The TuMYB46L-TuACO3 module regulates ethylene biosynthesis in einkorn wheat defense to powdery mildew

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Summary

- Powdery mildew disease, elicited by the obligate fungal pathogen Blumeria graminis f.sp. tritici (Bgt), causes widespread yield losses in global wheat crop. However, the molecular mechanisms governing wheat defense to Bgt are still not well understood.
- Here we found that TuACO3, encoding the 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase functioning in ethylene (ET) biosynthesis, was induced by Bgt infection of the einkorn wheat Triticum urartu, which was accompanied by increased ET content. Silencing TuACO3 decreased ET production and compromised wheat defense to Bgt, whereas both processes were enhanced in the transgenic wheat overexpressing TuACO3.
- TuMYB46L, phylogenetically related to Arabidopsis MYB transcription factor AtMYB46, was found to bind to the TuACO3 promoter region in yeast-one-hybrid and EMSA experiments. TuMYB46L expression decreased rapidly following Bgt infection. Silencing TuMYB46L promoted ET content and Bgt defense, but the reverse was observed when TuMYB46L was overexpressed.
- Hence, decreased expression of TuMYB46L permits elevated function of TuACO3 in ET biosynthesis in Bgt-infected wheat. The TuMYB46L-TuACO3 module regulates ET biosynthesis to promote einkorn wheat defense against Bgt. Furthermore, we found four chitinase genes acting downstream of the TuMYB46L-TuACO3 module. Collectively, our data shed a new light on the molecular mechanisms underlying wheat defense to Bgt.

Introduction

Common wheat (Triticum aestivum, AABBDD) is the most widely cultivated staple crop in the world (IWGSC. et al., 2018). Efficient and stable wheat production is essential for maintaining global food security. Powdery mildew disease, caused by the obligate biotrophic fungal pathogen Blumeria graminis f.sp. tritici (Bgt), leads to widespread yield reductions in many wheat-producing regions (Singh et al., 2016). The infection cycles of cereal powdery mildew fungi (including Bgt) are similar to each other (Hückelhoven & Panstruga, 2011; Wicker et al., 2013). Under favorable conditions, the conidium germinates and produces a primary germ tube and an appressorial germ tube (AGT). The AGT then develops into an appressorium from which an infection peg emerges. In a compatible interaction, the infection peg enters host cells and forms a haustorium. This structure absorbs nutrients from host cells and promotes rapid secondary hyphae growth on the leaves, leading to the formation of powdery mildew colonies and the production of a new generation of conidia. To manage the damages inflicted by Bgt, substantial efforts are being devoted to study the genetic and molecular mechanisms of wheat defense to powdery mildew disease in order to develop effective controlling measures (Yahiaoui et al., 2004; Bhullar et al., 2010; He et al., 2018; McNally et al., 2018; Xing et al., 2018; Zou et al., 2018).

To date, two layers of plant immunity to pathogen attacks have been elucidated (Bigeard et al., 2015; Cui et al., 2015). Pattern triggered immunity (PTI), initiated by recognition of pathogen associated molecular patterns by host cell surface receptors, is a basal level of defense. However, effector triggered immunity (ETI), augmented after detecting the avirulent effector of pathogen by host intracellular immunoreceptor molecules, is an intensified form of defense often with high race specificity. Many defense proteins, such as various types of pathogen-related (PR) proteins including chitinases, are induced upon the activation of PTI and ETI, and contribute...
to the limitation of pathogen growth. The mechanisms underlying the induction of defense genes are highly complex, which frequently involve phytohormone signaling processes and vary among different pathosystems (Pietrzak et al., 2012; Bigeard et al., 2015; Cui et al., 2015). Detailed characterization of these mechanisms is essential for completely understanding the genetic and molecular basis of plant defense to different pathogen attacks.

Through molecular and genetic analysis, ethylene (ET) biosynthesis and signal transduction have been found to regulate plant defense to diverse pathogens (Broekaert et al., 2006; Tintor et al., 2013; Broekgaarden et al., 2015). In Arabidopsis thaliana, ET biosynthesis was activated in response to the infection by the necrotroph Botrytis cinerea (Gravino et al., 2015); ET induction also was shown to play a positive role in defense against the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Guan et al., 2015). In both studies, the infection-induced ET biosynthesis was caused by increased activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS), a key enzyme required for ET biosynthesis in plant cells (Booker & Delong, 2015). In line with the above findings, a recent work shows that rice defense against the hemibiotrophic fungal pathogen Magnaporthe oryzae also involved the upregulation of ET production through the function of ACS. Apart from ACS, several transcriptional factor (TF) genes acting in the ET signaling pathway also have been demonstrated to regulate plant defense against pathogens. TaPIE1, a pathogen-induced ET responsive factor (ERF), was reported to positively regulate the resistance response to Rhizoctonia cerealis by activating the transcription of defense-related genes downstream of the ET signaling pathway (Zhu et al., 2014). Overexpression of GmERF5 was shown to enhance the resistance of soybean to Phytophthora sojae via positively regulating the expression of the defense genes PR10, PRI-1 and PR10-1 (Dong et al., 2015a). Activation of two TFs functioning in ET signaling in rice increased the resistance to M. oryzae by elevating the production of reactive oxygen species (ROS) and phytoalexins (Yang et al., 2017). Finally, ectopic expression of ERF-V, an ET-responsive element-binding factor gene of the AP2/ERF transcription factor gene family, conferred high resistance to Bgt infection in wheat (Xing et al., 2017). Despite the progress outlined above, there is still little molecular evidence for the function of ET biosynthesis and signaling in plant defense to obligate biotrophic fungal pathogens, such as powdery mildew fungi.

