Identification and characterization of a fusarium head blight resistance gene TaACT in wheat QTL-2DL

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Summary
Fusarium head blight (FHB) resistance in wheat is considered to be polygenic in nature. Cell wall fortification is one of the best resistance mechanisms in wheat against Fusarium graminearum which causes FHB. Metabolomics approach in our study led to the identification of a wide array of resistance-related (RR) metabolites, among which hydroxycinnamic acid amides (HCAAs), such as coumaroylagmatine and coumaroylputrescine, were the highest fold change RR metabolites in the rachis of a resistant near-isogenic line (NIL-R) upon F. graminearum infection. Placement of these metabolites in the secondary metabolic pathway led to the identification of a gene encoding agmatine coumaroyl transferase, herein referred to as TaACT, as a candidate gene. Based on wheat survey sequence, TaACT was located within a FHB quantitative trait loci on chromosome 2DL (FHB QTL-2DL) between the flanking markers WMC245 and GW608. Phylogenetic analysis suggested that TaACT shared closest phylogenetic relationship with an ACT ortholog in barley. Sequence analysis of TaACT in resistant and susceptible NILs, with contrasting levels of resistance to FHB, led to the identification of several single nucleotide polymorphisms (SNPs) and two inversions that may be important for gene function. Further, a role for TaACT in FHB resistance was functionally validated by virus-induced gene silencing (VIGS) in wheat NIL-R and based on complementation studies in Arabidopsis with act mutant background. The disease severity, fungal biomass and RR metabolite analysis confirmed TaACT as an important gene in wheat FHB QTL-2DL, conferring resistance to F. graminearum.

Introduction
Fusarium head blight (FHB) is a worldwide wheat disease caused by Fusarium graminearum that significantly affects yield and grain quality (Bai and Shaner, 1994, 2004; Dexter et al., 1996; Steiner et al., 2004). Many FHB QTL have been identified but the genes and their functions are largely unknown. Thus, there is an urgent need to identify the resistance genes underlying the QTL and to decipher the resistance mechanisms for their use in breeding programs. The FHB QTL on chromosome 2DL identified from Wuhan-I × Nyubai (Somers et al., 2003) is one of the major QTL conferring rachis resistance (type II resistance) by limiting the spread of pathogen from the initial point of infection. Despite significant efforts to identify and characterize the genes underlying FHB QTL using different tools, very few have led to new insights. Positional cloning of the QTL-Fhb1 region of chromosome 3B disclosed the presence of seven novel genes in the 261-kb region. Transgenic wheat lines were developed for four of these genes but none of the transgenic lines carrying these genes exhibited rachis resistance (Liu et al., 2008). Transcriptomic studies involving NILs with QTL-3BS and QTL-5A have also identified many differentially expressed genes (Schweiger et al., 2013). The RNA-Seq analysis of Wangshuibai and its FHB susceptible mutant with deletion of the QTL-Fhb1 region identified several differentially expressed genes but none were selected to have rachis resistance (Xiao et al., 2013). Recently, gene expression profiling of NILs containing QTL-2DL revealed eight candidate genes but only one gene was localized on the 2DL chromosome (Long et al., 2015). Although several candidate genes were identified in most of the studies, none could pinpoint and explain the resistance mechanisms. Also, there were only a few attempts towards functional characterization of the genes. The application of new tools could help in understanding the genetic determinants underlying the FHB QTL. The functional characterization of mapped QTL using an alternative approach, such as metabolomics, is considered one of the best tools to decipher the resistance mechanisms and genes underlying FHB resistance (Kushalappa and Gunnaiah, 2013). Such an approach has led to the identification of several RR metabolites in barley (Bollina et al., 2010; Kumaraswamy et al., 2011) and wheat (Gunnaiah et al., 2012). In potato, not only the RR metabolites but also their biosynthetic resistance genes were identified (Pushpa et al., 2013; Yogendra et al., 2014, 2015). Hydroxycinnamic acid amides (HCAAs), a class of several complex secondary metabolites produced in the phenylpropanoid pathway, are induced in plants in response to pathogens (Gunnaiah et al., 2012; Muroi et al., 2009, 2012; von Rönneack et al., 1998). HCAAs reduce pathogen advancement through their antimicrobial and cell wall reinforcement properties (Ishihara et al., 2008; Keller et al., 1996; Miyagawa et al., 1995; Schmidt et al., 1998). HCAAs such as coumaroylagmatine, coumaroylputrescine, feruloylagmatine and feruloylputrescine were identified...
as effective defence metabolites in Arabidopsis thaliana rosette leaves infected with Alternaria brassicicola (Muroi et al., 2009). HCAAs are biosynthesized by condensation of hydroxyxycinnamoyl-CoA thioesters produced from phenylalanine via the phenylpropanoid pathway with aromatic amines by amine-specific hydroxyxycinnamoyl transferases (Edreva et al., 2007; Facchini et al., 2002).

In this study, we identified several fold change differences in HCAA metabolites including coumaroylagmatine and coumaroylputrescine in FHB QTL-2DL NIL-R compared to the susceptible NIL (NIL-S). The TaACT gene responsible for biosynthesizing these high fold-change metabolites was found within the QTL-2DL region. The transcript expression, disease severity and fungal biomass as estimated by quantification of relative copy number of a housekeeping fungal gene were also studied. Further, functional characterization of TaACT was performed using VIGS in NIL-R and a complementation study in the Arabidopsis mutant lacking the functional AtACT gene.

