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LsNRL4 enhances photosynthesis and decreases leaf angles in lettuce

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Summary

Lettuce (*Lactuca sativa*) is one of the most important vegetables worldwide and an ideal plant for producing protein drugs. Both well-functioning chloroplasts that perform robust photosynthesis and small leaf angles that enable dense planting are essential for high yields. In this study, we used an F_2 population derived from a cross between a lettuce cultivar with pale-green leaves and large leaf angles to a cultivar with dark-green leaves and small leaf angles to clone *LsNRL4*, which encodes an NPH3/RPT2-Like (NRL) protein. Unlike other NRL proteins in lettuce, the LsNRL4 lacks the BTB domain. Knockout mutants engineered using CRISPR/Cas9 and transgenic lines overexpressing *LsNRL4* verified that *LsNRL4* contributes to chloroplast development, photosynthesis and leaf angle. The *LsNRL4* gene was not present in the parent with pale-green leaves and enlarged leaf angles. Loss of *LsNRL4* results in the enlargement of chloroplasts, decreases in the amount of cellular space allocated to chloroplasts and defects in secondary cell wall biosynthesis in lamina joints. Overexpressing *LsNRL4* significantly improved photosynthesis and decreased leaf angles. Indeed, the plant architecture of the overexpressing lines is ideal for dense planting. In summary, we identified a novel *NRL* gene that enhances photosynthesis and influences plant architecture. Our study provides new approaches for the breeding of lettuce that can be grown in dense planting in the open field or in modern plant factories. *LsNRL4* homologues may also be used in other crops to increase photosynthesis and improve plant architecture.

Introduction

Due to the continued expansion of urban areas, loss of soil fertility and climate change, agricultural land is increasingly becoming a scarce resource (Foley *et al.,* 2011; Lambin *et al.,* 2013; Pandey and Seto, 2015). Thus, to use agricultural land efficiently, we have a critical need to improve photosynthesis in crop plants that can produce high yields when grown in high-density conditions. Another approach to improve land-use efficiency is the deployment of plant factories. To make plant factories profitable and sustainable, it is critical to grow crops such as lettuce (*Lactuca sativa*) with high photosynthesis efficiencies and small leaf angles (Touliatos *et al.,* 2016).

Chloroplast, converting solar energy into biologically usable forms of energy, is one of the most critical organelles to improve land-use efficiency and crop yields (Russo *et al.,* 2019). Genes regulating chloroplast development and chlorophyll biosynthesis are part of a complex network that responds to light and hormones that can be exploited to improve photosynthesis and the production of biomass (Cackett *et al.,* 2022). For example, the *GOLDEN2-LIKE* genes (*GLKs*) regulate chloroplast development to increase photosynthesis and consequently, enhance both the biomass and grain yields in crops (Li *et al.,* 2020b; Yeh *et al.,* 2022). Each plant cell has many chloroplasts, which lack gene silencing or position effects, providing a perfect system for uniform and high levels of expression of introduced foreign genes (Daniell *et al.,* 2021).

Leaf angle, as a major component of ideal plant architecture, is an important agronomic trait that affects the efficiency of photosynthesis and crop yields (Dong *et al.,* 2018). For example, *AUXIN RESPONSE FACTORS* and *PUT ON WEIGHT1*, regulate leaf angle in response to auxin and brassinosteroids, respectively, and the development of compact plant architecture to increase yield in rice (Huang *et al.,* 2021; Zhang *et al.,* 2021). *ZmILI1* and *Upright Plant Architecture2* regulate leaf angle in response to cytokinins and brassinosteroids, respectively, decrease leaf angle to facilitate high-density planting and consequently, improve yields in maize (Ren *et al.,* 2020; Tian *et al.,* 2019). The secondary cell wall determines the mechanical strength of lamina joints and plays an important role in the regulation of leaf angle (Huang *et al.,* 2021). Although the mechanisms underlying leaf angle are intensively studied in monocots, factors that contribute to leaf angle in dicots are largely unknown (Jin *et al.,* 2021).

The NPH3/RPT2-Like (NRL) protein family is prevalent in algae and land plants. The NRL family has three main domains, including an N-terminal BTB (bric-a-brac, tram track and broad complex) domain, a central NPH3 domain and a C-terminal coiled-coil domain (Liscum *et al.,* 2014). The BTB domain interacts with Cullin3 *in vivo* to form an E3 ligase, with the BTB protein subunit and functions in substrate recognition (Figueroa *et al.,* 2005). The coiled-coil domain is proposed to facilitate the localization of NRLs to the plasma membrane and to bind phototropin (Inoue *et al.,* 2008). The NRL family is involved in

many important biological pathways, although the functions of the majority of NRL proteins remain unknown (Christie *et al.,* 2018). The NRL homologues studied so far are mainly involved in phototropism, petiole positioning, leaf expansion, chloroplast accumulation and the regulation of auxin transport (Cheng *et al.,* 2007; Christie *et al.,* 2018). For example, PHOTOTROPIC HYPOCOTYL 3 (NPH3) and ROOT PHOTOTROPISM 2 (RPT2), two members of the NRL family, are localized on the plasma membrane and interact with phototropin to modulate phototropism in Arabidopsis (Fankhauser and Christie, 2015; Liscum *et al.,* 2014). RPT2 and NRL PROTEIN FOR CHLOROPLAST MOVEMENT 1 (NCH1) mediate chloroplast accumulation in land plants (Suetsugu *et al.,* 2016). It remains unclear whether other NRLs contribute to chloroplast development.

The NRL4 and NRL12 proteins are two unique members of the NRL family in Arabidopsis because they lack the conserved BTB domain (Pedmale *et al.,* 2010). The lack of the BTB domain provides evidence that NRL4, NRL12 and their orthologs cannot interact with Cullin3 and therefore, do not contribute to the ubiquitin/proteasome pathway (Figueroa *et al.,* 2005). Hence, NRLs lacking the BTB domain and the NRLs containing the BTB domain may have different functions. Nonetheless, the impact of deficiencies in the BTB domain on the function of NRLs remains unknown. Therefore, to gain a better understanding of the NRL family, we have a critical need to study the functions of NRL proteins that lack the BTB domain.

Lettuce (*Lactuca sativa*) is one of the most popular vegetables worldwide. It has also been engineered to produce protein drugs, such as valuable pharmaceutical and edible vaccines (Daniell *et al.,* 2020; Daniell *et al.,* 2022; Power *et al.,* 2021; Singh *et al.,* 2021). For example, angiotensin-converting enzyme 2 was expressed in lettuce chloroplasts as an oral drug to attenuate pulmonary arterial hypertension or to trap the SARS-CoV-2 and decrease infectivity (Daniell *et al.,* 2020; Daniell *et al.,* 2022). In addition, lettuce leaves were expressed with the SARS-CoV-2 receptor-binding domain subunit of the surface-exposed spike glycoprotein for the oral vaccine against SARS-CoV-2 infection (Power *et al.,* 2021). In this study, we used map-based cloning to clone the gene *LsNRL4*, which plays important roles in the development and proliferation of chloroplasts. *LsNRL4* enhances the accumulation of chlorophyll and photosynthesis and decreases leaf angles to increase the yield of lettuce and meet the ever-increasing requirement of the production of lettuce leaf biomass. Our results provide new insights into the functions of the *NRL* gene family and contribute a new gene resource for the breeding of high-yielding lettuce and other crops for modern agriculture.

