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ORIGINAL ARTICLE

The leucine-responsive regulatory protein SCAB_Lrp modulates thaxtomin biosynthesis, pathogenicity, and morphological development in *Streptomyces scabies*

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Abstract

Streptomyces scabies is the best-characterized plant-pathogenic streptomycete, which is a special species among the large genus *Streptomyces*. The pathogenicity of *S. scabies* relies on the production of the secondary metabolite thaxtomin A. Little is known about the molecular mechanisms underlying the regulation of thaxtomin biosynthesis in *S. scabies* beyond the pathway-specific activator TxtR and the cellulose utilization repressor CebR. The leucine-responsive regulatory protein (Lrp) family modulates secondary metabolism in nonpathogenic streptomycetes. However, the regulatory relationship between the Lrp and pathogenic streptomycetes remains unknown. In this study, we demonstrated that SCAB_Lrp (SCAB_77931) from *S. scabies* significantly affects thaxtomin biosynthesis, pathogenicity, and morphological development. *SCAB_Lrp* deletion resulted in a dramatic decline in thaxtomin A production and a low-virulence phenotype of *S. scabies*. An in-depth dissection of the regulatory mechanism of SCAB_Lrp revealed that it positively regulates the transcription of the thaxtomin biosynthetic gene cluster by directly binding to the promoter of the cluster-situated regulator gene *txtR*. SCAB_Lrp also controls the morphological development of *S. scabies* by directly activating the transcription of *amfC*, *whiB*, and *ssgB*. SCAB_Lrp directly controls the transcription of its own gene by binding a specific sequence (5′-GGACAGTCGCCGTGCTACG-3′). Moreover, phenylalanine and methionine have been characterized as SCAB_Lrp effectors by strengthening the binding affinity and complex status between SCAB_Lrp and DNA. Our findings characterize a multifunctional regulatory protein, SCAB_Lrp, that controls secondary metabolism, pathogenicity, and sporulation in *S. scabies* and provide new insights into the complex regulatory network that modulates thaxtomin phytotoxins in pathogenic *Streptomyces*.

Jing Liu, Yunxia Wang, and Haoyang He contributed equally to this work.

KEYWORDS
leucine-responsive regulatory protein, morphological development, pathogenicity, SCAB_Lrp, *Streptomyces scabies*, thaxtomin

1 | INTRODUCTION

The genus *Streptomyces* is best known for producing various natural products with a wide range of biological functions. Nevertheless, rare species of this genus are phytopathogenic and cause pitted or raised scab lesions on root and tuber crops (Lerat et al., 2009; Loria et al., 2006). *Streptomyces scabies*, *S. acidiscabies*, and *S. turgidiscabies* are the best-studied representatives of the species that cause potato common scab. The phytotoxin thaxtomin A has been identified as a principal determinant of the pathogenicity of *S. scabies* and related species (Loria et al., 2008). Thaxtomin A is a member of a family of nitrated diketopiperazines containing phenylalanine residues and 4-nitrotryptophan (King & Calhoun, 2009). Thaxtomin A was identified as a repressor of cellulose biosynthesis in plants, although its precise target and molecular mode of action remain unclear (Wang et al., 2020). Furthermore, thaxtomin A at the nanomolar level causes serious seedling stunting and plant cell hypertrophy (Bischoff et al., 2009), thus has great potential for use as a natural bioherbicide (Wang et al., 2020).

The biosynthetic pathway of thaxtomin A and its analogues has been investigated through extensive genetic and biochemical studies (Bignell et al., 2014b; Li et al., 2019a, 2019b; Loria et al., 2006). The thaxtomin biosynthetic gene cluster (*txt* cluster) of thaxtomin is highly conserved and contains seven genes arranged within a region of approximately 18.3 kb (Huguet-Tapia et al., 2016). Among them, six genes (*txtA*, *txtB*, *txtC*, *txtD*, *txtE*, and *txtH*) encode thaxtomin biosynthetic enzymes, whereas *txtR* encodes the only cluster-situated regulator (CSR) that functions as an activator of thaxtomin biosynthesis (Joshi et al., 2007; Yang et al., 2011). The biosynthesis of thaxtomin A is under strict transcriptional control, involving at least five global regulators belonging to the *bld* gene family with unresolved mechanism (Bignell et al., 2014a) and a pleiotropic regulator, CebR (Francis et al., 2015), in addition to the CSR protein TxtR (Joshi et al., 2007).

As typical transcription regulators, the leucine-responsive regulatory protein (Lrp) regulator family is widespread and well-characterized in bacteria and archaea (Peeters & Charlier, 2010; Ziegler & Freddolino, 2021). Lrps consist of two domains: a helix-turn-helix (HTH) domain at the N-terminus for DNA binding and an αβ sandwich domain at the C-terminus for ligand response (Ziegler & Freddolino, 2021). Studies on Lrps have made significant advances in understanding the regulatory mechanism controlling antibiotic biosynthesis by *S. lincolnensis* SLCG_Lrp (Xu et al., 2020), *S. coelicolor* SCO3361 (Liu et al., 2017b), and *Saccharopolyspora erythraea* SACE_Lrp (Liu et al., 2017a, 2021) and SACE_5717 (Liu et al., 2019) in our laboratory, and *S. spiramyceticus* SSP_Lrp (Lu et al., 2019).

Among the *S. scabies* 87.22 genome, 10 Lrp genes were detected by bioinformatic analysis. However, the regulatory function of the Lrps regarding thaxtomin biosynthesis, morphological differentiation, and viability in *S. scabies* is unclear. In this study, we found that a novel protein SCAB_Lrp (SCAB_77931) among all Lrp homologues from *S. scabies* 87.22 exhibited the highest amino acid identity with previously reported actinomycete Lrps, implying that SCAB_Lrp may have similar regulatory functions to them. We therefore focused on SCAB_Lrp to investigate its function and mechanism in controlling thaxtomin production, morphological development, and pathogenicity.

2 | RESULTS

2.1 | Identification of an Lrp family homologue in *S. scabies*

The genomic sequence of *S. scabies* predicts that SCAB_77931 is a putative Lrp family homologue named SCAB_Lrp, exhibiting 30%–45% amino acid identity with previously reported actinomycete Lrps (Figure 1a). Specifically, SCAB_Lrp shares high similarities with SACE_5717 (45% identity) (Liu et al., 2019) and SACE_Lrp (36% identity) (Liu et al., 2017a) of *S. erythraea*, SLCG_Lrp (34% identity) of *S. lincolnensis* (Xu et al., 2020), SCO3361 (34% identity) of *S. coelicolor* (Liu et al., 2017b), and SSP_Lrp (30% identity) of *S. spiramyceticus* (Lu et al., 2019) (Figures 1b and S1). Furthermore, by analysing the amino acid sequences of SCAB_Lrp and the above actinomycete Lrps, we found their N-terminus with the helix-turn-helix DNA-binding motif was more conserved, while the C-terminus with the effector-binding domain was relatively variable (Figure 1b).

