INTRODUCTION

Potato (Solanum tuberosum) is one of the most important food and economic crops in the world. However, similar to other plants, potato yield and quality are severely impaired by various microbial pathogens during the life cycle. Phytophthora infestans, the causal agent of late blight, is a major threat to potato production. Late blight can occur at any time of the growing season, causing serious economic and output losses for field-grown potato. Given its lethality, wide host range, and broad geographical distribution, the pathogen is considered one of the most destructive pathogens in agricultural systems (Kamoun et al., 2015; Nowicki et al., 2012).

Plants have evolved an effective innate immune system to fight pathogens. Generally, the system includes two types: microbe- or pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Dodds and Rathjen, 2010;...
Jones and Dangl, 2006). As a consequence, current agricultural approaches to control disease caused by P. infestans are enriched, including regulating defence-related genes working downstream of PTI and ETI. Currently, a WRKY transcription factor WRKY1 from tomato has been described as a key positive regulator of defence against P. infestans by modulating reactive oxygen species (ROS) homeostasis and enhancing expression of defence-related genes (Li et al., 2015). Comparative transcriptome analysis revealed that another tomato gene, IncRNA16397, a long noncoding RNA, can induce GRX expression to reduce ROS accumulation and alleviate cell membrane injury, thus enhancing tomato resistance to P. infestans (Cui et al., 2017). In potato, basic leucine zipper transcription factor 61 functions in concert with a nonexpressor of pathogenesis-related genes 3-like protein (StNPR3L) to regulate the temporal activation of salicylic acid (SA) biosynthesis, which contributes to SA-mediated immunity against P. infestans (Zhou et al., 2018). Moreover, different potato cultivars show varying degrees of resistance to P. infestans, suggesting a need for characterization and identification of effective plant immune components to confer or increase plant resistance to the pathogen (Orłowska et al., 2012).

Plant nonspecific lipid transfer proteins (nsLTPs, PR-14) are small, soluble, and basic proteins that contain eight conserved cysteine residues (Wang et al., 2012). Generally, the term plant nsLTPs indicates that LTPs can associate with various phospholipids with broad specificity (Ostergaard et al., 1993). The first known plant nsLTP was isolated from potato tuber in 1975 by Kadar (Kader, 1975). In the past decade, nsLTPs were reported to be involved in abiotic stress and multiple developmental processes, such as seed storage, pollen tube adhesion, somatic embryogenesis, and cuticle synthesis (Chae et al., 2009; Guo et al., 2013; Jacq et al., 2017; Potocka et al., 2012; Xu et al., 2018). In addition, compelling evidence demonstrated that nsLTPs also play an important role in plant immunity. For instance, Arabidopsis DIR1, which encodes a nsLTP, plays a vital role in plant resistance to pathogen invasion (Maldonado et al., 2002). Arabidopsis azelaic acid-induced 1 (AZI1), a lipid transfer protein (LTP-related protein), is involved in signal transduction during bacterial infection (Pitzschke et al., 2014; Xu et al., 2011). Studies have shown that overexpression of pepper nsLTPs CALTP1 and CALTP2 enhanced resistance to oomycete and bacterial pathogens (Sarovar et al., 2009). In potato, only StLTPa7 was reported to be involved in the early phase of resistance to Ralstonia solanacearum (Gao et al., 2009). Thus, the biological functions and mechanisms of potato nsLTPs in regulating plant disease resistance remain unclear.

To fight against pathogen infection, plant resistance is often tightly controlled by coordinated actions of various phytohormones. Previous studies demonstrated that the expression of some nsLTP genes was induced by well-known antidisease hormones, such as SA, methyl jasmonate (MeJA), and abscisic acid (ABA) (Gao et al., 2016; Jung et al., 2006). As one of the most important hormones, SA is recognized as a signalling molecule that is primarily affected by responses to various biotrophic pathogens (Zheng et al., 2015). ABA is also reported as a crucial regulator of plant pathogen resistance. For example, exogenous application of ABA increased resistance of tomato against Alternaria solani (Song et al., 2011). Recent investigations have provided evidence that ABA plays a central role in regulating Pseudomonas syringae pv. tomato- and PAMP-triggered stomatal closure (Du et al., 2014; Zeng and He, 2010). ABA receptor pyrabactin resistance (PYR)/PYR-like (PYL)/regulatory component of the ABA receptor (RCAR) proteins play a central role in ABA perception and signalling (Cutler et al., 2010; Kim et al., 2010). PYR/PYL/RCAR receptors perceive ABA intracellularly and subsequently form ternary complexes with clade A protein phosphatases type-2C, thereby inactivating them. This process facilitates the activation of downstream targets of ABA signalling (Diaz et al., 2016; Nishimura et al., 2010; Rodriguez et al., 2014; Umezawa et al., 2009).

Mitogen-activated protein kinase (MAPK) cascades are conserved eukaryotic transduction signalling modules that play vital regulatory roles in plant development and stress responses. When the MAPK cascade pathway senses external stress stimuli or self-growth signals, MAPK kinase kinase directly activates MAPK kinase, which subsequently activates the target MAPK. Finally, activated MAPKs target and regulate substrate proteins, such as cytoplasmic/nuclear protein kinases, transcription factors, or other types of effector proteins (Rodriguez et al., 2010). Based on the phosphorylation and activation pathways noted above, extracellular signals are amplified and transmitted to target proteins and then stimulate various physiological and biochemical responses in the plant. Many studies have contributed to the understanding of MAPK-mediated stress adaptation. Mitogen-activated protein kinase 3 (MPK3) and mitogen-activated protein kinase 6 (MPK6) are strongly associated with biotic and abiotic stresses signalling (Rodriguez et al., 2010; Samajova et al., 2013). For example, in response to pathogen attack in Arabidopsis, MPK3/MPK6 phosphorylates the transcription factor WRKY33 to trigger synthesis of camalexin, a major antimicrobial phytoalexin (Mao et al., 2011). MPK3 targets v-myb avian myeloblastosis viral oncogene homolog transcription factor 44 to confer abiotic stress tolerance in a phosphorylation-dependent manner (Persak and Pitzschke, 2013). Furthermore, Pitzschke et al. (2014) reported a LTP-related hybrid proline-rich protein (HyPRP) as a direct component in the MAPK-mediated stress response.