Results

Disease severity in NILs

The disease severity in spikes of NILs was assessed. Dark brown discoloration due to fungal infection was observed at 3 days postinoculation (dpi) of spikelets in NIL-S, whereas it was observed only at six dpi in NIL-R. The uninoculated spikelets above and below the inoculated spikelets in NIL-S were diseased and bleached at nine dpi. At 15 dpi, almost all of the spikelets were diseased in NIL-S, whereas only a few were diseased in NIL-R (Figure 1). The proportion of spikelets diseased (PSD) (Figure 2a) and the area under the disease progress curve (AUDPC) (Figure 2b) at 15 dpi were significantly ($P < 0.05$) lower in NIL-R than in NIL-S (Figure 2c), confirming the discrimination of resistance based on disease severity. This indicated that the NIL-R was significantly more resistant than the NIL-S.

Metabolite profiling in NILs

The metabolite profiling of wheat rachis inoculated with F. graminearum at 72 h postinoculation (hpi) identified a wide range of metabolites. Among these metabolites, the abundance of two HCAAs, coumaroylagmatine and coumaroylputrescine was 28- and 9.5-fold higher in NIL-R than in NIL-S, respectively (Table 1). No other HCAAs were detected.

Histochemical localization of HCAAs

The induction of coumaroylagmatine and coumaroylputrescine in NIL-R was confirmed by specific staining and fluorescence at specific wave length of cross sections of infected rachis. Stronger chemifluorescence at 405 nm, the typical spectrum of HCAA, was observed in pathogen-infected NIL-R cells than in NIL-S, and also in mock-treated NIL-R and NIL-S (Figure 3). This high chemifluorescence was considered to be mainly due to HCAAs, as we did not find any other high fold-change phenolics or flavones, which also can be stained with Neu’s reagent.

Identification of candidate gene TaACT in the QTL-2DL

The two HCAA candidate metabolites identified here were mapped onto metabolic pathways to identify their biosynthetic enzymes. Agmatinecoumaroyl transferase (ACT) is a rate-limiting enzyme involved in the biosynthesis of these metabolites (Burhenne et al., 2003; Muroi et al., 2009, 2012). BLAST analysis positioned TaACT as the closest gene match for this enzyme within the presumed QTL interval on 2DL. The TaACT full-length gene, including 546-bp promoter region was sequenced using the genomic DNA from both resistant and susceptible NILs. Analyses of TaACT using both the genomic DNA and cDNA determined the TaACT to be of 1326 bp in length and were devoid of introns. Sequence comparison of TaACT from NIL-R and T. aestivum cv. Chinese spring showed that the gene sequence is highly conserved. Comparison of the DNA sequences between NIL-R and NIL-S revealed two inversions (2 bp) and 67 SNPs (Figs. S1 & S2). The conserved domain analysis of the encoded protein using the MOTIF Search tool (http://www.genome.jp/tools/motif/), revealed the presence of a transferase domain. The predicted protein consisted of two consensus motifs (Figure 4): (i) the HLVSD motif that starts at His-153 and is identical to the HXXXXD motif that is commonly found in the
transferredase family which are responsible for CoA-dependent acyl transfer (St-Pierre et al., 1998) and (ii) the DFGGGQP motif, that starts at residue Asp-387, which is a motif of unknown function (Burhenne et al., 2003). This protein also has a similar N-terminal-specific 15 amino acid sequence MKITVLSSRAVKPDY that is found in most other reported ACTs. BLASTP search of TaACT in UniProt database indicated 83% identity with barley ACT (HvACT) protein. Phylogenetic analysis further demonstrated a close relatedness of these orthologs (Figure 5). It revealed that plant acyltransferases form five evolutionary groups (Burhenne et al., 2003), and HvACT falls under the fifth group. This group defines plant acyltransferases that are involved in transferring acyl groups to the acceptors. For further confirmation of predicted protein size (~48 kDa), the TaACT was heterologously expressed in E. coli (BL21) cells. The protein extract from supernatant was used to run the SDS-PAGE. A protein band with molecular weight approximately 48 kDa was observed (Fig. S3), and it was comparable to the previously reported HvACT (Burhenne et al., 2003). Our results confirmed the presence of ACT gene within the QTL region on 2DL, thus suggesting a possible link of this gene to the induction of high levels of coumaroylagmatine and coumaroylputrescine following pathogen inoculation.

Expression of gene TaACT based on semi-quantitative PCR (semi-qPCR) and real-time quantitative PCR (RT-qPCR)

The expression of the gene TaACT was examined using semi-qPCR and RT-qPCR. Although semi-qPCR did not reveal any expression in mock-treated samples from NIL-R and NIL-S, at 72 hpi, a high expression was observed in NIL-R relative to NIL-S upon pathogen inoculation (Figure 6a). This suggested that the gene TaACT was induced in wheat rachis only after pathogen inoculation. A semi-qPCR of an increased template (cDNA) quantity from 20 to 100 ng revealed the difference (data not shown), and this was further confirmed by RT-qPCR, which indicated the expression to be 3.2-fold higher in NIL-R than in NIL-S (\(P < 0.05\)) (Figure 6b).

