Efficient expression and function of a receptor-like kinase in wheat powdery mildew defence require an intron-located MYB binding site

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Summary

The LRK10-like receptor kinases (LRK10L-RLKs) are ubiquitously present in higher plants, but knowledge of their expression and function is still limited. Here, we report expression and functional analysis of TtdLRK10L-1, a typical LRK10L-RLK in durum wheat (Triticum turgidum L. ssp. durum). The introns of TtdLRK10L-1 contained multiple kinds of predicted cis-elements. To investigate the potential effect of these cis-elements on TtdLRK10L-1 expression and function, two types of transgenic wheat lines were prepared, which expressed a GFP-tagged TtdLRK10L-1 protein (TtdLRK10L-1:GFP) from the cDNA or genomic DNA (gDNA) sequence of TtdLRK10L-1 under the native promoter. TtdLRK10L-1:GFP expression was up-regulated by the powdery mildew pathogen Blumeria graminis f. sp. tritici (Bgt) in both types of transgenic plants, with the scale of the elevation being much stronger in the gDNA lines. Both types of transgenic plants exhibited enhanced resistance to Bgt infection relative to wild type control. Notably, the Bgt defence activated in the gDNA lines was significantly stronger than that in the cDNA lines. Further analysis revealed that a putative MYB transcription factor binding site (MYB-BS, CAGTGA) located in TtdLRK10L-1 intron I was critical for the efficient expression and function of TtdLRK10L-1 in Bgt defence. This MYB-BS could also increase the activity of a superpromoter widely used in ectopic gene expression studies in plants. Together, our results deepen the understanding of the expression and functional characteristics of LRK10L-RLKs. TtdLRK10L-1 is likely useful for further dissecting the molecular processes underlying wheat defence against Bgt and for developing Bgt resistant wheat crops.

Introduction

Plants have evolved a sophisticated innate immune system to defend against pathogen attack, which functions in two forms, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006; Zhou and Zhang, 2020). Many plant receptor-like kinases (RLKs) have been shown to perceive and process the signals from invading pathogens in diverse pathosystems (Tang et al., 2017). They often serve as pattern-recognition receptors (PRRs) in PTI and are rapidly activated by specific pathogen effectors (Dardick et al., 2012; Tang et al., 2017; Zhou and Zhang, 2020; Zhou et al., 2019).

In general, a typical RLK protein contains a variable ectodomain responsible for ligand binding, a single transmembrane domain, an intracellular juxtamembrane domain, and a cytoplasmic kinase domain (Shiu and Bleecker, 2003). Based on the structural features of extracellular regions, the plant RLK family has been divided into 46 different subfamilies (Shiu and Bleecker, 2003; Shiu et al., 2004). According to the presence or absence of the RD motif, which consists of a highly conserved positively charged arginine (R) located adjacent to the key negatively charged catalytic aspartate (D) in the kinase domain, plant and animal RLKs can be classified as RD and non-RD kinases (Dardick and Ronald, 2006; Dardick et al., 2012). The function of RLKs in plant immune response involves specific phosphorylation events within and outside of the kinase domain, which leads to altered kinase activity and thus immune signal transduction (Dardick et al., 2012; Tang et al., 2017; Zhou and Zhang, 2020). To date, the function of several RLKs in pathogen resistance has been extensively studied in the model plants with simpler genomes, for example, rice and Arabidopsis (Tang et al., 2017; Zhou et al.,...
The examples include XA21 (LRR XII subfamily) and XA26 (LRR XII subfamily) from rice (Chen et al., 2014; Song et al., 1995; Sun et al., 2004; Tang et al., 2019) and PRSK (LRR XII subfamily) and FLS2 (LRR XII subfamily) from Arabidopsis (Dunning et al., 2007; Lu et al., 2011; Wang et al., 1996). In contrast, much less progress is made in understanding the RLKs functioning in the disease resistance of the crop plants with complex genomes.

The powdery mildew disease caused by *Blumeria graminis* f. sp. *tritici* (*Bgt*) severely reduces the grain yield and quality of worldwide wheat crops including hexaploid common wheat (*Triticum aestivum*, AABBD) and tetraploid durum wheat (*Triticum turgidum* L. ssp. *durum*, AABB) (Figueroa et al., 2018; Singh et al., 2016). Breeding and utilizing *Bgt* resistant wheat cultivars remain the most effective, economic and environmentally friendly approach to combat this disease (Singh et al., 2016; Zou et al., 2018). At present, two major types of powdery mildew resistance have been characterized, which are controlled by *R* genes and negative regulators of cellular defense, respectively (Zou et al., 2018). Some *Pm* resistance genes have been isolated by map-based cloning that encode classic *R* proteins, but the resistance mediated by them tends to be *Bgt* race-specific (Bourras et al., 2019; He et al., 2018; Hurni et al., 2013; Krattinger et al., 2009; Lu et al., 2020; Moore et al., 2015; Singh et al., 2018; Xie et al., 2020; Xing et al., 2018; Yahiaoui et al., 2004; Zou et al., 2018). On the other hand, the powdery mildew resistance conferred by mutations in the negative regulators, for example, *mildew resistance locus* (*MLO*) and enhanced disease resistance 1 (*EDR1*), appears to be broad-spectrum (Acevedo-Garcia et al., 2017; Wang et al., 2014; Zhang et al., 2017). In addition, several RLKs, namely *TaRLK1* and *TaRLK2* from wheat (Chen et al., 2016) and *RLK-V* and *LecRK-V* from *Hymindalia villosa* (Hu et al., 2018; Wang et al., 2018), have been shown to regulate wheat defence to *Bgt*. *TaRLK1* and *TaRLK2* are leucine-rich-repeat (*LRR*) RLKs; their overexpression in transgenic wheat leads to enhanced *Bgt* resistance via increased production of reactive oxygen species (*ROS*) in fungal penetration sites (Chen et al., 2016). *RLK-V* is a malectin-like/LRR-RLK that regulates both basal and *Pm*2-mediated resistance to *Bgt* with the involvement of elevated ROS accumulation (Hu et al., 2018). *LecRK-V* is a lecithin type receptor-like kinase, whose overexpression confers resistance to *Bgt* through enhancing ROS and cell death upon *Bgt* infection (Wang et al., 2018). *TaRLK1*, *TaRLK2*, *RLK-V* and *LeckR-V* are all RD kinases (Chen et al., 2016; Hu et al., 2018; Wang et al., 2018).