Results

Phenotypic and physiological characterization of pale-green cultivars of lettuce

The variations in chlorophyll levels in leaves were investigated in 240 lettuce accessions. Five accessions, all stem lettuce accessions, have pale-green leaves (Figure 1a). Each of the five accessions has a wild-type *LsGLK*. Moreover, their pale-green leaves are distinct from those of the *Lsglk* mutants (Zhang *et al.,* 2022).

To investigate the mechanism that contributes to the development of pale-green phenotypes, we quantified the chlorophyll content of leaves from a pale-green cultivar, S23, and a dark-green cultivar, S34 (Figure 1a-b). The chlorophyll and carotenoid contents of leaves from S23 were significantly lower than those from S34 (Figure 1c). Moreover, key photosynthetic parameters, including net photosynthetic rate (Pn), effective quantum yield of PSII (YII), photochemical quenching (qP) and photosynthetic electron transport rate (ETR) were significantly decreased in S23 relative to S34 (Figure 1d-g). The decreases in the total chlorophyll content and other traits associated with photosynthesis are consistent with the pale-green phenotype of S23.

Construction of a segregating population for the pale-green trait

To dissect the genetics underlying the pale-green leaves, we used an F_2 segregating population derived from a cross between S23 and S34. Individuals in the F_2 segregating population had either dark-green leaves or pale-green leaves, which is consistent with leaf color segregating as a qualitative trait in this population. Interestingly, amongst the 94 individuals that we initially screened, 73 individuals had dark-green leaves and small leaf angles. The remaining 21 individuals had pale-green leaves and large leaf angles (Figure S1a-d). Thus, the pale-green leaves and large leaf angles were segregated in a 3:1 ratio, as expected for a Mendelian trait ($\chi^2 = 0.355$, $P > 0.05$). These data are consistent with a mutation in a single gene or with mutations in two tightly linked genes leading to the development of pale-green leaves and large leaf angles.

We used bulked segregant analysis and RNA-seq to genetically map the gene controlling leaf color and leaf angle. From the F_2 population, 20 individuals with pale-green leaves were mixed to construct a 'pale-green-leaf' pool, and 20 individuals with dark-green leaves were mixed to construct a 'dark-green-leaf' pool. RNA was extracted from each pool and sequenced. The RNA-seq data were analysed, and the Δ(SNP-index) was calculated for each SNP. The average of Δ(SNP-index) of the SNPs in a 3-Mb sliding window with a 1-Mb step was plotted along the nine chromosomes of lettuce, and a single significant peak was detected on Chromosome 4 (Figure 2a, Tables S1, S2, S3). A marker (AGH305) around this peak co-segregated with leaf color and leaf angle in the F_2 family (Table S4). Therefore, we successfully constructed a population in which chlorophyll deficiency and enlarged leaf angles segregating as one locus.

Map-based cloning of the candidate gene controlling the development of pale-green leaves and large leaf angles

To genetically fine map and clone the gene controlling pale-green leaves and large leaf angles, we used two flanking markers, AGH318 (173.240 Mb) and AGH180 (183.607 Mb), to screen 3168 individuals from the F_2 family and obtained 90 recombinants (Figure 2b). We mapped the candidate gene to a 731-kb interval between markers AGH471 (177.262 Mb) and AGH424 (177.993 Mb) on Chromosome 4 by genotyping the recombinants with a series of markers from the candidate region (Figure 2b). Nine open reading frames (ORFs) were predicted in the candidate interval (Figure 2c; Table S5).

To identify additional polymorphisms between the two parents in the candidate interval, the DNA from both parents was re-sequenced. The re-sequencing data were mapped to the lettuce reference genome. The SNPs and the structural variations in the nine ORFs were analysed. No reads were

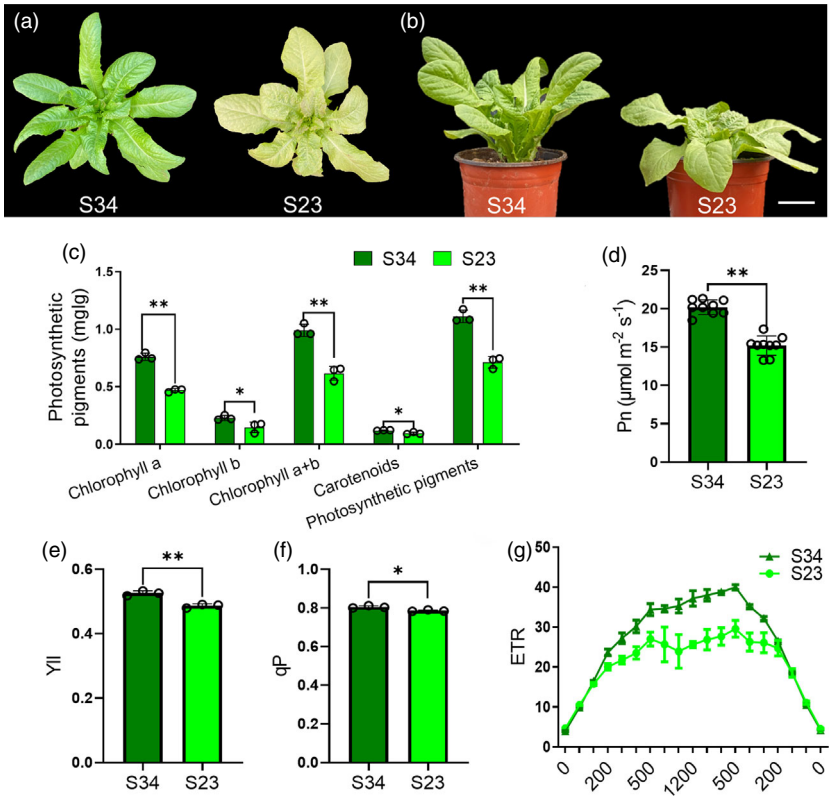

Figure 1 Phenotypic characterization of dark-green lettuce cultivar S34 and pale-green lettuce cultivar S23. (a-b) Top views (a) and side views (b) of cultivars S34 and S23. Bar = 5 cm. (c-f) Photosynthetic pigment contents (c), photosynthetic rates (Pn) (d), effective quantum yield of PSII (YII) (e) and photochemical quenching (qP) (f) in S34 and S23 (means ± SD; n = 3 to 9). Statistically significant differences were determined using a Student's t-test (*P < 0.05, **P < 0.01). (g) The photosynthetic electron transport rate (ETR) for cultivars S34 and S23 in different intensities of photosynthetically active radiation (x-axis; means ± SD; n = 3).

mapped to *LG4_398401* in the pale-green parent (i.e. S23), in striking contrast to a large number of reads mapped to *LG4_398401* in the dark-green parent (i.e. S34; Figure S2a). The re-sequencing data suggested that the deletion in parent S23 extended at least 467-Kb, which was further supported by four markers showing presence/absence polymorphism between the two parents (Figure S2a). The RNA-seq data from the two pools also showed that *LG4_398401* was not expressed in the 'pale-green-leaf' pool and that it was highly expressed in the 'dark-green-leaf' pool (Figure S2b). We used *LG4_398401*-specific primers to amply PCR products from the two parents. PCR products were amplified from S34 but not from S23 (Figure S2c). Therefore, *LG4_398401* is deleted in parent S23 and exhibits presence/absence polymorphism between the two parents. We hypothesize that *LG4_398401* may contribute to the development of dark-green leaves with small leaf angles.