2.2 | SCAB_Lrp positively regulates the production of thaxtomin A

SCAB_Lrp gene contains 495 nucleotides (nt) and encodes a novel Lrp family protein of 164 amino acids with an unresolved regulatory function in *S. scabies*. To clarify the relationship between SCAB_Lrp and thaxtomin A production, the *SCAB_Lrp* gene was disrupted with *tsr* replacement in *S. scabies* 87.22 by the method of homologous chromosomal recombination (Figure 2a), and the resulting mutant Δ*SCAB_Lrp* was confirmed by PCR (Figure 2b). Fermentation and high-performance liquid chromatography (HPLC) analysis showed that the production of thaxtomin A in Δ*SCAB_Lrp* was only approximately 48% of that in the parent strain 87.22 cultured in the oat bran broth (OBB) liquid medium (Figure 2c). To investigate the effect of *SCAB_Lrp* deletion on bacterial growth, the biomass curves of mutant Δ*SCAB_Lrp* and parent strain 87.22 were examined. The

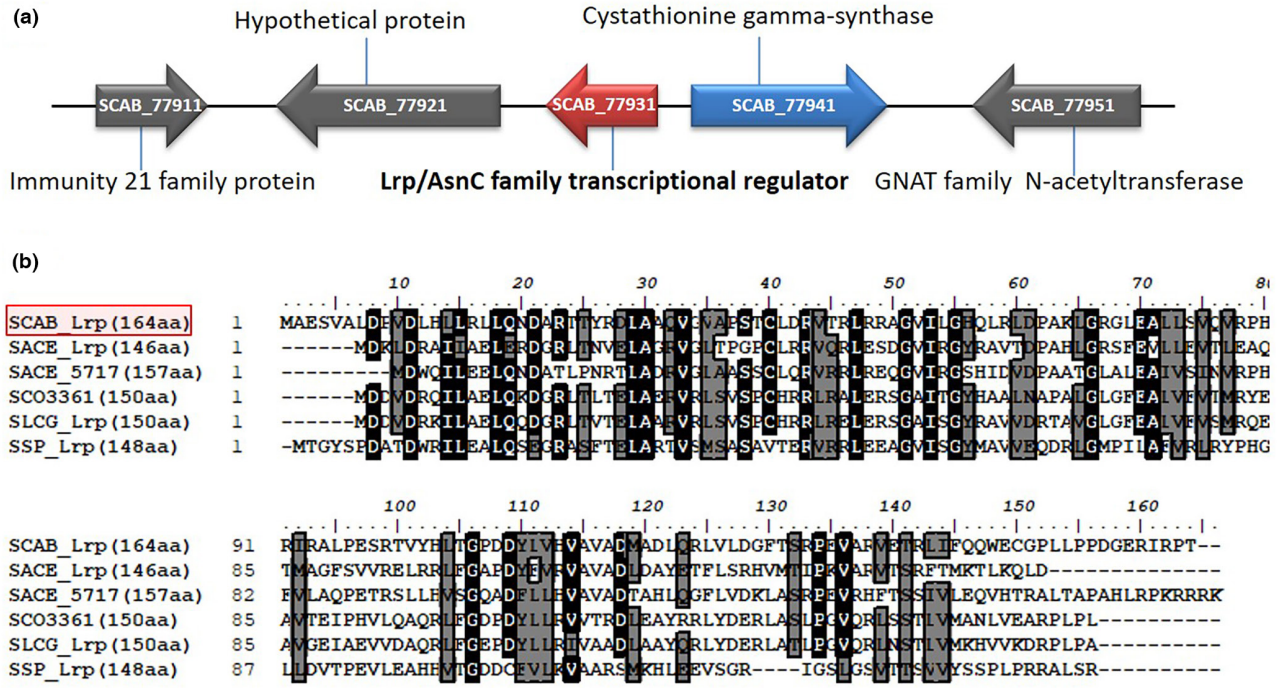

FIGURE 1 Identification of an Lrp family homologue in *Streptomyces scabies* 87.22. (a) Genetic locus organization of the SCAB_Lrp. (b) Sequence alignment of SCAB_Lrp and its homologues

results showed that Δ*SCAB_Lrp* accumulated a lower dry weight of mycelia than that of strain 87.22 (Figure 2d), and the yield of thaxtomin A in Δ*SCAB_Lrp* relative to the dry weight of mycelia was also decreased by 31%, demonstrating that the decreased thaxtomin yield in Δ*SCAB_Lrp* partly resulted from changes in cell growth. Thaxtomin A production and bacterial growth of complemented strain Δ*SCAB_Lrp/SCAB_Lrp* were restored to that of the parent strain 87.22 when a single copy of *SCAB_Lrp* was introduced into Δ*SCAB_Lrp* (Figure 2c,d). These results indicate that SCAB_Lrp of *S. scabies* is an important activator of thaxtomin A production and bacterial growth.

2.3 | SCAB_Lrp positively regulates virulence of *S. scabies*

Because thaxtomin A is the principal virulence determinant of pathogenic streptomycetes, we selected radish and *Arabidopsis thaliana* (ecotype Col-0) seedlings to evaluate the effect of *SCAB_Lrp* disruption on the virulence phenotype of *S. scabies*. Radish (Figure 3a) and *A. thaliana* (Figure 3b) seedlings were inoculated with the fermentation extract isolated from 87.22 and Δ*SCAB_Lrp*. After 3 days of growth, radish (Figure 3a) and *A. thaliana* seedlings (Figure 3b) inoculated with the fermentation broth of Δ*SCAB_Lrp* displayed reduced root and shoot stunting compared to those inoculated with 87.22. The average length of radish seedlings inoculated with Δ*SCAB_Lrp* was 3.18 cm, an increaseof 45% ($p < 0.05$) compared to 2.2 cm of seedlings inoculated with 87.22. Similarly, the average length of *A. thaliana* seedlings inoculated with Δ*SCAB_Lrp* was 1.24 cm, an increase of 48% ($p < 0.001$) compared to seedling length of 0.84 cm when inoculated with 87.22. In addition, a decreased capacity of Δ*SCAB_Lrp* to induce pitting and necrosis in potato tuber slices in comparison to 87.22 was observed (Figure 3c). Taken together, the disruption of *SCAB_Lrp* resulted in a low-virulence phenotype of *S. scabies*, demonstrating that SCAB_Lrp is the primary activator of plant pathogenicity through its effect on the production of thaxtomin and bacterial growth.

2.4 | SCAB_Lrp directly controls the expression of the *txt* cluster

To investigate the relationship between SCAB_Lrp and the *txt* cluster, transcriptional analyses of Δ*SCAB_Lrp* and 87.22 were performed during the thaxtomin production process. The *txt* cluster includes two polycistronic transcriptional units (*txtA* to *txtC* and *txtE* to *txtD*) and *txtR* (Figure 4a). *txtR* encodes only the CSR and is in the opposite orientation to *txtA* with an intergenic region of 1671 bp (Joshi et al., 2007). Three genes, *txtR*, *txtA*, and *txtE*, were selected for the reverse transcription-quantitative PCR (RT-qPCR) analyses. The results showed that the transcript amounts of *txtR*, *txtA*, and *txtE* were drastically decreased in Δ*SCAB_Lrp* compared to that in 87.22 (Figure 4b). These results demonstrate that SCAB_Lrp positively controls the transcription of thaxtomin biosynthesis genes in *S. scabies*.