Higher plants generally possess a large family of MYB TF genes, many of which have been found to regulate plant defense against pathogens. For example, the knockout of AtMYB46, an R2R3 type MYB TF originally found to regulate secondary cell wall biosynthesis in Arabidopsis (Zhong et al., 2007; Zhong & Ye, 2012), conferred enhanced resistance to B. cinerea through increasing the expression of cell wall remodeling genes encoding type III peroxidases and two defense-related genes coding for PR3 and PDF1.2a (Ramirez et al., 2011a,b). Another Arabidopsis R2R3 MYB TF, MYB15, positively regulates Arabidopsis basal immunity to the bacterial pathogen Pseudomonas syringae through promoting defense-induced lignification (Chezem et al., 2017). MYBS1, a MYB TF gene in rice, contributes to rice defense against M. oryzae through suppressing bsr-d1 gene expression, which resulted in elevated ROS accumulation and, thus, limited pathogen growth (W. Li et al., 2017). Finally, several MYB TF genes inducible by ET treatment, including MYB108 from cotton, and TaMYB4 and TaPIMP2 from wheat (Al-Attala et al., 2014; Cheng et al., 2016; Wei et al., 2017), are found to contribute to plant defense against pathogens. Nevertheless, there is still no detailed report on the regulation of plant pathogen defense by MYB TFs through upregulating the transcription of ET biosynthesis gene(s) and the resultant elevation of ET production.

In order to efficiently analyze the defense mechanisms to Bgt, we used the diploid einkorn wheat Triticum urartu (AA), which has a much smaller genome than that of common wheat, as an experimental host (Zhang et al., 2016; Ling et al., 2018). Triticum urartu accessions from around the world differed extensively in their response to Bgt infection; T. urartu genes and networks associated with different types of resistance responses to Bgt have been revealed by transcriptome comparison and selection sweep mapping analyses (Zhang et al., 2016; Ling et al., 2018). Further to the studies above, we here report the function of TuACO3 and TuMYB46L, encoding an active 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (ACO) enzyme and a R2R3 myeloblastosis (MYB) TF, respectively, in wheat defense against Bgt. We found that the expression of TuACO3 was increased by Bgt infection in diverse T. urartu accessions, and its protein promoted ET biosynthesis in Bgt-infected plants. Silencing TuACO3 lowered Bgt infection-induced ET production and weakened the defense against Bgt. Through yeast-one-hybrid (Y1H) assay and complementary molecular experiments, we identified TuMYB46L that could bind to the promoter region of TuACO3 and acted as a negative regulator of TuACO3 expression. TuMYB46L was rapidly downregulated by Bgt infection, thus permitting enhanced expression of TuACO3 in the infected plants. Therefore, TuMYB46L and TuACO3 comprise a functional gene module that regulates ET biosynthesis to promote einkorn wheat defense against Bgt. We further identified four chitinase genes that function downstream of the TuMYB46L-TuACO3 module through transcriptome comparison. Together, our data provide new information on the function of ET biosynthesis in wheat defense against Bgt and the transcriptional control of plant ACO gene in powdery mildew-infected plants.

Materials and Methods

Plant materials

The Triticum urartu accessions used in this work included PI428193, PI428202, PI428214, PI428220, PI428224 and G1812 (Zhang et al., 2016; Ling et al., 2018). They were all susceptible to the Blumeria graminis f.sp. tritici (Bgt) race E09. G1812 has previously been used for determining the genomic sequence of T. urartu (Ling et al., 2018). The common wheat cultivar Kenong 199 (KN199), highly susceptible to E09 (Wang
et al., 2014; Zou et al., 2018), was used for propagating Bgt and as a recipient for developing the transgenic wheat lines overexpressing TuACO3 or TuMYB46L (ACO, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase; MYB, myeloblastosis).

**Bgt infection and phenotyping**

Wheat seeds were germinated in the glasshouse at 20–22°C under a 16 h : 8 h, light : dark photoperiod for 1 wk to yield seedlings at the one-leaf stage. They were inoculated with E09 spores as described by Wang et al. (2014). At 72 h post-inoculation (hpi), the leaves were cut into 5-cm segments, and subjected to microcolony staining using Coomassie blue (Wang et al., 2014). For each inoculation, 10–15 leaves from ≥10 infected seedlings were examined, with the results used to calculate the percentage of microcolonies developing from the total number of examined Bgt spores. For observing the development of Bgt colonies, the infected leaves were photographed at 8 d post-inoculation (dpi), with Bgt colony area determined using IMAGEJ software (https://imagej.nih.gov/ij/).

**Quantification of ethylene content**

Ethylene (ET) content was measured using a gas chromatography instrument (Yin et al., 2015). For the samples without Bgt inoculation, the seedlings (30 for T. urartu and 10 for common wheat) at the one-leaf stage were transferred into a 50-ml Falcon tube with their roots immersed in water. After 24 h at 22°C, the tube was tightly sealed with a rubber cap for 24 h, with the emitted ET subsequently measured. In the case of Bgt inoculation, the desired seedlings were placed into the Falcon tube, followed by Bgt inoculation as described above, with the tube sealed immediately post-inoculation. The ET content was measured at 24 hpi, and repeated three times, with each measurement including three biological replicates.

**Gene expression analysis by qRT-PCR**

Gene expression levels were determined using the samples collected from at least 10 seedlings (including untreated controls and those treated for 24 h) by quantitative reverse transcription (qRT)-PCR with gene-specific primers (Supporting Information Table S1; Methods S1).

