GmBTB/POZ promotes the ubiquitination and degradation of LHP1 to regulate the response of soybean to *Phytophthora sojae*

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*Phytophthora sojae* is a pathogen that causes stem and root rot in soybean (*Glycine max* [L.] Merr.). We previously demonstrated that GmBTB/POZ, a BTB/POZ domain-containing nuclear protein, enhances resistance to *P. sojae* in soybean, via a process that depends on salicylic acid (SA). Here, we demonstrate that GmBTB/POZ associates directly with soybean LIKE HETEROCHROMATIN PROTEIN1 (GmLHP1) in vitro and in vivo and promotes its ubiquitination and degradation. Both overexpression and RNA interference analysis of transgenic lines demonstrate that GmLHP1 negatively regulates the response of soybean to *P. sojae* by reducing SA levels and repressing *GmPR1* expression. The WRKY transcription factor gene, *GmWRKY40*, a SA-induced gene in the SA signaling pathway, is targeted by GmLHP1, which represses its expression via at least two mechanisms (directly binding to its promoter and impairing SA accumulation). Furthermore, the nuclear localization of GmLHP1 is required for the GmLHP1-mediated negative regulation of immunity, SA levels and the suppression of *GmWRKY40* expression. Finally, GmBTB/POZ releases GmLHP1-regulated *GmWRKY40* suppression and increases resistance to *P. sojae* in GmLHP1-OE hairy roots. These findings uncover a regulatory mechanism by which GmBTB/POZ-GmLHP1 modulates resistance to *P. sojae* in soybean, likely by regulating the expression of downstream target gene *GmWRKY40*.

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Plants have sophisticated cell-autonomous defense mechanisms that combat microbial pathogens, including a waxy cuticle, anti-microbial compounds, and plant innate immunity systems. In general, the waxy cuticle and preformed anti-microbial compounds provide passive protection against pathogens rather than attacking a specific host, whereas plants rely on innate immunity to defend themselves against widespread diseases. These immunity responses arise via a regulatory network coordinating immune response proteins, transcriptional regulators, and other structural components. Regulation occurs at every level, from differential transcript accumulation and processing to protein modification and turnover. Thus, research on the regulatory components of plant defense responses can provide insights into the complex processes involved in plant immunity.

Ubiquitination is a common post-translational modification in which ubiquitin (Ub) is covalently bound to lysine residues in target proteins. Ubiquitination is carried out by Ub-activating (E1), Ub-conjugating (E2), and Ub-ligase (E3) enzymes, and often leads to target protein degradation mediated by the 26S proteasome. The BTB/POZ domain (Broad Complex, Tram-track, Bric-a-brac/Pox virus and Zinc Finger) is an evolutionarily conserved, NH2-terminal protein–protein interaction motif present in a variety of cytoskeletal modifiers and Ub ligase substrate recognition factors. Substrate specificity factors associate with cullin 3-based E3 ligases through BTB/POZ proteins. Therefore, BTB/POZ proteins function as a bridge between CUL3 (CUL3-RING E3 ligase) and substrate proteins and are essential for the ubiquitination process.

HP1 (HETEROCHROMATIN PROTEIN1) was first described in Drosophila melanogaster as a non-histone chromosomal protein that preferentially binds to constitutive heterochromatin on polytene chromosomes. HP1 orthologs are present in organisms ranging from yeasts to humans. Plants possess a single-copy gene for HP1, LIKE HETEROCHROMATIN PROTEIN1 (LHP1), which was initially identified in screens for inflorescence meristem function in Arabidopsis thaliana and is also referred to as TERMINAL FLOWER2 (refs. 22,23). To date, many plant LHP1 homologs have been identified. LHP1 encodes a highly evolutionarily conserved protein containing a chromo domain and a chromo shadow domain.

LHP1 proteins regulate several important growth and development processes in plants. Mutations in AtLHP1 cause a range of developmental defects, including reduced stability of the vernalized state, conversion of the shoot apical meristem to a terminal flower, curled leaves, and reduced root growth. LHP1 is also involved in auxin biosynthesis in Arabidopsis. In general, LHP1 proteins also function as transcriptional repressors, which play crucial roles in maintaining the transcriptionally silenced state of their targets. For example, AtLHP1 directly represses the expression of the floral promoter FLOWERING LOCUS T (FT) in vascular tissue before dusk and at night. The early-flowering phenotype of Arabidopsis lhp1 mutants results from increased expression of FT. These findings indicate that LHP1 represses the transcription of genes that function during different stages of reproductive development. Nevertheless, most studies of LHP1 performed to date in plants other than Arabidopsis were limited to examining the differences in protein expression profiles, whereas no in-depth study of gene expression, functions, or molecular mechanisms of plant LHP1s have been performed. In particular, the role of LHP1 in soybean (Glycine max [L.] Merr.) in response to biotic stress has not yet been evaluated.

GmBTB/POZ positively regulates the response of soybean to Phytophthora sojae, a destructive pathogen that causes stem and root rot in soybean; this response primarily depends on the salicylic acid (SA) signaling pathway. In the current study, we focused on soybean LIKE HETEROCHROMATIN PROTEIN1 (GmLHP1; NCBI protein no. XP_003548606), a GmBTB/POZ-interacting partner involved in the response to P. sojae infection. GmLHP1 was degraded in soybean inoculated with P. sojae, primarily through the 26S proteasome. Further analysis showed that GmBTB/POZ promotes the ubiquitination and degradation of GmLHP1 in vitro and in vivo. In addition, GmLHP1 inhibits the expression of GmWRKY40, a SA-inducible gene that functions downstream of SA biosynthesis. Therefore, we uncovered a potential role of the GmBTB/POZ–GmLHP1 regulatory module in plant pathogen resistance, providing insights into the mechanism underlying defense responses against P. sojae infection.