The *txt* cluster of *S. scabies* contains three regulatory regions: putative *txtR*, *txtA*, and *txtE* promoter regions (Li et al., 2021) (Figure 4a). To investigate whether SCAB_Lrp directly binds to the

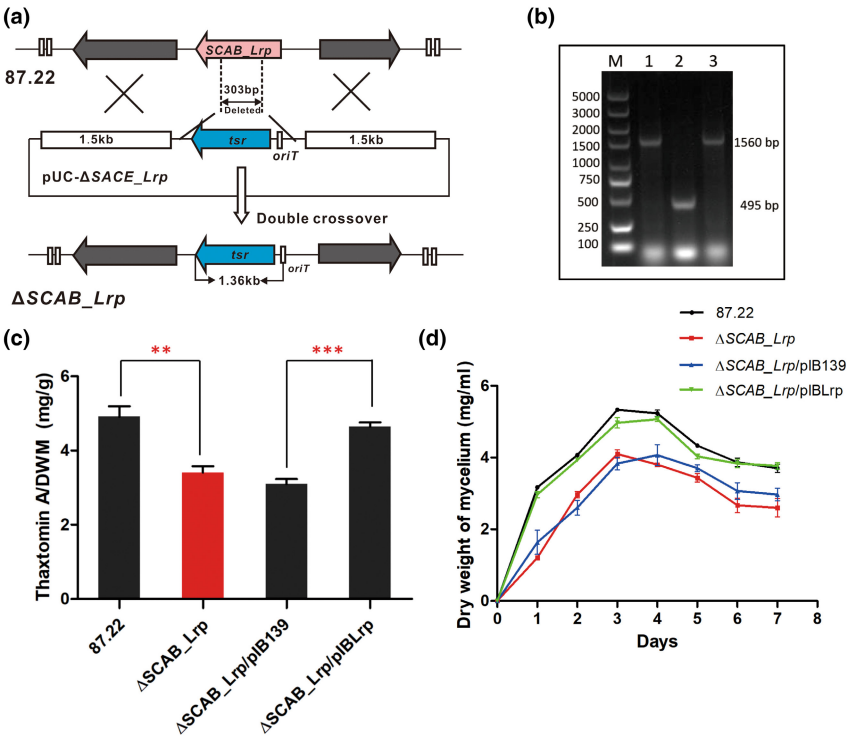

FIGURE 2 SCAB_Lrp positively regulates the production of thaxtomin A in *Streptomyces scabies* 87.22. (a) Schematic of deletion of *SCAB_Lrp* by homologous recombination in *S. scabies* 87.22. By the homologous chromosome recombination, a 303-bp fragment within the *SCAB_Lrp* gene was replaced by the *tsr* gene. (b) PCR confirmation of the *SCAB_Lrp* deletion mutant by the primers 77931-C-F/R. Lanes: M, 5000-bp DNA ladder; 1, the positive control, 1560 bp amplified from pUC-Δ*SCAB_Lrp*; 2, the negative control, 495 bp amplified from 87.22; 3, the sample, 1560 bp amplified from mutant Δ*SCAB_Lrp*. (c) HPLC analysis of thaxtomin A production in *S. scabies* 87.22 and its derivatives cultured for 7 days in oat bran broth (OBB). Mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{**}p < 0.01$, $^{***}p < 0.001$). (d) Growth curves of 87.22 and Δ*SCAB_Lrp*. The two strains were cultured in the OBB liquid medium and their dry weights of mycelia (DWM) were measured. Mean values of three replicates are shown, with the standard deviation indicated by error bars

regulatory regions of the *txt* cluster, electrophoretic mobility shift assays (EMSAs) were performed using purified His_6-tagged SCAB_Lrp (Figure 4c) and the corresponding probes (Table S2). The EMSA results showed that SCAB_Lrp could bind to the promoter region of the CSR gene *txtR*, and the SCAB_Lrp-P_{txtR} complex formed in a concentration-dependent manner (Figure 4d). However, no protein–DNA complex was formed when P_{txtA} or P_{txtE} was incubated with SCAB_Lrp (Figure 4d). We also used a biosensor system with green fluorescent protein (GFP) in *Escherichia coli* to verify the interaction of SCAB_Lrp with the probe P_{txtR} in vivo. As shown in Figure 2e, plasmid p*txtR*-E in which *egfp* gene was directly controlled by the promoters of *txtR* was transformed into *E. coli* DH5α as control. *SCAB_Lrp* gene driven by the promoter of the *aac(3)IV* gene was inserted into p*txtR*-E and transformed into *E. coli* DH5α. When *SCAB_Lrp* gene was inserted into the p*txtR*-E, the green fluorescence was enhanced by 76% compared to that without *SCAB_Lrp* (Figure 2f). However, when *SCAB_Lrp* gene was inserted into the p*txtA*-E or p*txtE*-E, the green fluorescence was almost unchanged compared to that without SCAB_Lrp (Figure 2f).

Taken together, these results demonstrated that SCAB_Lrp affects thaxtomin biosynthesis by directly activating the expression of the CSR gene *txtR* in *S. scabies*.

2.5 | SCAB_Lrp is directly involved in the morphological differentiation of *S. scabies*

To investigate the role of *SCAB_Lrp* disruption on morphological differentiation, 87.22 and Δ*SCAB_Lrp* spores were grown on a soy flour mannitol (SFM) agar for phenotypic observation. Compared with 87.22, Δ*SCAB_Lrp* showed significantly delayed spore formation, and the morphological 87.22 phenotype in Δ*SCAB_Lrp/SCAB_Lrp* was recovered (Figure 5a), indicating that SCAB_Lrp has a significant effect on the morphological development of *S. scabies*.

RT-qPCR assays were performed on three genes related to morphological differentiation of *Streptomyces*: *amfC* (*SCAB_49711*), *whiB* (*SCAB_55081*), and *ssgB* (*SCAB_53351*). The expression levels of *amfC*, *whiB*, and *ssgB* were dramatically decreased in Δ*SCAB_Lrp* compared to that in 87.22 (Figure 5b). Moreover, the binding affinity of SCAB_Lrp to the promoter regions of *amfC*, *whiB*, or *ssgB* was assessed by EMSAs, and SCAB_Lrp could directly bind to the promoters of these three genes to different extents (Figure 5c). In particular, there were two shifted bands in SCAB_Lrp incubated with the probes P_{amfC} and P_{whiB}, while only one shifted band appeared after SCAB_Lrp was incubated with P_{ssgB} (Figure 5c). Results from GFP reporter assays showed that when the *SCAB_Lrp* gene was