LRK10-like RLKs (LRK10-LRLKs) were firstly reported in wheat and relatives, but later studies indicate that these proteins are widespread in higher plants and constitute a distinct subfamily (Zhou et al., 2007). The complex regulation of LRK10 gene expression correlates with the presence of numerous putative cis-elements in the promoter and intronic regions (Zhou et al., 2007), although no experimental evidence has yet been obtained to validate the function of these cis-elements. Importantly, three LRK10-LRLKs (*TaRLK-R1*, -R2 and -R3) have been demonstrated to contribute positively to wheat hypersensitive resistance to the stripe rust fungus (*Puccinia striiformis* f. sp. *tritici*) (Zhou et al., 2007). However, it is still unknown if LRK10-LRLKs may participate in wheat defence response to *Bgt* infection.

From the information presented above, the main objectives of this work were to investigate if LRK10-LRLKs may function in wheat defence against *Bgt* and whether the intronic cis-elements may affect the expression and function of LRK10-LRLKs. Towards this end, we studied the expression and function of *TtdLRK10L-1* in wheat defence against *Bgt* infection. *TtdLRK10L-1* was identified in the durum wheat cultivar Stewart and resembled highly LRK10 in both amino acid sequence and primary structure. Like the genes encoding *TaRLK-R1*, -R2 and -R3, *TtdLRK10L-1* carried abundantly predicted cis-elements in its introns. Central to this study, we prepared two types of transgenic wheat lines that ectopically expressed GFP-tagged *TtdLRK10L-1* protein (i.e. *TtdLRK10L-1*:GFP) from the cDNA coding sequence of *TtdLRK10L-1* or its genomic open reading frame (ORF) under the native promoter. The results from analysing these transgenic plants, together with the data obtained from single-cell *Bgt* defence tests and transient luciferase (LUC) reporter assays, allowed us to reveal the contribution of *TtdLRK10L-1* to durum wheat resistance against *Bgt* infection as well as the positive role of an intronic putative MYB binding site (MYB-BS) in the expression and function of *TtdLRK10L-1*. Remarkably, the presence of this MYB-BS could also enhance the activity of a superpromoter, which was developed by combining a trimer of the octopine synthase transcriptional activating element and the activator-promoter region of mannopine synthase2 and has been highly useful for ectopic gene expression studies in both monocot and dicot plants (Lee et al., 2007). Thus, our work provides new insight into the function of *TtdLRK10L-1* and enriches the knowledge of gene expression regulation by intron-located cis-elements.

### Results

#### Structural and expression features of *TtdLRK10L-1*

Previously, we conducted a preliminary functional analysis of three LRK10-LRLKs (*TaRLK-R1*, -R2 and -R3) in hexaploid common wheat (Zhou et al., 2007). But further molecular and mechanistic studies of these RLKs are hampered by the difficulty in genetically transforming the common wheat varieties (*Swwon 11*, Chinese Spring, and Xiaoyan 54) used in the original work. To deepen our research, we decided to analyse LRK10-LRLKs in the spring durum wheat cultivar Stewart that is amenable to *Agrobacterium* mediated genetic transformation (He et al., 2010).

In the genomic sequence of the hexaploid wheat variety Chinese Spring (CS), seven LRK10 genes were annotated, which were located on 1A (*TraesCS1A02G018000* and *TraesCS1A02G018600*), 1B (*TraesCS1B02G020700* and *TraesCS1B02G022400*) and 1D (*TraesCS1D02G015900* and *TraesCS1D02G017800*) chromosomes, respectively (Table 1). Nucleic acid and amino acid sequence comparisons indicated that *TraesCS1A02G018000* corresponded to LRK10, whereas *TraesCS1B02G022400*,
TraesCS1D02G017800 and TraesCS1B02G020700 were TaLRK-R1, -R2 and -R3, respectively (Table 1). In the genome sequence of the durum wheat cultivar Svevo, four LRK10 genes have been annotated, which were located on 1A (TRITD1Av1G004220) and 1B (TRITD1Bv1G004010 and TRITD1Bv1G004020) chromosomes or remained to be assigned to a specific chromosome (TRITD0Uv1G102600) (Table 1). Three to four LRK10 genes were also present in the annotated genome sequence of T. urartu, Ae. tauschii or wild emmer wheat (Table 1). The deduced protein of TRITD1Av1G004220 was highly similar to LRK10 and TaLRK-R1, -R2 and -R3 in both amino acid sequence and primary structure (Figure S1). In phylogenetic analysis, TaRLK-R1, TaRLK-R2, TaRLK-R3 and TRITD1Av1G004220 clustered in different sub-branches (Figure S2), indicating that the four genes may have similar but not identical functions. Thus, we focused on TRITD1Av1G004220, which encodes TtdLRK10L-1, to facilitate subsequent experimentation.

The TtdLRK10L-1 cDNA sequence isolated from Stewart was 100% identical to that of TRITD1Av1G004220. The deduced protein of TtdLRK10L-1 contained a signal peptide, a cytochrome-rich extracellular domain, a transmembrane domain and an intracellular kinase domain containing the 12 subdomains shared by plant serine/threonine protein kinases (Figure S1). The genomic sequence of TtdLRK10L-1 had two introns and three exons, with the size of the first intron (intron I, 1015 bp) being substantially larger than that of the second intron (intron II, 191 bp) (Figure 1a). When analysed in the NEW PLACE database (Higo et al., 1999), multiple putative cis-elements were found in the two introns of TtdLRK10L-1 (Table S1). These cis-elements were likely involved in transcription factor binding, abiotic stress response, tissue-specific expression and phytohormone induction. Notably, an MYB-BS containing the consensus MYB transcription factor recognition sequence (TAACGT) and two W-box transcription factor binding elements were present in only intron I (Table S1). Like what was recorded for LRK10-RLKs by previous studies (Feuillet et al., 1997, 1998; Zhou et al., 2007), TtdLRK10L-1 expression was induced by light and highly abundant in wheat leaf tissues (Figure S3).

**Development and characterization of two types of transgenic lines**

To facilitate further expression and functional investigations, we prepared two types of transgenic Stewart wheat lines using TtdLRK10L-1 native promoter. Three homozygous and independent cDNA lines (CL-1, CL-2 and CL-3) expressed a TtdLRK10L-1-GFP transgene from the cDNA coding sequence devoid of introns I and II; another three homozygous and independent gDNA lines (GL-1, GL-2 and GL-3) expressed the same transgene but from the genomic sequence containing both introns (Figure 1b). Real-time TaqMan PCR assays showed that CL-1, CL-2, CL-3, GL-1, GL-2 and GL-3 each harbour a single copy of TtdLRK10L-1-GFP transgene (Figure S4). Consistent with these results, genomic PCR, RT-PCR and protein blot assays revealed the presence and active transcription and translation of TtdLRK10L-1-GFP in the cDNA and gDNA transgenic lines (Figure 1c). Notably, the protein level of TtdLRK10L-1-GFP was higher in GL-1, GL-2 and GL-3 than in CL-1, CL-2 and CL-3 (Figure 1c, lower panel). Confocal microscopy indicated that TtdLRK10L-1-GFP was targeted to the plasma membrane in the leaf epidermal cells of both cDNA and gDNA transgenic lines (Figure 1d). Consistent with this finding, the TtdLRK10L-1-GFP fusion protein expressed in wheat mesophyll protoplasts was located in the plasma membrane (Figure S5).