**Single-cell functional assay**

Single-cell functional tests, based on transient overexpression (TOE) of cloned cDNA or transiently induced gene silencing (TIGS) in wheat epidermal cells, were accomplished essentially as described in previous publications (Douchkov et al., 2005; Shen et al., 2007). The reporter plasmid contained the expression cassette of β-glucuronidase gene. The effector constructs were pUbi: TuACO3, pUbi:TuMYB46L, pUbi:TuACO3as or pUbi: TuMYB46Las. The former two constructs transiently expressed the full-length cDNA of TuACO3 and TuMYB46L, respectively, whereas the latter two plasmids contained a short segment from TuACO3 or TuMYB46L coding sequence in an antisense orientation (Methods S1).

**Immunoblotting assay**

Immunoblotting was carried out using either the antibody to a recombinant TuACO3 protein or the FLAG tag (Sigma, F7425). The recombinant TuACO3 was prepared by expressing a histidine-tagged protein in Escherichia coli, with the purified protein used to raise a mouse polyclonal antibody as described by Qin et al. (2008). More details are described in Methods S1.

**Virus-induced gene silencing**

Virus-induced gene silencing (VIGS) was carried out using the barley stripe mosaic virus (BSMV) vector (Yuan et al., 2011). Two constructs, pCaBS-γ:ACO3as and pCaBS-γ:MYB46Las harboring a short antisense fragment derived from the coding sequence of TuACO3 (122 bp) or TuMYB46L (130 bp), were prepared. Together with pCaBS-α and pCaBS-β, the two recombinant viruses, BSMV:ACO3as and BSMV:MYB46Las, were formed, and used to silence endogenous TuACO3 and TuMYB46L in G1812 leaf cells, respectively. The two viruses also were used to silence the orthologous genes of TuACO3 or TuMYB46L in the common wheat cultivar KN199 because the VIGS-inducing fragments carried by BSMV:ACO3as and BSMV:MYB46Las were conserved in the respective orthologs (Methods S1).

**Overexpression of TuACO3 and TuMYB46L in common wheat**

The constructs pUbi:TuACO3-FLAG and pUbi:TuMYB46L-FLAG, expressing TuACO3-FLAG and TuMYB46L-FLAG fusion proteins, respectively, were prepared with the vector pHZ206 (carrying a maize ubiquitin gene promoter) and the primers listed in Table S1. They were introduced into the immature embryos of KN199 by biolistic transformation (Wang et al., 2014). Homozygous T2 lines were selected from selfed T1 individuals, with the expression of TuACO3-FLAG or TuMYB46L-FLAG identified by immunoblotting using the antibodies recognizing FLAG tag or TuACO3. In total, 12 and 17 independent lines overexpressing TuACO3-FLAG or TuMYB46L-FLAG were identified. TuACO3-OX1 and -OX2 (overexpressing TuACO3-FLAG) and TuMYB46L-OX1 and -OX2 (overexpressing TuMYB46L-FLAG) were used as representatives for more detailed analysis. These transgenic lines resembled wild-type (WT) KN199 in their growth and development in the glasshouse (Fig. S1). The transgenic plants and the WT segregate controls (WT57 and WT59) were inoculated with Bgt, followed by examination of microcolonies and colonies as detailed above. At least 30 plants were inoculated with Bgt per line per experiment. Measurement of ET content, either before or after Bgt inoculation, was carried out as described above. We were unable to overexpress TuACO3 and
**TuMYB46L** in *T. urartu* because of the difficulty to genetically transform this species.

**Yeast-one-hybrid (Y1H) assay**

A Y1H library was generated using the vector pGADT7 and the SMART cDNA Library Construction Kit (634901; Clontech, Dalian, China). The cDNAs used for library construction were reverse-transcribed from total RNAs extracted from the G1812 leaves collected at 0, 4 and 24 hpi with *Bgt*. This library was screened using the 2-kb promoter region of *TuACO3* cloned in the bait vector pABAi (pABAi:TuACO3pro). The positive clones obtained from the initial screen were analyzed by DNA sequencing, with those carrying in frame inserts subjected to a second screen. To validate the binding of TuMYB46L to the *TuACO3* promoter region, the cDNA coding sequence of TuMYB46L was cloned into pGADT7, generating pGADT7:TuMYB46L. This construct was co-transformed with pABAi:TuACO3pro into the yeast strain Y1H Gold. Positive binding of TuMYB46L to *TuACO3* promoter region was indicated by abundant growth of transformants on the SD/-Leu/-Ura/+AbA medium. As controls, pABAi:TuACO3mpro was prepared by mutating each of the four predicted MYB46 binding sites (E1–E4) within the 2-kb promoter region of *TuACO3* into AAAAAA; pGADT7:EVC (empty vector control) and pGADT7:GFP (expressing green fluorescent protein, GFP) also were used to test the binding specificity (Methods S1).

**Chromatin immunoprecipitation (ChIP)-quantitative (q) PCR analysis**

Approximately 1 × 10^7 protoplasts, isolated according to Wang *et al.* (2014), were obtained from c. 150 G1812 seedlings. The pUbi:TuMYB46L-FLAG plasmid (20 μg) and the control vector pHZ206 (20 μg) (see above) each were transformed into c. 1 × 10^6 protoplasts using polyethylene glycol. After 18 h of culture in the dark, the protoplasts were collected and treated with 1% formaldehyde at room temperature for 15 min to promote protein-chromatin cross-linking (Bao *et al.*, 2014). The nuclei were then isolated, lysed and used for preparing chromatin complexes. The resulting complexes were ultrasonically treated to obtain a suitable input, in which the chromosomal DNA had been fragmented to a size of c. 500 bp, for ChIP. The anti-FLAG antibody (F7425; Sigma, Shanghai, China) was used to precipitate the chromatin, which were subsequently analyzed by qPCR with 20 different primer sets (Table S1) covering the 2-kb promoter region of *TuACO3*. As control, a mock precipitation with normal mouse IgG was executed. Two independent ChIP-qPCR experiments were performed with similar results obtained.