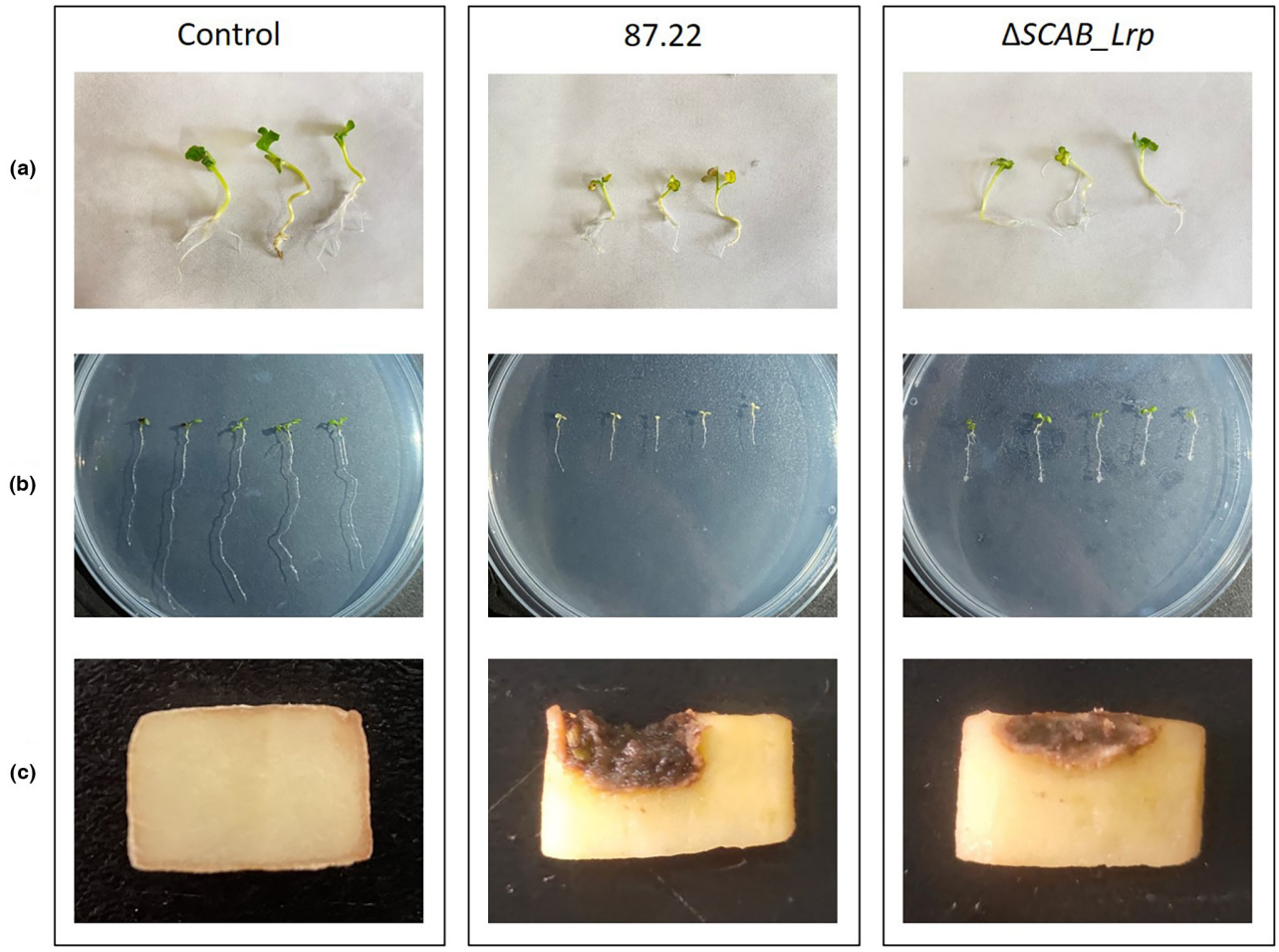

FIGURE 3 SCAB_Lrp positively regulates virulence of *Streptomyces scabies* 87.22. (a) Phenotype of radish seedlings grown for 6 days in the presence of 87.22 and its mutant Δ*SCAB_Lrp*. (b) Phenotype of *Arabidopsis thaliana* seedlings grown for 3 days in the presence of 87.22 and its mutant Δ*SCAB_Lrp*. (c) Phenotype of potato tuber slices inoculated with 87.22 and its mutant Δ*SCAB_Lrp* for 7 days

expressed in the presence of p*amfC*-E, p*whiB*-E and p*ssgB*-E, the green fluorescence was enhanced by 111%, 58%, and 82% compared to that without *SCAB_Lrp* (Figure 5d). These results suggest that SCAB_Lrp positively controls sporulation by directly activating the expression of the genes *amfC*, *whiB*, and *ssgB*.

Based on the above findings, SCAB_Lrp was identified to directly regulate thaxtomin biosynthesis and control the morphological development of *S. scabies*.

2.6 | Identification of the binding site and the amino acid effector of SCAB_Lrp

Based on the regulatory model of the Lrp, we hypothesized that SCAB_Lrp might regulate the transcription of its own gene, so the expression level of *SCAB_Lrp* in Δ*SCAB_Lrp* was examined. Through measuring the remaining 99-bp 5′-region of *SCAB_Lrp* gene, the transcript amount of *SCAB_Lrp* in Δ*SCAB_Lrp* was found to be slightly increased compared to that in 87.22 at 24 h but dramatically decreased at 48 h (Figure 6a). Furthermore, in vitro, EMSAs results showed that an evidently shifted band appeared after SCAB_Lrp incubation with probe P_{77931} (the promoter region of *SCAB_Lrp*), confirming that SCAB_Lrp could specifically bind to the promoter of its own gene with the highest affinity compared to the other probes mentioned above (Figure 6b).

SCAB_Lrp shares the highest similarity with SACE_5717 of *S. erythraea*. The DNA-binding site of SACE_5717 (5′-GAACGTTC GCCGTCACGCC-3′) has previously been reported (Liu et al., 2019) and this was used to search for a SCAB_Lrp potential binding site. In sequence BLAST analysis, a 19 bp highly similar sequence (5′-GGACAGTCGCCGTGCTACG-3′) was identified in P_{77931}, which lay in the *SCAB_Lrp* promoter region (named as site OP) (Figure 6b). To detect whether SCAB_Lrp directly interacts with the site OP, EMSAs were performed using SCAB_Lrp and the mutated probe $P_{d(siteOP)}$ derived from P_{77931} with the site OP deleted. The SCAB_Lrp-P_{77931} complex completely disappeared when SCAB_Lrp was incubated with $P_{d(siteOP)}$, indicating that SCAB_Lrp specifically binds to its own promoter by directly interacting with the OP site (Figure 6b).

Lrp transcriptional regulators respond to various amino acids (Ziegler & Freddolino, 2021). To investigate the potential effectors of SCAB_Lrp, a series of EMSAs was conducted using SCAB_Lrp