| Species (Genotype) | Chromosome | Locus | Correspondence to the genes investigated | Size of genomic ORF (bp) | Size of intron I (bp) | Size of intron II (bp) |
|--------------------|------------|-------|------------------------------------------|------------------------|----------------------|------------------------|
| Common wheat (Chinese Spring) | 1A | TraesCS1A02G018000 | - | 4491 | 2430 | 156 |
| | 1A | TraesCS1A02G018600 | LMK10 | 2950 | 831 | 181 |
| | 1B | TraesCS1B02G020700 | TaLRK-R3 | 3177 | 1096 | 185 |
| | 1B | TraesCS1B02G022400 | TaLRK-R1 | 2925 | 824 | 178 |
| | 1D | TraesCS1D02G015300 | - | 3081 | 1007 | 169 |
| | 1D | TraesCS1D02G015900 | - | 3381 | 1315 | 182 |
| | 1D | TraesCS1D02G017800 | TaLRK-R2 | 2912 | 820 | 178 |
| Durum wheat (Svevo) | 1A | TRITD1Av1G004220 | TtdLRK10L-1 | 3108 | 1015 | 191 |
| | 1B | TRITD1Bv1G004010 | - | 3489 | 1141 | 178 |
| | 1B | TRITD1Bv1G004020 | - | 2725 | 598 | 186 |
| | Un | TRITD0Uv1G102600 | - | 2724 | 593 | 181 |
| Wild emmer wheat (Zavitan) | 1A | TRIDC1AG001890 | - | 3431 | 1339 | 178 |
| | 1A | TRIDC1AG001960 | - | 2761 | 616 | 186 |
| | Un | TRIDC0UG001810 | - | 2740 | 598 | 186 |
| | Un | TRIDC0UG021350 | - | 3269 | 1111 | 184 |
| T. urartu (G1812) | 1A | TuG1812G0100000266 | - | 3183 | 594 | 350 |
| | Un | TuG18125002479500 | - | 2878 | 592 | 348 |
| | Un | TuG18125003465600 | - | 2718 | 599 | 181 |
| Ae. tauschii (ALB78) | 1D | AET1Gv20037000 | - | 3437 | 1361 | 168 |
| | 1D | AET1Gv20040600 | - | 3018 | 847 | 178 |
| | 1D | AET1Gv20047000 | - | 2741 | 588 | 185 |

ORF, open reading frame. Un, unassigned to specific chromosomes.
Higher transgene expression and stronger Bgt defence in gDNA transgenic lines

The cDNA and gDNA transgenic lines were inoculated with Bgt at the seedling stage. Quantitative RT-PCR assay showed that the transcript level of endogenous TtdLRK10L-1 was gradually increased by Bgt infection (Figure S6). Likewise, the TtdLRK10L-1-GFP transgene was significantly up-regulated by Bgt infection at 24 and 48 hours post-inoculation (hpi), with the magnitude of the elevation being much stronger in the three gDNA lines (GL-1, GL-2 and GL-3) (Figure 2a). The protein level of TtdLRK10L-1:GFP was also enhanced by Bgt infection, with the scale of the increase being larger for the gDNA lines GL-1, GL-2 and GL-3 (Figure 2b). At 72 hpi, both the cDNA and gDNA transgenic lines developed fewer Bgt microcolonies than the controls, which included wild type (WT) Stewart and a transgene null segregant (TNS) (Figure 2c). The six transgenic lines also had decreased Bgt colonies on their leaves than WT Stewart and TNS at seven days post-inoculation (dpi) (Figure 2d and e). Importantly, GL-1, GL-2 and GL-3 consistently exhibited significantly lower Bgt growth than CL-1, CL-2 and CL-3 (Figure 2c, d and e), indicating the occurrence of more potent Bgt defence in the three gDNA transgenic lines compared to the three cDNA lines.

Oxidative bursts at Bgt penetration site (type a) or throughout a Bgt-infected cell (type b) constitute an important early event of strong host cell defence to powdery mildew infection (Chen et al., 2016; Hu et al., 2018; Wang et al., 2018). The two types of oxidative bursts can be reliably detected by histological staining of H2O2 accumulation with 3,3’-diaminobenzidine (DAB) (Figure 3a). At 24 hpi, the percentages of cells showing the two types of oxidative bursts were lowest in WT Stewart (4.17% and 0.66%) and TNS control (4.34% and 0.83%) plants, intermediate in the three cDNA transgenic lines (CL-1: 12.17% and 2.16%; CL-2: 11.17% and 3.5%; CL-3: 11.67% and 3.66%), and highest in the three gDNA lines (GL-1: 16.50% and 7.67%; GL-2: 18.50% and 6.50%; GL-3: 16.50% and 7.00%) (Figure 3b). On the other hand, the percentage of cells with no oxidative burst (type c), which indicates a susceptible response, was highest in WT Stewart and TNS control plants, intermediate in the three cDNA lines, and lowest in the three gDNA lines (Figure 3b).