LG4_398401 encodes an NPH3/RPT2-like (NRL) protein lacking a BTB domain

The *LG4_398401* gene encodes an NPH3/RPT2-like (NRL) protein. The lettuce reference genome encodes 34 NRL proteins. The Arabidopsis genome encodes 33 NRL proteins. A phylogenetic tree for the 67 NRL proteins encoded by the lettuce and Arabidopsis genomes was constructed (Figure S3). The phylogenetic tree indicates that *LG4_398401* encodes a protein that is orthologous to NRL4 from Arabidopsis. For convenience, we hereafter refer to *LG4_398401* as *LsNRL4*.

LsNRL4 and its ortholog *NRL4* from Arabidopsis encode NRL proteins lacking the N-terminal BTB domain (Figure 2d). To explore the evolutionary significance of the lack of the BTB domain in some NRLs, the amino acid sequences of the NRLs from 78 representative land plants were examined. Although the NRLs are present in all 78 land plants included in this study, the NRLs lacking the BTB domain can be found only in angiosperms (Figure S4a). Moreover, the NRLs lacking the BTB domain maintain obvious orthologous relationships, which is consistent with NRL4 proteins performing conserved functions in different plant species (Figure S4b).

We determined the proportion of cultivated and wild lettuce (*Lactuca serriola*) that contain null alleles of *LsNRl4*. Re-sequencing and/or sequencing of PCR products showed that the 488 accessions of wild lettuce included in this study had intact alleles of *LsNRL4* (Figure S5). Therefore, the loss of *LsNRL4* most likely occurred after domestication. We also investigated the *LsNRL4* genotypes in 198 lettuce cultivars (Zhang *et al.*, 2017). Amongst them, only five stem lettuce cultivars have the null allele of *LsNRL4* (Figure S5). We conclude that the deletion of *LsNRL4* is unique to stem lettuce and is likely associated with the breeding and development of stem lettuce.

Expression pattern and subcellular localization of LsNRL4

We then analysed the expression pattern and subcellular localization of LsNRL4. We quantified the relative expression levels of *LsNRL4* using qRT-PCR and found that *LsNRL4* is expressed at the

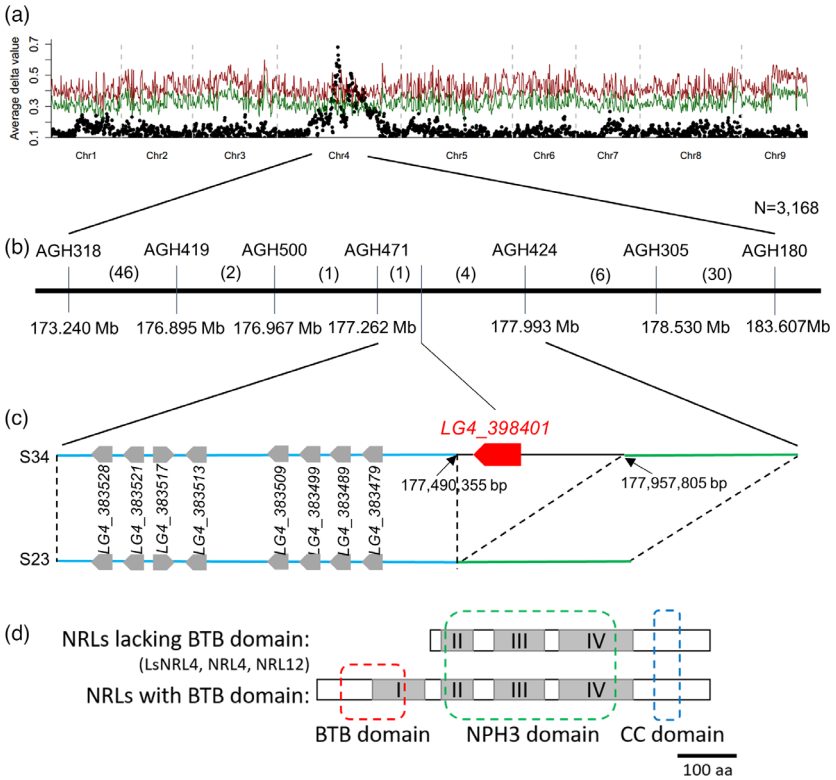

Figure 2 Map-based cloning of *Lsnrl4*. (a) BSR-seq showing a single locus on Chromosome 4. The *y*-axis represents the average of Δ(SNP-index) amongst the SNPs in a 3-Mb sliding window with a 1-Mb step. The *x*-axis represents the nine chromosomes of lettuce. The red and green lines indicate 99% and 95% confidence intervals (i.e. $P = 0.01$ and $P = 0.05$), respectively. (b) Genetic mapping of the candidate gene. The numbers in the parentheses refer to the number of recombinants between the two neighbouring markers from 3168 progeny. (c) Nine open reading frames present in the candidate interval. The dashed lines and gene names show the microsynteny between cultivars S34 and S23. (d) Diagram for the protein structure of NRLs with or without the BTB domain. The grey boxes indicate the conserved regions I to IV.

highest levels in leaves and lamina joints and at low levels in flowers, stems, roots and seeds (Figure 3a). *In situ* hybridization was carried out to investigate the expression patterns of *LsNRL4* in the two parents. We found that *LsNRL4* was expressed mainly in the mesophyll cells of leaves in S34 and that its expression was undetectable in the leaves of S23 due to the absence of the *LsNRL4* gene (Figure 3b). Then, we transformed the fusion gene *LsNRL4-GFP* driven by the CaMV 35S promoter into S23. Fluorescence emitted from the LsNRL4-GFP fusion protein was detected only in the plasma membrane, but not in the nuclei or chloroplasts, which indicates that LsNRL4 is a cell membrane-localized protein in lettuce (Figure 3c). The subcellular localization of LsNRL4 is consistent with that of NPH3, RPT2 and NCH1, which contain the BTB domain (Suetsugu *et al.*, 2016).

Genetic demonstration of *LsNRL4* function

To test whether the deletion of *LsNRL4* leads to the development of pale-green leaves and large leaf angles in lettuce, we transformed S23 with a transgene that uses the CaMV 35S promoter to drive the expression of the *LsNRL4* gene. We obtained three positive transformants (*LsNRL4*-OX #1, #2, #3) that produced dark-green leaves and developed smaller leaf angles relative to S23 (Figure 4a-b). The total photosynthetic pigment content was significantly higher in the *LsNRL4*-OX leaves than in S23 (Figure 4c). The expression level of the *LsNRL4* gene was significantly higher in the *LsNRL4*-OX plants than in S23 (Figure 4d). The expression of the N-terminus Myc-tagged LsNRL4 protein was detected in the *LsNRL4*-OX plants (Figure 4e). Furthermore, the elevated levels of chlorophyll and small leaf angles co-segregated with the transgene in a T_1 population (Figure S6).

We also used CRISPR/Cas9 technology to test whether *LsNRL4* is required for the accumulation of chlorophyll in S34. We transformed S34 with a recombinant CRISPR/Cas9 vector that expresses a sgRNA that specifically binds the coding region of the *LsNRL4* gene. Two knockout mutants with modified *LsNRL4* sequences were obtained (Figure 4f). The homozygous knockout mutants *Lsnrl4*-KO #1 and #2 produced pale-green leaves that accumulated lower levels of chlorophyll than S34 and resembled the leaves of S23, and the leaf angles of the *Lsnrl4*-KO plants were significantly larger than the leaf angles of S34 (Figure 4a-c). We conclude that loss-of-function mutations in *LsNRL4* lead to the development of pale-green leaves and large leaf angles in lettuce.