Functional characterization of TaACT using VIGS

At 72 hpi of F. graminearum, the silenced plants NIL-R+BSMV_{TaACT} (BSMV (Barley Stripe Mosaic Virus) + TaACT insert) showed significant (\(P < 0.05\); 74.29%) reduction in TaACT transcript abundance as compared to nonsilenced plants NIL-R+BSMV_{0} (BSMV + without TaACT insert) confirming the silencing of the target gene in wheat rachis (Figure 7a). Metabolite analysis revealed that the relative

**Table 1** Fold change in abundance of resistance-related (RR) metabolites detected in wheat rachis following F. graminearum inoculation

| Observed Mass (ppm) | AME | Name                          | Molecular formula | Retention time (min) | Fold change in NIL-R relative to NIL-S | Matched fragmentation patterns | Category | References                  |
|---------------------|-----|-------------------------------|-------------------|----------------------|----------------------------------------|-----------------------------|----------|-----------------------------|
| 276.1592            | 2.3 | Coumaroylagmatine C14H20N402  | 12.2              | 28.7                 | 119.0, 147.0, 218.1, 233.17, 231.3, 258.1, 260.0 | HCAAs                      | Muroi et al. (2009); Wen et al. (2014); Gorzelka et al. (2014) |
| 234.1373            | 2.2 | Coumaroylputrescine C13H18N202 | 10.1              | 9.5                  | 93.1, 119.1, 147.0, 162.2, 191.2, 204.1, 218.1, 233.3 | HCAAs                      | Muroi et al. (2009); Moheb et al. (2011); Wen et al. (2014); |
The abundance of coumaroylagnatine and coumaroylputrescine was significantly reduced in plants NIL-R + BSMV TaACT compared to NIL-R + BSMV0 plants by 6.4- and 3.2-fold respectively (Figure 7b). The fungal biomass in NIL-R + BSMV TaACT was significantly higher than in NIL-R + BSMV0 (1.86 folds, \( P < 0.05 \)) (Figure 7c). whereas the fungal biomass in NIL-R + BSMV TaACT was 1.47-fold lower than in NIL-S, indicating possible involvement of other resistance genes associated with the QTL-2DL.

**Functional characterization of TaACT based on complementation study in Arabidopsis**

Metabolite profiling of the inflorescence of Arabidopsis plants revealed significantly \( (P < 0.05) \) higher abundances of coumaroylagnatine (4.1-fold) and coumaroylputrescine (2.5-fold) in plants over-expressing the TaACT from NIL-R (TaACT NIL-R) than plants over-expressing NIL-S (TaACT NIL-S) (Figure 8a-1). Further, the inflorescence of transgenic Arabidopsis in plants was inoculated with *F. graminearum* and the number of plants with inflorescence diseased was assessed at six dpi (Figure 8a-2). The mean number of plants with diseased inflorescence of 25 plants and for four replicates was significantly \( (P < 0.01) \) reduced in plants over-expressing NIL-R TaACT than in plants over-expressing NIL-S TaACT (Figure 8b). The fungal biomass reduction in inflorescence of Arabidopsis also followed a same trend. The fungal biomass was 3.8-fold higher in TaACT NIL-S than in TaACT NIL-R over-expressing plants.
Figure 5 Evolutionary analysis of TaACT. Protein sequences used for phylogenetic analysis are as follows: HV – agmatine coumaroyl transferase [Hordeum vulgare—AAO73071.1], TaACT – wheat agmatinecoumaroyltransferase (Triticum aestivum—KT962210), OS – Putative anthranilate N-benzoyltransferase [Oryza sativa Japonica Group—AA74310.1], NT – hydroxycinnamoyl transferase [Nicotiana tabacum—AJ507825], AT – hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase [Arabidopsis thaliana—NM_124270], IB – N-hydroxycinnamoyl/benzoyltransferase [Ipomoea batatas—AB035183], DC – anthranilate N-hydroxycinnamoyl/benzoyltransferase [Dianthus caryophyllus—Z84383], TC2 – 2-debenzoyl-7,13-deacetyl-baccatin III-2-O-benzylo transferase [Taxus cuspidate—AAG38049.1], TC1 – 10-deacetyl-baccatin III-10-O-acetyl transferase [Taxus cuspidate—AAF27621.1], TC3 – taxadene acetyl transferase [Taxus cuspidate—AAF34254.1], TC5 – 3′-N-debenzoyl taxol N-benzoyltransferase [Taxus Canadensis—AAM75818.1], CR – deacetyl vindoline 4-O-acetyltransferase [Catharanthus roseus—AAC99311.1], PS – salutaridinol 7-O-acetyltransferase [Papaver somniferum—AAG73661.1], FA – alcohol acetyltransferase [Fragaria ananassa—AAC183130.1], CB – acetyl-CoA:benzyl alcohol acetytransferase [Clarkia concinna—AAFO47748.1], CB – acetyl-CoA: benzyl alcohol acetyltransferase [Clarkia breviflora—AAC18062.1], SS – malonyl-CoA: anthoxygen 5-O-glucoside-6′-O-malonyl transferase [Salvia splendens—AAL50566.1], PF – malonyl-CoA:anthoxygen 5-O-glucoside-6′-O- malonyl transferase [Perilla frutescens—AAL50565.1], GT – Anthoxygen 5-aromatic acetyltransferase [Genitana triflora—BAA74428.1].

