWRKY10*-silenced wheat leaves (Figure 3e), with a 50% reduction on *BSMV:TaWRKY10_1*- and a 46% reduction on *BSMV:TaWRKY10_2*-infected plants when compared to the BSMV:00 control. *BSMV:TaWRKY10_1* was more efficient at silencing *TaWRKY10* than *BSMV:TaWRKY10_2*. Strikingly, the spore production in the *BSMV:TaWRKY10*-silenced Santiago lines was equivalent to spores produced from BSMV:00 control Lili plants (Figure 3e). These data indicate that *TaWRKY10* may play a role in determining the susceptibility phenotypes of cvs Lili and Santiago. Furthermore, silencing *TaWRKY10* enhanced Lili resistance to STB.

3.3 | Sensitivity of wheat cultivars to JA

It is well established that JA is a growth regulator as well as a key hormone in defence signalling (Chini et al., 2016; Goossens et al., 2016). In order to assess if JA could have a role during the immune response against STB, we treated Lili and Santiago with JA and infected them with *Z. tritici*. Our data indicate that both cultivars showed increased resistance to STB when treated with JA (Figure 4a; Figure S2).

JA is a pivotal hormone in the control of root growth (Staswick et al., 1992). To ascertain if the JA response was different in Lili compared to Santiago, we germinated seeds of both cultivars on MS agar plates, then transferred 2-day-old seedlings to plates that were supplemented with 10 μM JA to monitor root growth daily. As shown in Figure 4b,c, seedlings of Lili showed root growth inhibition after 24 hr (40% root growth inhibition) compared to Santiago. Lili roots barely elongated at all for the first 24 hr of JA treatment, indicating dramatic JA-mediated root growth inhibition response. At 48 hr after JA treatment, there was similar root growth inhibition in both cultivars. Santiago roots remained unresponsive to JA and elongated at rates comparable to untreated seedlings in the first 24 hr of JA treatment, after which they exhibited growth inhibition at 48 hr. Our data indicates that Lili was more sensitive to JA than Santiago.

JA treatment is also known to induce anthocyanin accumulation in plants (Chini et al., 2016) and this was observed to a much greater extent in Lili compared to Santiago, with 3-fold and 1.8-fold more accumulation, respectively, compared to their controls (Figure 4d,e).

Our data demonstrate that Lili is more responsive to JA, resulting in greater root growth inhibition and with more pronounced anthocyanin accumulation compared to Santiago. The evidence indicates that the observed sensitivity to JA between the cultivars might explain the greater resistance to STB in Lili.

To ascertain whether *TaWRKY10* silencing leads to any changes in morphology, we analysed plant height and root length in the silenced lines and compared it to empty vector controls. Plants from both varieties were pregerminated on filter paper for 3 days and

subsequently moved to sand-filled pots, and grown for 10 days before BSMV inoculation. Seven weeks after germination, the sand was washed away, the roots and the shoots were photographed, and the length quantified via ImageJ software. No significant difference was observed in plant height in 7-week-old *TaWRKY10*-silenced plants compared to controls (data not shown). However, in cv. Santiago we observed a root growth inhibition of 25% (Figure S3) compared to empty vector controls. A similar phenotype was also observed in Lili (Figure S4). This evidence suggests a role for *TaWRKY10* in regulating root growth as well as immunity against STB.

3.4 | Response of JA signalling markers in wheat cultivars

To investigate the molecular basis of the differential JA responses in Lili and Santiago, we performed RT-qPCR analysis of JA marker genes (Goossens et al., 2016) in these cultivars. Established molecular markers for JA biosynthesis, perception, and signalling were analysed. JA biosynthesis was monitored by analysing expression of genes encoding allene oxide synthase (*TaAOS*) and lipoxygenase 2 (*TaLOX2*). JA perception was monitored by analysing gene expression of the JA receptor coronatine-insensitive 1 (*TaCOI1*) and signal