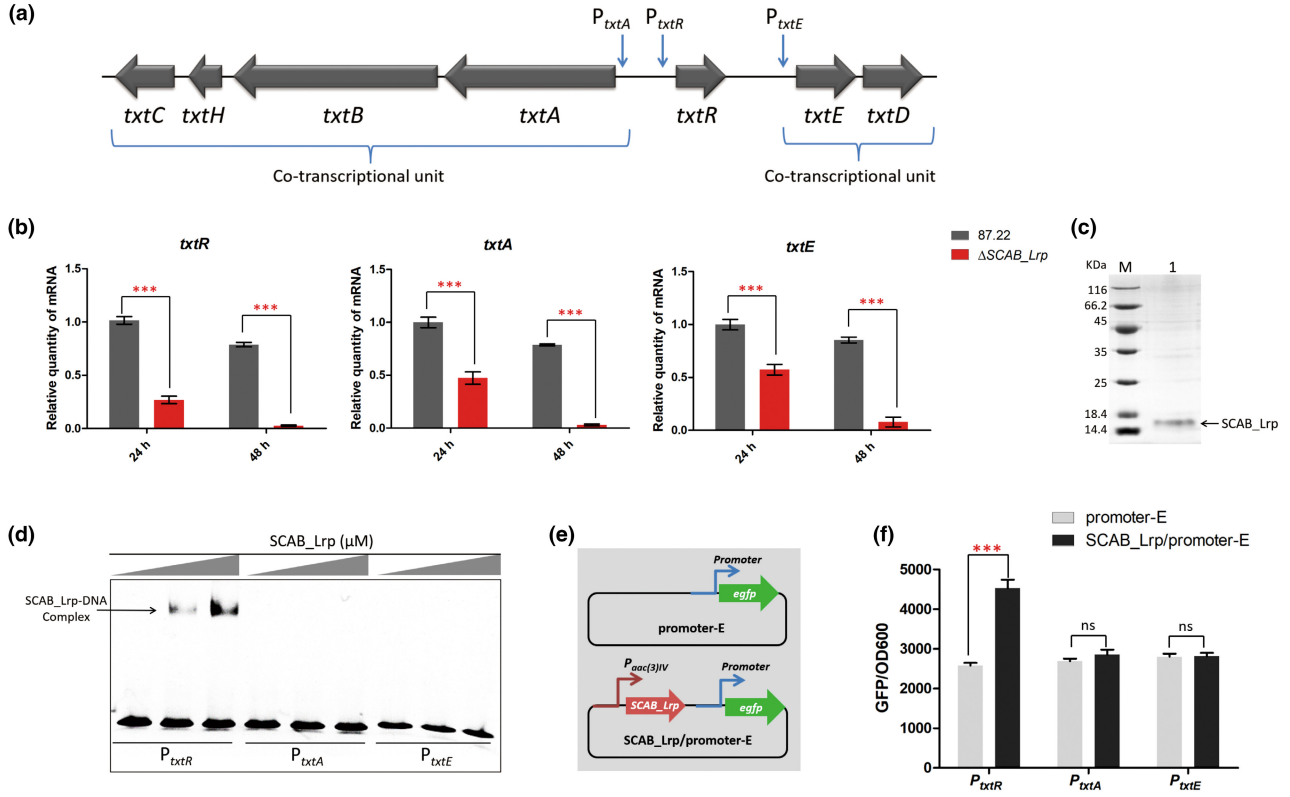

FIGURE 4 SCAB_Lrp directly controls the expressions of the *txt* biosynthetic cluster. (a) Organization of cotranscriptional units in the thaxtomin biosynthetic gene cluster. (b) Effects of *SCAB_Lrp* disruption on transcriptional levels of *txtR*, *txtA*, and *txtE*. Reverse transcription-quantitative PCR was used to quantify the amounts of transcripts in 87.22 and Δ*SCAB_Lrp* cultured for 24 and 48 h in liquid oat bran broth medium. Mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{***}p < 0.001$). (c) SDS-PAGE analysis of purified His_6-SCAB_Lrp. (d) Electrophoretic mobility shift assays of binding of SCAB_Lrp to P_{txtR}, P_{txtA} and P_{txtE}. The amounts of SCAB_Lrp used were 0, 1 and 2 μM. The probe P_{txtR} represents the promoter region of *txtR*, P_{txtA} represents the promoter region of *txtA-txtB-txtH-txtC*, and P_{txtE} represents the promoter region of *txtE-txtD*. (e) An illustration of the GFP reporter plasmids in biosensor system. (f) Detection of the interaction of SCAB_Lrp with the promoters of *txtR*, *txtA*, and *txtE* using the relative fluorescence (GFP/OD_{600}) in *Escherichia coli*. The mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{***}p < 0.001$, ns, not significant)

and P_{77931} present with different protein amino acids (Figure S2). The results showed that adding methionine (Figure 6c) and phenylalanine (Figure 6c) could enhance the binding affinity between the SCAB_Lrp and P_{77931} probes, resulting in a new upper-shifted band of the protein–DNA complex. However, we did not identify any amino acids that could reduce the binding activity of SCAB_Lrp to P_{77931} (Figure S2). We also used the above biosensor system with GFP to assess the interaction of SCAB_Lrp with its effectors in vivo. Results showed that the green fluorescence was significantly enhanced by 36% and 59% in DH5α/pLrp-*77931*-E when 10 mM methionine and phenylalanine was added to the culture medium, respectively (Figure 6d). As the control, the green fluorescence showed no difference in DH5α/p*77931*-E after the addition of 10 mM methionine or phenylalanine (Figure 6d). Moreover, we found that similar results occurred in DH5α/pLrp-*txtR*-E, DH5α/pLrp-*amfC*-E, DH5α/pLrp-*whiB*-E, and DH5α/pLrp-*ssgB*-E, and the green fluorescence was enhanced to different degrees when 10 mM methionine and phenylalanine were added to the culture medium (Figures 6e and S3). Taken together, these findings suggest that methionine and phenylalanine are indeed the effectors of SCAB_Lrp.

3 | DISCUSSION

The most important regulator of thaxtomin production is the TxtR protein, which acts as a transcriptional activator and induces transcription of the *txt* cluster (Joshi et al., 2007). TxtR is the primary regulator of thaxtomin production and virulence (Joshi et al., 2007). Another important regulator, CebR, has been characterized as a primary inhibitor of thaxtomin A biosynthesis via direct binding to two sites in the *txt* cluster (Francis et al., 2015). Cellobiose and cellotriose have been identified as ligands for CebR and mediate its binding affinity to DNA, thereby improving the expression of the genes in *txt* cluster (Francis et al., 2015). In this study, the third important regulator, SCAB_Lrp, of the Lrp family protein from *S. scabies*, was characterized as a master activator of thaxtomin biosynthesis and morphological development by directly modulating the transcription