(Figure 8c). This suggests that some of the inversions and SNPs found in NIL-S were responsible for the reduced functionality and resistance. Although the TaACT gene from NIL-R can be used to replace nonfunctional genes in commercial wheat cultivars based on genome editing, the use of specific nucleotide replacement requires further studies to validate the functions of these SNPs.

Discussion
The present study reports an integrated approach of metabolomics, gene sequencing information, gene expression histological studies and heterologous protein expression to identify and confirm the presence of the candidate gene TaACT in the FHB QTL-2DL region. Further, for the first time in wheat our study reports the functional characterization of TaACT through VIGS and a complementation study in Arabidopsis. Taken together, our results revealed the presence of the candidate gene TaACT in FHB QTL-2DL in NIL-R and its resistance functions against FHB. Its role in resistance and its application in breeding are discussed.

FHB resistance in NIL-R is due to high fold induction of HCAAs
Resistance in plants is controlled by hierarchies of resistance (R) genes, including regulatory genes and the regulated genes that biosynthesize RR metabolites and proteins, which directly suppress the pathogen development through their antimicrobial and cell wall reinforcement properties (Kushalappa et al., 2016a). Resistance in wheat to the spread of F. graminearum through rachis is mainly due to the production of phytoalexins that are antimicrobial, and/or to the deposition of phenylpropanoids to reinforce the cell wall, thus preventing further progress of the pathogen in plant (Gunniaiah et al., 2012). Coumaroylagmatine and coumaroylputrescine types of HCAAs were induced in high fold-change in resistant NIL. The reinforcement of cell walls by these compounds was confirmed by histochemical localization of HCAAs. Deposition of feruloyl-3′-methoxytyramine, feruloyltyramine and p-coumaroyltyramine has been reported from onion cells at the infection sites, following inoculation with Botrytis allii (Ishihiara et al., 2008). Different HCAAs including coumaroylagmatine and coumaroylputrescine were proved to be antimicrobial and highly induced in Arabidopsis thaliana rosette leaves infected with Alternaria brassicicola (Muroi et al., 2009) and in transgenic torenia expressing AtACT to resist Botrytis cinerea, and arthropod herbivores (Muroi et al., 2012). Accumulation of coumaroylagmatine and its antimicrobial effect was reported in barley leaves infected with Erysiphe graminis f. sp. hordei (von Röpneck et al., 1998). HCAAs such as p-coumaroyl serotonin and feruloyl serotonin were detected in rice leaves infected with Bipolaris oryzae (McLusky et al., 1999). Several HCAAs, such as feruloylputrescine, p-coumaroyltyramine, N-feruloyltyramine, 4-coumaroyl-3-hydroxy agmatine, feruloylagmatine, coumaroylagmatine, terrestriamidine and feruloylserotonin, were induced in potato leaves against Phytophthora infestans (Keller et al., 1996; Pushpa et al., 2013; Yogendra et al., 2014). In our study, the cell wall reinforcement in NIL-R was also associated with lower disease severity and fungal biomass relative to NIL-S. Accordingly, our results suggest that coumaroylagmatine and coumaroylputrescine are responsible for resistance in QTL-2DL against the spread of F. graminearum through rachis.

TaACT induced high levels of coumaroylagmatine and coumaroylputrescine
The coumaroylagmatine and coumaroylputrescine responsible for resistance were searched in metabolic pathway networks to identify their respective biosynthesizing enzymes and genes. This led to the identification of the ACT gene, which is also known to catalyse the last step of biosynthesis of these two HCAAs (Burhenne et al., 2003; Muroi et al., 2009, 2012). We temporarily located the FHB QTL-2DL on wheat 2DL chromosome and the gene encoding functional TaACT within the QTL-2DL region. This gene was sequenced and confirmed by comparing with the sequences and conserved protein domains of the previously characterized HvACT gene (Burhenne et al., 2003). Closest phylogenetic relationship with HvACT confirmed TaACT is a member of the group five acyltransferases, which is involved in transferring acyl groups to the acceptors. Further, the
recombinant protein was expressed in E. coli, and the protein size was similar to the protein size of barley HvACT. In barley and wheat, the coexistence of hydroxycinnamoyl agmatines and putrescines has been demonstrated, and they are induced by the same biological stimuli (Fester et al., 1999; Ogura et al., 2001). Our report here further confirms that the TaACT in wheat QTL-2DL is responsible for the production of high abundance of coumaroylagmatine and coumaroylputrescine. In addition, based on semi-qPCR and qRT-PCR studies, it was confirmed that TaACT was expressed only after pathogen invasion. Similarly in Arabidopsis, the AtACT gene was highly expressed after pathogen inoculation (Muraoi et al., 2009).