Results

GmLHP1 interacts with GmBTB/POZ. We previously demonstrated that GmBTB/POZ positively regulates the response of soybean to P. sojae infection and GmBTB/POZ interacted with GmLHP1 (LIKE HETEROCHROMATIN PROTEIN1) in a bimolecular fluorescence complementation (BiFC) assay. In soybean, there are two genes encoding copies of LHP1 (LHP1-1 and LHP1-2). In the current study, we focused on LHP1-1, namely GmLHP1 (NCBI protein no. XP_003548606; Glyma.16G079900) which contains two highly conserved structural domains: a chromo domain and a chromo shadow domain (Supplementary Fig. 1). Firstly, in a Y2H assay, yeast cells co-expressing pGBD-GmLHP1 + pGAD-GmBTB/POZ or pGAD-GmBTB/POZ + pGAD-GmLHP1, but not pGAD-GmLHP1 + pGAD or pGAD-GmBTB/POZ + pGAD, grew well on SD/-Trp/-Leu/-His/-Ade (QDO) screening medium and showed α-galactosidase activity (Fig. 1a), indicating that GmLHP1 interacts with GmBTB/POZ in yeast cells.

We performed an in vitro pull-down assay to validate the interaction between GmLHP1 and GmBTB/POZ. GmLHP1-His, GmBTB/POZ-GST, and GST alone were detected in whole-cell lysates (Input). GmLHP1 fused with a His tag was not detected in the control sample (GST protein alone), whereas GmLHP1-His was pulled down via GmBTB/POZ-GST (Fig. 1b), suggesting that GmLHP1 directly interacts with GmBTB/POZ. We further confirmed the interaction between GmLHP1 and GmBTB/POZ using firefly luciferase complementation imaging (LCI). The results confirmed that GmLHP1 interacts with GmBTB/POZ in planta (Fig. 1c). Furthermore, these assays indicated that GmLHP1 interacts with GmBTB/POZ in the nucleus (Fig. 1c). Therefore, these three different methods indicated that GmLHP1 directly interacts with GmBTB/POZ both in vitro and in vivo.

GmBTB/POZ promotes the ubiquitination and degradation of GmLHP1. BTB/POZ proteins are a bridge between CUL3-RING E3 ligase and substrate proteins, and they are essential for the Ub process. Since our protein interaction assays between GmLHP1 and GmBTB/POZ suggested that GmLHP1 is a potential substrate of GmBTB/POZ, we speculated that GmBTB/POZ plays a role in the ubiquitination and degradation of GmLHP1. To explore this possibility, we performed in vitro protein degradation assays. Specifically, protein extracts from the WT soybean were incubated with the His-tagged GmLHP1 (GmLHP1-His) proteins purified from Escherichia coli Rosetta (DE3) cells at 22 °C. Then, we performed an immunoblot assay using anti-His antibody to measure the abundance of GmLHP1-His protein. GmLHP1-His was unstable in WT soybean protein extracts; clear GmLHP1-His degradation was observed beginning at 0.5 h, and it was almost completely degraded by 3 h (Fig. 2a). However, treating the samples with 100 μM of the proteasome inhibitor MG132 significantly repressed the degradation process (Fig. 2a). This observation suggests that GmLHP1 is normally degraded by the 26S proteasome in the absence of GmBTB/POZ.
Fig. 1 GmLHP1 interacts with GmBTB/POZ. a GmLHP1 interacts with GmBTB/POZ in yeast cells. The yeast cells were selected on SD medium lacking Leu and Trp (QDO), and interaction was assessed based on their ability to grow on selective medium lacking Leu, Trp, His, and Ade (QDO) or SD medium lacking Leu, Trp, His, and Ade (QDO) but containing X-a-Gal for 3 days at 30 °C. The combination of pGBD-P53 + pGAD-SV40 was used as a positive control and pGBD-Lam + pGAD-SV40 as a negative control. X-a-Gal represents 5-bromo-4-chloro-3-indolyl-β-D-galactoside. b In vitro pull-down assays showing the interactions of GmLHP1 with GmBTB/POZ. His-tagged proteins were incubated with immobilized GST or GST-tagged proteins, and immunoprecipitated fractions were detected by anti-His antibody. c Interaction between GmLHP1 and GmBTB/POZ in LCI assays. The combination of Fts2-nLUC + Gfp-ccLUC was used as a positive control.

GmLHP1 negatively regulates plant immunity. LHP1 plays an important role in plant responses to environmental stimuli. In addition, LHP1 interacts with various proteins to perform distinct roles in different cell types; for example, LHP1 interacts with Polycomb Group proteins to repress the expression of developmentally important genes. LHP1 also interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types. LHP1 interacts with various proteins to perform distinct roles in different cell types.

We investigated P. sojae resistance in the roots of these transgenic plants. At 96 h of post-inoculation (hpi), the roots of all three GmLHP1OE soybean lines exhibited more serious symptoms than WT roots, including watery and rotting lesions (Fig. 3a). By contrast, the three GmLHP1RNAlA soybean lines displayed almost no visible lesions compared to WT roots (Fig. 3a). We analyzed the relative biomass of P. sojae in soybean roots based on the transcript level of P. sojae TEF1 (EU079791). P. sojae biomass was significantly higher (** p < 0.01) in the GmLHP1OE lines and significantly lower (** p < 0.01) in the GmLHP1RNAl lines compared to WT plants (Fig. 3b). Similar results were obtained for GmLHP1OE and GmLHP1RNAl transgenic soybean hairy roots, which were generated by high-efficiency Agrobacterium rhizogenes-mediated transformation (Fig. 3f, g, j). These results indicate that overexpressing GmLHP1 in soybean increases susceptibility to P. sojae and that silencing this gene improves resistance to P. sojae.
SA plays major roles in regulating basal defense responses during plant immunity\(^4\) and acts as a crucial signaling element in systemic acquired resistance (SAR) signaling pathways\(^47,48\). SA mediates SAR, which limits the growth of biotrophic and necrotrophic virulent pathogens and favors long-term protection against a broad spectrum of microorganisms\(^49,50\). Increased endogenous SA levels trigger SAR by inducing the expression of pathogenesis-related (PR) genes, such as \(\text{PR1}\), which is considered to be an effector gene for SAR\(^48\). To determine whether GmLHP1 also regulates the SA signaling pathway, we analyzed the SA contents and expression levels of GmPR1 (AF136636) in GmLHP1OE, WT, and GmLHP1RNAi soybean plants. Both SA levels and GmPR1 expression levels were significantly lower (**\(P < 0.01\)) in GmLHP1OE plants and higher (**\(P < 0.01\)) in GmLHP1RNAi plants compared to WT (Fig. 3c, d). In addition, both SA levels and GmPR1 expression levels were significantly reduced (**\(P < 0.01\)) in GmLHP1-OE transgenic hairy roots vs. the control. However, SA levels and GmPR1 expression levels were significantly higher (**\(P < 0.01\)) in GmLHP1-RNAi vs. control hairy roots (Fig. 3h, i). The results suggest that GmLHP1 regulates defense responses against \(P. sojae\) by affecting SA levels and GmPR1 expression.