**Electrophoretic mobility shift assay (EMSA)**

The cDNA coding sequence of TuMYB46L was cloned into the pET-32a vector to express a recombinant TuMYB46L-HIS tag protein in the *E. coli* Rosetta (DE3) strain, which was purified to homogeneity using Ni-NTA Agarose (30210; Qiagen, Beijing, China). Three oligonucleotide probes (E1, E3 and E4, 39 bp each; Table S1) were synthesized and labeled with biotin at the 3’ end (Invitrogen, Beijing, China). An EMSA was performed using a LightShift Chemiluminescent EMSA kit (20148; Thermo Scientific, Beijing, China) according to the supplier’s instructions. The biotin-labeled probes each were incubated in a reaction mixture (1× binding buffer, 2.5% glycerol, 50 mM KCl, 5 mM MgCl₂, and 10 mM EDTA) with or without TuMYB46L protein at room temperature for 20 min. The following controls were included in the experiment to test the binding specificity: WT competitor (unlabeled E1, E3 and E4), mutant competitor (mutated and unlabeled E1, E3 and E4, with their MYB46 binding site changed to AAAAAA, Table S1), and mutant probe (mutated and labeled E1, E3 and E4). The EMSA assay was carried out five times with identical findings made.

**RNA sequencing and GO analysis of differentially regulated genes**

Total RNA was extracted from the leaf samples collected from control G1812 seedlings and those treated with *Bgt* (at 24 hpi) or ET (at 24 h post-treatment). They were then subjected to RNA sequencing as reported by Dong *et al.*, (2015b). Three independent biological replicates were sequenced for each treatment at each time point. Two criteria were used to compute differentially expressed genes (DEGs), (1) a log2 ratio > 1.0 and (2) the expression difference was consistently observed in between different biological replicates. Gene ontology (GO) enrichment analysis of the DEGs shared by the two treatments was accomplished using the software agriGO (http://bioinfo.cau.edu.cn/agriGO).

**Analysis of chitinase genes and chitinase enzyme assay**

The effects of ET treatment (10 ppm) or *Bgt* infection (for 24 h) on the expression of four chitinase genes were validated by qRT-PCR with gene-specific primers (Table S1). Transcript levels of the four genes in the G1812 leaf samples infected by BSMV: EVC, BSMV:ACO3as or BSMV:MYB46Las were evaluated by qRT-PCR. Total chitinase activities were assayed with a commercial kit (Solarbio, BC0820) following the manufacturer’s guidelines (Methods S1).

**Sequence information and raw data**

Sequence information for *TuACO3*, *TuMYB46L*, *TaACO3*, *TaMYB46L*, VIGS-induced fragments and the DEGs identified by RNA sequencing, as well as the raw data used for calculating the means (± SE), are provided in Dataset S1.

**Results**

**TuACO3** enhances wheat defense against *Bgt* via increasing ET biosynthesis

In our previous study (Zhang *et al.*, 2016), a gene encoding a putative ACO was found induced by *Bgt* infection in multiple
T. urartu accessions. This gene (TuG1812G0600003491), located on chromosome 6A of T. urartu (Ling et al., 2018), is orthologous to rice OsACO3 based on phylogenetic analysis (Fig. S2). Because of the poor understanding of ET function in plant defense against obligate biotrophic fungal pathogens and the fact that ACO catalyzes the last step of ET biosynthesis (Kende, 1993; Booker & Delong, 2015), we decided to analyze TuACO3 further to gain insights into the function of ET in wheat defense against Bgt.

Using qRT-PCR, we validated the induction of TuACO3 by Bgt in G1812 and another five diverse T. urartu accessions (Figs 1a, S3a). Immunoblot analysis revealed increased accumulation of native TuACO3 in G1812 leaf tissues at 24 hpi (Fig. 1b). Concomitant to this increase, ET production was significantly upregulated (by 50.2%) at 24 hpi (Fig. 1c). Similar rises of ET production were found in the other five T. urartu accessions at 24 hpi (Fig. S3b). In single-cell functional assays, Bgt infection level, as measured by the haustorium index, was decreased by overexpressing TuACO3, but uplifted by knocking down TuACO3 expression via TIGS (Fig. 1d). Consistent with these findings, lowering TuACO3 expression via TIGS (Fig. 1d). Consistent with these findings, lowering TuACO3 expression via TIGS (Fig. 1e–h). Based on the reference genome sequence of Chinese Spring (CS) (International Wheat Genome Sequencing Consortium et al., 2018), the orthologous gene of TuACO3 in common wheat was TaesCS6a02G325600 (TuACO3) (Fig. S3c). Silencing of this gene in the common wheat cultivar KN199 also increased the susceptibility to Bgt (Fig. S3d,e).

In order to further examine the function of TuACO3 in ET biosynthesis and Bgt defense, we analyzed two independent common wheat transgenic lines (TuACO3-OX1, and -OX2) designed to overexpress TuACO3 with a C-terminal FLAG tag. In both lines, TuACO3-FLAG was confirmed to be overexpressed using immunoblotting with the antibodies to FLAG or TuACO3 biosynthesis and promoted susceptibility to Bgt in G1812 (Fig. 1e–h). Based on the reference genome sequence of Chinese Spring (CS) (International Wheat Genome Sequencing Consortium et al., 2018), the orthologous gene of TuACO3 in common wheat was TaesCS6a02G325600 (TuACO3) (Fig. S3c). Silencing of this gene in the common wheat cultivar KN199 also increased the susceptibility to Bgt (Fig. S3d,e).