Functional validation of TaACT

To further assess the effect of TaACT on resistance to spread of F. graminearum through rachis, it was silenced in NIL-R. The transcript abundance of TaACT and its respective metabolites coumaroylagmatine and coumaroylputrescine abundances were significantly reduced after silencing. The fungal biomass increased in silenced NIL-R relative to nonsilenced. In the same way, transient gene silencing of MYB10 decreased the flavonoid/phenylpropanoid metabolism in strawberry (Medina-Puche et al., 2014). Silencing of hydroxycinnamoyl-CoA:hydroxycinnamoyl transferase gene in N. benthamiana stems inhibited the

Figure 6 Gene expression analysis of TaACT gene in wheat rachis at 72 hpi, (a) gene expression of TaACT based on Semi-qPCR; (b) gene expression of TaACT in pathogen inoculated treatments (RP, SP; the mock inoculated did not show any expression, thus not presented) based RT-qPCR. TaActin gene was used as internal standard. RM = resistant mock, RP = resistant pathogen, SM = susceptible mock, SP = susceptible pathogen treatments.

Figure 7 Effect of TaACT silencing in NIL-R inoculated with F. graminearum or mock solution inoculation: (a) relative transcript expression of TaACT; (b) relative metabolite abundances of coumaroylagmatine and coumaroylputrescine in silenced (NIL-R+BSMV\textsubscript{TaACT}) and nonsilenced (NIL-R+BSMV\textsubscript{0}) NIL-R; (c) biomass (as relative gene copy number based on RT-qPCR) of F. graminearum in wheat rachis of silenced (NIL-R+BSMV\textsubscript{TaACT}), nonsilenced (NIL-R+BSMV\textsubscript{0}) NIL-R and NIL-S.
accumulation of a lignin polymer, dimethoxylated syringyl, affecting the cell wall reinforcement (Hoffmann et al., 2004). Similarly, the virus-induced silencing of StWRKY1 significantly reduced the abundance of N-feruloyltyramine, N-feruloyloctopamine, feruloylputrescine and feruloylagmatine types of HCAAs compromising late blight resistance by reducing reinforcement of secondary cell walls in potato (Yogendra et al., 2015). Knock-down of FcWRKY70 in kumquat down-regulated ADC (arginine decarboxylase) abundance and decreased putrescine level accompanied by compromised dehydration tolerance (Gong et al., 2015).

The TaACT gene function was also proved based on a complementation study using act mutant Arabidopsis lines which lacked the ability to biosynthesize coumaroylagmatine and coumaroylputrescine. Mutant Arabidopsis plants overexpressing the TaACT from NIL-R had higher abundances of coumaroylagmatine and coumaroylputrescine metabolites as compared to plants overexpressing TaACT from NIL-S. In addition, the former also resulted in decreased disease severity and amount of fungal biomass, thus confirming that this difference is mainly due to polymorphic sequences of TaACT. Model plants have been used to prove the functions of several genes. Transgenic A. thaliana expressing a barley UDP-glucosyltransferase exhibited resistance to the mycotoxin deoxynivalenol (Shin et al., 2012). Transgenic expression of polygalacturonase-inhibiting proteins in Arabidopsis and wheat increased the resistance to flower pathogen F. graminearum (Ferrari et al., 2012). Also, overexpression of PvPGIP2 was shown to be effective against a wheat foliar pathogen, B. sorokiniana due to increased polygalacturonase-inhibiting proteins (Janni et al., 2008). Taken together, these results provide compelling evidence and support that TaACT is one of the candidate genes responsible for FHB resistance in wheat QTL-2DL, through deposition of HCAAs to reinforce secondary cell walls, thus preventing further spread of pathogen throughout rachis.

In conclusion, we have identified here the TaACT gene located in wheat QTL-2DL and confirmed its resistance function against FHB under greenhouse conditions. The FHB resistance of the TaACT should also be effective under field conditions. Stacking of a few genes with similar FHB resistance effects should significantly reduce FHB under field conditions. The TaACT therefore can be used in FHB resistance breeding programmes either through development of genic markers (Kage et al., 2015) or through replacement of a nonfunctional Taact gene in commercial cultivars with functional TaACT genes based on genome editing (Kushalappa et al., 2016b). Pyramiding of a few genes should result in a resistant cultivar that reduces application of fungicide.

**Experimental procedures**

**Plant material and experimental layout**

The NILs carrying resistant and susceptible alleles of QTL-2DL were derived from a cross BW301 × HC374 (McCartney et al., 2007). The BW301 is a FHB susceptible hard red spring wheat line from Western Canada, and HC374 is a resistant line derived from Wuhan-1. The experiment was laid out in a randomized complete block design (RCBD) with two genotypes (resistant and susceptible NILs), two inoculations (pathogen and mock solution) and five replications over time to include sufficient block effect. Initially, five seeds were planted per pot, and later each pot was maintained with only three plants. The plants were grown in the greenhouse which was maintained at 23 ± 2 °C with 16 h of light and 8 h of dark.
Pathogen production and inoculation

An isolate of *F. graminearum* (GZ-3639, obtained from Dr. R.H. Proctor, USDA) was grown on potato dextrose agar (PDA) plates and incubated at 26 °C for 4 days. For spore production, *F. graminearum* was further subcultured on Rye B agar media and kept inverted by exposing the plates to near UV light for a period of 3 days. Macroconidia were harvested from 7-day-old cultures, spore concentration was determined using a haemocytometer (American Scientific, Ohio, USA), and the final concentration was adjusted to 1 × 10^5 macroconidia/mL (Chammarthi et al., 2014). At 50% anthesis (GS = 65), three alternate spikelet pairs of ten spikes per replicate were individually inoculated with 10 μL of fungal spore suspension containing of 1 × 10^5 macroconidia/mL or mock solution (water) using a syringe with an autodispenser (GASTIGHT 1750DAD, Reno). Inoculated plants were covered with water-sprayed polythene bags to maintain high moisture content. At 48 hpi, the bags were removed.