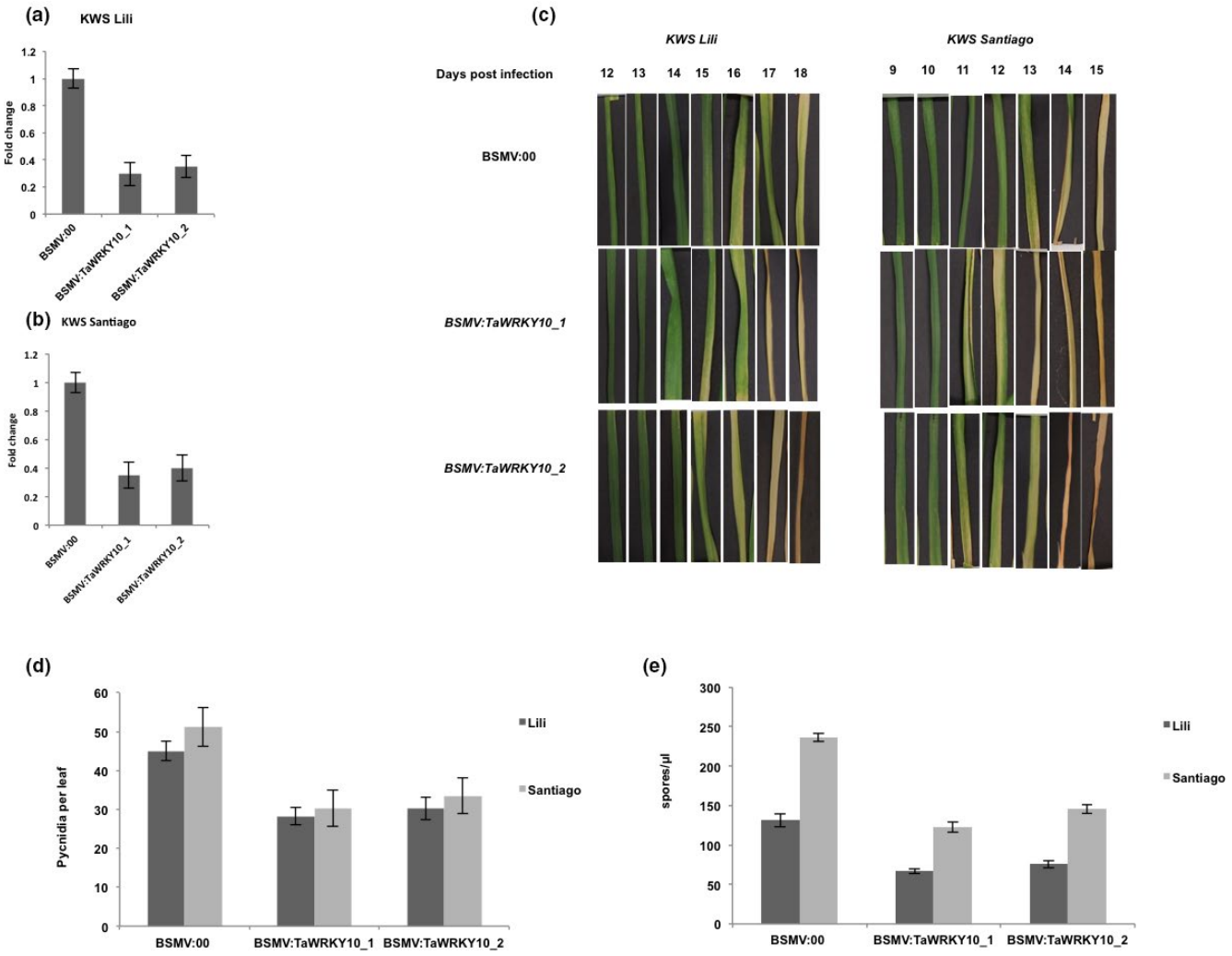

FIGURE 3 Silencing *TaWRKY10* led to increased resistance to Septoria tritici blotch (STB). (a,b) Quantitative reverse transcription-PCR data showed the expression of *TaWRKY10* gene reduced in *BSMV:TaWRKY10_1* and *BSMV:TaWRKY10_2*, compared to BSMV:00 empty vector control in both wheat cultivars Lili and Santiago. The relative expression is normalized to BSMV:00-treated Lili. Error bars, ± *SEM* of raw data. (c) Images of leaves of BSMV:00 mock-silenced, *BSMV:TaWRKY10_1*- and *BSMV:TaWRKY10_2*-silenced plants for Lili and Santiago from day 12 to day 15 after *Zymoseptoria tritici* inoculation. (d) The number of pycnidia produced on the leaves of *TaWRKY10*-silenced plants compared to mock-silenced plants. Error bars, ±*SEM* of raw data. Student's *t* test showed significance difference of the silenced lines compared to their own control (Lili *BSMV:TaWRKY10_1* $p = 3.5 \times 10^{-6}$, Lili *BSMV:TaWRKY10_2p* $= 7.0 \times 10^{-4}$; Santiago *BSMV:TaWRKY10_1* $p = 2.5 \times 10^{-9}$ Santiago *BSMV:TaWRKY10_2* $p = 7.8 \times 10^{-7}$) (e) Spore washes performed 32 days after *Z. tritici* infection on *TaWRKY10*-silenced plants. Error bars, ±*SEM* of raw data. Student's *t* test showed significance difference of the silenced lines compared to their own control (Lili *BSMV:TaWRKY10_1* $p = 5.7 \times 10^{-10}$, Lili *BSMV:TaWRKY10_2p* $= 3.2 \times 10^{-8}$; Santiago *BSMV:TaWRKY10_1* $p = 8.3 \times 10^{-13}$ Santiago *BSMV:TaWRKY10_2* $p = 2.3 \times 10^{-9}$) [Colour figure can be viewed at wileyonlinelibrary.com]