Effect of intron I on TtdLRK10L-1 mediated defence against Bgt

The findings that the three gDNA lines displayed higher expression of TtdLRK10L-1-GFP transgene and stronger Bgt defence than the three cDNA lines indicated the potential involvement of introns in regulating TtdLRK10L-1 expression and function. To test this possibility, the cDNA coding sequence and the genomic ORF of TtdLRK10L-1, as well as the four mutants with only intron I, intron II, intron I with mutant MYB binding site (intron I/mMYB-BS) or intron I with the two W-box elements mutated (intron I/mW-box) (Figure 4a), were used to perform single-cell Bgt defence assays in one-week-old leaves of Stewart seedlings. Bgt haustorium index (HI) in the transiently transformed cells, which is...
Figure 2  Comparative analysis of TtdLRK10L-1 cDNA (CL-1, −2 and −3) and gDNA (GL-1, −2 and −3) transgenic lines in their response to powdery mildew (Bgt) infection. (a) Expression levels of TtdLRK10L-1-GFP transgene in the cDNA and gDNA transgenic lines before Bgt inoculation (0 h) and at two time points (24 h and 48 h) post-Bgt inoculation as assessed by qRT-PCR. Each data point was the mean (± SD) of the expression values obtained from four separate plants. The assay was repeated three times with similar results generated. (b) Detection of TtdLRK10L-1:GFP fusion protein (~97 kD) in the six transgenic lines at 0 and 48 h post-Bgt inoculation by immunoblotting with a GFP specific antibody. The relative intensities of TtdLRK10L-1:GFP bands, reflecting TtdLRK10L-1:GFP accumulation levels, are quantified using ImageJ (https://imagej.nih.gov/ij/) and shown above the graph. Equal loading of protein samples was demonstrated by Ponceau S staining of Rubisco large subunit (~55 kD) or by immunodetection of plant actin protein (~43 kD). The data shown were representative of three independent experiments. (c) Percentage of microcolonies formed from the total number of germinated spores of Bgt determined for the six transgenic lines and the two control materials, WT Stewart and transgene null segregant (TNS), at 72 h post-Bgt inoculation. Each data point was the mean (± SD) of three separate Bgt inoculation assays, with at least 600 germinated spores examined per genotype per assay. (d) Bgt colonies developed on the leaf surface of the six transgenic lines and the two controls (WT Stewart and TNS) at seven days post-inoculation. The images displayed were representative of three independent inoculation experiments. Scale bars, 1 cm. (e) Quantification of the percentages of leaf areas with Bgt colonies using ImageJ in the different leaf samples in (d). In (a), (c) and (e), each data point was the mean (SD) of three separate measurements. The means were analysed using Duncan’s multiple comparison test, with those marked by different letters being statistically significant (P < 0.05).

Figure 3  Analysis of Bgt-induced oxidative bursts in the cDNA (CL-1, −2 and −3) and gDNA (GL-1, −2 and −3) transgenic lines and the two control materials, WT Stewart and transgene null segregant (TNS), at 48 h post-pathogen inoculation. (a) Three types of cellular oxidative bursts as revealed by staining with 3,3’-diaminobenzidine. Type a, oxidative burst at Bgt penetration site. Type b, oxidative burst distributed throughout the cell. Type c, no detectable oxidative burst. Scale bars, 50 μm. (b) Quantitative comparison of the percentages of cells showing types a, b or c oxidative bursts in the transgenic and control lines. Each mean (± SD) was calculated with the data obtained from four separate plants. The means were statistically compared using Duncan’s multiple comparison test, with those marked by different letters being significantly different (P < 0.05). The staining assay was performed twice with similar results obtained.
an effective indicator of the defence to Bgt infection (Shen et al., 2003, 2007; Zhang et al., 2016), was scored, with the HI values of the examined constructs statistically compared. As shown in Figure 4b, the highest HI values were obtained for pHZ206(VC) and pUbi:TtdLRK10L-1intron II, whereas the lowest HI values were recorded for pUbi:TtdLRK10L-1gDNA, pUbi:TtdLRK10L-1introntmW-box and pUbi:TtdLRK10L-1introntmMYB-BS, with the HI values determined for pUbi:TtdLRK10L-1gDNA and pUbi:TtdLRK10L-1introntmMYB-BS being intermediate. Together, these data indicated that lacking intron I (as in pUbi:TtdLRK10L-1intron II) substantially lowered TtdLRK10L-1 mediated Bgt defence, removing intron II (pUbi:TtdLRK10L-1introntmW-box) or mutating the W-boxes in intron I (pUbi:TtdLRK10L-1introntmW-box) had no significant effect on TtdLRK10L-1 function in Bgt defence, and mutating the MYB-BS in intron I (pUbi:TtdLRK10L-1introntmMYB-BS) further debilitated the promotion of Bgt defence by TtdLRK10L-1.

Enhancement of superpromoter activity by the putative MYB-BS in TtdLRK10L-1 intron I

Further to the above experiment, we examined if the MYB-BS (CAGTTA) in TtdLRK10L-1 intron I might increase the activity of the superpromoter (SP), which is less susceptible to silencing and has been extensively used in the ectopic gene expression studies of plants (Lee et al., 2007). For this investigation, the T-DNA vector SP1300:LUC, which carries a superpromoter directed LUC expression cassette, was used to create the desired reporter constructs. These constructs harboured TtdLRK10L-1gDNA, TtdLRK10L-1introntmMYB-BS or TtdLRK10L-1introntmW-box upstream of the coding sequence of LUC (Figure S7). After confirming in-frame fusion with the LUC coding sequence in each construct, three sets of agroinfiltration assays were executed in tobacco (Nicotiana benthamiana) leaves. In the first set, the LUC signals elicited by SP:TtdLRK10L-1gDNA-LUC, SP:TtdLRK10L-1introntmW-box-LUC, SP:TtdLRK10L-1introntmMYB-BS-LUC or SP:TtdLRK10L-1gDNA-LUC were compared (Figure 5a, d), which showed that the presence of intron I (as in SP:TtdLRK10L-1gDNA-LUC and SP:TtdLRK10L-1introntmW-box-LUC) was necessary and sufficient for achieving a higher level of LUC signals. In the second set, the LUC signals produced by SP:TtdLRK10L-1gDNA-LUC, SP:TtdLRK10L-1introntmMYB-BS-LUC, SP:TtdLRK10L-1introntmW-box-LUC or SP:TtdLRK10L-1gDNA-LUC were compared (Figure 5b, e), which indicated that the existence of MYB-BS, but not the W-box elements, was required for the higher LUC signals elicited by SP:TtdLRK10L-1gDNA-LUC. In the third set, the LUC signals derived from SP:TtdLRK10L-1introntmW-box-LUC, SP:TtdLRK10L-1introntmMYB-BS-LUC or SP:TtdLRK10L-1introntmW-box-LUC were evaluated (Figure 5c, f). The result of this set, together with that obtained in the second set (Figure 5b, e and f), confirmed the importance of the MYB-BS, but not the W-box elements, in yielding the higher LUC signals by SP:TtdLRK10L-1gDNA-LUC or SP:TtdLRK10L-1introntmW-box-LUC. Considering that SP was used to create all six examined cassettes, it was logic to suggest that the putative MYB-BS in TtdLRK10L-1 intron I enhanced the activity of SP in SP:TtdLRK10L-1gDNA-LUC, SP:TtdLRK10L-1introntmW-box-LUC, thus enabling them to produce higher levels of LUC signals than SP:TtdLRK10L-1gDNA-LUC, SP:TtdLRK10L-1introntmW-box-LUC and SP:TtdLRK10L-1introntmMYB-BS-LUC, in which the normal MYB-BS (CAGTTA) was neither absent nor mutated to AAAAA.