Sample collection, metabolite extraction and metabolite analysis using liquid chromatography-high-resolution mass spectrometry (LC-HRMS)

At 72 hpi, ten inoculated spikes per replicate were harvested. The spike region, with three inoculated and three uninoculated (a total of six) pairs of spikelets was retained. Spikelets and rachis samples were separately collected, immediately frozen in liquid nitrogen and stored at −80 °C until further use. Of ten, five rachis samples (remaining five were used for histochemical analysis) were ground in liquid nitrogen, and the metabolites were extracted in 60% ice-cold aqueous methanol. The extract was sonicated for 15 min at 25 °C, centrifuged, the supernatant was filtered and then 5 μL of the clear sample extract was used for metabolite analysis using LC-HRMS at (IRCM, Montreal, Canada), by following the previously established protocol (Bollina et al., 2010).

Data processing using MZmine software and statistical analysis

The output data Xcaliber RAW files from LC-HRMS were converted into mzXML format. Converted files were imported to bioinformatic tool Mzmine2 for peak de-convolution, peak detection and spectral filtering (Katajamaa et al., 2006). The observed monoisotopic masses (negative ionization) and their respective abundances (relative intensity) were imported to MS Excel. The relative peak intensities of monoisotopic masses of metabolites were subjected to Student’s t-test (SAS v 9.3) in pairwise treatment combinations (RP vs RM, RM vs SM, SP vs SM and RP vs SP, where RP = resistant NIL inoculated with pathogen, RM = resistant NIL inoculated with mock solution, SP = susceptible NIL inoculated with pathogen, SM = susceptible NIL inoculated with mock solution). Treatment significant metabolites with P < 0.05 were retained for further analysis. The metabolites significantly higher in abundance in resistant than susceptible NIL were considered as RR metabolites. Further, these metabolites were grouped into RR constitutive (RRC = RM > SM) and RR induced (RRI = (RP > RM) > (SP > SM)) metabolites. The fold-change in abundance of metabolites in NIL-R was calculated relative to NIL-S (NIL-R/NIL-S) (Gunnaiah et al., 2012). Only the selected high fold-change RRI metabolites were prioritized for further candidate gene identification.

Putative identification of metabolites

The RR metabolites were putatively identified based on three criteria: (i) accurate mass match with the metabolites reported in several databases such as PlantCyc, METLIN, KNApSACK, LIPID-MAPS, NIST and KEGG, with an accurate mass error, AME ≤ 5 ppm (Kushalappa and Gunnaiah, 2013; Tohge and Fernie, 2010); (ii) fragmentation pattern mass match with databases and literature (Matsuda et al., 2009); (iii) in silico confirmation of fragmentation based on Massspec scissors in ChemSketch (ACD labs, Toronto) (Matsuda et al., 2009).

Disease severity and quantification of fungal biomass

To evaluate rachis resistance in wheat genotypes, two NILs with resistant and susceptible alleles were planted in RCBD with three biological replications at 3-day intervals to include sufficient block effect. At the 50% anthesis (GS = 65), a single pair of spikelets in the middle of the spike with five spikes per replicate was point inoculated with 10 μL of spore suspension (1 × 10^5 macroconidia/mL). The number of diseased spikelets was recorded at 3-day intervals until 15 dpi. Spikelets with both dark brown discoloration and bleaching symptoms were considered as diseased. From these data, the PSD (proportion of spikelets diseased = number of spikelets diseased/total number of spikelets in spike) and the AUDPC were calculated (Hamzehzarghani et al., 2008). The PSD among observations was analysed for significance based on ANOVA using SAS program (SAS v 9.3).

To quantify the fungal biomass, a separate experiment was conducted with two treatments (NIL-R and NIL-S) and three biological replications over time. At the 50% anthesis (GS = 65), three alternate pairs of spikelets in the middle of the spike with five spikes per replicate was point inoculated with 10 μL of spore suspension (1 × 10^5 macroconidia/mL). Samples were collected at six dpi, and the fungal biomass was quantified in rachis samples as relative gene copy number of the fungal housekeeping gene *Tri6*. Genomic DNA was isolated from rachis samples using a DNeasy Plant Mini Kit (Qiagen, Canada), and DNA quality was assessed by gel electrophoresis in 1% agarose gel and quantified by nano-drop (Thermo-Scientific Canada). Equal quantities of DNA (20 ng) were used for the relative fungal biomass quantification in the NILs under study. RT-qPCR was performed using fungal-specific gene (*Tri6*) primers. The abundance of *Tri6* gene was normalized with *TaActin*, and the results obtained from RT-qPCR experiment were used to estimate the fungal biomass (Kumar et al., 2015).