In order to further examine the function of TuACO3 in ET biosynthesis and Bgt defense, we analyzed two independent common wheat transgenic lines (Fig. 2a). Compared to WTS7, a WT segregate control line, ET production was elevated by c. 1.7-fold in TuACO3-OX1 and 1.4-fold in TuACO3-OX2 (Fig. 2b). When infected by Bgt, the two OX lines showed significantly reduced fungal colonies on the leaf surface (Fig. 2c–f). These results demonstrated that the transgenically expressed TuACO3 functioned in ET biosynthesis, with concurrent improvement in Bgt defense.

The above findings prompted us to test if applying ET, or the chemicals known to stimulate or inhibit ET biosynthesis, may alter wheat defense against Bgt (Methods S1). This test was conducted using both G1812 and the common wheat variety KN199. Exogenous application of ET or ACC (a precursor of ET biosynthesis) significantly increased the resistance to Bgt in both G1812 and KN199, whereas the reverse was found when aminoethoxyvinylglycine (AVG, an inhibitor of ET biosynthesis) was applied (Fig. 3). The increased resistance against Bgt by exogenous ET was additionally confirmed using five more T. urartu accessions (Fig. S4). Together, the analyses presented above corroborate with each other, and suggest that TuACO3, induced by Bgt infection, promotes wheat defense against Bgt through significantly upregulating the content of ET.

TuMYB46L is a negative regulator of TuACO3 expression

In order to identify the TF(s) controlling Bgt infection-induced expression of TuACO3, we screened a Y1H library using TuACO3 promoter region as bait. This led to the identification of six genes whose proteins could bind to the promoter of TuACO3 in two independent Y1H screens (Table S2). One of them, namely TuMYB46L (TuG1812G0500001166), was predicted to encode a MYB TF carrying a putative R2R3 MYB domain (Fig. S5a), whose full-length protein showed 35.2% identity to AtMYB46, a previously characterized MYB TF regulating secondary cell wall biosynthesis and host defense against B. cinerea in Arabidopsis (Zhong et al., 2007; Ko et al., 2009; Ramirez et al., 2011a,b; Zhong & Ye, 2012; Kim et al., 2014). Consistent with the identification of TuMYB46L by Y1H, the 2-kb promoter region of TuACO3 was found to carry four cis-elements highly similar to the consensus binding site of AtMYB46, ACC(A/T) A(A/C)/(T/C) (Zhong & Ye, 2012) (Fig. S5b). We therefore focused on analyzing the potential regulation of TuACO3 transcription by TuMYB46L.

The specific binding of TuMYB46L to TuACO3 promoter was verified by including additional controls (Fig. 4a). The binding was not observed when the four putative MYB46 recognition sites in the promoter region of TuACO3 were all mutated to AAAAAA; neither was it observed when free GFP was used as prey and WT TuACO3 promoter region as bait. In the effectortransporter reporter assay conducted in Nicotiana benthamiana leaves, expression of TuMYB46L, but not free GFP, suppressed the promoter activity of TuACO3 (Fig. 4b,c). TuMYB46L did not suppress the activity of the mutant promoter of TuACO3 (with all four MYB46 recognition sites changed to AAAAAA) as effectively as it did for the WT promoter of TuACO3 (Fig. 4b, c). Phylogenetic analysis validated the relatedness of TuMYB46L to its homologs (including AtMYB46) from monocot and dicot plants (Fig. S5c). However, the predicted proteins of TuMYB46L and its close cereal homologs (ZmMYB46 and OsMYB46) were considerably larger than those of AtMYB46 and the homologs from dicot plants (Fig. S5c). As anticipated, TuMYB46L was targeted to the nucleus when transiently expressed as a YFP fusion protein in N. benthamiana leaf cells (Methods S1; Fig. S5d).

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The expression of TuMYB46L was strongly and rapidly decreased by Bgt infection in all six T. urartu accessions examined (Fig. 4d). Silencing TuMYB46L expression by BSMV-mediated VIGS resulted in a significantly greater induction of TuACO3 at 24 hpi of Bgt, and substantially more ET was produced in TuMYB46L silenced plants relative to their controls (Fig. 4e–g). The Bgt microcolonies recorded for TuMYB46L silenced plants were substantially fewer than those found for the controls (Fig. 4h,i). In agreement with these results, the defense against Bgt was compromised by overexpressing TuMYB46L, but strengthened after silencing TuMYB46L by TIGS, in the single-cell transfection assays conducted with T. urartu leaves (Fig. S6a, b). BSMV-mediated VIGS also was conducted for the orthologous gene of TuMYB46L in common wheat
(Trac5A02G101000, TaMYB46L), which led to substantial increase of TaACO3 transcript level and considerable decrease of the susceptibility to Bgt (Fig. S6d–f).

We further analyzed two independent transgenic lines (TuMYB46L-OX1 and TuMYB46L-OX2) overexpressing a TuMYB46L-FLAG tag protein in the common wheat cultivar KN199. Expression of TuMYB46L-FLAG in the transgenic lines was confirmed by immunoblotting (Fig. 5a). Infection with Bgt significantly upregulated ET production in WTS9, a corresponding WT segregate control line, but this upregulation was diminished in TuMYB46L-OX1 and TuMYB46L-OX2 (Fig. 5b). Consistently, substantially more Bgt microcolonies and stronger Bgt colony growth were observed on TuMYB46L-OX1 and TuMYB46L-OX2 leaves than on those of WTS9 (Fig. 5c–f). These findings, together with the foregoing results (Fig. 4), indicate that TuMYB46L is a negative regulator of TaACO3 expression, and that its downregulation is required for the enhancement of TaACO3 expression, ET content and Bgt defense in wheat.