Here, we demonstrate that potato nonspecific transpor protein 10 (StLTP10) functions as a positive regulator in increasing plant resistance to P. infestans. Overexpression of StLTP10 led to the decrease of ROS generation and the up-regulated expression of defence-related genes after P. infestans infection. Moreover, our data suggests that StLTP10 and PYL4 collaborate to regulate stomatal closure on pathogen attack. Additionally, the wound-induced protein kinase (WIPK), an MPK3-like protein, acts as a positive regulator of StLTP10 abundance. All together, our findings reveal the molecular mechanism of StLTP10 in P. infestans defence and suggest that StLTP10 may act as an integrator of multiple disease-resistant pathways.

## RESULTS

### 2.1 StLTP10 is induced by P. infestans and phytohormones

Late blight, caused by P. infestans, is the most devastating disease in potato production. Previous studies have revealed that nsLTPs
play important roles in disease resistance in other plants. To test whether potato nslTPs play a similar role during P. infestans attack, an RNA-Seq (PRJNA635213) analysis was performed in potato plants sprayed with P. infestans for 2 days. Among the differentially expressed genes (DEGs), the CL12476Contig1 gene that encodes a nslTP was significantly induced by P. infestans (Figure S1). Next, we identified the orthologue of CL12476Contig1 using a BLAST search against the potato genome, and found that the similarity between CL12476Contig1 and StLTP10 (XM_006367335.2) was up to 99.13% (E value = 2 \times 10^{-177}). The expression level of StLTP10 in potato plants suggested that StLTP10 was also significantly induced by P. infestans (Figure S2a). Thus, we cloned StLTP10 for further study. Sequence analysis showed that StLTP10 encodes a protein with 114 amino acids that contains two conserved pentapeptides and one signal peptide of 24 amino acid residues at the N-terminus (Figure 1a,c). Multiple sequence alignments and phylogenetic analysis demonstrated that StLTP10 is a typical nslTP (Figure 1a,b,d). To investigate the potential biological functions of StLTP10, we examined its induced expression patterns in potato plants exposed to various defence-related phytohormones. It was found that StLTP10 expression was significantly enhanced after ABA, SA, and MeJA treatments and peaked at different time points for each separate treatment (Figure S2b,c,d). These results indicate that StLTP10 may play a crucial role in the plant defence response to disease.

2.2 | Silencing of StLTP10 results in reduced resistance to P. infestans

To explore whether StLTP10 participates in potato late blight resistance, the Agrobacterium-mediated virus-induced gene silencing (VIGS) approach was used to silence StLTP10 in potato plants. Twenty-one days after Agrobacterium infiltration, StLTP10 transcript abundance was typically reduced (Figure S3a). Next, we examined the role of StLTP10 in the defence response to P. infestans using detached leaf inoculation assays. At 5 days after inoculation, TRV2::StLTP10 leaves showed strong signs of necrotic lesions, whereas TRV2::00 leaves exhibited only mild disease (Figure S3a,b). To further confirm this result, we investigated the biomass level of pathogen in these leaves by quantifying the P. infestans-specific PiO8 element. The PiO8 content in TRV2::StLTP10 leaves was significantly higher than that in the TRV2::00 leaves (Figure S3c). These data indicate that the StLTP10-silenced potato plants were more susceptible to P. infestans than the control. ROS are involved in plant–pathogen interactions. Low levels of ROS are involved in the response to pathogen infection as signalling molecules, but large amounts of ROS in cells may damage the cell membrane and enhance susceptibility to pathogens. As previous described in tomato, SpWRKY1 and IncRNA16397 can reduce ROS accumulation to enhance plant resistance against P. infestans (Cui et al., 2017; Li et al., 2015). Thus, we assessed the accumulation of H₂O₂ and O₂⁻, the two main forms of ROS, in these P. infestans-inoculated leaves via 3,3′-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining. As shown in Figure S3a, TRV2::StLTP10 leaves had higher accumulation of H₂O₂ and O₂⁻ than TRV2::00 leaves. Furthermore, we performed quantitative reverse transcription RT-PCR (RT-qPCR) assays to analyse the expression patterns of several well-known ROS scavenging-related genes (APX, CAT, GST, and SOD), and found that the expression levels of these genes were lower in TRV2::StLTP10 leaves than in TRV2::00 leaves (Figure S3d).

To further investigate the mechanism underlying the reduced resistance of StLTP10-silenced potato plants, we also measured the transcription of defence-related genes that are associated with the SA response (NPR1, PR1, PR2, and PR5) in potato leaves, and found that the expression of these genes was also down-regulated in TRV2::StLTP10 leaves at different degrees compared with TRV2::00 leaves (Figure S3e). These results suggest that the reduced resistance of StLTP10-silenced potato plants to P. infestans may be associated with the down-regulated expression levels of ROS scavenging- and defence-related genes.