Discussion

Owing to their wide presence in higher plants, LRK-RLKs may well play important roles in the growth and development of plants and their responses to biotic and abiotic responses, although there is still little functional information on these RLKs. Further to the original observations made on LRK10L-RLKs in wheat and related Triticeae species (Cheng et al., 2003; Feuillet et al., 1997, 1998;
Zhou et al., 2007), we here studied in more detail the expression and function of TtdLRK10L-1, a typical LRK10L-RLK from durum wheat. TtdLRK10L-1 functions in wheat defence against Bgt via positively regulating oxidative burst. As revealed by functional genomics studies (Fu et al., 2016; Li et al., 2016; Xin et al., 2012; Xing et al., 2017; Zhang et al., 2012; Xing et al., 2017; Zhang et al., 2016), numerous genes and multiple defence processes are activated in wheat responses to Bgt infection in both compatible and incompatible interactions, whose dissection is essential for understanding and improving wheat resistance to powdery mildew disease. Based on the evidence gathered here, we suggest that TtdLRK10L-1 functions in wheat defence against Bgt infection by promoting oxidative burst. As shown by analysing the expression of TtdLRK10L-1-GFP transgene directed by the native promoter (Figures 1 and 2), Bgt infection can trigger the active transcription and translation of TtdLRK10L-1, with the resultant proteins targeted to the plasma membrane, a cellular location likely conducive for the proper action of TtdLRK10L-1. Compelling evidence for the function of TtdLRK10L-1 in Bgt defence comes from the finding that the expression of TtdLRK10L-1-GFP transgene, from either the cDNA coding sequence or the genomic ORF, strongly decreased Bgt growth and development relative to that observed for WT Stewart and TNS controls (Figure 2). Because transgene expression was directed under the native promoter, it is highly likely that the functional information obtained from analysing TtdLRK10L-1-GFP reflects the authentic action of endogenous TtdLRK10L-1. Thus, TtdLRK10L-1, a canonic LRK10L-RLK by its primary structure, joins the previously described LRR-RLKs (TaRLK1 and TaRLK2), mal-ectin-like/LRR-RLK (RLK-V) and lectin-RLK (LecRK-V) in being able to positively contribute to wheat defence to Bgt. Since TtdLRK10L-1 is a putative non-RD kinase, and TaRLK1, TaRLK2, RLK-V and LecRK-V are all predicted to be RD kinases (Chen et al., 2016; Hu et al., 2018; Wang et al., 2018), we deduce that a wide

Figure 5 Effect of the putative MYB binding site (MYB-BS) in TtdLRK10L-1 intron I on superpromoter activity analysed using LUC reporter assays conducted with the leaves of Nicotiana benthamiana. (a-c) LUC signals generated in three sets of reporter assays that were initiated by different combinations of the constructs expressing WT or mutant TtdLRK10L-1 ORFs (as explained in Figure 4a). The ORFs were all expressed under the direction of the superpromoter. (d-f) Quantitative analysis of LUC signals generated in the assays depicted in (a), (b) and (c), respectively. Each data point was mean (± SD) of the LUC signals generated from four different plants. The means were statistically compared using Duncan’s multiple comparison test, with those labelled by different letters being significantly different (P < 0.05). The whole dataset shown was typical of three independent experiments.

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spectrum of RLKs may participate in wheat defence processes to Bgt infection.

Oxidative burst, which is important in the process of plant defence, was observed in TaRLK1, TaRLK2, RLK-V and LeRLK-V mediated wheat defence to Bgt infection (Chen et al., 2016; Hu et al., 2018; Wang et al., 2018). Consistently, we found that the enhanced Bgt defence response seen in the two types of transgenic lines was associated with increased oxidative bursts at both the Bgt penetration sites and the entire Bgt-infected cells, with the degree of the increase positively correlating with the amount of TtdLRK10L-1:GFP protein and the level of Bgt defence (Figures 1 and 3). Thus the regulation of oxidative burst is an integral component of the Bgt defence processes regulated by TtdLRK10L-1. A plant NADPH oxidase, known as respiratory burst oxidase homolog D (RBOHD), has been shown to play a crucial role in pathogen infection-induced oxidative burst (Coutu and Zipfel, 2016; Kimura et al., 2017, 2020). Some plant kinases, including RLKs, have been shown to directly phosphorylate RBOHD, thereby regulating the production of ROS in response to pathogen attack or PAMP treatment (Kadota et al., 2014; Kimura et al., 2017, 2020; Lee et al., 2020; Li et al., 2014; Zhang et al., 2018). For example, Arabidopsis CRK2, an RLK with a cysteine-rich ectodomain, directly phosphorylated a serine residue (S703) at the C-terminal region of RBOHD, thus stimulating ROS burst in the cells infected by the bacterial pathogen Pseudomonas syringae pv tomato DC3000 (Pst DC3000); functional deficiency of CRK2 impaired ROS production and Arabidopsis defence to Pst DC3000 (Kimura et al., 2020). As TtdLRK10L-1 extracellular domain is also rich in cysteine, and ROS burst and Bgt defence were both enhanced in the TtdLRK10L-1:GFP expressing transgenic wheat plants, it will be interesting to examine if TtdLRK10L-1 may modulate the phosphorylation status, and thus the function of RBOHD, in further research.

An intronic MYB-BS is required for efficient expression and function of TtdLRK10L-1 in wheat defence against Bgt

Intron-mediated regulation (IMR) is an important mechanism for controlling gene expression levels in eukaryotic organisms (Gallegos and Rose, 2015, 2019; Laxa, 2017; Shaul, 2017). In higher plants, recent studies suggest that IMR has two forms, intron-mediated enhancement (IME) and intron-mediated suppression (IMS). A well-analysed example of IME concerns the binding of two MYB transcription factors (PHR1 and PHL1) to the PHR1-binding site located in the first intron of AtP5CS1 (Aleksza et al., 2017), which encodes Δ1-pyrroline-carboxylate synthetase crucial for proline biosynthesis from glutamic acid (Fichman et al., 2015). This IME regulates the expression and function of AtP5CS1 in phosphate depletion-induced proline accumulation in Arabidopsis (Aleksza et al., 2017). A thoroughly studied example of IMS relates to the intronic cis-element SE1, which recruits trans-acting repressors to suppress the expression of rice Eu1 gene that encodes a gibberellic acid (GA) deactivating enzyme; loss of SE1 promotes Eu1 expression, leading to a decrease of bioactive GA and a dwarfing plant phenotype (Xie et al., 2018). To our knowledge, neither IME nor IMS has previously been analysed in detail for LRK10L members or other plant RLK genes. Here, by comparatively analysing two types of transgenic lines developed with the absence or presence of TtdLRK10L-1 introns in the native promoter directed transgene (Figures 1–3), coupled with additional single single-cell Bgt defence tests and LUC reporter assays (Figures 4 and 5), we stepwiscely delineated a putative MYB-BS (CAGTTA) in the first intron of TtdLRK10L-1 that is required for efficient expression and function of TtdLRK10L-1 in wheat defence to Bgt infection. However, further work is needed to identify the trans-acting factor(s) that can bind to the MYB-BS. As demonstrated by previous studies (e.g. Aleksza et al., 2017; Xie et al., 2018), this future goal is achievable using a combination of molecular genetic and biochemical approaches.