transduction through expression of the genes encoding jasmonate-insensitive1 (*TaJIN1-TaMYC2*) and jasmonate-ZIM-domain (*TaJAZ*). Downstream response markers were monitored by analysing expression of genes encoding plant defensin 1.1 (*TaPDF1*) and tyrosine aminotransferase (*TaTAT1*).

Tissue samples from leaves and roots of JA-treated or mock-treated Lili and Santiago were used to extract mRNA at 0, 24, and 48 hr time points, where we observed the greatest differential JA response in root growth. We observed a down-regulation in the expression of genes that affect JA biosynthesis between the two cultivars after JA treatment, possibly due to a negative feedback mechanism (Figure 5a–d). Intriguingly, gene expression of JA receptor *TaCOI1* was up-regulated dramatically in both shoot and root in Lili at 24 hr after the treatment, by 6-fold and 8-fold, respectively (Figure 5e), while in Santiago *TaCOI1* was not up-regulated even 48 hr after JA treatment (Figure 5f). The JA signal transduction marker gene *TaMYC2* was not up-regulated significantly in either cultivar. The JA signalling repressor *TaJAZ* was up-regulated 14-fold and 10-fold in Lili shoot and root tissues, respectively, but only 7-fold and 4-fold in Santiago shoot and root tissues 48 hr after JA treatment (Figure 5g,h,i,l). The downstream JA response gene *TaTAT1* was more active in Lili at 24 hr after JA treatment. Moreover, a second JA marker gene, *TaPDF1*, showed enhanced transcription in Lili at 48 hr after JA treatment (Figure 5m,n,o,p). Our data indicate that JA