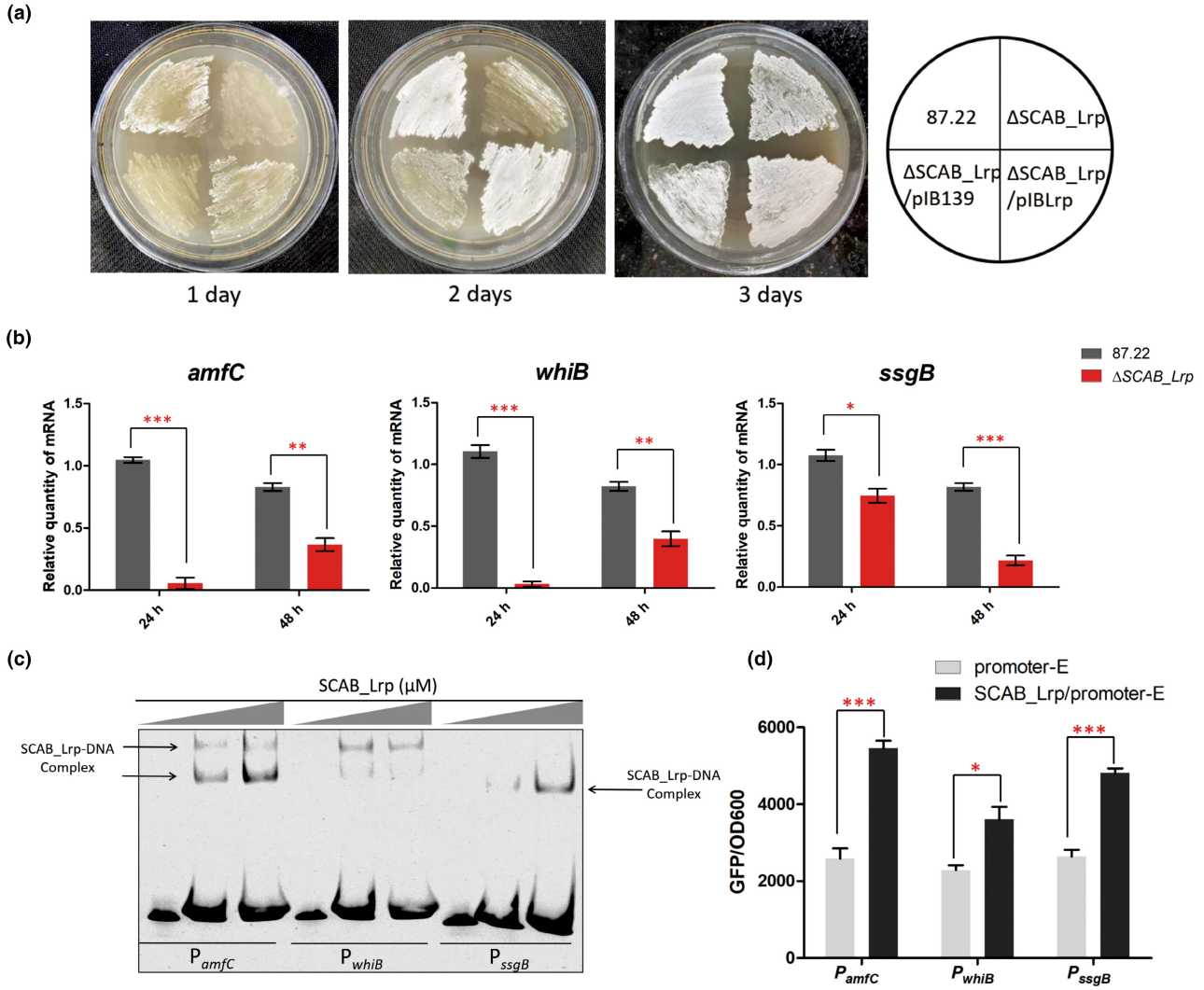

FIGURE 5 SCAB_Lrp is directly involved in the morphological differentiation of *Streptomyces scabies* 87.22. (a) Phenotypes of 87.22, Δ*SCAB_Lrp*, Δ*SCAB_Lrp*/pIB139, and Δ*SCAB_Lrp*/pIBLrp grown on soy flour mannitol agar for 1–3 days. (b) Effects of *SCAB_Lrp* disruption on transcriptional levels of genes *amfC*, *whiB*, and *ssgB* involved in morphological differentiation. Reverse transcription-quantitative PCR was used to quantify the amounts of transcripts in 87.22 and Δ*SCAB_Lrp* cultured for 24 and 48 h in liquid oat bran broth medium. Mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$). (c) Electrophoretic mobility shift assays of binding of SCAB_Lrp to P_{amfC}, P_{whiB}, and P_{ssgB}. P_{amfC}, P_{whiB}, and P_{ssgB} represent the promoter regions of *amfC*, *whiB*, and *ssgB*. The amounts of SCAB_Lrp used were 0, 1, and 2 μM. (d) Detection of the interaction of SCAB_Lrp with the promoters of *amfC*, *whiB*, and *ssgB* using the relative fluorescence (GFP/OD_{600}) in *Escherichia coli*. The mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{*}p<0.05$, $^{***}p<0.001$)

of the CSR gene *txtR* and sporulation-associated genes *amfC*, *whiB*, and *ssgB*, as summarized in Figure 7. Our findings broaden our limited insight into the molecular mechanisms underlying the role of transcriptional regulators in thaxtomin biosynthesis and morphogenesis in *S. scabies*.

Previous studies have revealed that Lrp family proteins are crucial regulators of diverse biological processes, such as amino acid metabolism, and respond to various amino acids in various bacterial species (Ziegler & Freddolino, 2021). Recently, significant advances in understanding the regulatory mechanism controlling antibiotic biosynthesis by Lrp regulators have been made (Liu et al., 2017a, 2017b, 2019, 2021; Lu et al., 2019; Xu et al., 2020). Furthermore, this study demonstrated that a novel Lrp family protein, SCAB_Lrp, from plant-pathogenic *S. scabies*, is directly involved in thaxtomin biosynthesis, pathogenicity, and sporulation. Thus far, this study has established a concrete regulatory relationship between Lrp family regulators and thaxtomin phytotoxins for the first time, expanding the function of the Lrp on secondary metabolism and morphogenesis from nonpathogenic to pathogenic in the large genus *Streptomyces*.

The plant-pathogenic bacterium *Erwinia amylovora* causes the devastating fire blight disease in apple and pear trees (Geier & Geider, 1993; Pusey, 2000). Lrp from *E. amylovora* Ea1189 is involved in the virulence expression and several virulence-associated

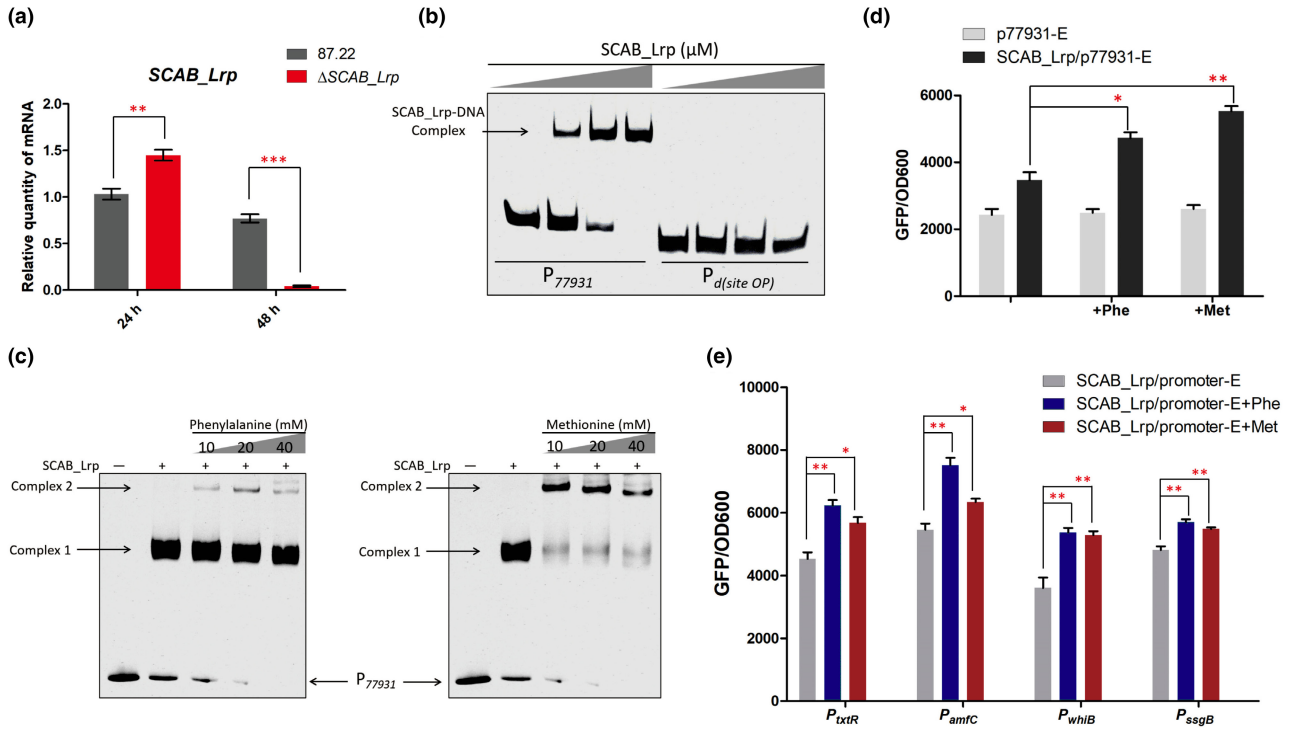

FIGURE 6 Identification of the binding site and the amino acid effector of SCAB_Lrp. (a) The transcriptional level of *SCAB_Lrp* in 87.22 and Δ*SCAB_Lrp* by reverse transcription-quantitative PCR analysis. Mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{**}p<0.01$, $^{***}p<0.001$). (b) Electrophoretic mobility shift assay (EMSA) of binding of SCAB_Lrp to the probes P_{77931} and $P_{d(site\ OP)}$. The probe P_{77931} represents the promoter region of *SSAB_Lrp* and the $P_{d(site\ OP)}$ is derived from P_{77931} by deleting the site OP. The amounts of SCAB_Lrp used were 0, 250, 500, and 1000 nM. (c) EMSAs of binding affinity of SCAB_Lrp to the probe P_{77931} in presence of phenylalanine and methionine. The amount of SCAB_Lrp used was 500 nM. (d) Detection of the relative fluorescence (GFP/OD_{600}) after adding phenylalanine and methionine in *Escherichia coli* DH5α/p*77,931*-E and DH5α/pLrp-*77931*-E. The mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{*}p<0.05$, $^{**}p<0.01$). (e) Detection of the relative fluorescence (GFP/OD_{600}) after adding phenylalanine and methionine in *E. coli* DH5α/pLrp-promoter-E strains. The mean values of three replicates are shown, with the standard deviation indicated by error bars (Student's *t* test; $^{*}p<0.05$, $^{**}p<0.01$)