LsNRL4 enhances chloroplast coverage and photosynthesis

The chlorophyll content in *LsNRL4* genotypes, such as S34 and the *LsNRL4*-OX plants, was significantly higher relative to the *Lsnrl4* genotypes, such as S23 and the *Lsnrl4*-KO plants. We fixed leaf tissue with glutaraldehyde and used differential interference contrast (DIC) microscopy to quantify the numbers and sizes of chloroplast and the amount of space in the cell devoted to all chloroplasts (i.e. chloroplast coverage) in S34, S23, *LsNRL4*-OX and *Lsnrl4*-KO plants (Figure 5a). We found that chloroplasts were larger in the *Lsnrl4* genotypes than in the *LsNRL4* genotypes. However, the number of chloroplasts per cell plan area and chloroplast coverage in the *Lsnrl4* genotypes were significantly reduced compared to the *LsNRL4* genotypes (Figure 5 b-d).

We also investigated the ultrastructure of chloroplasts in S23, S34, *LsNRL4*-OX and *Lsnrl4*-KO plants using transmission electron microscopy. In S34 and *LsNRL4*-OX plants, the chloroplasts have well-developed thylakoid membrane systems with numerous grana. In contrast, the chloroplasts from S23 and the *Lsnrl4*-KO plants are enlarged and have fewer thylakoid membranes and

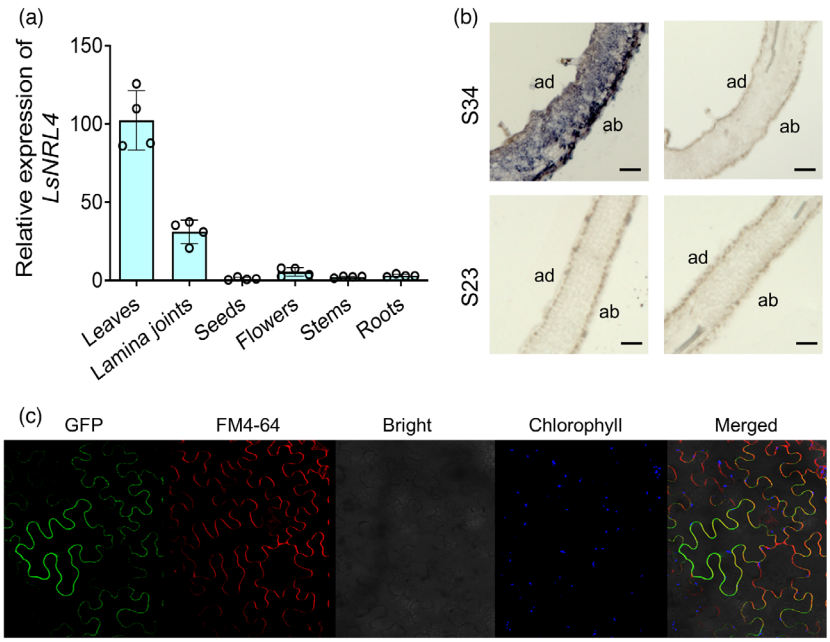

Figure 3 Expression pattern of *LsNRL4* and subcellular localization of LsNRL4-GFP. (a) Relative expression of *LsNRL4* in different tissues (means ± SD; $n = 4$). (b) *In situ* hybridization of *LsNRL4* transcripts in S34 and S23. Antisense probe was used in the left panel, and sense probe was used in the right panel. Bars = 50 μm. 'ab' and 'ad' represent the abaxial and adaxial domains of leaves, respectively. (c) Subcellular localization of LsNRL4-GFP in lettuce leaf cells. The imaging was performed using a confocal laser scanning microscope. The plasma membrane was labelled with FM4-64.

fewer grana thylakoids (Figure 5e). Thus, the loss of *LsNRL4* gene function leads to abnormal chloroplast development.

To determine whether the reduced chlorophyll content and the impaired chloroplast development in the *Lsnrl4* genotype affect photosynthesis, we measured key photosynthetic parameters in S23, S34, *LsNRL4*-OX and *Lsnrl4*-KO plants. The Pn, YII, qP and ETR in the *Lsnrl4* genotype were significantly decreased relative to the *LsNRL4* genotype (Figure 5f-i). We conclude that *LsNRL4* promotes chloroplast coverage and chlorophyll content and consequently, enhances photosynthetic capacity.

LsNRL4 and *LsGLK* independently promote the accumulation of chlorophyll in lettuce leaves

A recent study reported that the *LsGLK* gene is attenuated by the insertion of a CACTA transposon in some cultivars. This transposon insertion explains 29.2% of the variation in total photosynthetic pigments in a natural lettuce population (Zhang *et al.*, 2022). We investigated the photosynthetic pigments from 60 accessions that had a wild-type *LsGLK* (Figure 6a). In these accessions, *LsNRL4* explained 78.0% of the variation in chlorophyll *a*, 56.9% of the variation in chlorophyll *a* + *b*, 68.4% of the variation in carotenoids and 65.1% of the variation in total photosynthetic pigments. Therefore, the loss of *LsNRL4* has a significant influence on chlorophyll content in lettuce, and similar to *LsGLK*, the *LsNRL4* gene plays important roles in the variation in chlorophyll content in lettuce.

To determine the genetic relationships between *LsNRL4* and *LsGLK*, the photosynthetic pigment content of four genotypes, *LsNRL4*/*LsGLK*, *Lsnrl4*/*LsGLK*, *LsNRL4*/*Lsglk* and *Lsnrl4*/*Lsglk*, from a natural population was measured in each genotype from three randomly chosen cultivars (Figure 6b). The results showed that compared to wild type, the photosynthetic pigment content of the *LsNRL4*/*Lsglk* and *Lsnrl4*/*LsGLK* mutants was reduced by 45.5% and 33.9%, respectively. And the photosynthetic pigment content of the *Lsnrl4*/*Lsglk* (i.e. the double mutant) was reduced by 51.9%, leading to more severe pale-green phenotypes than the single mutant.

We also quantified chloroplast development using DIC microscopy in different *LsNRL4* and *LsGLK* genotypes. Our results indicate that the loss of *LsNRL4* decreased the number of chloroplasts per cell but increased the average size of individual chloroplasts (Figure 6c-e). In contrast, *LsGLK* mainly regulates the size of individual chloroplasts but has no obvious effects on the number of chloroplasts per cell (Figure 6c-e). Chloroplast coverage was reduced in *Lsnrl4* and *Lsglk* single mutants and was further reduced in the *Lsnrl4 Lsglk* double mutant (Figure 6f). We conclude that *LsNRL4* and *LsGLK* both promote the accumulation of chlorophyll and the proliferation of chloroplasts and that the loss of function of either gene leads to decreases in the chlorophyll content of lettuce leaves. Moreover, because the double mutants accumulate significantly less chlorophyll and allocate significantly less cellular space to chloroplasts relative to the single mutants, we conclude that *LsNRL4* and *LsGLK* contribute to different mechanisms that promote the accumulation of chlorophyll and the proliferation of chloroplasts.