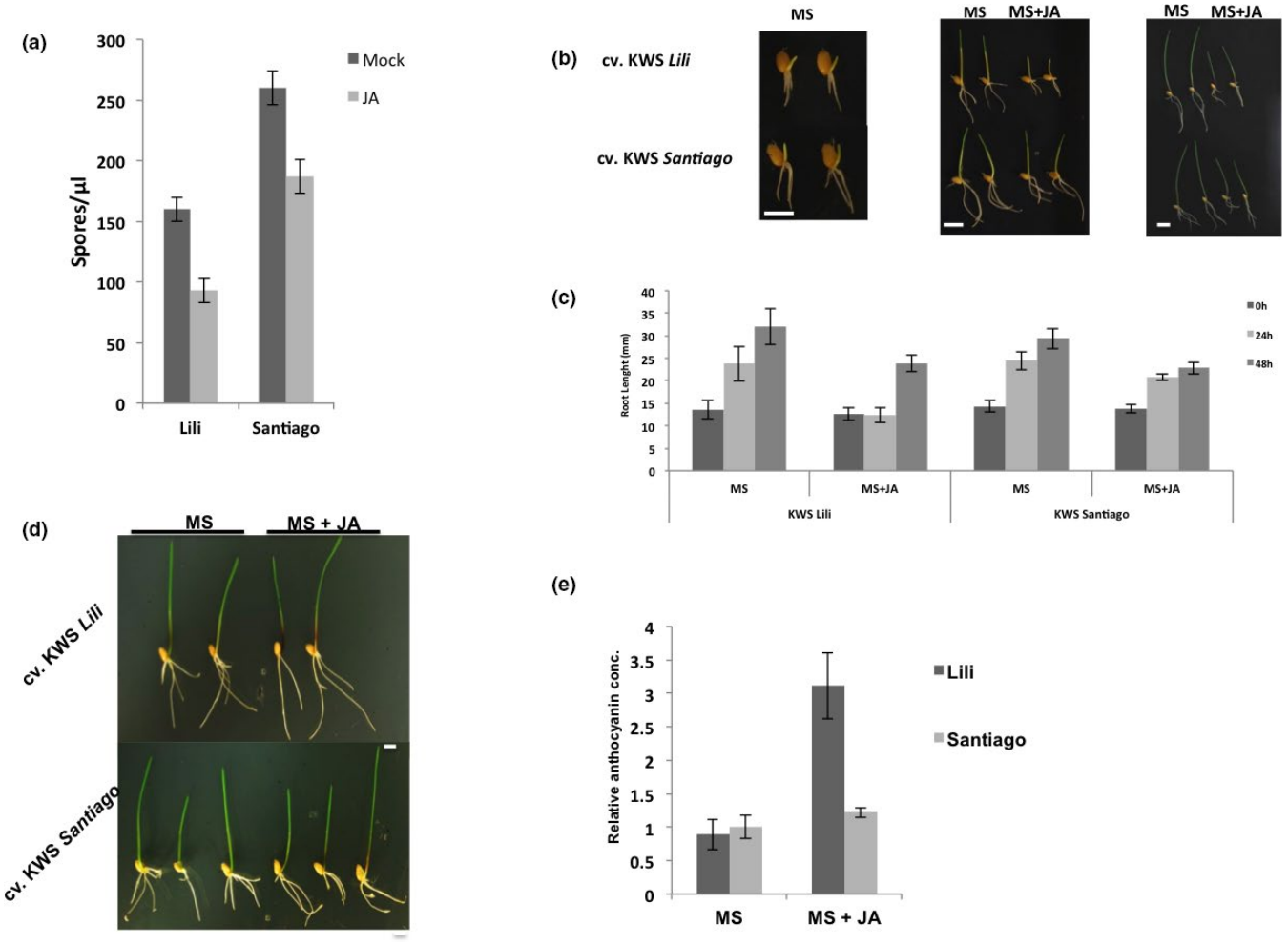

FIGURE 4 Wheat cv. Lili is more sensitive to jasmonic acid (JA) compared to cultivar Santiago. (a) Wheat leaves were treated with either JA or mock treated every 4 days and spores counted at the end of the infection. Treated leaves displayed a statistically significant decrease of *Zymoseptoria tritici* sporulation compared to their mock-treated control (Lili $p = 1.6 \times 10^{-3}$; Santiago $p = 4.6 \times 10^{-3}$). Biological and technical replication was performed similarly to previously described experiments in this work. (b) White light pictures of wheat seedlings of cvs Santiago and Lili exposed to JA compared to their controls. Seeds were germinated on Murashige and Skoog medium (MS) for 2 days, imaged at 0 hr and then moved either to MS or MS supplemented with 10 µM JA. Pictures were taken 24 and 48 hr after treatment. (c) Quantification of root growth during JA treatment. Data were obtained analysing white light pictures with ImageJ software. Student's *t* test did not show a statistical difference of Lili root length from 0 to 24 hr of JA treatment ($p = 0.95$) whereas a statistically significant increase in root length was observed for Santiago in the same time frame ($p = 2.2 \times 10^{-3}$). (d) White light picture of seedlings after 5 days of JA treatment. (e) Relative quantification of anthocyanin accumulation in seedlings via spectrophotometer analysis. Error bars, ± *SEM* of raw data for three different biological replicates. Student's *t* test showed significant difference for Lili treated compared to its own control ($p = 3.6 \times 10^{-5}$); Santiago did not show any significant increase in anthocyanin content upon JA treatment ($p = 0.9$) [Colour figure can be viewed at wileyonlinelibrary.com]

perception and response is dampened in Santiago compared to Lili. Taken together with expression differences in *TaWRKY10* between the cultivars, our data suggest that there may be a link between JA responses and *TaWRKY10* gene expression.