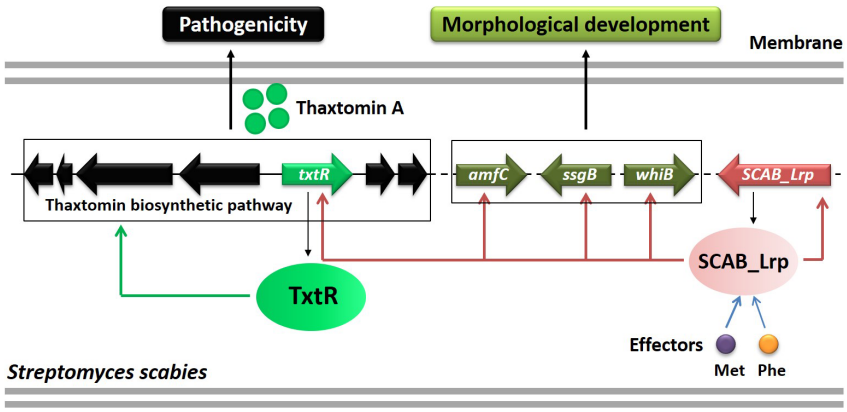

FIGURE 7 Proposed model of the regulatory roles of SCAB_Lrp in control of thaxtomin biosynthesis, pathogenicity, and morphological development in *Streptomyces scabies* 87.22

traits, including biofilm formation, levansucrase activity, and production of the exopolysaccharide amylovoran (Schachterle & Sundin, 2019). In our study, SCAB_Lrp from *S. scabies* 87.22 is implicated in bacterial growth and production of thaxtomin A. These findings indicated that the role of regulatory mechanisms of Lrp regulators on pathogenicity and virulence might be numerous and complex in different pathogenic bacteria. More interestingly, Lrp is directly regulated at the posttranscriptional level by the small RNA ArcZ through destabilization of Lrp mRNA in *E. amylovora* Ea1189 (Schachterle & Sundin, 2019), prompting us to explore further the topology of Lrp in the virulence regulatory network in *S. scabies*.

Previous studies have shown that the secondary or hierarchical regulatory function of Lrp, which directly controls other regulators, is rare. Our previously reported Lrp SCO3361 directly regulated the *actII-ORF4* of CSR gene to control the biosynthesis

of actinorhodin in *S. coelicolor* (Liu et al., 2017b), which is very similar to the case of SCAB_Lrp in *S. scabies* (Figure 4). SSP_Lrp directly modulates three regulatory genes to control the biosynthesis of spiramycin and bitespiramycin in *S. spiramyceticus* (Lu et al., 2019). Recently, we reported that SACE_Lrp directly controls the MarR family protein SACE_6745, which plays an essential regulatory role in erythromycin biosynthesis and export (Liu et al., 2021).

Regarding morphological development, SCAB_Lrp directly activated the expression of *amfC*, *whiB*, and *ssgB*, thereby affecting the morphological development of *S. scabies* (Figure 5). In contrast, in *S. coelicolor*, SCO3361 activates the transcription of *amfC*, *whiB*, and *ssgB* but only directly binds to the *amfC* promoter region (Liu et al., 2017b). Furthermore, two shifted bands appeared after SCAB_Lrp incubation with the *amfC* promoter region (Figure 5c), whereas only one shifted band appeared after SCO3361 incubation with the *amfC* promoter region (Liu et al., 2017b). These findings indicate that the regulatory model of the role of Lrps in morphological development may vary in the large genus *Streptomyces*.

The Lrp family proteins generally utilize amino acids as structural effectors (Ziegler & Freddolino, 2021). In our previous studies, SLCG_Lrp (Xu et al., 2020), SCO3361 (Liu et al., 2017b), and SACE_Lrp (Liu et al., 2017a) responded to bidirectional amino acid effectors, either increasing or decreasing the DNA-binding affinity of the protein. SACE_5717 (Liu et al., 2019) and SCAB_Lrp exhibited only a one-directional response to their respective effectors. Notably, tryptophan, tyrosine, and arginine decreased the DNA-binding affinity of SACE_5717 (Liu et al., 2019), whereas methionine and phenylalanine increased the DNA-binding affinity of SCAB_Lrp, resulting in a new protein–DNA complex (Figure 6c). Although SCAB_Lrp shares the highest similarity to SACE_5717 (45% identity), it responds to different amino acids, probably because the ligand-binding domain in its C-terminal is not conserved to interact with a wide variety of effectors (Figure 1). These findings demonstrate the sophisticated mechanism by which Lrps respond to different amino acid effectors. The response of SCAB_Lrp to methionine and phenylalanine may have more important biological significance, and the plant pathogenicity of *S. scabies* could be altered by changing the concentration of methionine and phenylalanine in the environment to coordinate the affinity between SCAB_Lrp and its target.

4 | EXPERIMENTAL PROCEDURES

4.1 | Bacterial strains and cultivation conditions

The plasmids, *E. coli* strains, *S. scabies*, and its derivatives used in present work are listed in Table S1. *E. coli* strains were cultured in liquid or on solid Luria Bertani (LB) medium at 37°C. *E. coli* DH5α was used for plasmid construction, *E. coli* ET12567 (pUZ8002) was used as the donor host for plasmid conjugation to *S. scabies*, and *E. coli* BL21 (DE3) was used for overproduction of SCAB_Lrp protein. *S. scabies* and its derivatives were cultured at 28°C on SFM solid medium for sporulation and phenotypic observation, or in tryptic soy broth (TSB) liquid medium for growth of mycelia and seed culture (Kieser et al., 2000). In addition, OBB liquid medium was used for thaxtomin production (Francis et al., 2015). The antibiotics were added at the following concentrations where required: ampicillin 100 μg/ml, apramycin 100 μg/ml, kanamycin 50 μg/ml, and chloramphenicol 25 μg/ml for *E. coli* strains; thiostrepton 25 μg/ml, nalidixic acid 50 μg/ml, and apramycin 100 μg/ml for *S. scabies* strains.

4.2 | Deletion mutant construction

To generate the *SCAB_Lrp* deletion mutant in *S. scabies* 87.22, two 1500 bp flanking fragments of *SCAB_Lrp* were prepared by PCR with the corresponding primers (Table S2). The two fragments were treated with *Kpn*I/*Eco*RI and *Xba*I/*Hin*dIII, respectively, and then cloned into the vector pUCTSR (Han et al., 2011) to obtain recombinant plasmid pUC-Δ*SCAB_Lrp*. Subsequently, pUC-Δ*SCAB_Lrp* was transferred into *S. scabies* 87.22 by conjugation with *E. coli* ET12567 (pUZ8002). By the homologous chromosomic recombination, a 303-bp fragment of the *SCAB_Lrp* gene was replaced by the thiostrepton resistance gene (*tsr*) in *S. scabies* 87.22, with 99-bp initial part of *SCAB_Lrp* gene remained. The mutant Δ*SCAB_Lrp* with thiostrepton resistance was confirmed by PCR with the corresponding primers (Table S2).