Judging from the LUC reporter assays, the intron I located MYB-BS of TtdLRK10L-1 can significantly enhance the activity of the superpromoter (Lee et al., 2007). This is remarkable because Lee et al. (2007) showed that it was not possible to increase the activity of this superpromoter using the leader intron of maize ubiquitin gene (Ubil) in either transiently transected protoplasts or stably transformed plants, although Ubil could effectively elevate the activity of pUbil. The same study also found that superpromoter activity could not be increased by an intron from the ST-LS1 gene of potato either. Given these results, we speculate that the putative MYB-BS and Bgt may be employed to further increase the efficacy and versatility of the widely used superpromoter. 

Apart from intron-located cis-elements, other factors have also been reported to affect the expression and function of plant genes including those encoding RLKs. For example, the maelcienlike/eucine-rich repeat RLK, RLK-V, has two alternatively spliced transcripts, with only one of them being functional in regulating wheat defence to Bgt (Hu et al., 2018). The 3′-untranslated region (3′-UTR) may also contain different types of cis-elements (including miniature inverted-repeat transposable element) capable of regulating plant gene expression at post-transcriptional and/or translational levels (Bernardes and Menossi, 2020; Shen et al., 2017). Because of these facts, it will be interesting to investigate whether alternative mRNA splicing and 3′-UTR may affect the expression and function of TtdLRK10L-1 in further research. Finally, it is worth noting that we used TtdLRK10L-1: GFP fusion protein to investigate the function of TtdLRK10L-1 in this work. Although many studies in the past have used GFP fusions to examine the function of plant RLKs (e.g. Campos et al., 2020; Fábregas et al., 2013; Park et al., 2013), there is a possibility that GFP fusion proteins of RLKs may differ from the native proteins to some extent. Consequently, further work is necessary to validate the function of TtdLRK10L-1 in wheat defence to Bgt using the wheat lines expressing native TtdLRK10L-1 protein.

In summary, this work has improved the understanding of LRK10L-RLKs through discovering the function of TtdLRK10L-1 in wheat defence against powdery mildew disease and by revealing the importance of an intron-located MYB-BS in enhancing the expression of TtdLRK10L-1. TtdLRK10L-1 may provide a new avenue for more systematically dissecting the molecular and physiological processes functioning in wheat defence against Bgt. This gene might also be useful for developing Bgt resistant wheat crops using molecular breeding methods including transgenic breeding and marker assisted selection of elite allele. In line with this view, we found TtdLRK10L-1 orthologs in both wild emmer wheat and 10 diverse common wheat cultivars sequenced by the 10 + wheat genomes project (http://www.10wheatgenomes.com/) (Figure S8). Interestingly, there are molecular variations in exons and introns, as well as the putative MYB-BS, among TaLRK10L-1 alleles from the 10 common wheat cultivars (Figure S8), indicating the possibility of selecting superior alleles for breeding applications. Lastly, the putative MYB-BS and its hosting intron in TtdLRK10L-1, which showed a strong IME effect, may be
harnessed to boost ectopic gene expression in future plant biotechnology research.

**Experimental procedures**

**Plant materials**

The spring type durum wheat cultivar Stewart and a laboratory strain of *N. benthamiana* were used in this work. Wheat plants were raised in a greenhouse under a 16-h light/8-h dark photoperiod, a day/night temperature cycle of 24/18 °C, and 60-70% relative humidity. The wheat plants were cultured in a growth room with the following environmental settings, a 16-h light/8-h dark photoperiod, a day/night temperature cycle of 23/20 °C, 60-70% relative humidity, and a light intensity of approximately 130 μmol m⁻² s⁻¹.

**DNA and RNA extractions and PCR assays**

Genomic DNA was prepared using a CTAB-based protocol as described previously (Saghai-Maroof et al., 1984). Total RNA was isolated from the desired samples using TRIzol Reagent (Roche, Indianapolis, IN, USA) and treated with RNase-free DNase (Qiagen, Hilden, Germany) to eliminate contaminating genomic DNA according to the manufacturer’s instructions. Genomic PCR was conducted using the high-fidelity *FastPfu* DNA Polymerase (Transgen, Beijing, China). For RT-PCR, first-strand cDNA was synthesized using GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). The resulting cDNAs were subjected to RT-PCR or qRT-PCR assays as described previously (Zhou et al., 2007). *FastStart FastPfu* DNA Polymerase was used in all RT-PCR assays. The qRT-PCR was performed using three independent RNA preparations as biological replicates with a LightCycler 480 Real-time PCR system (Roche). The relative transcript levels of *TtdLRK10L-1* and *TtdLRK10L-1-GFP* were measured using the 2⁻ΔΔCT method (Livak and Schmittgen, 2001). Details of the oligonucleotide primers used in PCR assays are listed in Table S2.

**Bioinformatic search of LRK10 genes, isolation of *TtdLRK10L-1* and cis-element prediction**

It is well known that LRK10 genes are located on group 1 chromosomes and exist as a multigene family in wheat and relatives (Feuillet et al., 1997, 1998; Zhou et al., 2007). To efficiently identify *TtdLRK10L-1* and its homologs in this work, we used the genomic sequence information of common wheat (IWGSC et al., 2018), durum wheat (Maccarferi et al., 2019), wild emmer wheat (Avni et al., 2011), and *Aegilops tauschii* (Luo et al., 2017). The last two species are progenitors of the A or D subgenomes of hexaploid wheat (Marcussen et al., 2014). Hence, the deduced amino acid sequence of LRK10 (GenBank accession U51330.1) was used to search the genomic databases of Chinese Spring (common wheat), Svevo (durum wheat), Zavitan (wild emmer wheat), G1812 (T. urartu) and ALB78 (Ae. tauschii) at the Triticaceae Multi-omics centre (http://t202.194.139.32), with the homologous genes identified using stringent identity (≥ 80%) and coverage (≥ 95%) cut-offs. The deduced amino acid sequences of LRK10 homologs and *Arabidopsis* PRSK were aligned and used for constructing a phylogenetic tree using the neighbour-joining method installed in the MEGA 5 package (Tamura et al., 2011). Subsequently, the cDNA coding sequence and genomic ORF of *TtdLRK10L-1* were isolated from Stewart by RT-PCR and genomic PCR assays, respectively (see above). The New PLACE database (http://www.dna.affrc.go.jp/PLACE/;action=newplace; Higo et al., 1999) was used to predict putative cis-elements in the two introns of *TtdLRK10L-1* (Table S1).