2.3 | Overexpression of StLTP10 leads to increased resistance against P. infestans

To further verify the role of StLTP10 in P. infestans resistance, we generated StLTP10-overexpression and StLTP10-RNAi lines of potato (Figure S4). Then, four transgenic potato lines with significantly increased expression levels (denoted OE1 and OE2) or dramatically decreased expression levels (denoted RNA1 and RNA2) of StLTP10 were chosen for further analysis (Figure 2a,b). P. infestans treatment was carried out in the detached leaves of these selected plants. At 5 days postinoculation (dpi), the leaves of OE and RNAi plants exhibited the least and most disease symptoms in the surrounding areas of P. infestans infection, respectively, as demonstrated by the lesion diameter and biomass level of P. infestans (Figure 2a,c,d). Moreover, DAB and NBT staining results showed that the ROS content in leaves of OE plants was lower than that in leaves of wild-type (WT) and RNAi plants (Figure 2a). Furthermore, we found that the expression levels of ROS scavenging- and defence-related genes were up-regulated in OE lines and down-regulated in RNAi lines compared with WT (Figure 2e,f). Together, our results support the idea that StLTP10 plays a crucial role in increasing plant resistance against P. infestans through scavenging excess ROS produced after P. infestans infection and up-regulating the expression levels of defence-related genes.

Signal peptides play important roles in the localization and function of proteins (Yang et al., 2017). To investigate whether the N-terminus signal peptide of StLTP10 contributes to P. infestans resistance, the pBI121-based expression vectors were used for expression of full-length StLTP10 or StLTP10-∆SP (lacking the signal peptide) in Nicotiana benthamiana leaves by agroinfiltration. At 1 day after infiltration, the leaves expressing StLTP10 or StLTP10-∆SP were detached for a P. infestans inoculation assay. We observed that
the leaves expressing full-length StLTP10 exhibited more disease resistance than the vector control leaves, while leaves expressing StLTP10-∆SP displayed similar disease symptoms as control leaves, as demonstrated by the lesion diameter and biomass of P. infestans, indicating that the disease resistance function of StLTP10 requires the signal peptide (Figure S5).

2.4 | StLTP10 interacts with ABA receptor PYL4

To identify the possible partners of StLTP10 involved in disease defense, we used StLTP10 as the bait and screened a yeast two-hybrid (Y2H) library composed of cDNA from potato leaves. We found that the ABA receptor PYL4 might interact with StLTP10 (Figure 3a). To verify this interaction in plant cells, we conducted luciferase complementation (LUC) assays in N. benthamiana leaves. Strong LUC activity signals were detected only when StLTP10-nLUC and PYL4-cLUC or StLTP10-cLUC and PYL4-nLUC were coexpressed (Figure 3b). Next, this interaction was confirmed using bimolecular fluorescence complementation (BiFC) assays in vivo. A specific yellow fluorescent protein (YFP) signal was observed in the plasma membrane of plant cells when StLTP10-nYFP and PYL4-cYFP were transiently coexpressed in N. benthamiana leaves, but no fluorescence was detected when StLTP10-nYFP and cYFP or nYFP and PYL4-cYFP were coexpressed (Figure 3c). Furthermore, a glutathione S-transferase (GST) pull-down assay in vitro also suggested that StLTP10 interacted with PYL4 (Figure 3d). These results demonstrate that StLTP10 physically interacts with PYL4.

PYR/PYLs represent a large family of hormone receptors in plants and function diversely in ABA signalling (Ma et al., 2009). We examined the interactions between StLTP10 and other tested potato PYR/PYLs in Y2H assays, and found that the interaction between StLTP10 and PYL4 is specific (Figure S6).

2.5 | StLTP10 recruits PYL4 to membranes and positively regulates ABA signalling

To clarify the biological function of the interaction between StLTP10 and PYL4, we first explored the subcellular localization of StLTP10 and PYL4 using red fluorescent protein (RFP) and green fluorescent protein (GFP). StLTP10-RFP, StLTP10-∆SP-RFP, and PYL4-GFP fusion
proteins were separately expressed in N. benthamiana epidermal cells by agroinfiltration. The fluorescence analysis showed that StLTP10-∆SP-RFP could colocalize with PYL4-GFP in the cytoplasm and nucleus; however, the StLTP10-RFP protein was mainly detected in the plasma membrane (Figure 4a). Interestingly, compared with PYL4-GFP expression alone, which was mostly localized to the cytoplasm and nucleus, more PYL4-GFP protein was mobilized to the plasma membrane after coexpressing with StLTP10 (Figure 4b,c). These results suggest that StLTP10 protein may recruit PYL4 to the membranes.

A family of lipid-binding C2-domain ABA-related (CAR) small proteins in Arabidopsis recruit PYRs/PYLS to membranes and positively regulate ABA sensitivity (Rodriguez et al., 2014). To investigate whether StLTP10 affects ABA signalling through regulating PYL4 subcellular localization, we first measured the expression of key ABA-responsive genes, that is, ABI3, ABI4, ABI5, and RAB18, in WT, OE, and RNAi potato plants without ABA treatment, and observed an increased induction of these genes in OE plants compared with WT and RNAi plants (Figure 4d–g). Next, we analysed the ABA sensitivity of these lines with respect to ABA-mediated inhibition of root growth. We cut potato seedlings into nodal segments and transferred them to Murashige and Skoog (MS) medium lacking or supplemented with 0.5 μM ABA. After 15 days, we found that the OE lines showed enhanced sensitivity to ABA-mediated inhibition of root growth compared with WT lines, whereas RNAi lines showed reduced sensitivity to ABA (Figure 4h,i). Taken together, these results indicate that StLTP10 acts as a positive regulator in ABA signalling, probably by affecting the subcellular localization of PYL4.