LsNRL4 decreases leaf angle by regulating secondary cell wall biosynthesis

Leaf angle is determined by the mechanical properties of lamina joint tissues. The sclerenchyma tissue of lamina joints is largely responsible for providing the mechanical strength necessary to maintain leaf erectness (Zhou *et al.*, 2017). We examined the sclerenchyma tissues in the lamina joints of S23, S34 and *LsNRL4*-OX. The cross sections revealed that the thickness of the secondary cell walls of the sclerenchyma cells of S23 was significantly reduced relative to S34 and the *LsNRL4*-OX plants

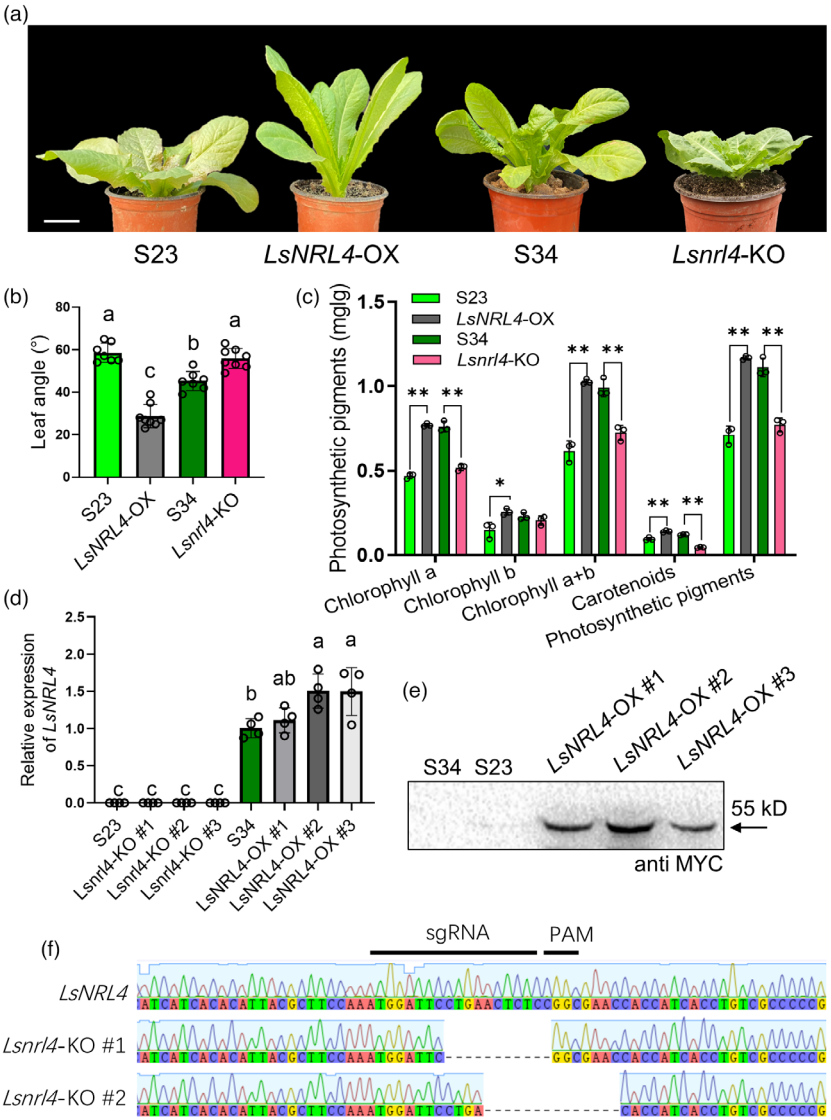

Figure 4 Functional analysis of the *LsNRL4* gene. (a) Front view of S23, *LsNRL4*-OX, S34 and *Lsnrl4*-KO plants. Bar = 5 cm. (b-d) Leaf angles (means ± SD; $n = 7$) (b), photosynthetic pigment contents (means ± SD; $n = 3$) (c) and expression of *LsNRL4* (means ± SD; $n = 4$) (d) in S23, *LsNRL4*-OX, S34 and *Lsnrl4*-KO plants. Different letters indicate statistically significant differences based on a one-way ANOVA analysis, followed by Tukey's multiple comparison test ($P < 0.05$). (e) Expression of an N-terminus Myc-tagged LsNRL4 protein in *LsNRL4*-OX plants. (f) DNA sequences deleted from *LsNRL4* in the *Lsnrl4*-KO plants.

(Figure 7a-b). These data indicate that the mechanical strength of the sclerenchyma tissue was attenuated in the lamina joints of S23. We conclude that the increased leaf angle in cultivar S23 results from defective sclerenchyma tissue.

To determine how *LsNRL4* influences the formation of sclerenchyma tissue, we analysed co-expression networks in 240 accessions from natural populations of lettuce (Zhang *et al.,* 2017). We found that 665 genes were co-expressed with the *LsNRL4* gene (Table S6). Gene ontology (GO) analysis using the 665 co-expressed genes and 1421 DEGs from BSR-seq showed that the GO terms related to 'cell wall organization,' 'lignin metabolic process,' 'cellulose metabolic process' and 'pectin metabolic process' were significantly enriched ($P < 0.00017$ to 2.1×10^{-8}; Figure 7c; Tables S7-S9). We chose *LsCOMT*, *LsCAD* and *LsF5H* for further analysis, which encode key enzymes for the lignin biosynthesis (Wu *et al.,* 2019). The expression of these three genes was significantly reduced in S23 relative to S34 (Figure 6d). Consistently, the accumulation of lignin in the lamina joints of leaves from S23 was considerably reduced relative to S34 (Figure 6e). Overexpressing the *LsNRL4* gene led to increases in the expression of *LsCOMT*, *LsCAD* and *LsF5H*, and consequently, led to the accumulation of more lignin in the lamina joints of the *LsNRL4*-OX plants (Figure 7d-e). Therefore, we conclude that the loss of *LsNRL4* affects the expressions of a large number of genes associated with cell wall biosynthesis and leads to the development of defective sclerenchyma tissue and consequently, large leaf angles.

Discussion

The *NPH3/RPT2-Like* (*NRL*) gene family

In this study, we cloned a gene that belongs to the *NPH3/RPT2-Like* (*NRL*) gene family that controls chlorophyll content, chloroplast development and secondary cell wall development. Particular NRL proteins coordinate different aspects of the signalling activated by phototropins, which are blue-light receptors (Christie *et al.,* 2018). Phototropins are present in green algae and flowering plants and their functions are highly conserved (Li *et al.,* 2015). The NRL proteins serve as effectors in phototropin signalling and are also conserved in algae and plants (Christie *et al.,* 2018). Compared to green algae, phototropin signalling in land plants is complicated in that it contributes to phototropism, chloroplast movement, leaf positioning and leaf flattening. Consistent with these observations, a large number of NRL proteins are present in land plants to