3.5 | TaWRKY10 silencing and JA signalling

Given that *TaWRKY10* silencing led to altered immune responses in wheat, and cultivars that showed different *TaWRKY10* levels had different JA responses, we wanted to explore the possibility that *TaWRKY10* expression could be linked to JA signalling. We performed RT-qPCR analysis of JA marker genes on 4-week-old *TaWRKY10*-silenced plants. Well-established molecular markers for JA signalling were chosen for gene expression analysis. We analysed genes affecting biosynthesis (*TaLOX1*), hormone perception (*TaCOI1*), signal transduction (*TaMYC2*), and downstream response (*TaPDF1*) markers. The gene expression analysis shown in Figure 6 demonstrates that there was no significant difference in expression of *TaLOX1* genes encoding components of JA biosynthesis between the *TaWRKY*-silenced lines and nonsilenced control (Figure 6a). Surprisingly, we detected a 4-fold increase in *TaCOI1* transcript in the *TaWRKY10*-silenced lines (Figure 6b). We also noticed that *TaCOI1* transcript was present at 10-fold higher level in Lili compared to Santiago in the empty vector controls. We also observed an up-regulation of the JA-responsive transcriptional activator *TaMYC2* (Goossens et al., 2016) in the *TaWRKY10*-silenced lines (Figure 6c). Not surprisingly, *TaPDF1*, a downstream target gene of *TaMYC2*, was also up-regulated in the *TaWRKY10*-knockdown plants (Figure 6d). Our data indicate that *TaWRKY10* acts as a repressor of *TaCOI1* gene expression. We confirmed the presence of W-boxes *cis*-elements in the *TaCOI1* promoter region (Figure S5).

We demonstrated that manipulating *TaWRKY10* gene expression in wheat cultivars led to altered JA perception via changes in the JA receptor *COI1* gene expression. Because JA is a major hormone that regulates defence against necrotrophic pathogens, our data provide a plausible mechanistic explanation for the differential *Z. tritici* susceptibility phenotypes observed in the wheat cultivars with altered *TaWRKY10* expression.