2.6 | StLTP10 plays a positive role in regulating stomatal closure

Stomatal closure establishes an important layer of plant immunity. Previous elegant studies revealed that ABA signalling is essential to stomatal closure for pathogen defence (Du et al., 2014; Melotto et al., 2006). Based on the above results and previous studies, we speculated that StLTP10 participates in ABA-induced stomatal movement. To test our hypothesis, we examined the stomatal behaviour of WT, OE, and RNAi leaves after treatment with 50 μM ABA. Our analysis revealed that the stomatal closure was more sensitive to
ABA in OE leaves than in WT leaves after 2 hr treatment. In contrast, RNAi leaves showed impaired stomatal closure in response to ABA (Figure S7). These results demonstrate that StLTP10 plays a positive role in ABA-induced stomatal closure. *Pseudomonas syringae* pv. *tomato* DC3000 (Pst DC3000)-induced stomatal closure is well studied and requires the plant hormone ABA (Melotto et al., 2006). To determine whether StLTP10 also plays an important role in stomatal closure during pathogen attack, we spray-inoculated plants leaves with Pst DC3000. Our data revealed that the OE leaves exhibited enhanced resistance to Pst DC3000, while the RNAi leaves were more susceptible than WT leaves (Figure S8a,c). Significantly, we noticed that stomatal closure was more susceptible in OE than in WT leaves after spray-inoculation of Pst DC3000 for 3 hr; however, the RNAi leaves exhibited disrupted stomatal closure after inoculation (Figure S8b). The results imply that StLTP10 is involved in regulating stomatal closure during pathogen attack. As a pathogenic oomycete, *P. infestans* enters plants through multiple routes: entry into the leaves may occur when zoospores or germ tubes pass through stomata or other natural openings (Hoegen et al., 2002; Judelson and Ah-Fong, 2019; Li et al., 2015). We therefore incubated leaves with *P. infestans* and investigated the stomatal response. After 2 hr of incubation, the stomatal aperture decreased more dramatically in OE leaves than that in WT, whereas the stomatal closure was largely compromised in RNAi leaves (Figure S9a,b). Farrell et al. (1969) demonstrated that the invasion of potato leaves by *P. infestans* generally results in stomata opening abnormally wide, and the hyphae were seen to have grown towards and through these apertures. We herein observed a similar phenomenon that the hyphae preferred to enter through the stomata of RNAi leaves that were still open wide (Figure S10). Based on these results, we initially measured the biomass of pathogen in these leaves to assess the effect of stomatal closure, and observed least *P. infestans* in OE leaves. However, the biomass of *P. infestans* in RNAi leaves was increased approximately two-fold compared with that in WT (Figure S9c). These data suggest that StLTP10 may exert its role through regulating the ABA signalling pathway and then affecting stomatal closure to reduce pathogen invasion.
2.7 StLTP10 cooperates with PYL4 to positively regulate stomatal closure after *P. infestans* infection

*Arabidopsis* ABA receptor RCAR3 mediated ABA signalling positively regulates the preinvasive defence response against pathogens (Lim et al., 2014). To investigate whether PYL4 cooperates with StLTP10 to prevent pathogen invasion during the early stage, we first identified the function of PYL4 under *P. infestans* infection by transiently overexpressing PYL4 in *N. benthamiana* leaves. The results showed that the PYL4-expressing leaves exhibited enhanced resistance to *P. infestans*, which is similar to the effect noted in StLTP10-expressing leaves (Figure S11). In addition, we found that the leaves co-over-expressing StLTP10 and PYL4 were more resistant to *P. infestans* compared with leaves overexpressing each gene individually. We hypothesized that the effect of these two genes on disease resistance may be related to their interaction. We then examined the changes of protein expression levels with or without *P. infestans* infection in *N. benthamiana* leaves. Compared with the control, StLTP10 not only affected membrane localization of PYL4 under normal conditions but also promoted the accumulation of PYL4 in the cytoplasm when the leaves were infected by *P. infestans*, thus causing increased disease resistance of leaves (Figure S12).
To further explore whether PYL4 is also required for stomatal movement of potato leaves after *P. infestans* infection, we generated PYL4-silencing lines (TRV2::PYL4) using VIGS in potato plants. After 2 hr of incubation, we found that the stomatal closure of TRV2::PYL4 leaves was impaired in a manner similar to that observed in StLTP10-RNAi1 leaves, suggesting that PYL4 also plays an essential role in regulating stomatal closure during pathogen attack (Figure 5a). To explore the possible link between StLTP10 and PYL4, we generated double down-regulated lines (TRV2::PYL4/StLTP10-RNAi1) by silencing PYL4 in StLTP10-RNAi1. We observed that the stomatal phenotype showed no differences between double down-regulated lines and single down-regulated lines under normal conditions or after *P. infestans* infection (Figure 5a,b). Furthermore, PYL4 was knocked down in StLTP10-OE1 lines (TRV2::PYL4/StLTP10-OE1) to determine if the effect of StLTP10 on stomatal closure was dependent on PYL4. After inoculation, the stomatal movement of TRV2::PYL4/StLTP10-OE1 leaves was largely similar to that of the StLTP10-RNAi1 leaves (Figure 5a,b). The results suggest that these two proteins do function closely. Based on the results of pathogen biomass (Figure 5c), we conclude that the increased resistance of StLTP10-OE lines against *P. infestans* is probably related to the more tightly closed stomata.