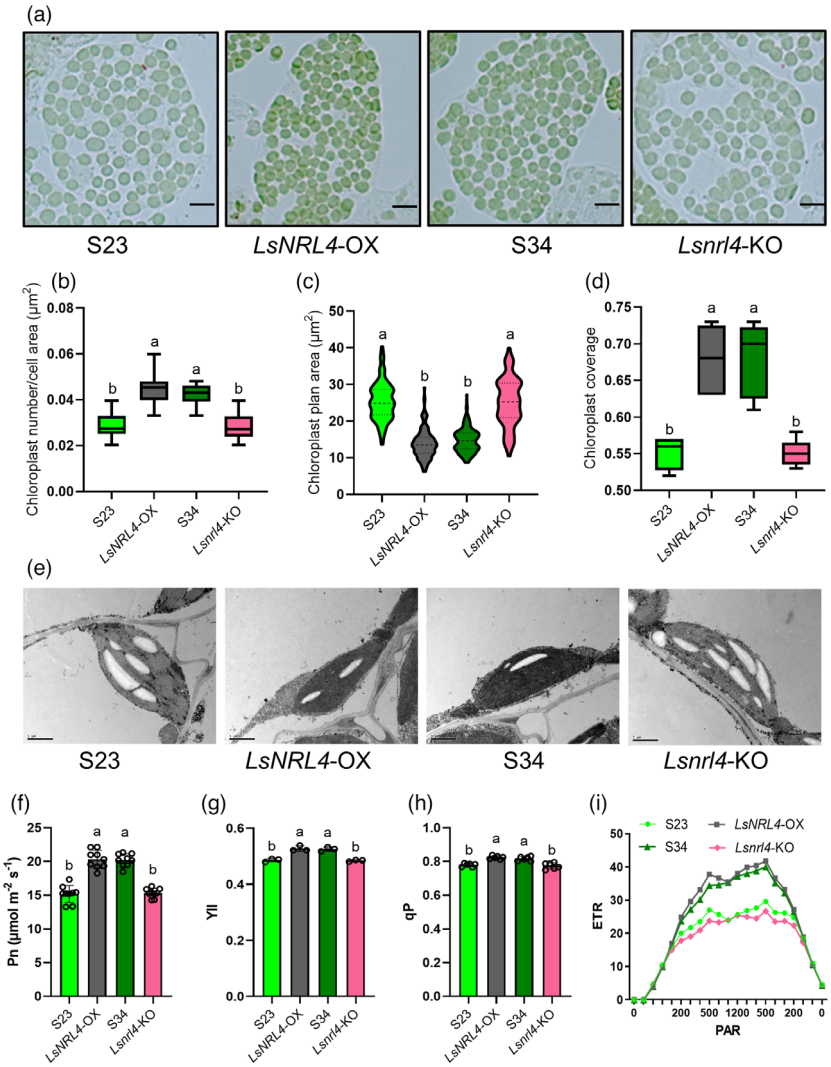

Figure 5 *LsNRL4* promotes chloroplast coverage and photosynthesis. (a) Glutaraldehyde-fixed mesophyll cells from S23, *LsNRL4*-OX, S34 and *Lsnrl4*-KO plants. Bars = 20 μm. (b-d) Quantification of chloroplast number per cell plan area (means ± SD; n = 30) (b), chloroplast plan area (means ± SD; n = 150) (c) and chloroplast coverage (means ± SD; n = 10) (d) in S23, *LsNRL4*-OX, S34 and *Lsnrl4*-KO plants. (e) Transmission electron microscopy of mesophyll cells from S23, *LsNRL4*-OX, S34 and *Lsnrl4*-KO plants. Bars = 1 μm. (f-i) Pn (f), YII (g), qP (h) and ETR (i) in S23, *LsNRL4*-OX, S34 and *Lsnrl4*-KO plants (means ± SD; n = 3 to 9). Different letters indicate statistically significant differences based on a one-way ANOVA analysis, followed by Tukey's multiple comparison test ($P < 0.05$).

facilitate the regulation of these biological processes by phototropins (Suetsugu *et al.,* 2016).

Most of the NRL proteins have BTB domains. The BTB domains of NPH3 and RPT2 can form a heterodimer in yeast. Moreover, the BTB domain of NPH3 can serve as a substrate adapter in a Cullin3-based E3 ubiquitin ligase in Arabidopsis (Roberts *et al.,* 2011). However, NRL4 and NRL12 from Arabidopsis and LsNRL4 from lettuce lack the BTB domain (Pedmale *et al.,* 2010). The impact of these deficiencies in the BTB domain remains unclear because prior to this study, there were no reports on the biological functions of NRL proteins lacking the BTB domain. Prior to this study, the only information available on members of the *NRL* genes that do not encode proteins with BTB domains was that *NRL4* and *NRL12* are expressed in leaves and flowers in Arabidopsis, according to the TAIR database.

In this study, we showed that LsNRL4 is associated with the plasma membrane and promotes the development of chloroplast and secondary cell walls. The loss of *LsNRL4* leads to the development of pale-green leaves and large leaf angles. However, the biochemical functions of LsNRL4 remain to be studied. We hypothesize that LsNR4 may not interact with Cullin3 or target the phototropin light-activated kinase for ubiquitination due to its lack of a BTB domain. Instead, the conserved C-terminal consensus sequence of LsNRL4 may contribute to its biochemical function. Two serine residues, S744 and S746, located in the conserved C-terminal domain of Arabidopsis NPH3 are required for NPH3 to promote phototropism and to influence the positioning of petioles (Sullivan *et al.,* 2021). Phosphorylation of serine residue S744 in Arabidopsis NPH3 leads to its interaction with 14–3-3 proteins, which play important roles in phototropin signalling. (Sullivan *et al.,* 2021). Similar to NPH3, LsNRL4 has a conserved C-terminal domain, including the third to last residue (i.e. S744 in NPH3), which, in turn, may enable the binding of 14–3-3 proteins and the development of normal leaf angles in lettuce.