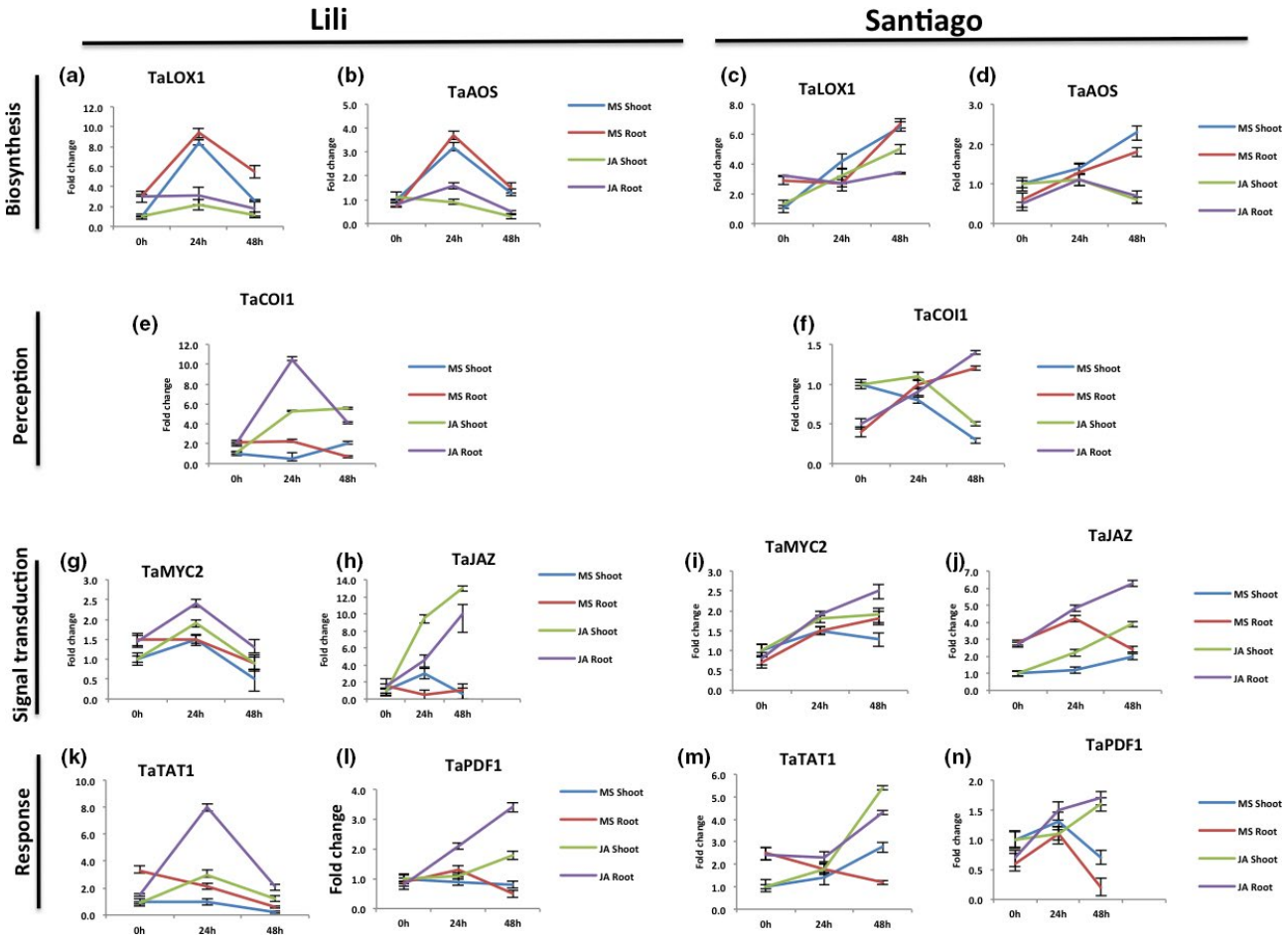

FIGURE 5 Jasmonic acid (JA) response in wheat cultivars Lili and Santiago. (a–d) Quantitative reverse transcription-PCR data indicating down-regulation in marker gene expression 24 hr after JA treatment. (e,f) Expression of receptor *TaCOI1*; (g,i) JA signal transduction marker gene *TaMYC2*; and (h,l) JA signalling repressor *TaJAZ*. (m–p) JA response genes *TaTAT1* and *TaPDF1*. Error bars, ± *SEM* of raw data for three biological replicates. Comparable results were observed on all three repeats of the experiment [Colour figure can be viewed at wileyonlinelibrary.com]