GmLHP1 regulates the transcription of \(GmWRKY40\) via two mechanisms. LHP1 is a nucleus-localized protein that generally functions as a transcriptional repressor in both plants and animals\(^21,33,34,51\). To examine the subcellular localization of GmLHP1, we analyzed the expression of the \(GmPR1\) (AF136636) in GmLHP1OE, WT, and GmLHP1RNAi soybean plants. Both SA levels and GmPR1 expression levels were significantly lower (**\(P < 0.01\)) in GmLHP1OE plants and higher (**\(P < 0.01\)) in GmLHP1RNAi plants compared to WT (Fig. 3c, d). In addition, both SA levels and GmPR1 expression levels were significantly reduced (**\(P < 0.01\)) in GmLHP1-OE transgenic hairy roots vs. the control. However, SA levels and GmPR1 expression levels were significantly higher (**\(P < 0.01\)) in GmLHP1-RNAi vs. control hairy roots (Fig. 3h, i). The results suggest that GmLHP1 regulates defense responses against \(P. sojae\) by affecting SA levels and GmPR1 expression.
Fig. 3 GmLHP1 negatively regulates plant immunity. a Disease symptoms in the roots of wild-type (WT), GmLHP1OE, and GmLHP1RNAi soybean plants at 96 h after inoculation with P. sojae. b Relative biomass of P. sojae in WT, GmLHP1OE, and GmLHP1RNAi soybean plants based on P. sojae TEF1 (EU079791) transcript levels. c SA contents in leaves of transgenic and WT soybean. FW, fresh weight. d Relative GmPR1 expression levels in transgenic and WT soybean plants. The expression level of the control sample (WT plants) was set to 1. e qRT-PCR analysis of relative GmLHP1 expression in transgenic soybean hairy roots. Empty vector (EV) transgenic hairy roots were used as controls, and the expression level of the control sample (EV) was set to 1. f Relative biomass of P. sojae in GmLHP1-transgenic hairy roots based on P. sojae TEF1 (EU079791) transcript levels. g Typical infection symptoms of GmLHP1-OE and EV soybean hairy roots at 48 h after P. sojae inoculation. h Total SA content in GmLHP1-OE and GmLHP1-RNAi hairy roots. FW, fresh weight. i Relative GmPR1 expression levels in GmLHP1-OE, GmLHP1-RNAi, and EV hairy roots. The expression level of the control sample (EV) was set to 1. j Typical infection symptoms of GmLHP1-RNAi and EV hairy roots at 48 h after P. sojae inoculation. The housekeeping gene GmEF1 was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and the results were statistically analyzed using Student’s t-test (*P < 0.05, **P < 0.01). Bars indicate the standard deviation of the mean (n = 3).
and GmLHP1OE transgenic soybean plants after 6 weeks of growth in the field. RNA-Seq analysis identified 422 differentially expressed genes (DEGs) with >2.0-fold differences in expression in GmLHP1-OE vs. WT plants under non-stress conditions (false discovery rate (FDR) \( **P < 0.01 \)). Among the 422 DEGs, 253 were significantly upregulated and 169 were significantly downregulated (Supplementary Fig. 5a and Fig. 4a). Gene ontology (GO) analysis revealed that these genes are primarily enriched in the GO terms plant response to biotic and abiotic stress, hormone stimulus, transference activity, transport, and other metabolic processes (Supplementary Fig. 5b).

We examined the expression of several downregulated stress-related DEGs in GmLHP1OE and GmLHP1RNAi soybean plants by qRT–PCR analysis. Examples of these genes include immunity signaling genes such as GmMEKK2 (Glyma.17G173000), GmWRKY40 (Glyma.15G003300), and GmCPK2 (Glyma.11G206300) and defense-associated genes such as GmNAC90 (Glyma.11G182000), GmNAC29 (Glyma.02G109800), GmERF104 (Glyma.20G070000), GmBLH35 (Glyma.13G101100), GmMYB70 (Glyma.17G237900), and GmMLP34 (Glyma.09G102400). GmWRKY40 expression was dramatically reduced in GmLHP1OE vs. WT plants. Notably, in GmLHP1RNAi soybean plants, GmWRKY40 expression significantly increased \( (**P < 0.01) \) compared to the WT, while none of the other genes showed markedly altered expression (Fig. 4b). These findings indicate that the regulation of GmWRKY40 expression likely plays a role in GmLHP1-mediated defense responses.