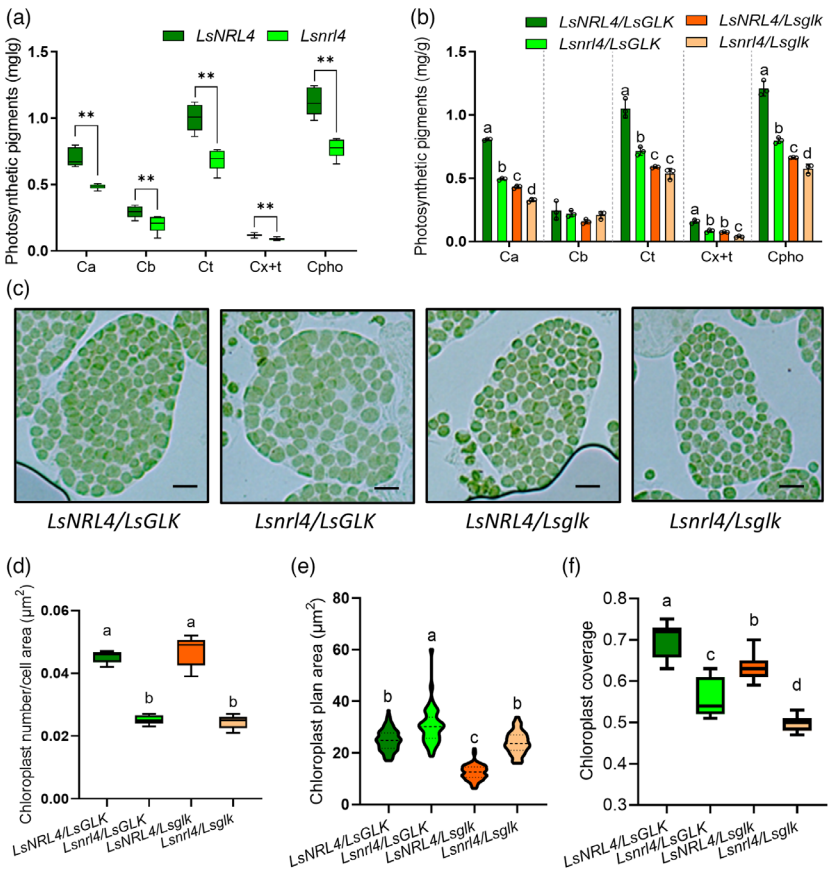

Figure 6 *LsNRL4* and *LsGLK* independently promote the accumulation of chlorophyll and the proliferation of chloroplasts in lettuce leaves. (a) Photosynthetic pigment content of *LsNRL4* genotypes and *Lsnrl4* genotypes from 60 accessions in genetic backgrounds containing wild-type *LsGLK*. Statistically significant differences were determined using a Student's *t*-test (**$P < 0.01$). (b) Photosynthetic pigment contents from different *LsNRL4* and *LsGLK* genotypes (means ± SD; $n = 3$). (c) Glutaraldehyde-fixed mesophyll cells from different *LsNRL4* and *LsGLK* genotypes. Bars = 20 μm. (d-f) Quantification of Chloroplast number per cell plan area (means ± SD; $n = 50$) (d), chloroplast plan area (means ± SD; $n = 100$) (e) and chloroplast coverage (means ± SD; $n = 10$) (f) from different *LsNRL4* and *LsGLK* genotypes. Different letters refer to statistically significant differences based on a one-way ANOVA analysis followed by Tukey's multiple comparison test ($P < 0.05$).

LsNRL4 contributes to the development of chloroplasts and photosynthesis

The loss of *LsNRL4* leads to abnormal chloroplast development, decreases in chlorophyll content, enlarged chloroplasts and less cellular space devoted to chloroplasts in leaves. A complex network that responds to light and hormones controls chloroplast development and the accumulation of photosynthetic pigments (Cackett *et al.,* 2022). A mechanism that drives increases in chloroplast volume determines how much space in the cell is devoted to chloroplasts. When chloroplasts reach a particular threshold size, chloroplast division is activated and breaks up the chloroplast compartment into many small chloroplasts. Thus, when the chloroplast division machinery is knocked out, mesophyll cells contain one enlarged chloroplast that occupies as much cellular space as all of the chloroplasts of wild-type mesophyll cells combined (Cackett *et al.,* 2022; Larkin *et al.,* 2016). In *Lsnrl4*, chloroplasts are 70.3% larger than wild type and are fewer in number, which indicates that chloroplast division is attenuated. Also, in *Lsnrl4*, the amount of cellular space allocated to chloroplasts is reduced 44.0% relative to wild type. In contrast, when all of the *REDUCED CHLOROPLAST COVERAGE* (*REC*) from Arabidopsis are knocked out, the amount of cellular space allocated to chloroplasts is reduced 50.0% relative to wild type (Larkin *et al.,* 2016). The striking decrease in the accumulation of photosynthetic pigments, small increase in the size of the individual chloroplasts relative to chloroplast division mutants, smaller reduction in the amount of cellular space allocated to chloroplasts in *Lsnrl4* relative to the *rec* mutants and the precedents for NRL proteins contributing to signalling are consistent with LsNRL4 influencing a network that controls chloroplast development. Consistent with numerous chloroplast defects in *Lsnrl4*, we found that the photosynthesis capacity decreased in *Lsnrl4*. A total of 26 genes that were differentially expressed in the *LsNRL4* and *Lsnrl4* genotypes were associated with chlorophyll metabolic and biosynthetic processes (Table S9). Our results showed that LsNRL4 plays important roles in the accumulation of chlorophyll and the development and proliferation of chloroplasts.

Previous studies have shown that in land plants, two members of the NRL gene family, *RPT2* and *NCH1*, redundantly mediate the chloroplast accumulation response, which refers to the movement of chloroplasts towards low fluence rate light (Suetsugu *et al.,* 2016; Wang *et al.,* 2021). Although RPT2 and NCH1 contain four conserved regions including the BTB domain, the LsNRL4 protein in lettuce lacks the BTB domain. Therefore, LsNRL4 may use a different mechanism to influence chloroplasts. In this study, we discovered obvious decreases in the accumulation of chlorophyll and in the development and proliferation of chloroplasts in *Lsnrl4*, which lacks the entire *LsNRL4* gene. It remains to be investigated how LsNRL4 regulates the development and proliferation of chloroplasts in lettuce.

LsNRL4 contributes to the formation of secondary cell walls and leaf angles

The loss of *LsNRL4* in lettuce attenuates secondary cell wall (SCW) biosynthesis in the sclerenchyma tissue of lamina joints. The lamina joint connects the leaf to the stem and is considered to be the most important tissue governing leaf angle (Zhou *et al.,* 2017). In rice and maize leaves, the leaf angle largely

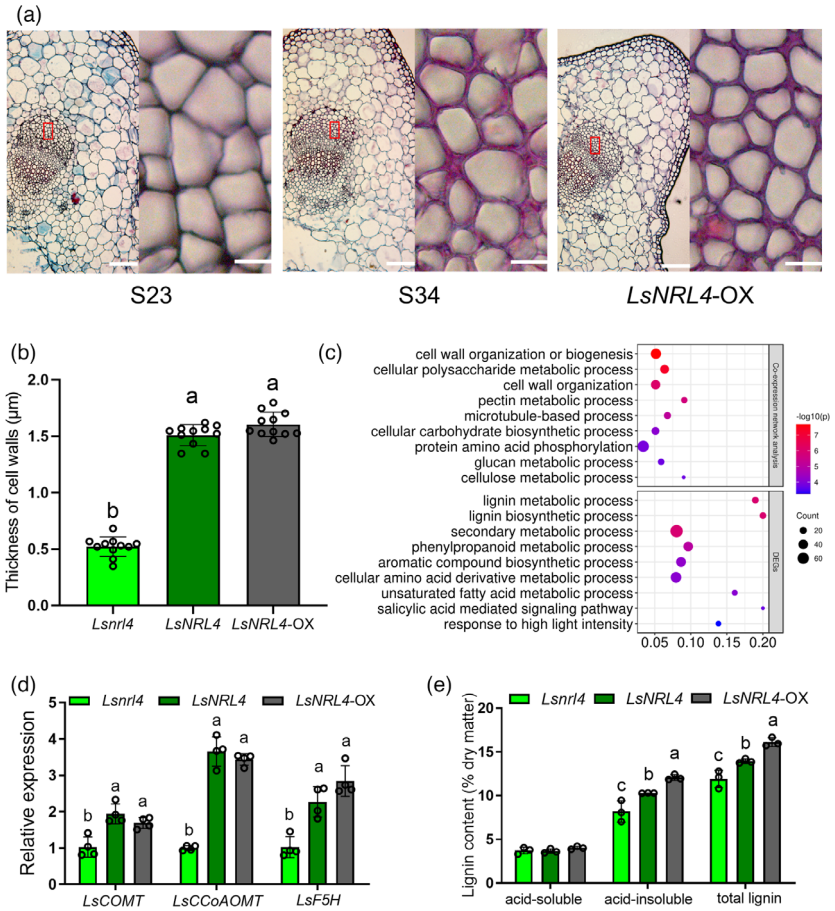

Figure 7 Influence of *LsNRL4* on secondary cell wall biosynthesis and leaf angles. (a) Cross sections showing the anatomical structures of sclerenchyma tissue in the lamina joints of S34, S23 and *LsNRL4*-OX plants. Lignin was stained using sarranine. High magnification images (right panels) of sclerenchyma tissue indicated with red rectangles (left panels) are shown. Bars = 50 μm (left panel). Bars = 10 μm (right panel). (b) Cell wall thickness in sclerenchyma cells (means ± SD; n = 11). (c) Gene ontology enrichment analysis of genes co-expressed with *LsNRL4* and DEGs from BSR-seq. (d) Relative expression of *LsCOMT*, *LsCAD* and *LsF5H* in S23, S34 and *LsNRL4*-OX plants (means ± SD; n = 4). (e) Lignin content of the lamina joints from S23, S34 and *LsNRL4*-OX plants (means ± SD; n = 3). Different letters indicate statistically significant differences based on a one-way ANOVA analysis, followed by Tukey's multiple comparison test ($P < 0.05$).