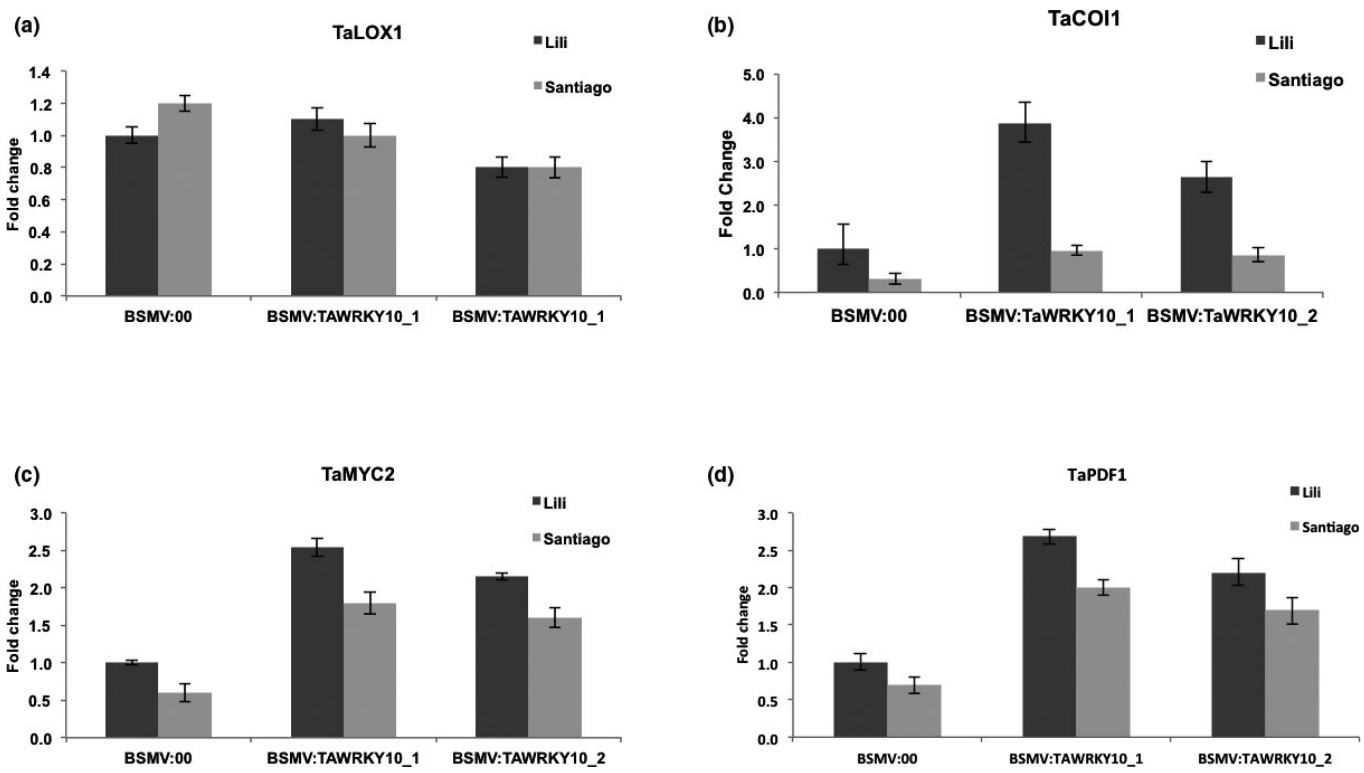

FIGURE 6 *TaWRKY10* gene silencing affects jasmonic acid (JA) signalling in wheat cultivars Lili and Santiago. Quantitative reverse transcription-PCR data shows the expression of (a) *TaLOX1*, a gene involved in JA biosynthesis pathway; (b) the JA receptor *TaCOI1*; and marker genes (c) *TaMYC2* and (d) *TaPDF1*. Error bars, ± *SEM* of raw data for three biological replicates

4 | DISCUSSION

STB is one of the most devastating wheat foliar diseases worldwide. Currently all elite wheat cultivars show varying degrees of susceptibly to *Z. tritici*, and STB is solely controlled by fungicide treatment. In recent years, outbreaks of fungicide-resistant strains of *Z. tritici* have become more prevalent, indicating that new control measures are vital for sustainable wheat production. A better understanding of wheat defence mechanisms could lead to the discovery of novel components that could be exploited as STB resistance breeding targets.

The WRKY domain-containing transcription factor gene family is universally present in plants and is an integral part of plant defence signalling pathways (Bakshi & Oelmüller, 2014). Several recent studies confirm the role of WRKY transcription factors as major regulators of immune responses in crop plants (Lee et al., 2018; Satapathy et al., 2018).

Here, we identified *TaWRKY10* as a novel component of wheat immunity against STB. Among the *TaWRKY*s identified to date in wheat, *TaWRKY10* shows homology to only a single WRKY factor from *H. vulgare*, indicating that *TaWRKY10* is a Pooideae-specific gene in *T. aestivum*, and is only the second such gene to be characterized (Perochon et al., 2015). Interestingly, the other Pooideae-specific gene, *TaFROG*, is also reported to be involved in immunity against a wheat pathogen, suggesting that these genes may have evolved to play a common role in wheat.

Two different cultivars, Santiago and Lili, were selected to understand the role of *TaWRKY10* in more detail. Initially, we demonstrated that these varieties show different degrees of resistance to STB. Lili (lower *TaWRKY10* transcript levels) is more resistant to STB than Santiago (higher *TaWRWKY10* transcript levels).

To assess if *TaWRKY10* expression level decrease has direct correlation with wheat defence against *Z. tritici*, we performed gene silencing experiments to down-regulate *TaWRKY10* expression. Silencing *TaWRKY10* in both Lili and Santiago cultivars led to reduced sporulation but an earlier onset of the *Z. tritici* necrotrophic phase, similar to that observed for another negative regulator of immunity, *TaR1*, in wheat (Lee et al., 2015). Thus, for both of the cultivars the resistance to STB increased as a consequence of silencing *TaWRKY10*, suggesting that *TaWRKY10* may be a contributing factor during STB response.