### 2.8 WIPK positively regulates StLTP10 abundance

To further identify the core components of StLTP10 involved in plant resistance against *P. infestans*, we rescreened the Y2H library composed of cDNA from potato leaves. WIPK, which is a homologue of the well-known MPK3 required for both development and biotic stress responses, was our focus in further studies. We conducted Y2H, BiFC, LCI, and coimmunoprecipitation (Co-IP) assays to confirm this interaction (Figure 6a–d). We then examined whether WIPK plays a role in resistance to *P. infestans*. As shown in Figure 6e, WIPK expression was significantly induced in potato plants by *P. infestans*. WIPK-overexpressing leaves exhibited susceptibility to *P. infestans* similar to that noted for vector controls (Figure 6f,g). However, co-overexpression of StLTP10 and WIPK...
significantly enhanced leaf resistance to *P. infestans* compared with over-expressing StLTP10 alone (Figure 6f,g). In Arabidopsis, MPK3 can interact with AZI1, an LTP-related hybrid proline-rich protein (HyPRP), and act as a positive regulator of AZI1 protein levels (Pitzschke et al., 2014). Thus, we conclude that WIPK may play a positive role in StLTP10-mediated resistance against *P. infestans* by controlling StLTP10 abundance.

To examine the hypothesis, we conducted cell-free assays in vector control and WIPK-overexpressing *N. benthamiana* leaves. Recombinant StLTP10-GST was purified and incubated with supernatant obtained from these *N. benthamiana* leaves. We then used anti-GST antibodies to detect StLTP10-GST protein levels at different time points. As shown in Figure 7a, StLTP10-GST levels
were reduced over time, while this decrease was much slower in WIPK-overexpressing leaves than that in vector control leaves (Figure 7a,b). The results suggest that WIPK can inhibit the degradation of StLTP10. To further investigate the degradation pathway of StLTP10, leaves were treated with 100 μM MG132 (an inhibitor of the 26S proteasome degradation pathway). We observed that MG132 dramatically inhibited the StLTP10 degradation. Moreover, no significant difference was noted between WIPK-overexpressing and vector control leaves (Figure 7a). These results indicate that WIPK inhibits StLTP10 degradation through the 26S proteasome degradation pathway.

We further tested whether WIPK inhibited in vivo degradation of StLTP10 based on a method reported in previous studies using agroinfiltration in N. benthamiana (Liu et al., 2010; Zhao et al., 2013). The coinfiltration experiment was performed using Agrobacterium carrying constructs to express StLTP10 and increasing amounts of the Agrobacterium that promotes WIPK expression. After 3 days, samples were collected for detection of both protein and RNA levels of transfected construct. As the amount of WIPK-FLAG increased, StLTP10 protein levels also increased (Figure 7c). In this experiment, GFP abundance was measured as an internal control and was not affected by increasing the amount of WIPK (Figure 7c,d). Additionally, both Actin and StLTP10 mRNAs were analysed by RT-PCR to ensure equal levels of StLTP10 were expressed in different coinfiltrations. Taken together, these results illustrate that WIPK positively regulates StLTP10 protein abundance, thereby enhancing the ability of StLTP10 to increase plant resistance against P. infestans (Figure 8).

3 | DISCUSSION

Plant nsLTPs has been extensively reported to be involved in biotic stress tolerance. Unfortunately, only a small number of nsLTPs functions have been elucidated in potato (Gangadhar et al., 2016; Gao et al., 2009). In this study, we characterized the potato StLTP10 and investigated its molecular and biological functions in resistance to P. infestans. Compared to untransformed WT plants, overexpression of StLTP10 increased P. infestans resistance in transgenic potato plants, which was mainly demonstrated by smaller lesion size and lower pathogen biomass. However, knockdown of StLTP10 in potato plants resulted in enhanced susceptibility to P. infestans, suggesting that StLTP10 is necessary for potato disease resistance. Our further investigations revealed that StLTP10 may act as an integrator...
of multiple resistance signalling pathways to play a positive role in pathogen defence (Figure 8).

In plants, one of the most rapid and earliest cellular responses following successful pathogen recognition is an oxidative burst involving ROS generation (Zeng et al., 2015). During pathogen attack, low levels of ROS can act as signalling molecules in response to pathogen infection, but late massive ROS generation may damage the cell membrane, lead to cell death, and enhance susceptibility to pathogen. ROS-scavenging mechanisms have been suggested to protect plants against P. infestans by increasing expression levels of ROS-scavenging-related genes and using antioxidative enzymes (Cui et al., 2017; Li et al., 2015). In our study, we found that the leaves of StLTP10-overexpressing lines accumulated less ROS than those of WT plants after P. infestans infection, as shown by DAB and NBT staining, while the leaves of StLTP10-silenced and StLTP10-RNAi lines displayed the opposite phenotypes. Correspondingly, ROS-scavenging-related genes were significantly up-regulated in StLTP10-overexpressing lines but down-regulated in the other two lines compared with WT plants. These results suggest that StLTP10 may reduce the accumulation of ROS by up-regulating ROS-scavenging-related genes, thus increasing plant disease resistance. In addition, the expression of defence-related genes is induced to increase the plant defence response. Well-studied PR genes for SA signalling are typically marker genes in pathogen resistance assays (Seo et al., 2008). Notably, it was found that StLTP10-overexpressing plants exhibited increased expression of key marker genes of SA signalling compared with WT and StLTP10-RNAi plants. The results of our analysis indicate that StLTP10 may participate in the SA-mediated defence pathways. The data support the notion that StLTP10 overexpression causes increased plant resistance to P. infestans by enhancing the expression levels of ROS-scavenging- and defence-related genes.