**Development of transgenic lines and characterization of transgene copy number**

Two constructs, pNP:*TtdLRK10L-1*_{DNA}-GFP and pNP:*TtdLRK10L-1*_{GFP}, were developed for transforming Stewart using the dual binary pGreenII-UB/pSoup vector system as reported in previous studies (He et al., 2010; Helliens et al., 2000; Wang et al., 2016). For developing pNP:*TtdLRK10L-1*_{DNA}-GFP, the native promoter (NP, 1082 bp) and cDNA coding sequence (1899 bp, without stop codon) were amplified by PCR and cloned in two steps into an in-house prepared plasmid vector pAct1::GFP that carried a GFP expression cassette under the direction of nice Act1 gene promoter. The first step replaced the Act1 promoter by NP, and the second step fused *TtdLRK10L-1* cDNA sequence with that of GFP. Subsequently, the NP:*TtdLRK10L-1*_{DNA}-GFP fragment was amplified using high-fidelity PCR with *attB* containing primers, followed by cloning into the Gateway compatible vector pGreenII-UB through BP and LR clonase reactions (Hartley et al., 2000), thus yielding pNP:*TtdLRK10L-1*_{DNA}-GFP. The construct pNP:*TtdLRK10L-1*_{DNA}-GFP was prepared similarly except that *TtdLRK10L-1* gDNA sequence (3105 bp, without stop codon) was used to fuse with GFP. After confirming in-frame fusion between *TtdLRK10L-1* CDNA (gDNA) coding sequence with that of GFP by DNA sequencing, pNP:*TtdLRK10L-1*_{DNA}-GFP and pSoup vector (pAL154) were co-transformed into the *Agrobacterium* strain AGL1 for wheat transformation. The T₀ positive plants were identified and propagated through T₁ to T₄. Eventually, three independent T₄ homozygous CDNA lines (CL-1, −2 and −3) and another three independent T₄ homozygous gDNA lines (GL-1, −2 and −3) were obtained with sufficient seeds for the different analyses performed in this work. A TNS plant identified in the T₂ population was kept as a negative control in the study. The primers used in the cloning experiment are listed in Table S2.

Real-time TaqMan assays were conducted to analyse transgene copy number in the six transgenic lines, with WT Stewart and the TNS line employed as negative controls. The assays were executed following the method detailed by Gadaleta et al. (2011) with some modifications. Briefly, two primers and one TaqMan probe, specific for *TtdLRK10L-1*_{DNA}-GFP and *TtdLRK10L-1*_{GFP} fusion cistrons (Figure S4), were designed and synthesized. The amplicon generated by the two primers was 86 bp; the TaqMan probe was 15 bp and labelled by FAM and BHQ1 moieties at its 5′- and 3′-ends, respectively (Table S2). Two standard calibration curves (Figure S4), suitable for determining transgene copy number via real-time absolute quantification of DNA, were developed using serial dilutions of the purified plasmids of pNP:*TtdLRK10L-1*_{DNA}-GFP or pNP:*TtdLRK10L-1*_{DNA}-GFP. TaqMan reactions were carried out in the StepOne Plus Real-time PCR System (Thermo Fisher, Rockford, IL, USA) using 96 well reaction plates. Triplicate samples were analysed for each transgenic line, and the mean Ct value obtained was compared to that produced by a known starting amount of DNA in the calibration curve to infer transgene copy number in the analysed transgenic material (Figure S4). Two independent sets of TaqMan assays were performed with highly similar results obtained.
Detection of TtdLRK10L-1:GFP fusion protein in transgenic wheat plants

Two sets of immunoblotting assays were performed to detect the accumulation of TtdLRK10L-1:GFP in the two types of transgenic wheat with WT Stewart and the TNS line as negative controls. The first set used leaf samples from the plants grown under normal conditions, whereas the second set used those collected at different time points post-Bgt inoculation. Total membrane proteins were isolated from each leaf sample (c. 0.7 g) using the Minute Plasma Membrane Protein Isolation Kit for Plants (Invent, Plymouth, MN, USA) following the supplier’s instruction. The resultant membrane proteins were separated on 12% SDS-PAGE gel, and subsequently electrophobbed onto Amersham Protran 0.45 µm NC membrane (GE Healthcare, Chicago, IL, USA). TtdLRK10L-1:GFP was detected using a GFP specific antibody (Roche Diagnostics, catalog number: 11814460001) at 1:5000 dilution. The blots were then developed with HRP-conjugated secondary antibody at 1:5000 dilution and the SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA). Detection of actin proteins, which used an Anti-Plant-actin Mouse Monoclonal Antibody (Easybio, Beijing, China), served as a control for equal protein loading. Plasma membrane targeting of TtdLRK10L-1:GFP in the leaf cells of the two types of transgenic wheat grown under normal conditions was detected under a Zeiss LSM 710 confocal microscope.

Subcellular localization of TtdLRK10L-1

The full-length TtdLRK10L-1 cDNA (TtdLRK10L-1cDNA) and genomic ORF (TtdLRK10L-1gDNA) without stop codon were each fused with GFP coding sequence to express TtdLRK10L-1:GFP fusion protein in wheat protoplasts under the direction of maize ubiquitin gene promoter pubi in the plasmid vector pH2202. The cloning step used the restriction enzymes BamHI and KpnI and employed an In-Fusion HD Cloning kit (Clontech, Mountain View, CA, USA). The resulting constructs (pUBi:TtdLRK10L-1cDNA-GFP, pUBi:TtdLRK10L-1gDNA-GFP) and pH2202 (pUBi:GFP) were each transformed into the mesophyll protoplasts prepared from the wheat cultivar Kenong199. The plasma membrane was stained with 50 µM FM 4-64 (Invitrogen, CA, USA) according to the method detailed by Ueda et al. (2001) with some modifications. Briefly, after 24 h incubation in W5 solution, transformed wheat protoplasts were collected by centrifugation, gently resuspended in WS solution containing 50 µM FM 4-64, and placed on ice for 10 min. The stained protoplasts were washed and resuspended in the WS solution without FM 4-64. After incubation for 30 min at room temperature, the protoplasts were visualized under a Zeiss LSM 710 confocal microscope.

Bgt inoculation and histochemical detection of H2O2

The Bgt race 09, maintained on the susceptible wheat cultivar Kenong 199, was used to inoculate the seedlings of the six Bgt inoculated line and subjected to microcolony staining using Coomassie Brilliant Blue R 250 (Wang et al., 2014). For each line, at least 2000 germinated Bgt spores were examined with three independent replicates, which were used to calculate the percentage of microcolonies formed from the total number of germinated Bgt spores. Bgt colonies developed in the infected leaves of the compared lines were photographed at 7 dpi. The Bgt inoculation experiment was repeated three times with highly similar results obtained.