To explore how GmLHP1 regulates the expression of GmWRKY40, we performed a dual effector–reporter assay using GmLHP1 as the effector and the luciferase gene under the control of 2.0 kb of the GmWRKY40 promoter as the reporter. The effector construct harbored GmLHP1 expressed under the control of the 35S promoter (p35S: Flag-GmLHP1). We transformed the reporter construct (p35S: REN-pGmWRKY40: LUC) and the effector construct (p35S: Flag-GmLHP1) into healthy soybean hairy roots via high-pressure hydrotropism (Supplementary Fig. 2k) and qRT–PCR (Fig. 5c) and the GmWRKY40-RNAi transgenic hairy roots were examined by immunoblotting (Supplementary Fig. 2k) and qRT–PCR (Fig. 5c) and the GmWRKY40-RNAi transgenic hairy roots by analysis with QuickStix Kit for LibertyLink bar strips (Supplementary Fig. 2l) and qRT–PCR (Fig. 5c). After 2 d of incubation with P. sojae zoospores, GmWRKY40-RNAi OE hairy roots displayed almost no visible lesions (Fig. 5a), whereas the GmWRKY40-RNAi lines exhibited enhanced wilting symptoms and chlorosis compared to the control (Fig. 5b). We also analyzed the relative biomass of P. sojae in infected hairy roots after 2 days of incubation with P. sojae zoospores. The biomass of P. sojae was significantly lower \( (**P < 0.01) \) in the roots of GmWRKY40-RNAi lines but significantly \( (**P < 0.01) \) higher in the roots of GmWRKY40-RNAi lines compared to the control (Fig. 5d).

GmWRKY40 expression was significantly induced by SA, suggesting that GmWRKY40 functions downstream of SA biosynthesis as a component of SA signaling. To determine whether GmWRKY40 also participates in the SA signaling pathway, we measured SA content and GmPR1 expression in the transgenic hairy roots. GmPR1 was expressed at significantly higher levels \( (**P < 0.01) \) in GmWRKY40-OE lines but at significantly lower levels \( (**P < 0.01) \) in GmWRKY40-RNAi lines compared to the control (Fig. 5f). However, there was no significant difference in SA level between the GmWRKY40 lines and control hairy roots (Fig. 5e). These results suggest that GmWRKY40 functions as a SA-induced gene downstream of SA biosynthesis and enhances the expression of SA-marker gene GmPR1 in response to P. sojae infection.
Nuclear localization of GmLHP1 is required for its functionality. To determine the region(s) responsible for the nuclear localization of GmLHP1 and analyze whether the nuclear localization of GmLHP1 is required for its functionality, we firstly analyzed the nuclear localization signal (NLS) regions of GmLHP1 using NLS Mapper software. Three putative NLS regions (NLS1 to NLS3) were identified (Fig. 6a, left column), NLS1 (IRRKR-EVQY, amino acids 116–128) is located at the conserved CD domain, and the other two are located at the hinge region: a bipartite NLS2 (GKHRK-LERS, amino acids 165–188) and NLS3 (RCRGS-VKRF, amino acids 324–339). Then, we constructed the GmLHP1 deletion mutants (GmLHP1-1 to 8), each fused with GFP at its C terminus, and analyzed its subcellular localization (Fig. 6a).
that the localization of GmLHP1-1 (amino acids 108–448), containing all NLS regions but lacking the N-terminal part (amino acids 1–107), and GmLHP1-2 (amino acids 1–373), containing all NLS regions but lacking the CSD domain, both were localized in the nucleus of the transformed cell and were indistinguishable from that of the intact protein (amino acids 1–448). The results showed that the absence of N-terminal part alone or the CSD domain alone does not change the nuclear localization of GmLHP1. GmLHP1-3 (amino acids 340–448), containing the conserved CSD domain region, green fluorescent signal was dispersed in the entire cell of protoplasts similar to that displayed by GFP alone, further indicating that the conserved CSD domain has no specific nuclear targeting properties. GmLHP1-4 (amino acids 1–323), containing the conserved CD domain and NLS2 regions but lacking NLS3 and the CSD domain, was localized in the nucleus of the transformed cell, while GmLHP1-5 (amino acids 189–448), which containing NLS3 and the CSD domain, green fluorescent signal was dispersed in the entire cell of protoplasts similar to that displayed by GFP alone, suggesting that the region (amino acids 189–448) of GmLHP1 is not required for the nuclear localization of GmLHP1 and the putative NLS3 region is non-functional. On the basis of GmLHP1-5 deletion mutant sequence (amino acids 189–448), GmLHP1-6 (amino acids 165–448) which added NLS2 region was localized in the nucleus, suggesting NLS2 region has specific nuclear targeting properties. Furthermore, we found that the region encompassing residues 108 to 164 (GmLHP1-7), corresponding to NLS1 region, retained the nucleolus-targeting localization property, indicating the putative NLS1 may also be functional, like the NLS2 region. To verify this prediction, we finally constructed the GmLHP1-8 deletion mutants (amino acids 1–115~129–164~189–448), which deleted the NLS1 and NLS2, and after transformation, we analyzed its subcellular localization. GmLHP1-8 was detected in the entire cell of protoplasts similar to that displayed by GFP alone control. Together, the results showed that both NLS1 and NLS2 regions are required for the nuclear targeting properties of GmLHP1.

**Fig. 5 GmWRKY40 also functions downstream of SA biosynthesis and enhances the expression of SA-marker gene GmPR1 in response to P. sojae.**