TuMYB46L binds to cis-elements in the promoter region of TaACO3

Among the four putative MYB46 binding sites in the 2-kb sequence upstream of the initiation codon of TaACO3, the first one (E1, ACCAAAG) was located near the ATG codon, and the
third (E3, ACCTAAA) and fourth (E4, ATTTGGT) were more distant (Figs 6a, S5b). We first performed ChIP-qPCR assays to test the binding of TuMYB46L to different regions of TuACO3 promoter. A TuMYB46L-FLAG tag protein was transiently expressed in T. urartu leaf protoplasts, followed by ChIP with the antibody against FLAG. Co-precipitating DNAs were quantified by qPCR using primer sets covering the 2 kb promoter. Compared to the control in which the FLAG antibody was replaced by normal mouse IgG, the inclusion of FLAG antibody led to the precipitation of substantially more DNA fragments carrying E1 or E3 + E4, but not that harboring E2, with the average enrichment obtained for the two regions being 3.5-fold and 2.3-fold, respectively (Fig. 6b).

We thus conducted EMSA to validate the binding of E1, E3 and E4 by a bacterially expressed, histidine-tagged TuMYB46L protein. As anticipated, the TuMYB46L-HIS tag protein was able to bind all three elements in repeated EMSA experiments (Fig. 6c). The binding was specific because it was not found with the mutant probes, but it was not affected by the mutated competitor probes (Fig. 6c).

Multiple processes and genes function in ET-mediated defense against Bgt

In order to gain insight into the biological processes and genes functioning in ET-mediated wheat defense against Bgt, we carried out two types of transcriptome assays. The first type used the G1812 leaf samples without or with Bgt inoculation for 24 h, and the second employed the G1812 leaves without or with ET treatment for 24 h. For Bgt treatment, the up- and down-regulated genes were 2307 and 1516, respectively; for ethylene treatment the differentially expressed genes were 1406 (up-regulated) and 1222 (down-regulated), respectively (Fig. 7a). The up-regulated and down-regulated genes shared by the two treatments amounted to 691 and 559, respectively (Fig. 7b). For the shared upregulated genes, the significantly enriched biological processes were related to chitin catabolism,
cellulose biosynthesis and isoprenoid biosynthesis; for the shared
downregulated genes, the highly enriched biological processes
included oxidation reduction and metal ion transport (Table S3).

Because previous studies have shown the increase of host chiti-
nase gene expression in fungal-infected plant tissues and the
involvement of ET in the induction process (Jacobs et al., 1999;
Akagi et al., 2011), we further analyzed the four chitinase genes
(i.e., TuG1812G0100002418, TuG1812G0200004053,
TuG1812G0200004054 and TuG1812G0300003072, Ling
et al., 2018) that were upregulated by both
Bgt and ET treat-
ments (Table S4). The fold-induction of the four genes varied
approximately from three to nine (after
Bgt infection) or two to
four (post ET application) (Table S4). Their upregulated expres-
sion after Bgt or ET treatment was confirmed by qRT-PCR
(Fig. 7c). Importantly, the expression of the four chitinase genes
all were repressed in the T. urartu leaf tissues in which TuACO3
was silenced by BSMV-mediated VIGS, but promoted in those
with downregulated TuMYB46L expression (Fig. 7d).

Consistent with the foregoing results, total chitinase activities
were substantially elevated by Bgt infection (1.5-fold) or ET
treatment (1.3-fold) in T. urartu (Fig. 7e). Moreover, total chiti-
nase activity levels in the two TuACO3 overexpression transgenic
lines were significantly higher than that of the control (WTS7)
(Fig. 7f). By contrast, total chitinase activities in the two
TuMYB46L overexpression lines were substantially lower relative
to those of the control WTS9 (Fig. 7f).

Discussion

In this work, we studied the functions of TuACO3 and
TuMYB46L, and discovered a previously uncharacterized gene
module (i.e. TuMYB46L-TuACO3; ACO, 1-aminocyclo-
propane-1-carboxylic acid (ACC) oxidase; MYB, myeloblastosis)
that regulates ethylene (ET) biosynthesis and content in einkorn
wheat defense against Blumeria graminis f.sp. tritici (Bgt). The
findings were made primarily in the einkorn wheat
Triticum urartu, and subsequently confirmed in common wheat by barley
stripe mosaic virus (BSMV)-mediated virus-induced gene silenc-
ing (VIGS) and using the KN199 transgenic lines overexpressing
TuACO3 or TuMYB46L.

The expression and function of TuACO3 are enhanced in
Bgt-infected wheat

Although ACO has long been known to catalyze the last step of
ET biosynthesis (Spanu & Boller, 1989; Kende, 1993; Booker &
DeLong, 2015), it is only recently that several studies have
uncovered the importance of transcriptional control in the func-
tion of ACO during cotton fiber development, apple fruit ripen-
ing and Arabidopsis root development (Li et al., 2014; T. Li et al.,
2017; Hu et al., 2019; Park et al., 2018). A number of studies
have recorded changes in the expression of various ACO gene
members accompanying the infections by different pathogens
(Shan & Goodwin, 2006; Yu et al., 2011; Vilanova et al., 2017),
but the function of the concerned ACO and the underlying regulatory mechanism were generally not analyzed using molecular genetic means.