4.3 | Gene complementation

To construct the complementation strain Δ*SCAB_Lrp*/pIB-*SCAB_Lrp*, full-length *SCAB_Lrp* gene of 495-bp was prepared by PCR from the 87.22 genomic DNA with the corresponding primers (Table S2). The obtained fragment was inserted into the vector pIB139 (Wilkinson et al., 2002) by *Nde*I/*Xba*I sites to construct plasmid pIB-*SCAB_Lrp*. By conjugation, pIB-*SCAB_Lrp* and pIB139 were introduced into the mutant Δ*SCAB_Lrp*, generating the complemented strains Δ*SACE_Lrp*/*SACE_Lrp* and Δ*SACE_Lrp*/pIB139, respectively, by apramycin-screening and PCR with the corresponding primers (Table S2).

4.4 | Determination of thaxtomin A production

Quantified 10^7 spores of *S. scabies* 87.22 and its derivatives were inoculated into 50 ml of TSB liquid medium with shaking at 28°C, 220 rpm for 2 days as a seed culture, and then 5 ml of seed culture was transferred into 50 ml of OBB liquid medium at 28°C with shaking at 220 rpm for 7 days. For thaxtomin A analysis, the extracts were isolated and analysed by an HPLC system with a Wondasil C18 Superb column (4.6 × 150 mm, 5 μm; GC Sciences) as previously described (Francis et al., 2015; Jourdan et al., 2016). The production of thaxtomin A was quantified by a standard curve generated using an authentic thaxtomin A standard (AbMole BioScience).

4.5 | Determination of cell growth

The biomass of *S. scabies* 87.22 and mutant Δ*SCAB_Lrp* were measured as previously described (Wu et al., 2014) to determine the effect of *SCAB_Lrp* deletion on cell growth. Cultivation of 87.22 and Δ*SCAB_Lrp* was as described in Section 4.4. One millilitre of culture samples was collected once a day and followed by centrifugation. Cell pellets were washed twice with sterile water and dried until constant weight. The dry weight of cell pellets was measured with a precise analytical balance.

4.6 | RNA extraction and RT-qPCR experiment

Total cells from *S. scabies* 87.22 and mutant Δ*SCAB_Lrp* were prepared by centrifugation, and total RNA was extracted using TransZol reagent (TransGen Biotech) at 24 and 48 h of growth in OBB liquid medium. Briefly, isolated RNA was treated with DNase I (TransGen Biotech) and cDNA synthesis was achieved by reverse transcription with a cDNA synthesis kit (TransGen Biotech). Quantitative real-time PCR was performed on the Applied Biosystems QuantStudio 6 Flex system, and all operation procedures were performed in accord with the manufacturer's instructions (TransGen Biotech). The 16S rRNA gene from *S. scabies* served as an internal control to normalize samples. All primers used for RT-qPCR assay are listed in Table S2.

4.7 | SCAB_Lrp protein overproduction and purification

The coding sequence of the *SCAB_Lrp* (*SCAB77931*) gene was amplified from the *S. scabies* 87.22 genome by PCR using the corresponding primers (Table S2), and inserted into the *Hin*dIII/*Nde*I sites of pET28a to construct pET-*SCAB_Lrp* plasmid. pET-*SCAB_Lrp* was transferred into *E. coli* BL21 (DE3) for SCAB_Lrp overproduction. Overproduction and purification of His_6-tagged SCAB_Lrp were carried out as previously described (Liu et al., 2017a).

4.8 | EMSA

The promoter regions of *txtR* (*SCAB_31801*), *txtA* (*SCAB_31791*), *txtE* (*SCAB_31831*), *amfC* (*SCAB_49711*), *whiB* (*SCAB_55081*), *ssgB* (*SCAB_53351*), and *SACB_Lrp* (*SCAB_77,931*) were amplified from 87.22 genomic DNA by PCR with the respective primers (Table S2). The probes of PCR products were purified, quantified, and then used for EMSA (Hellman & Fried, 2007). All probes were individually incubated with His_6-tagged SCAB_Lrp in binding buffer as described previously (Liu et al., 2017a).

For analysis of the potential amino acid-effectors of SCAB_Lrp, 20 natural amino acids were individually incubated with His_6-tagged SCAB_Lrp protein and the probe P_{77931} as described previously (Liu et al., 2017a).

4.9 | Plant virulence bioassays

Virulence assays of *S. scabies* strains on radish seedlings were prepared as described previously (Jourdan et al., 2016). Germinated radish seeds were placed into six agar wells formed in a deep 1.5% water agar plate and were inoculated each with 200 μl of fermentation extract, which was isolated from *S. scabies* 87.22 or mutant Δ*SCAB_Lrp* as previously described (Francis et al., 2015, Jourdan et al., 2016). The plates were incubated at 21 ± 2°C under a 16 h photoperiod for 6–7 days. Virulence assays of *S. scabies* strains on *Arabidopsis thaliana* (ecotype Col-0) seedlings were prepared as described previously (Francis et al., 2015). *A. thaliana* seedlings were transferred and inoculated with 200 μl of fermentation samples of 87.22 or Δ*SCAB_Lrp*. The plates were incubated at 21 ± 2°C, with a 12 h photoperiod, for 3–4 days. The virulence phenotypes of *S. scabies* strains on potato tuber slices were assessed as described previously (Loria et al., 1995). Quantified 10^7 spores of *S. scabies* 87.22 and mutant Δ*SCAB_Lrp* were cultured on SFM plates until well sporulated. Agar plugs with mature spores were prepared and then inverted onto the potato tuber slices. The tuber slices were then incubated at 23 ± 2°C in a dark and moist incubator for 7–8 days.

4.10 | GFP reporter assay

For construction of the GFP reporter plasmids, a fragment containing the putative promoter region of *txtR* was amplified from the 87.22 genome by PCR with PtxtR-F and PtxtR-egfp-R primers (Table S2), and the enhanced green fluorescent protein gene (*egfp*) fragment was amplified from pUPW-EGFP (Liu et al., 2017a) by PCR with PtxtR-egfp-F and egfp-R primers. The two fragments were together used as templates for an overlapping PCR with PtxtR-F and egfp-R primers (Table S2) to obtain the P*txtR-egfp* fragment, then digested with *Bam*HI/*Eco*RI and joined into pKC1139 to create the control plasmid p*txtR*-E. To evaluate the regulatory effect of SCAB_Lrp on *txtR* gene, P*aac(3)IV* promoter was amplified from pIB139 (Wilkinson et al., 2002) using primers Paac(3)IV -F and Paac(3)IV-Lrp-R (Table S2) and the *SCAB_Lrp gene* was amplified from the 87.22 genome using primers Paac(3)IV-Lrp-F and Lrp-R (Table S2), respectively. The two fragments were together used as templates for an overlapping PCR with Paac(3)IV -F and Lrp-R primers (Table S2) to obtain the P*aac(3)IV-SCAB_Lrp* fragment, then digested with *Xba*I/*Hin*dIII and joined into p*txtR*-E to create the plasmid pLrp-*txtR*-E. The above method was also used to obtain the other reporter plasmids with corresponding primers listed in Table S2. These plasmids were transformed into *E. coli* DH5α. Green fluorescence was detected by excitation at 485 nm, and emission at 510 nm (Molecular Devices). All fluorescence values were normalized to growth rates (OD_{600}).

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CONFLICT OF INTEREST

All authors declare they have no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

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

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