a Typical phenotypes of WRKY40-OE and EV soybean hairy roots after 48 h of P. sojae inoculation. Bars, 0.5 cm. b Typical phenotypes of WRKY40-RNAi and EV soybean hairy roots after 48 h of P. sojae inoculation. Bars, 0.5 cm. c qRT-PCR analysis of relative GmLHP1 expression in transgenic soybean hairy roots. Soybean hairy roots transformed with empty vector (EV) were used as controls; the expression level of the control sample (EV) was set to 1. d Relative biomass of P. sojae in GmLHP1-transgenic soybean hairy roots based on the transcript level of P. sojae TEF1 (EU077971). e SA contents in WRKY40-OE, WRKY40-RNAi, and EV hairy roots. FW, fresh weight. f Relative expression level of GmPR1 in WRKY40-OE, WRKY40-RNAi, and EV hairy roots. The expression level of the control sample (EV) was set to 1. The housekeeping gene GmEF1 was used as an internal control to normalize the data. The experiment was performed on three biological replicates, each with three technical replicates, and the results were statistically analyzed using Student’s t-test (*P < 0.05, **P < 0.01). Bars indicate the standard deviation of the mean (n = 3).
To analyze whether the nuclear localization of GmLHP1 is necessary for its functionality, we investigated the P. sojae resistance in GmLHP1-8-OE transgenic soybean hairy roots. After 2 days of incubation with *P. sojae* zoospores, there was no significant phenotype difference between EV and GmLHP1-8-OE soybean hairy roots (Fig. 6b). In accordance with this, the relative biomass of *P. sojae* in infected EV and GmLHP1-8-OE soybean hairy roots have no significant difference (Fig. 6c). Furthermore, the SA levels in GmLHP1-8-OE soybean hairy roots were not significantly downregulated compared to that in EV soybean hairy roots (Fig. 6d). To further determine whether the changes of GmLHP1 nuclear localization have an effect on the suppression of *GmWRKY40* expression by GmLHP1, we also analyzed the expression levels of *GmLHP1-8* and *GmWRKY40* in GmLHP1-8-OE soybean hairy roots (Fig. 6e, f). *GmWRKY40* expression was not significantly suppressed in the GmLHP1-8-OE soybean hairy roots. These results indicated that the nuclear localization of GmLHP1 is required for its functionality.

GmBTB/POZ releases GmLHP1-regulated *GmWRKY40* suppression in GmLHP1-OE soybean lines. We also analyzed the expression levels of *GmWRKY40* in GmBTB/POZ-OE and GmBTB/POZ-RNAi soybean plants. As shown in Fig. 7a, GmWRKY40 was upregulated in GmBTB/POZ-OE plants and downregulated in GmBTB/POZ-RNAi plants compared to the WT. These results indicate that GmBTB/POZ is also involved in regulating *GmWRKY40* transcription. To further explore the role of GmBTB/POZ in GmLHP1-mediated suppression of *GmWRKY40* expression, we generated GmLHP1-OE and GmBTB/POZ-OE/GmLHP1-OE transgenic soybean hairy roots and used hairy roots transformed with EV as a negative control. After measuring GmBTB/POZ and GmLHP1 transcript levels to evaluate the efficiency of GmBTB/POZ and GmLHP1 expression (Fig. 7b, c), we measured *GmWRKY40* transcript levels in EV, GmLHP1-OE, and GmBTB/POZ-OE/GmLHP1-OE hairy roots by qRT-PCR. GmWRKY40 expression was significantly suppressed in the GmLHP1-OE lines, but this effect was inhibited in GmBTB/POZ-OE/GmLHP1-OE hairy roots (Fig. 7d). These results suggest that GmBTB/POZ releases GmLHP1-mediated suppression of *GmWRKY40* expression, likely by inducing the degradation of GmLHP1.

GmBTB/POZ increases resistance to *P. sojae* in GmLHP1-OE soybean lines. Since GmBTB/POZ directly interacts with GmLHP1 to induce its degradation and weakens GmLHP1-mediated *GmWRKY40* suppression, we investigated whether GmBTB/POZ modifies GmLHP1-regulated *P. sojae* defense responses by quantifying *P. sojae* biomass in EV control, GmLHP1-OE, and GmBTB/POZ-OE/GmLHP1-OE transgenic soybean hairy roots at 48 hpi. As expected, *P. sojae* biomass was significantly (**P < 0.01**) higher in GmLHP1-OE hairy roots than...
in EV hairy roots (Fig. 7e). However, the overexpression of GmBTB/POZ resulted in a significant reduction in P. sojae biomass (Fig. 7e). These results indicate that GmBTB/POZ modulates GmLHP1-mediated P. sojae defense responses in soybean, possibly by regulating the expression of the downstream target gene GmWRKY40.

Regulatory mechanism of GmBTB/POZ to GmLHP1 is independent of exclusive or predominant nuclear localization of GmBTB/POZ. To test whether the nuclear localization of GmBTB/POZ is required for the regulatory mechanism of GmBTB/POZ to GmLHP1, we first analyzed the NLS regions of GmBTB/POZ using NLS Mapper software\(^5\)\(^6\),\(^7\). However, no putative NLS region was identified. We further constructed the GmBTB/POZ deletion mutants, each fused with GFP at its C terminus, and analyzed its subcellular localization (Fig. 7f). Transient expression into Arabidopsis protoplasts showed that the full-length GmBTB/POZ protein (amino acids 1–258) was localized to the nucleus, which has also been demonstrated by Zhang et al.\(^3\)\(^5\), while all the GmBTB/POZ deletion mutants (GmBTB/POZ-1 to 7) green fluorescent signal was dispersed in the entire cell of protoplasts similar to that displayed by GFP alone. These results suggested that the integrity of GmBTB/POZ may be required for the nuclear-targeting localization of GmBTB/POZ, the nuclear localization of GmBTB/POZ may not be controlled by a specific region.

Then, we take the deletion mutant GmBTB/POZ-1, in which the nuclear localization has been changed and the protein sequence is the nearest to the full-length GmBTB/POZ protein, to analyze whether the nuclear localization of GmBTB/POZ is required for the ubiquitination-regulatory of GmBTB/POZ to GmLHP1 by in vitro cell-free degradation assay and in vivo
ubiquitination assay. The results suggested that GmBTB/POZ-1 could promote the ubiquitination of GmLHP1 in vitro and in vivo (Fig. 7g, h). To further explore whether the change of GmBTB/POZ nuclear localization has an effect on the GmLHP1-mediated suppression of GmWRKY40 expression, we also measured GmWRKY40 transcript levels in EV, GmLHP1-OE, and GmLHP1-OE/GmBTB/POZ-1-OE soybean hairy roots (Fig. 7k), while GmBTB/POZ-1 and GmLHP1 transcript levels were tested to evaluate the efficiency of GmBTB/POZ-1 and GmLHP1 expression (Fig. 7i, j). GmWRKY40 expression was significantly suppressed in the GmLHP1-OE lines, but the effect was inhibited in GmLHP1-OE/GmBTB/POZ-1-OE soybean hairy roots (Fig. 7k), suggesting GmLHP1-mediated suppression of GmWRKY40 expression. Taken together, these results indicated that the ubiquitination-regulatory of GmBTB/POZ to GmLHP1 may be independent of exclusive or predominant nuclear localization of GmBTB/POZ.