Based on the data gathered here, we suggest that the function of TuACO3 is enhanced in Bgt-infected wheat, which contributes to host defense against Bgt via significantly increasing ET...
biosynthesis and content. Importantly, we validated the promotion of wheat defense against *Bgt* by *TuACO3* via elevating ET biosynthesis through analyzing two independent common wheat transgenic lines overexpressing *TuACO3* (Fig. 2). Thus, our work has generated molecular and functional evidence for the positive contribution of an *ACO* gene to plant defense against pathogen infection through elevating ET concentration.

Before this work, there has been some evidence for the involvement of ET in plant defense against powdery mildew fungi. The *Arabidopsis* mutant cev1 displays constitutively active jasmonic acid/ET induced defense responses and shows increased resistance to three different powdery mildew pathogens (Ellis & Turner, 2001). In grapevine, powdery mildew infection and artificial treatment with ethephon induce an overlapping set of defense-related proteins (Jacobs et al., 1999). The present work demonstrates that ET biosynthesis evidently is upregulated in *Bgt*-infected wheat and contributes to host defense against *Bgt*. Although this contribution did not offer a complete protection to the disease, it probably reduced disease severity because silencing *TuACO3* not only decreased ET production, but also increased the development of powdery mildew colonies in *Bgt*-infected wheat (Fig. 1e–h). Furthermore, we showed that endogenous application of ET enhanced wheat resistance to *Bgt* and that inhibition of ET biosynthesis by aminoethoxyvinylglycine (AVG) aggravated susceptibility to *Bgt* (Fig. 3). Consequently, our work suggests that ET-mediated defense can be activated by an adapted powdery mildew pathogen in a compatible interaction in wheat. This provides a new molecular insight into the role of ET in plant defense to biotrophic fungal pathogens.

Consistent with the increased *TuACO3* expression and ET production discussed above, the transcript level of one 1-aminocyclopropane-1-carboxylic acid synthase (*ACS*) gene (i.e. *TuACS2*), which functions immediately upstream of *ACO* (Booker & DeLong, 2015), was elevated in *Bgt*-infected *T. urartu* plants (Fig. S7). The activity of *TuACS2* may enhance the generation of the precursor ACC for ET biosynthesis in *Bgt*-invaded wheat tissues. However, further work is needed to validate this possibility.

Because the *ACO* gene family is well conserved in higher plants (Booker & DeLong, 2015; Sun et al., 2017; Park et al., 2018), and powdery mildew diseases occur in diverse plant species (Glawe 2008), it is worth investigating whether the enhancement...
of plant defense by ACO, as revealed here, also may happen in other powdery mildew pathosystems. Considering the limited understanding on most of the powdery mildew diseases recorded to date (Glawe 2008), molecular and functional analysis of ACO may provide a generally useful entry point for studying host defense mechanisms to different powdery mildew fungi.

Downregulation of TuMYB46L is required for elevated expression of TuACO3 after Bgt infection

Judging from the molecular genetic data obtained (Figs 4, 5), we suggest that downregulation of TuMYB46L is a prerequisite for the elevated expression of TuACO3 in Bgt-infected wheat and its subsequent function in increasing ET biosynthesis. Central to the action of TuMYB46L is its ability to bind to multiple cis-elements in the promoter region of TuACO3 (Fig. 6). The rapid decline of TuMYB46L expression following Bgt infection diminishes the binding of its protein to the promoter of TuACO3, which permits transcriptional upregulation of TuACO3 in Bgt-infected wheat. Thus, TuMYB46L is likely a negative regulator of TuACO3 expression and function under normal growth conditions.

As a vital phytohormone, the biosynthesis of ET must be tightly regulated according the environmental conditions to which the plants are exposed (Booker & DeLong, 2015; Broekgaarden et al., 2015). Failure to adjust ET biosynthesis and signaling lessens plant adaptation to harmful environments (Guo & Ecker, 2003; Booker & DeLong, 2015; Broekgaarden et al., 2015). Therefore, TuMYB46L regulates ET biosynthesis through controlling the expression of TuACO3 according to the presence or absence of Bgt infection in wheat. The significance of the TuMYB46L-TuACO3 module in wheat defense against Bgt via regulating ET production is clearly demonstrated in this work. Further study is needed to examine if the negative regulation of TuACO3 by TuMYB46L also may be required for efficient plant growth under normal environmental conditions.

Among the homologs of TuMYB46L (Fig. S5c), only AtMYB46 from Arabidopsis has so far been shown to affect plant response to pathogen challenge (Ramírez et al., 2011a,b). The knockout plants of AtMYB46 showed increased disease resistance to Botrytis cinerea because of changed cell wall integrity and more efficient induction of the genes involved in cell wall remodeling or pathogen defense than WT plants (Ramírez et al., 2011a,b). In view of the relatedness of TuMYB46L to AtMYB46, on the one hand, it will be interesting to investigate if the contribution of the TuMYB46L-TuACO3 module to wheat defense against Bgt also may involve changed cell wall integrity and enhanced expression of cell wall remodeling genes, in addition to the upregulation of TuACO3 expression and ET production observed in...
this work. On the other hand, it also may be worthwhile to investigate the potential existence of an AtMYB46-AtACO module and its possible contribution to Arabidopsis defense against pathogens. In a preliminary analysis, we found that AtMYB46 could suppress the promoter activity of AtACO4, which is the closest Arabidopsis homolog of TuACO3 (65.8% protein identity), and that the promoter region of AtACO4 also carries five predicted AtMYB46 binding sites (Fig. S8). Whether the suppression of AtACO4 promoter activity by AtMYB46 may take part in the regulation of AtACO4 transcription during Arabidopsis pathogen defense is an important question for future research. Also important for future study is to examine if...
TuMYB46L may regulate secondary cell wall development as this is a shared function among MYB46 homologs (Zhong et al., 2011; Xie et al., 2018); TuMYB46L may resemble AtMYB46 and the MYB46 homolog from birch in being able to regulate multiple plant processes (Ramírez et al., 2011a,b; Guo et al., 2017). Nevertheless, certain functional difference between TuMYB46L and AtMYB46 is to be expected because the identity level of their full-length proteins (35.2%) is not high and their proteins differ substantially in size (Fig. S5a,c).