According to previous studies, the PYR/PYL/RCAR ABA receptors play a central role in ABA perception and signalling (Ruszala et al., 2011). Here, we found that StLTP10 physically interacts with ABA receptor PYL4. In Arabidopsis, the C2-domain ABA-related (CAR) family of small proteins is involved in recruitment of PYR/PYL/RCAR receptors to the membrane and regulates ABA sensitivity to elicit plant responses to a changing environment (Diaz et al., 2016; Rodriguez et al., 2014; Qin et al., 2019). In this study, the interaction between StLTP10 and PYL4 was detected in the plasma membrane. Compared with PYL4-GFP expression alone, which was localized to the cytoplasm and nucleus, coexpression of PYL4 and StLTP10 caused PYL4 to be primarily localized to the plasma membrane. Moreover, our results showed that StLTP10 overexpression enhanced ABA-mediated downstream signalling events. This result suggests that StLTP10 may facilitate ABA regulatory pathways by interacting with PYL4 and recruiting it to the plasma membrane.
Previous studies showed that stomata are the preferred and major routes of entry into plants for many pathogens, and plants have accordingly evolved mechanisms to actively regulate stomatal aperture to prevent pathogen invasion (Du et al., 2014; Melotto et al., 2006). The plant hormone ABA and its downstream signal transduction components play central roles in regulating stomatal closure during plant immunity (Kumar et al., 2012; Melotto et al., 2006). For example, stomata of ABA-deficient aba3-1 mutant and ost1 kinase mutant plants exhibit a significantly compromised ability to respond to bacteria compared with Col-0 plants (Leon-Kloosterziel et al., 1996; Mustilli et al., 2002). A homologous NAC transcription factor JA2 and a WRKY transcription factor SpWRKY1 in tomato regulate ABA-mediated stomatal closure to prevent pathogen invasion (Du et al., 2014; Li et al., 2015). Based on our results, we hypothesized that StLTP10 may be involved in ABA-mediated stomatal closure via interaction with the ABA receptor PYL4. After treatment with the same concentration of ABA, the stomata of StLTP10-overexpressing leaves were more susceptible to ABA than those of WT leaves, while the stomata of StLTP10-RNAi leaves exhibited a significantly compromised ability to respond to ABA, demonstrating that StLTP10 plays a positive role in ABA-induced stomatal closure. Previous reports have demonstrated that P. infestans can enter the leaf either via stomata or by penetrating the periclinal wall of epidermal cells, and the colocalization of some defence proteins in guard cells may function as a preformed defence barrier (Judelson and Ah-Fong, 2019; Hoegen et al., 2002; Li et al., 2015). Recently, Boevink et al. (2020) suggested that the sporangiophores of P. infestans commonly emerge through stomata, and this must involve manipulation of stomatal regulation. In this context, our results show that StLTP10 exhibits a cooperative action with PYL4 to regulate stomatal closure during P. infestans infection. Moreover, we found that the hyphae preferred to grow towards and through the stomata of StLTP10-RNAI leaves that were still open wide; however, only a few hyphae were seen to pass through the stomata of StLTP10-OE leaves compared with the control. Both our observations and measurement of hyphal growth showed that there was least P. infestans biomass in StLTP10-OE leaves, which may be related to the closed stomatal. In line with our results, it has been reported that the invasion of potato leaves by P. infestans causes abnormal stomata opening, and it is through these apertures that infectious hyphae grow and sporangiophores emerge, which is of major importance as a causative factor (Farrell et al., 1969). According to the above, it is reasonable to speculate that the effective stomatal closure caused by overexpression of StLTP10 may not only prevent pathogen invasion but also inhibit the production of sporangiophores through the stomata during P. infestans attack. This also supports a notion that the StLTP10-PYL4 module is required for the host to close stomata as a defence mechanism in response to P. infestans infection.

MAPK cascade pathways are an important part of plant development and immune responses. In Arabidopsis, MPK3 is particularly associated with numerous abiotic and biotic stress responses. For example, the lipid transfer protein AZI1 is phosphorylated and controlled by MPK3, and functional MPK3 appears to be required for the full extent of AZI1-conferred robustness (Pitzschke et al., 2014). Moreover, MPK3 enhances tolerance to drought stress via ABA-mediated stomatal closure and it is essential to both stomatal and apoplastic immunity (Gudesblat et al., 2007; Su et al., 2017). Here, our study demonstrated that StLTP10 interacts with WIPK, a homolog of MPK3. In addition, co-overexpression of StLTP10 and WIPK significantly enhanced leaf resistance to P. infestans compared with overexpressing StLTP10 alone. Possible explanations for this phenomenon include the notion that StLTP10 needs to be regulated by WIPK to become fully functional. Then, in vitro and in vivo protein degradation experiments suggested that WIPK could regulate StLTP10 abundance, thereby playing a positive role in StLTP10-mediated resistance against P. infestans. However, how WIPK regulates StLTP10 remains obscure. WIPK belongs to the MAPK family, and members of this family activate or inhibit protein activity mainly through phosphorylation. It is reasonable to hypothesize that WIPK may act as a protein kinase to phosphorylate StLTP10. To verify this hypothesis, in vitro and in vivo kinase assays must be performed. It will be interesting to identify the site(s) of phosphorylation of StLTP10 and to test whether this phosphorylation is a prerequisite for StLTP10 abundance. Thus, substantial additional research is needed to answer these questions.