Physical localization of QTL-2DL and identification of *TaACT* gene

The presence of SSR markers, WMC245, GPW8003, GWM539 and GWM608 was used to define the interval for QTL-2DL. Sequence of WMC245 was retrieved from available GrainGenes database, and markers GPW8003, GWM539 and GWM608 were sequenced in our laboratory. We directly sequenced the PCR products and subjected to BLASTN (Altschul et al., 1990) search on the international wheat genome-sequencing consortium (IWGSC) wheat genome database for temporary physical localization of the QTL (we considered only the best 2DL BLAST hits). High fold change RRI metabolites were mapped in metabolic pathways to identify the catalytic enzymes and their coding genes. Sequences of these genes were searched by BLAST against IWGSC database to confirm their colocalization within the predicted QTL-2DL region. Contigs identified as the best hits
were retrieved from the database, and gene prediction was performed using the SoftBerry’s FGENESh program (Solovyev et al., 2006) to study gene structure. The identified gene was amplified using gene-specific primers designed using the NCBI Primer-BLAST tool (Ye et al., 2012).

Cloning, sequencing and sequence analysis of TaACT gene

Genomic DNA was isolated from the rachis samples of NILs using DNeasy Plant Mini Kit (Qiagen, Canada) and used for the amplification of full-length TaACT gene using gene-specific primers. PCR was performed as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min followed by a final extension at 72 °C for 10 min. PCR products were separated on a 1% agarose gel. A band size corresponding to ~1350 bp was then purified from the gel, cloned into the pGEM-T Easy vector (Promega, Canada) and Sanger sequenced (Genome Quebec, McGill University). The sequence of TaACT (Accession No. KT962210) was deposited to the NCBI database. The sequences from both the NILs were aligned to identify sequence variations at the genic region using MultAlin (Corpet, 1988). MOTIF Search tool (http://www.genome.jp/tools/motif/) was used to see the presence of conserved domains in translated amino acid sequences. The Phylogey.fr (http://www.phylogeny.fr/) program was used to perform a multiple sequence alignment and to construct the phylogenetic tree.

RNA isolation and candidate gene expression based on semi-qPCR and RT-qPCR

Total plant RNA was isolated from five biological replicates using RNeasy plant mini kit (Qiagen, Canada). Purified total RNA was isolated from five biological replicates using RNeasy plant mini kit (Qiagen, Canada). The level of expression was determined for RNA (1 ²l) using RNeasy plant mini kit (Qiagen, Canada). Total plant RNA was isolated from five biological replicates semi-qPCR and RT-qPCR RNA isolation and candidate gene expression based on phylogenetic tree.

Cloning, sequencing and sequence analysis of TaACT gene

Genomic DNA was isolated from the rachis samples of NILs using DNeasy Plant Mini Kit (Qiagen, Canada) and used for the amplification of full-length TaACT gene using gene-specific primers. PCR was performed as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 2 min followed by a final extension at 72 °C for 10 min. PCR products were separated on a 1% agarose gel. A band size corresponding to ~1350 bp was then purified from the gel, cloned into the pGEM-T Easy vector (Promega, Canada) and Sanger sequenced (Genome Quebec, McGill University). The sequence of TaACT (Accession No. KT962210) was deposited to the NCBI database. The sequences from both the NILs were aligned to identify sequence variations at the genic region using MultAlin (Corpet, 1988). MOTIF Search tool (http://www.genome.jp/tools/motif/) was used to see the presence of conserved domains in translated amino acid sequences. The Phylogey.fr (http://www.phylogeny.fr/) program was used to perform a multiple sequence alignment and to construct the phylogenetic tree.

Histochemical localization of HCAAs

The five rachis samples in the region of inoculated spikelets, of ten collected at 72 hpi in the metabolomics study were stored at −20 °C for further use. For cryosectioning, the samples were prepared by embedding tissues in cryomoulds containing Shandon CRYOMATRIX (Richard-Allan Scientific, Kalamazoo). Cryosectioning (15 μm) was carried out using a cryotome (Leica, CM1850, Canada) machine at −20 °C, and the cross sections were collected on slides coated with 5% 3-aminopropyltriethoxysilane (APES) solution. Sections were washed with distilled water and stained with Neu’s reagent (1% 2-amino ethyl diphenylborinate (Sigma Aldrich, Canada) prepared in absolute methanol) for 5 min and mounted in 15% glycerol. The stained samples were observed under a confocal microscope (Nikon, Eclipse E800, USA) for HCAA chemiluminescence, with excitation at 405 nm with emission filter HQ442/45.

Expression and purification of recombinant protein in E. coli

The coding region of TaACT gene was amplified from cDNA using primers containing EcoRI and BglII restriction sites. PCR product was digested with EcoRI and BglII, the fragment was ligated into the pTRC-HisB vector (Invitrogen, Canada). Recombinant vector and empty vector without cloned gene were transformed into E. coli BL21 cells and grown in Luria Bertani medium at 28 °C to an optical density of 0.6 at 600 nm (A600). The induction of expression was carried out at 18 °C for 15 h by the addition of isopropyl-1-thio-D-galactopyranoside (IPTG) to a final concentration of 1 mM. After 15 h, 1 ml sample from both recombinant and empty suspension was collected, centrifuged at 10 000 g, processed further and the expression was confirmed on 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Remaining cells were immediately pelleted down by centrifugation at 4 °C. Cell pellet was resuspended in 1X LEW buffer (50 mM NaH2PO4, 300 mM NaCl, pH 8.0), and cells were lysed by adding lysozyme (1 mg/mL), incubating at 4 °C for 1 h, followed by sonication. The supernatant was collected after centrifugation at 12 000 g for 20 min at 4 °C, and purification was achieved using nickel nitritriacetic acid (Ni-NTA) column (Affymetrix, Canada). Purified protein fraction was detected by Coomassie Brilliant Blue Staining after electrophoresis in 12% SDS-PAGE. The average protein size of TaACT was predicted using ExPasy-Compute pI/Mw tool (http://web.expasy.org/compute_p/i/).