The relationships among ET biosynthesis and signaling, cell wall development, organ growth and pathogen defense are very complex, and may involve growth-defense trade-offs (Ramírez et al., 2011a,b; Souza et al., 2017; Xie et al., 2018). The finding of the participation of an Arabidopsis ACS protein (ACS5) in cell wall biosynthesis independent of ET concentration adds further complexity to the interactions (Xu et al., 2008). However, we demonstrated here that the TuMYB46L-TuACO3 module increased ET production in Bgt-infected wheat, and that exogenous application of ET or ACC enhanced wheat defense against Bgt. Thus, further research on the TuMYB46L-TuACO3 module may help to improve understanding of the interplays among ET biosynthesis and signaling, cell wall development and function, plant growth and pathogen defense.

Chitinase genes participate in ET-mediated defense against Bgt

Through transcriptome comparison and validation by quantitative reverse-transcription (qRT)-PCR, the expression of four different chitinase genes was confirmed to be upregulated by both Bgt infection and ET treatment (Fig. 7a–c; Table S4). Furthermore, the four genes were depressed by silencing TuACO3 but elevated after suppressing TuMYB46L (Fig. 7d), suggesting that they act downstream of the TuACO3-TuMYB46L module. This proposition is additionally supported by (1) increased total chitinase activities in the wheat plants infected by Bgt or treated with ET (Fig. 7e) and in those overexpressing TuACO3 (Fig. 7f), and (2) reduced total chitinase activities in the wheat lines overexpressing TuMYB46L (Fig. 7f).

Plant chitinase isoymes are potent players in host defense against fungal pathogens (Pusztahelyi, 2018), and past studies have reported the involvement of ET in the induction of host chitinase gene expression in fungal-infected plants (Jacobs et al., 1999; Akagi et al., 2011). This work improves the understanding of chitinase gene induction by a pathogenic fungus (Bgt) through identifying a specific host gene module (TuMYB46L-TuACO3) that produces the endogenous ET signal needed for the induction. The next challenge is to reveal how ET orchestrates chitinase gene expression. In this context, it is interesting to note that overexpression of certain ET responsive factors (ERFs) can enhance the expression of pathogenesis-related proteins including chitinase (Berrocal-Lobo et al., 2002; Akagi et al., 2011; Zhu et al., 2014). It also will be interesting to test if reactive oxygen species (ROS) and additional pathogenesis-related genes may be involved in the defense processes regulated by TuMYB46L-TuACO3 in further research.

Apart from chitinase genes, our transcriptome analysis also detected upregulation of the genes involved in the biosynthesis of cellulose or isoprenoids in Bgt-infected or ET-treated wheat plants (Table S3). On the one hand, there is now increasing evidence that alterations in cellulose biosynthesis and cell wall integrity modulate plant defense against pathogens (Caño-Delgado et al., 2003; Hernández-Blanco et al., 2007; Souza et al., 2017). On the other, the phytoalexins derived from phenylpropanoid metabolism are active defense compounds against plant pathogens (Ahuja et al., 2012; Liu et al., 2015). Therefore, elevated expression of the genes functioning in cellulose or isoprenoid biosynthesis may help to strengthen the defense against Bgt in wheat. We noted that the 2-kb promoter region of the three upregulated cellulose synthase genes, as well as the two upregulated phenylalanine ammonia lyase genes, each carried two or three putative MYB46 binding sites (Table S5). We are now in the process of investigating if these genes also may be regulated by the TuMYB46L-TuACO3 module.

In summary, this study establishes that the TuMYB46L-TuACO3 module regulates ET biosynthesis to promote einkorn wheat defense against Bgt. The insights obtained improve our knowledge on wheat defense mechanisms to Bgt and on the ACO enzyme that is critical in generating the ET signal for plant adaptation to changing environment (Booker & DeLong, 2015). Our work points to the possibility of controlling wheat powdery mildew disease through manipulating ET concentration, and may stimulate further research into the regulation and function of ET biosynthesis in plant defense against diverse powdery mildew fungi.

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Author contributions

HZ, KZ, and DW designed the project; HZ, LD and XH performed the experiments; HZ, LD, HJ and CY performed the analyses; KZ, YH, BL, HQ, QS and JZ contributed to data collection; and HZ and DW wrote the manuscript. HZ and LD contributed equally to this work.

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Fig. S8 Suppression of *AtACO4* promoter activity by *AtMYB46*.

Methods S1 Additional description of methods.

Table S1 PCR primers used in this study.

Table S2 List of six *T. urartu* genes found in two independent Y1H screens.

Table S3 Significantly enriched biological processes identified by GO analysis.

Table S4 Expression changes of the four chitinase genes upregulated by both ET treatment and *Bgt* infection.

Table S5 Presence of putative MYB46 recognition sites in the 2-kb promoter region of the five genes related to cellulose or isoprenoid synthesis.

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