Our findings indicate that the potato StLTP10 functions as a positive regulator in plant resistance to P. infestans and our data support the notion that StLTP10 has pleiotropic effects on biotic stress responses. The molecular mechanisms of StLTP10 in pathogen defence may occur through up-regulating the expression of stress-responsive genes in ROS-scavenging and SA-signalling pathways. Our study highlights that in the early stage of pathogen infection, StLTP10 interacts with ABA receptor PYL4, probably to participate in ABA-mediated stomatal closure, thus reducing pathogen invasion. Notably, WIPK was revealed as a positive regulator of StLTP10 abundance. To a large extent our results provide new functional and mechanistic findings of pathogen resistance via nsLTPs in potato and serve as an important scientific basis for pathogen prevention strategies.

## 4 | EXPERIMENTAL PROCEDURES

### 4.1 | Plant materials and growth conditions

The plants used in these experiments include potato (Solanum tuberosum 'Eshu 3', originally named Favorita, also known as Potato Holland 15, which the parentage is ZPC50-3535XZPC5-3, one of the main early maturity varieties in China imported from the Netherlands in 1981) and N. benthamiana. Potato seedlings grown from nodal segments cultured on MS basal medium containing 2% (wt/vol) sucrose and 0.8% (wt/vol) agar were transplanted into standard plastic pots containing peat-vermiculite (3:1, vol/vol). N. benthamiana seeds were germinated in flat trays and the
young seedlings were then transplanted into standard plastic pots filled with peat-vermiculite mixture. The plants were grown at 25°C, 65% relative humidity (RH), and 16/8 hr light/dark regime in growth chambers.

4.2 | Plant treatment and *P. infestans* inoculation

To analyse expression patterns of *StLTP10* under various defence-related hormone treatments, 4-week-old potato seedlings were sprayed with ABA (100 μM), SA (10 mM), and JA (100 μM) following Gao’s method (Gao et al., 2009).

The *P. infestans* isolate EC1 was cultured on rye B agar medium in the dark at 18°C. After 2 weeks, the sporangia were collected with sterile distilled water and incubated at 4°C for 2 hr to release zoospores. The zoospores were quantified under a haemocytometer and the concentration was adjusted to 50,000 zoospores/ml. For spray inoculation, the whole plants or detached leaves were sprayed with *P. infestans* zoospores suspension until run-off according to Cui et al. (2018). For drop inoculation, the surfaces of detached leaves were inoculated with 15 μl of *P. infestans* zoospores suspension and then placed at 18°C in sealed boxes with 100% RH environment, and kept in weak light. The diameters of lesions were measured at 5 dpi and scored as described by Wang et al. (2019). The biomass of inoculated leaves was extracted with cetyltrimethylammonium bromide solution at 5 dpi. The biomass level of *P. infestans* was measured by quantitative PCR (qPCR) with the *P. infestans*-specific PiO8 element, and the *Nbactin* or *StEF-1a* gene was used to normalize the level of *N. benthamiana* or potato.

DAB and NBT staining were performed to measure the levels of H2O2 and O2·− as described by Li et al. (2015).

4.3 | Stomatal aperture measurement

For stomatal aperture measurement before and after inoculation with *P. infestans*, the detached leaves were floated on stomatal opening buffer (25 mM MES, pH 6.15, 10 mM KCl) under light for 3 hr to fully open the stomata, as described by Su et al. (2017). They were then treated with *P. infestans* zoospore suspension, Pst DC3000 (OD600 = 0.1), or ABA (50 mM) for the indicated period of time. Stomatal aperture was measured using ImageJ (National Institutes of Health) software. The measurement of in planta Pst DC3000 growth was performed according to previously described protocols (Li et al., 2014).

4.4 | RNA extraction and RT-qPCR

Total RNA samples of plants or leaves were extracted using TRIzol reagent (Invitrogen). Subsequently, 1 μg of total RNA was used for first-strand complementary DNA (cDNA) synthesis (EasyScript First-Strand cDNA Synthesis SuperMix; Transgen). The target gene expression patterns for assays were analysed by RT-qPCR using the SuperReal PreMix Plus (Tiangen) in triplicate for each sample. The potato *StEF-1a* gene or *N. benthamiana* Nbactin gene was used as an internal reference gene for normalization. All primer sequences are shown in Table S1.

4.5 | Sequence analyses, vector construction, and genetic transformation of *StLTP10*

Amino acid sequence alignment and the phylogenetic tree of *StLTP10* were produced by DNAMAN and Molecular Evolutionary Genetics Analysis (MEGA) software 4.0, respectively. The plasmids used for *StLTP10*, *StLTP10ΔStP*, PYL4, and WIPK overexpression were constructed on the basis of the pBI121 vector. The full-length coding sequences (CDS) of these genes were digested with BamHI and SacI, and were cloned into BamHI/SacI-cut pBI121, which placed them under the control of the CaMV 35S promoter. The RNA interference (RNAi) plasmids were also constructed based on the pBI121 vector. A pair of long and short nonconserved fragments of *StLTP10* were inserted into pBI121 vector. These plasmids were mobilized into *A. tumefaciens GV3101::pMP90(RK)* (Hellens et al., 2000) using the freeze–thaw method. For transient overexpression in *N. benthamiana* leaves, *A. tumefaciens* (OD600 = 1.0) was introduced into leaf epidermal cells of 4-week-old *N. benthamiana* by infiltration. *A. tumefaciens* with pBI121 vector was used as a control. For genetic transformation of *StLTP10*, the constructs were individually introduced into potato via *A. tumefaciens*-mediated stem segment transformation according to a previously described method (Khaire et al., 2010) with some modifications. After obtaining T0 kanamycin-resistant plants, the presence of the transgene in the regenerated plantlets was further confirmed using PCR with a specific 35S forward primer (FP) (5′-CGCCAATCCACTTCC-3′) and *StLTP10* reverse primer (RP) (5′-TAGCGGCTGATTTCAAGC-3′). In addition, the expression level of *StLTP10* in these selected transgenic lines was further examined by RT-qPCR.