Virus-induced gene silencing of TaACT

The PCR product amplified from cDNA using VIGS primers (listed in Table S1) was used to construct the silencing vector. The VIGS primers were designed using the NCBI Primer-BLAST tool (Ye et al., 2012). A 271-bp fragment of the TaACT gene with efficient siRNA generation and no off target genes in the modified viral genome using siRNA Scan tool (http://bioinfo2. noble.org/RNAiScan.htm) and a BLAST search of fragment against GenBank database was chosen. The PCR product was cloned into the pGEM-T Easy Vector (Promega Corp., WI), confirmed by sequencing and excised using NotI (New England Biolabs, MA, USA), thereby generating NotI ends. These fragments were subsequently cloned into the NotI site of pSL038-1, a plasmid encoding a modified BSMV γ-genome segment with a cloning site downstream of the γb gene (Cakir and Scofield, 2008). Clones containing the fragments in the γ-vector were sequenced to confirm their identity and subsequently used for gene silencing.

The procedures for in vitro transcription of α-, β- and γ-RNAs of the BSMV genome were the same as described by Scofield et al. (2012). In vitro synthesized BSMV RNAs were three times rub inoculated on both flag leaf and spikes at growth stage 50–55 (Zadoks et al., 1974) with a solution containing 1 : 1 : 1 μL (α-, β- and γ-RNAs) + 22.5 μL of abrasive FES buffer (1% sodium pyrophosphate, 1% bентонит, 1% селитра, 2 μM glyoxylate, 0.06 μM dipotassium phosphate). Ten spikes per replicate were infected with each of BSMV + TaACT insert and BSMV:0 respectively with five biological replicates. Construct BSMV:0 and BSMV + PDS insert served as negative and positive controls respectively. An experiment involving VIGS resulted in photo-bleaching symptoms in the wheat spikes 12 days after the viral inoculation (Fig. S4). At
13 days postviral infection, ten spikes each in the negative control and test treatment were inoculated with 10 μL of *F. graminearum* spore suspension (1 × 10^5 macroconidia/mL) and covered with plastic bags to maintain high humidity. At 48 hpi with *F. graminearum*, plastic covers were removed and samples were collected from five spikes at 72 hpi for metabolite analysis and RT-qPCR to measure the transcript abundance of TaACT. Similarly, total DNA isolated from six dpi samples collected from remaining five spikes was used in the fungal biomass study. The fungal biomass was quantified in NIL-R+BSMV, NIL-R+BSMV-TaACT and NIL-S.

**TaACT functional complementation study in Arabidopsis**

The TaACT alleles from NIL-R and NIL-S were overexpressed in an Arabidopsis *AtAct* mutant (AT5g61160) background (obtained from TAIR) for their FHB resistance function validation. Homozygous T1 lines for four transgenic events were identified by examining the segregation for hygromycin resistance. Progenies derived from these transgenic events were 100% hygromycin resistant, and homozygous T2 lines derived from these were used for further testing. Samples were collected from 6-week-old plants for targeted metabolite analysis with three biological replicates. The detailed procedure followed for development of transgenic plants is given in supplementary data (Procedure S1).

A set of 25 T2 plants each with TaACT of NIL-R or NIL-S in four replicates over time were inoculated with *F. graminearum* at flowering stage to determine the effect of *F. graminearum* infection. The open flowers of 6-week-old plants were inoculated by placing 10 μL droplets of spore suspension (1 × 10^5 macroconidia/mL). Inoculated plants were covered with plastic bags for 3 days to maintain high humidity, and the symptom development was monitored. The inoculated flowers were diseased in four dpi, and by six dpi, the disease spread to uninoculated flowers within the inflorescence. At six dpi, the number of plants with diseased (dead) inflorescence was assessed (Figure 8a-2) (Ferrari et al., 2015). Following this, the samples were collected and fungal biomass was quantified using the same method as in wheat, but with protodermal factor 2 (AtPDF2) as the Arabidopsis reference gene at six dpi with three biological replicates. The data for each study were analysed using a Student t-test.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Comparison of promoter DNA sequence variation between NIL-R, NIL-S and Chinese spring TaACT.

Figure S2 Comparison of DNA sequence variation between NIL-R, NIL-S and Chinese spring TaACT. Green underlined indicates 5′ and 3′ regions.

Figure S3 Purification of bacterial expressed TaACT.

Figure S4 Silencing of the phytoene desaturase (PDS) gene.

Table S1 List of primers used in the experiments.

Procedure S1 Detailed procedure followed for development of transgenic Arabidopsis plants over-expressing TaACT.