TaWRKY10 mRNA levels were down-regulated in response to *Z. tritici* approximately 2–3 days prior to the onset of the necrotrophic stage. Silencing *TaWRKY10* enhanced *TaCOI1* expression. It has been previously shown that up-regulating *COI1* expression enhances JA signalling (Goossens et al., 2016). Therefore, our data indicates that *TaWRKY10* is a negative regulator of JA signalling, as silencing *TaWRKY10* enhances *TaCOI1* expression. Because *TaWRKY10* is a negative regulator of JA signalling, this suppression of gene

expression in response to *Z. tritici* indicates a response by wheat to up-regulate JA-mediated defence.

Hormone signalling pathways play a pivotal role in plant response to environmental stimuli. JA is one of the main hormones involved in plant–pathogen interactions, and the presence of JA receptor *COI1* is necessary to propagate the hormonal signal and thus the plant response (Chini et al., 2016; Goossens et al., 2016). Despite the wealth of knowledge of hormone signalling pathways in the model dicot *A. thaliana*, little is known about hormone signalling in monocots, especially in wheat. The current study sheds new light into regulation of JA signalling in wheat.

It has been previously reported that different WRKY transcription factors play opposite roles in the JA-mediated defence against the same necrotrophic pathogen. For example, overexpressing a wild grape WRKY transcription factor in *Arabidopsis* led to down-regulation of JA response after *B. cinerea* inoculation, leading to increased susceptibility (Wang et al., 2017), whereas overexpressing *Populus trichocarpa WRKY40* in poplar trees led to increased JA level and increased resistance to *B. cinerea* (Karim et al., 2015). This evidence indicates species specificity and functional diversity within the WRKY transcription factor gene family.

The inhibitory effect of JA on root growth is well established (Staswick et al., 1992). We demonstrated that silencing *TaWRKY10* led to root growth inhibition, mimicking the effect of enhanced JA signalling on root growth inhibition. Taken together with *TaWRKY10*-silenced plants exhibiting increased defence, our data demonstrate a negative role in JA signalling both in defence and also in growth control for *TaWRKY10*.

Moreover, we demonstrated that the down-regulation of *TaWRKY10* transcript level led to the up-regulation of the JA receptor *TaCOI1* gene transcription, establishing an inverse relationship between the expression patterns of these two genes. *COI1* transcript abundance is directly correlated with JA sensitivity in rice (Yang et al., 2012). In the current study we demonstrated that silencing *TaWRKY10* resulted in the up-regulation of *TaCOI1* transcription, which might lead to the activation of the JA signalling pathway as demonstrated in other studies (Goossen et al., 2015) and in our observation of both root growth inhibition and increased resistance to STB. Therefore, it is likely that *TaWRKY10* exerts a negative effect on JA signalling in wheat by suppressing *TaCOI1* gene expression. Because *TaWRKY10* only exists in the subfamily Pooideae, this may be a novel mechanism for regulating JA signalling in wheat, but further work is needed to understand the mechanistic basis of how *TaWRKY10* regulates *TaCOI1* gene expression.

Until now there have been no studies that examine the sensitivity of different wheat varieties to phytohormones. We have been able to demonstrate that a more rapid JA perception may be regulated by *TaWRKY10*-mediated suppression of the JA receptor gene expression and is coupled with best disease resistance performance. Our data suggest that breeding programmes that target improved JA perception mechanisms could lead to novel wheat germplasm better suited to withstand *Z. tritici* infection, resulting in greater yield.

ACKNOWLEDGEMENTS

We acknowledge Fenella Croley for critical reading of the manuscript. A.C., L.M., J.L., and A.S. were funded by Biotechnology and Biological Research Council of the UK.

CONFLICT OF INTEREST

The authors declare there is no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article.

ORCID

Ari Sadanandom https://orcid.org/0000-0003-0058-9479

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Campanaro A, Srivastava AK, Zhang C, et al. TaWRKY10 transcription factor is a novel jasmonic acid signalling regulator involved in immunity against Septoria tritici blotch disease in wheat. *Plant Pathol*. 2021;70:1397–1408. https://doi.org/10.1111/ppa.13388