Histochemical detection of H2O2 was carried out at 24 hpi of Bgt. For each of the 8 examined lines, 8 to 10 leaves detached from 4 different plants were stained with 3,3′-diaminobenzidine (Bio Basic Inc., Shanghai, China) following the procedure described by Chen et al. (2016). The division of oxidative burst into three types was performed according to Wang et al. (2018). Two independent H2O2 detection experiments were accomplished with highly similar findings made.

Single-cell Bgt defence tests

Four mutant TtdLRK10L-1 sequences, that is, TtdLRK10L-1intronicI, TtdLRK10L-1intronicIImMYB-BS, TtdLRK10L-1intronicIIntron-1, synthesized commercially (Genewiz Ltd., Suzhou, China). In intron IMYB-BS, the putative MYB-BS (CAGTTA) unique to intron I was mutated to AAAAA, while in intron WMW-box, the two predicted W-box (GGTCA) elements specifically present in intron I were both changed to AAAAA. The four mutants, as well as the cDNA coding sequence (TtdLRK10L-1cDNA) and genomic ORF (TtdLRK10L-1gDNA) of TtdLRK10L-1, were each transferred into the plasmid vector pHZ206 that contained an empty pubi cassette. This step used the restriction enzymes BamHI and KpnI and employed an In-Fusion HD Cloning kit (Clontech, Mountain View, CA, USA). The resultant constructs, pUBi:TtdLRK10L-1cDNA, pUBi:TtdLRK10L-1gDNA, pUBi:TtdLRK10L-1intronicI, pUBi:TtdLRK10L-1intronicIImMYB-BS, pUBi:TtdLRK10L-1intronicIIntron-1, as well as an empty vector control (VC) pHZ206 and a reporter construct expressing the marker gene b-glucuronidase (Zheng et al., 2020), were used to conduct single-cell Bgt defence tests by biolistic delivery of plasmid DNA into the epidermal cells of one-week-old Stewart leaves as detailed previously (Shen et al., 2003, 2007; Wang et al., 2014). Following Bgt inoculation, the interactive epidermal cells, which not only were successfully transfected by plasmid constructs but also had germinating Bgt spores, were microscopically examined for haustorium growth. Three independent replicates were checked for each construct, with at least 40 interactive cells observed in each replicate. The cells with haustorium growth were recorded and used to calculate a haustorium index against the total number of interactive cells examined. Three separate single-cell Bgt defence tests were executed with similar sets of HI data generated.

LUC reporter assays

The four mutants of TtdLRK10L-1 as well as its cDNA and gDNA coding regions were amplified by PCR, digested with XbaI and KpnI, and cloned into the binary vector SP1300:LUC, which carried a superpromoter directed LUC expression cassette in pCAMBIA1300 plasmid. This superpromoter, developed using octopine synthase transcriptional activating element and the activator-promoter region of mannopine synthase2, has a stronger activity than the enhanced double CaMV 35S (Lee et al., 2007). The cloning step, executed using the In-Fusion HD Cloning kit (Clontech, Mountain View, CA, USA), produced pSP: TtdLRK10L-1cDNA, pSP:TtdLRK10L-1gDNA, pSP:TtdLRK10L-1intronicI, pSP:TtdLRK10L-1intronicIImMYB-BS, and pSP: TtdLRK10L-1intronicIIntron-1WMW-box (Figure 5). The six constructs were separately introduced into the cells of Agrobacterium tumefaciens (strain GV3101), and then used to perform three sets of reporter assays (Figure 5) as outlined by Yang et al. (2017).
Briefly, GV3101 cells carrying the above constructs were grown in LB medium, harvested and resuspended, followed by infiltration into the leaves of 5-week-old *N. benthamiana* plants. The infiltrated leaves were sprayed with 100 µM luciferin (Promega) at 48 h post-infiltration and kept in dark for 5 min before recording luminescence using the Nightshade LB 985 in vivo Plant Imaging System (Berthold Technologies, Bad Wildbad, Germany). Three independent biological replicates were examined for each set of the assay, with each replicate involving the examination of four leaves from four separate plants. The LUC reporter assays were repeated three times with highly similar data obtained.

**Statistical analysis**

Numerical values were presented as means ± SD. Statistical comparison of the means was performed using Duncan’s multiple comparison tests in the SAS software package (version 9.2, SAS Institute, Cary, NC, USA).

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**Conflicts of interest**

The authors declare no conflicts of interest.

**Author contributions**

K.Z., D.W. and G.L. perceived and designed the project. T.X. and Y.Y. conducted the main experiments. H.Z., X.H., H. J. and Z.X. participated in data analysis, Bgt infection phenotype scoring, and Bgt culture maintenance. W.Q. developed the vector SP1300:LUC and assisted the preparation of derivative constructs. L.X. took part in the development of transgenic lines. T.X. and X.J. conducted subcellular localization of TtdLRK10L-1. T.X., D.W. and K.Z. wrote the manuscript.

**Accession number**

The promoter, cDNA and genomic DNA sequences of TtdLRK10L-1 from the durum wheat cultivar Stewart have been deposited in GenBank under the accession number MN597454.

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Tengfei Xia et al.

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Supporting information

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

Figure S1 Comparison of the deduced amino acid sequences of TtdLRK10L-1 and representative homologs from common wheat.
Figure S2 Phylogenetic tree of TtdLRK10L-1 and its homologs annotated in the genomes of common wheat and closely related Triticeae species.
Figure S3 Investigation of TtdLRK10L-1 expression profiles in Steward by qRT-PCR assays.
Figure S4 Analysis of transgene copy number using real-time TaqMan PCR assays.
Figure S5 Subcellular localization of TtdLRK10L-1 in wheat protoplasts.
Figure S6 Up-regulation of TtdLRK10L-1 expression in response to Bgt infection.
Figure S7 Schematic representation of the T-DNA region in the transformation constructs SP:TtdLRK10L-1\(\text{DNA-LUC}\), SP:TtdLRK10L-1\(\text{intron}\)\(1\)-LUC, SP:TtdLRK10L-1\(\text{intron}\)\(2\)-LUC, SP:TtdLRK10L-1\(\text{intron}\)\(1\text{MYB-BS}\)-LUC and SP:TtdLRK10L-1\(\text{intron}\)\(1\text{MYB-B}\)-LUC.
Figure S8 Presence of TtdLRK10L-1 orthologs in wild emmer wheat and common wheat.

Table S1 Cis-elements predicted in introns I and II of TtdLRK10L-1
Table S2 A list of PCR primers and the TaqMan probe in this study.