We further investigated the expression kinetics of GmBTB/POZ, GmLHP1, GmWRKY40, and GmPR1 in response to P. sojae. As shown in Supplementary Fig. 6, GmBTB/POZ was rapidly induced by P. sojae infection, with transcript levels peaking at 24 h. By contrast, GmLHP1 was downregulated after P. sojae infection and reached a peak within 24 h. GmBTB/POZ and GmLHP1 exhibited the opposite expression patterns in response to P. sojae. GmWRKY40 transcription was not significantly altered during the first 9 h of infection but reached a peak at 48 h. GmPR1 showed the slowest response to P. sojae infection, reaching a peak at 72 h. These findings support the notion that GmBTB/POZ and GmLHP1 play key roles in the response of soybean to P. sojae at both the transcriptional and post-translational levels.

Discussion

Many soybean genes respond to P. sojae infection35,58-62. The characterization of such genes has helped elucidate the genetic mechanisms underlying defense against P. sojae infection61-63. However, knowledge about the regulator components in plant–pathogen interaction model and plant immunity has remained fragmented. In the present study, we demonstrated that GmLHP1 is an important component of the GmBTB/POZ-mediated SA and immune signaling pathway, providing evidence that the linkage between GmBTB/POZ and GmLHP1 is involved in the response of soybean to P. sojae attack.

Protein ubiquitination is a key mechanism that regulates immune responses64. BTB/POZ functions as a Ub ligase by forming a complex with CRL3 (ref. 17). We previously demonstrated that GmBTB/POZ positively regulates disease resistance in plants, which primarily depends on SA signaling35. However, the components involved in GmBTB/POZ-mediated SA and defense response signaling had been unknown. In the current study, we demonstrated that GmBTB/POZ interacts with GmLHP1 in vitro and in vivo. In vitro protein degradation and in vivo ubiquitination assays suggested that GmBTB/POZ contributes to the Ub-mediated degradation of GmLHP1 through the 26S proteasome system (Fig. 2).

In addition to the roles of LHP1 in regulating flowering time and root development33,34,42,65,66, its potential roles in plant responses to abiotic and biotic stress have been receiving increasing attention. LHP1 interacts with different proteins in different cell types to perform distinct functions42,43. In soybean, LHP1 interacts with GmPHD6 to regulate the expression of genes involved in salt tolerance36. Along with the observation that GmBTB/POZ interacts with and ubiquitinates GmLHP1, these findings prompted us to investigate whether GmLHP1 is also involved in the response of soybean to P. sojae infection. In agreement with our speculation, overexpression and RNA interference analysis of transgenic soybean plants and hairy roots revealed that GmLHP1 negatively regulates the defense responses of soybean to P. sojae infection (Fig. 3). We also analyzed the SA content and expression levels of SA-marker gene GmPR1 in GmLHP1-OE, WT, and GmLHP1-RNAi soybean plants. Compared to WT plants, SA content and GmPR1 transcript levels were significantly lower in GmLHP1-OE plants but higher in GmLHP-1 RNAi plants (Fig. 3c, d). Similar results were obtained for GmLHP1-OE and GmLHP1-RNAi transgenic soybean hairy roots (Fig. 3h, i).

SA mediates the plant immune response SAR, a long-lasting, broad-spectrum resistance response to a variety of pathogenic fungi, bacteria, and viruses48,50,57. SA is characterized by increased endogenous SA levels and the increased expression of PR genes, such as PR1, which are considered to be effector genes for SAR48. Germinating soybean in red light improves resistance to Pseudomonas putida 229 by regulating SA levels and upregulating PR1 (ref. 68). Consistent with this, our findings suggest that GmLHP1 negatively regulates the response of soybean to P. sojae, possibly by suppressing SA levels and GmPR1 gene expression. Our study provides clear evidence for the linkage between a BTB/POZ-mediated ubiquitination pathway and a plant LHP1-associated defense system. Such a linkage has not been previously reported for any plant species.

LHP1 represses the transcription of numerous genes, including FLOWERING LOCUS C (FLC) and the floral organ identity genes AGAMOUS (AG) and APETALA3 (AP3)29,69,70. It has also been reported that GmPHD6 could form a complex with LHP1 to bind to the GAL4 element through BD-GmPHD6 and to activate gene expression in soybean, indicating that LHP1 could also function as the coactivator in transcriptional complex66. However, in the current study, a series of physiological and biochemical assays showed that GmLHP1 could directly target and suppress the expression of GmWRKY40. In a transient expression assay in yeast cells using a GAL4-responsive reporter system, GmLHP1 alone did not activate the transcription of the reporter gene (Supplementary Fig. 4b). RNA-Seq showed that various stress-related genes, including GmWRKY40, were significantly downregulated in GmLHP1-OE transgenic soybean plants (Fig. 4a). Furthermore, qRT-PCR analysis indicated that the changes in GmWRKY40 expression were much more pronounced in GmLHP1-OE and GmLHP1-RNAi vs. WT plants: GmWRKY40 expression was dramatically reduced in GmLHP1-OE vs. WT plants, and in GmLHP1-RNAi soybean plants, GmWRKY40 expression significantly increased (***p < 0.01) compared to the WT, while none of the other genes showed markedly altered expression (Fig. 4b). A dual effector–reporter system using GmLHP1 as the effector and the luciferase gene under the control of the GmWRKY40 promoter as the reporter, as well as ChIP-qPCR assays, demonstrated that GmLHP1 directly binds to the GmWRKY40 promoter and suppress its expression (Fig. 4c–f).