4.6 | *Agrobacterium*-mediated VIGS

The method for VIGS was performed according to Brigneti et al. (2004). The fragments of *StLTP10*, PYL4, and *StPDS* (phytoene desaturase, *PDS*) were amplified by PCR with the gene-specific primers shown in Table S1, and cloned into the pTRV2 VIGS vector. The recombinant vectors and pTRV1 plasmid were transformed into *A. tumefaciens GV3101::pMP90(RK)*. The *Agrobacterium* suspensions (OD600 = 1.0) containing pTRV1 and pTRV2 were mixed 1:1 and infiltrated into the leaves of 3-week-old potato plantlets after incubation for 3 hr. The silencing level of potato genes was tested 21–24 days
postagroinfiltration when the control pTRV2-StPDS plants were showing signs of bleaching.

**4.7 | Y2H, BiFC, LCI, and GST pull-down assays**

A Y2H assay was used to verify StLTP10-interacting proteins following the Yeast Protocols Handbook (Clontech). The CDS of StLTP10 was introduced into pGBKKT7 as bait, while PYL4 or WIPK was introduced into pGADT7 as prey. These plasmids were then cotransformed into the Y2H Gold yeast strain by the lithium acetate method. The transformed yeast cells were assayed for growth on synthetic double dropout (DDO, SD/-Ade/-His/-Leu/-Trp) medium and positive clones were grown on the selective quadruple dropout (QDO, SD/-Ade/-His/-Leu/-Trp with X-a-gal) medium.

For the BiFC assay, StLTP10 and PYL4 or WIPK were cloned into the pSPYNEx35S or pSPYCE-35S vector to generate a fusion with the N-terminal or C-terminal fragment of YFP, respectively. These vectors were then coinjected into the leaves of *N. benthamiana* using *A. tumefaciens* (OD<sub>600</sub> = 1.0) infiltration. After 48 hr of growth, the YFP signal was observed using an LSM 880 laser scanning confocal microscope (Carl Zeiss) with the excitation and emission wavelengths set to 514 and 520–560 nm, respectively.

For the LCI assay, the coding sequences of genes were cloned into pcAMB1300-nLUC/cLUC vectors, respectively. The experiment was performed by transient expression in *N. benthamiana* leaves through Agrobacterium-mediated infiltration as previously described (Chen et al., 2018). Two days after infiltration, these leaves were spray with 0.2 mM luciferin (Promega) uniformly before detection. Luciferase activity was measured using a low-light cooled charge coupled device imaging apparatus (Lumina II).

For the GST pull-down assay, plasmids pGEX-4p-3, pGEX-4p-3::PYL4, pET30a, and pET30a::StLTP10 were introduced into *Escherichia coli* BL21 (DE3). The expression of proteins was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 22°C for 12 hr. Equal amounts of GST-PYL4 and His-StLTP10 sonicated lysates were mixed with high-affinity GST resin (GenScript) and incubated for 2 hr at 4°C with constant rotation. The bound proteins were then eluted with fresh glutathione elution buffer. Afterwards, the proteins were separated by 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-GST or anti-His antibody (Cowin Biotech Co., Ltd).

**4.8 | Subcellular localization**

The coding regions of StLTP10, StLTP10-ΔSP, and PYL4 without stop codons were fused into binary vector pcambia1300-RFP or pBI121-GFP to produce red or green fluorescent protein fusion constructs driven by the CaMV 35S promoter. Then, the constructs were transformed into *A. tumefaciens* GV3101::MP90(RK) and the leaves of *N. benthamiana* were used for transient expression. The fluorescent signals were observed with an LSM 880 laser scanning confocal microscope (Carl Zeiss) after 48 hr of growth.

**4.9 | Co-IP assay**

To detect the interaction of StLTP10 and WIPK, StLTP10-Myc and WIPK-FLAG were first expressed in *N. benthamiana*. Proteins were extracted with extraction buffer containing 100 mM pH 7.4 Tris-HCl, 1 mM EDTA, 75 mM NaCl, 0.05% SDS, 0.1% Triton X-100, 10% glycerol, and protease inhibitor cocktail Roche. Then the Co-IP assay was performed as previously described (Zhu et al., 2014). The protein extracts were incubated with magnetic beads (Dynabeads Protein A; Invitrogen) and anti-Myc antibody (Sigma; 1:500 dilution) at 4°C for 6–8 hr. These beads were then recovered by centrifugation at 1,000 x g for 30 s and washed four times with extraction buffer. About 50 µl of sample buffer containing 50 mM pH 6.8 Tris-HCl, 2% SDS, 6% glycerol, 100 mM dithiothreitol, and 0.02% bromophenol blue was added and resuspended. After boiling for 10 min at 95°C, the proteins were loaded onto 12% SDS-PAGE gels for western blot analysis and detected immunoblotting by anti-FLAG-tag (1:1,000 dilution) and anti-Myc (Sigma 1:500 dilution) antibodies.

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**CONFLICT OF INTEREST**

The authors declare no competing interests.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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