WRKY family genes are involved in SA signaling pathways. Several WRKY genes are associated with SA biosynthesis; for example, the Arabidopsis wrky54 wrky70 double mutant has strongly increased SA levels71. In addition, several WRKYs are induced by SA and function downstream of SA the biosynthesis pathway. SA induces the rapid expression of WRKY genes in a number of plants72–74. In Arabidopsis, 49 of the 72 WRKY genes examined were differentially regulated in plants after treatment with SA72. In the current study, we determined that GmWRKY40 contains the WRKY domain, a highly conserved structural domain (Supplementary Fig. 7). GmWRKY40 expression was significantly induced by SA, and the amount of histochemical GUS staining in soybean hairy roots under SA treatment was clearly higher relative to mock (H2O) conditions (Fig. 4g, h). Moreover, whereas SA levels in GmWRKY40 transgenic hairy
roots were not significantly different from those of the control (Fig. 5e), GmWRKY40 expression enhanced the expression of SA-marker gene GmPR1 (Fig. 5f). These results suggest that GmWRKY40 functions as a SA-induced gene in the SA signaling pathway downstream of SA biosynthesis. We also demonstrated that GmLHP1 participates in the SA signaling pathway and inhibits SA accumulation. Meanwhile, exogenous SA application weakened the inhibition of GmWRKY40 expression in GmLHPIOE soybean plants, suggesting that GmLHP1-mediated suppression of GmWRKY40 expression might also occur via impaired SA accumulation (Fig. 4i, g). These findings indicate that GmWRKY40 is a GmLHP1 target and that at least two types of mechanisms (directly repressed GmWRKY40 expression and impaired SA accumulation) contribute to the regulation of GmWRKY40 expression by GmLHP1.

Specific WRKY transcription factors function in plant defense responses by affecting the expression of PR1 (ref. 75). For instance, Arabidopsis WRKY18 and WRKY70 activate the expression of genes including PR1 and increase resistance to pathogens76,77. Consistent with this finding, in the current study, GmWRKY40 expression enhanced resistance to P. sojae (Fig. 5a–d) and increased the transcript level of GmPR1 (Fig. 5f). Thus, perhaps GmLHP1 represses the expression of GmWRKY40, thereby negatively regulating resistance to P. sojae. Thereinto, the nuclear localization of GmLHP1 is required for the GmLHP1-mediated negative regulation of immunity, SA levels, and the suppression of GmWRKY40 expression (Fig. 6a–f).

More importantly, GmWRKY40 was upregulated in GmBTB/POZ-OE soybean lines and downregulated in GmBTB/POZ-RNAi lines compared to WT plants, indicating that GmBTB/POZ also affects the transcription of GmWRKY40. Analysis of soybean hairy roots co-transformed with GmBTB/POZ and GmLHP1 indicated that GmBTB/POZ released GmLHP1-regulated GmWRKY40 suppression and increased resistance to P. sojae in GmLHP1-OE hairy roots.

Finally, we demonstrated that GmBTB/POZ and GmLHP1 are both involved in regulating P. sojae resistance and GmWRKY40 expression but play opposite roles in this process. Specifically, we propose that GmBTB/POZ and GmLHP1 function together in SA and immune signaling pathways and that GmBTB/POZ recruits and degrades GmLHP1, thereby regulating the expression of downstream target gene GmWRKY40 in soybean. The expressions of GmLHP1 and GmBTB/POZ are inversely regulated during P. sojae infection (Supplementary Fig. 6). These findings strongly suggest that GmBTB/POZ and GmLHP1 play key roles in the response of soybean to P. sojae at both the transcriptional and post-translational levels.

Taken together, based on previous and current findings, we propose a model explaining how the GmBTB/POZ–GmLHP1 complex regulates the response of soybean to P. sojae infection (Fig. 8). According to our model, GmBTB/POZ and GmWRKY40 act as positive regulators, but GmLHP1 acts as a negative regulator, of the response of soybean to P. sojae infection. GmLHP1 functions as an upstream regulator to repress GmWRKY40 expression by directly suppressing its promoter activity and impairing SA accumulation, thus inhibiting plant defense responses. Moreover, P. sojae induces the transcription of GmBTB/POZ, whereas GmLHP1 is downregulated during P. sojae infection. The high levels of GmBTB/POZ recruit and degrade GmLHP1, thereby releasing its suppressive effect on GmWRKY40 expression, thus increasing the defense response to P. sojae. This study provides compelling evidence for the role of the GmBTB/POZ–GmLHP1 complex in modulating the response of soybean to P. sojae infection. Furthermore, it has been previously proved that LHP1 plays a central role in regulating flowering time, and Arabidopsis loss-of-function lhp1 mutants exhibit photoperiod-independent early flowering compared to WT plants23,78. In our study, we also observed that GmLHP1RNAi soybean plants showed early flowering compared with WT plants under artificial long-day conditions (Supplementary Fig. 8). However, whether GmBTB/POZ–GmLHP1 complex is also involved in flowering-regulatory, as well as the underlying genetic and molecular mechanisms still require further exploration.

Methods

Plant materials and pathogen inoculation. "Dongnong 50", a soybean ( Glycine max) cultivar susceptible to P. sojae race 1, was obtained from the Key Laboratory of Soybean Biology in the Chinese Ministry of Education, Harbin, and used for gene transformation experiments and expression analysis. "Suinong10", a soybean cultivar with gene-for-gene resistance against P. sojae race 1, the predominant race in Heilongjiang, China79, was used for the gene isolation and gene expression kinetics experiments. The coding sequences (CDS) of GmLHP1 (Glyma.15G003300) and GmWRKY40 (Glyma.15G003300) were amplified by PCR using CDNA derived from leaves of "Suinong10" soybean as the template. The seeds were grown in a growth chamber at 25 °C and 70% relative humidity under a 16 h light/8 h dark cycle. N. benthamiana plants for the LCI assays and dual-luciferase assays were grown at 22 °C under a 16 h light/8 h dark photoperiod with a light intensity of 120 µE m−2 s−1.

Phytophthora sojae race 1 (PSR01) was isolated from infected soybean plants in Heilongjiang, China79, and cultivated at 25 °C for 7 days on V8 juice agar in a polystyrene dish.

In vitro pull-down assay. To produce the GmLHP1-His fusion protein, the CDS of GmLHP1 was cloned into the pET29b (+) expression vector. The recombinant fusion plasmids were transformed into E. coli Rosetta (DE3) cells. The fusion proteins were purified at 4 °C and quantified according to the pET System Manual. To produce the GmBTB/POZ-GST protein, the CDS of GmBTB/POZ was inserted into the pGEX-4T-1 expression vector and expressed in Rosetta (DE3) cells. The target protein was purified with GST resin (GE Healthcare; 17-0756-01). Pull-down was performed as described by Yang et al.80. The pulled-down proteins were eluted with boiling, separated by 12% SDS-PAGE, and detected by immunoblotting using anti-GST (Abmart, code number M20007S) and anti-His antibodies (Abmart, code number M20001S), respectively.

Firefly LCI assay. The CDS of GmLHP1 and GmBTB/POZ were fused with the N-terminal and C-terminal parts of the luciferase reporter gene, respectively. Agrobacteria harboring the pCAMBIA1300-GmLHP1 Luc and pCAMBIA1300-GmBTB/POZLuc constructs were co-infiltrated into N. benthamiana leaves,
which were subsequently sprayed with luciferin (1 mM luciferin and 0.01% Triton X-100) and photographed using Chemiluminescence imaging (Tanon 5200) at 72 h after infiltration.

**BiFC assays and subcellular localization analysis.** For interaction studies, the gene sequences were cloned into serial pSiAT vectors encoding N- or C-terminal-enhanced yellow fluorescent protein fragments. To determine the subcellular localization of target proteins, the target gene sequences were ligated into the pCAMBIA1302 vector under the control of the 35S promoter, generating the recombinant plasmid. The resulting constructs were used for transient assays via PEG transfection of Arabidopsis protoplasts as described by Yoo et al.81. Transfected cells were imaged using a TCS SP2 confocal spectral microscopy imaging system (Leica, Solms, Germany).

**In vitro cell-free degradation assays.** Total proteins were extracted from WT and transgenic hairy roots with 100 µg of soybean total proteins and 100 ng of GmLHP1-His proteins purified from E. coli Rosetta (DE3) cells. For the proteasome inhibitor experiments, 100 µM MG132 was added to the total proteins 60 min prior to the cell-free degradation experiment. The reactions were incubated at 22 °C. The mixed solutions were collected at the designated time point (0, 0.5, 1, and 3 h) and examined using an anti-His antibody (Abmart, code number M20018S). The quantified results were analyzed using ImageJ software (https://imagej.nih.gov/ij/index.html).

**In vivo ubiquitination assay.** To detect ubiquitination of GmLHP1 in vivo, a plant binary expression vector system was constructed and used to generate GmTB/POZ-GmLHP1 (domain -C) OE. GmLHP1 OE (domain -C) OE, or GmLHP1 OE (domain -C) OE transformed hairy roots were treated with 100 µM MG132 for 8 h prior to protein extraction. GmLHP1-Flag protein was immunoprecipitated using anti-Flag-Tag Mouse mAb (Agarose Conjugated) (Abmart, code number M20018S). The eluted proteins were detected using anti-Flag antibody (Abmart, code number M20008M) and anti-UBI antibody (Abcam, code number ab19169).

**Plasmid construction and genetic transformation of soybean.** To produce the GmLHP1 overexpression and GmLHP1-Flag fusion constructs, the CDS of GmLHP1 and Flag sequence (ATGGACTACAAGGATGACGATGACAAG) overexpression and GmLHP1-Flag fusion constructs, the CDS of GmLHP1 soybean hairy roots by high-efficiency A. rhizogenes-mediated transformation. The transgenic hairy roots were treated with 100 µM MG132 for 8 h prior to protein extraction. GmLHP1-Flag protein was immunoprecipitated using anti-Flag-Tag Mouse mAb (Agarose Conjugated) (Abmart, code number M20018S). The eluted proteins were detected using anti-Flag antibody (Abmart, code number M20008M) and anti-UBI antibody (Abcam, code number ab19169).

**Transgenic soybean lines with degradation buffer.** Each reaction contained 500 µg of soybean total proteins and 100 ng of GmLHP1-His proteins purified from E. coli Rosetta (DE3) cells. For the proteasome inhibitor experiments, 100 µM MG132 was added to the total proteins 60 min prior to the cell-free degradation experiment. The reactions were incubated at 22 °C. The mixed solutions were collected at the designated time point (0, 0.5, 1, and 3 h) and examined using an anti-His antibody (Abmart, code number M20018S). The quantified results were analyzed using ImageJ software (https://imagej.nih.gov/ij/index.html).

**Data availability.** All data supporting the findings of this study are available in the main text and its Supplementary Information. All the source data for figures in Figures and Supplementary Information are presented in Supplementary Data 1 and 2. Raw images of the western blots are provided in Supplementary Fig. 9. Raw RNA sequencing data are available at the NCBI Sequence Read Archive (SRA) under accession PRJA762619. Gene sequences, involvement of stress conditions were used for RNA-Seq analysis. The accession numbers of genes are as follows: GmLHP1 (Glyma.16G079900), GmTB/POZ (Glyma.04G224490), GmMEKK2 (Glyma.17G173000), GmWRKY40 (Glyma.15G003000), GmCBP2 (Glyma.11G206300), GmNAC90 (Glyma.11G182000), GmNAC29 (Glyma.02G19800), GmERF104 (Glyma.20G070000), GmLHIE15 (Glyma.13G101000), GmMYB70 (Glyma.17G237900), and GmM3P4 (Glyma.09G102400).

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Author contributions
S.Z., P.X., and C.Z. designed the experiments. C.Z., Q.C., H.W., and H.G. performed the experiments. C.Z., X.F., X.C., M.Z., W.W., B.S., L.S., and J.W. analyzed the data. S.Z., P.X., and C.Z. wrote the manuscript. All of the authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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