depends on cell division, cell expansion and cell wall composition in the joints of rice and maize leaves (Feng *et al.,* 2016). Notably, most of the genes that are known to influence the leaf angle are either transcriptional regulators or signalling components that respond to a variety of phytohormones (Cao *et al.,* 2022). Here, we showed that LsNRL4, an atypical NRL protein, promotes the development of small leaf angles by promoting SCW biosynthesis. The GO enrichment of genes that were co-expressed with *LsNRL4* and the genes that were differentially expressed in *LsNRL4* relative to *Lsnrl4* indicates that LsNRL4 promotes the development of small leaf angles by serving as a key regulator of SCW biosynthesis. This is the first report that members of the NRL family contribute to SCW biosynthesis. To gain a comprehensive understanding of the functions of the NRL family, the mechanisms used by LsNRL4 to regulate SCW biosynthesis, need to be investigated.

LsNRL4 may regulate the defence system

We found many genes associated with biotic stress tolerance in the list of genes that were differentially expressed in *LsNRL4* relative to *Lsnrl4* (Table S9). These DEGs include genes that contribute to salicylic acid signalling, systemic resistance and defence responses. Consistent with these findings, recent studies reported that *StNRL1* is a plant susceptibility gene that enables the late blight pathogen *Phytophthora infestans* to infect potatoes (Garcia-Ruiz *et al.,* 2021; Yang *et al.,* 2016). The StNRL1 protein enhances pathogen infection by helping to degrade StSWAP70, an immune regulator (He *et al.,* 2018). Homodimerization of StNRL1 is required for StNRL1 to bind and help degrade StSWAP70. Mutations in the BTB domain of the StNRL1 protein attenuate its homodimerization activity and abolish its interaction with StSWAP70 and thus, prevent the degradation of StSWAP70 and reduce *P. infestans* infection (He *et al.,* 2018; Naqvi *et al.,* 2022). However, we suggest that because LsNRL4 lacks the BTB domain, LsNRL4 and StNRL1 may use different mechanisms to influence the defence response. It will be interesting to investigate how LsNRL4 contributes to the defence response and whether LsNRL4 helps lettuce tolerate biotic stress.

Furthermore, LsNRL4 induces increases in the thickness of the secondary cell walls, which is the first defensive barrier against plant pathogens (Miedes *et al.,* 2014). Indeed, secondary cell

wall thickening limits the spread of pathogen infection (Miedes *et al.,* 2014; Yogendra *et al.,* 2015). For example, OsMYB30 induces cell wall thickening in sclerenchyma cells and inhibits the penetration of *Magnaporthe oryzae* in rice leaves at an early stage of infection by inducing increases in the expression of genes associated with lignin biosynthesis (Li *et al.,* 2020a). In this study, we showed that LsNRL4 up-regulates the expression of genes associated with lignin biosynthesis. Lignin is considered to be one of the main components of the plant cell wall that can reduce the infiltration of pathogens (Liu *et al.,* 2018). The increased accumulation of lignin in the genotypes containing *LsNRL4* may not only support the optimal development of leaf angles and chloroplasts but may also promote disease resistance in lettuce.

LsNRL4 contributes to an ideal plant architecture for dense planting

We found that the leaf angles of the *LsNRL4* genotype were smaller than the leaf angles of the *Lsnrl4* mutant. Leaf angle is an important agronomic trait for plant architecture. Upright leaves can enhance photosynthetic capacity when plants are grown in high-density conditions and thus, increase grain yields for cereal crops (Cao *et al.,* 2022). For leafy vegetables, small leaf angles contribute to a compact architecture that reduces the amount of space required to grow each plant, enables dense planting and promotes vegetable production. *LsNRL4* could be exploited to produce new lettuce cultivars with upright leaf architectures. Orthologs of *NRL4* may reduce leaf angle in other crops and consequently, help to increase yields when crops are densely planted.

Experimental procedures

Plant materials and growth conditions

Lactuca materials used in this study were described previously (Zhang *et al.,* 2017). Stem lettuce cultivars S23 and S34 were crossed to generate the F_1 hybrids. The F_1 hybrids were self-crossed to generate an F_2 population. All plants were planted in a field at Huazhong Agricultural University, Wuhan, China.

Bulked segregant analysis and RNA-seq

A bulked segregant analysis in combination with RNA-seq was used for the map-based cloning. As described in the Results, total RNA was extracted from the two pools of tissue and sequenced using the Illumina Hiseq2500 platform (Personalbio, China). Approximately 4.05 and 4.69 Gb clean data were obtained for pale-green and dark-green pools, respectively, and the sequencing data were mapped to the lettuce reference genome using the Bowtie software (Langmead *et al.,* 2009; Reyes-Chin-Wo *et al.,* 2017). SNP calling was performed using SAMtools (Li *et al.,* 2009). The Δ(SNP-index) was calculated by subtracting the SNP-index value (allele frequency) of the pale-green pool from the dark-green pool. The average of Δ(SNP-index) of the SNPs in a 3-Mb sliding window with a 1-Mb step was plotted along the nine chromosomes of lettuce. The primers used in the genetic mapping are shown in Table S10.

Construction of the overexpression and knockout lines

To construct lines that overexpress *LsNRL4*, the coding sequence of *LsNRL4* was amplified from cDNA prepared from S34 and was inserted into pH7LI9 (with an N-terminus Myc-tag). *Agrobacterium tumefaciens* GV3101 was transformed with this vector using a thermal stimulation method and then, used to transform S23. For knockout assays, a CRISPR/Cas9 vector that expresses sgRNA specific to the coding region of *LsNRL4* was constructed. GV3101 was transformed with this construct and then used to transform stem lettuce cultivar S34. All primers used for vector construction are shown in Table S10. Lettuce was transformed as described previously (Curtis *et al.,* 1994).

Quantitative RT-PCR analysis

Total RNA was extracted from leaves using RNAiso plus (Takara, Japan). cDNA was synthesized using TransScript One-Step SuperMix (TransScript, China). The qRT-PCR analysis followed the guidelines and protocols described previously (Udvardi *et al.,* 2008). *Ubiquitin* (*LG4_16296*), a housekeeping gene, was used as an internal standard. The relative expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method. Statistically significant differences were calculated using a Student's *t*-test. The primers used in the qRT-PCR analysis are shown in Table S10.

Evaluation of photosynthetic pigment content

Photosynthetic pigments (i.e. chlorophylls and carotenoids) were extracted from 2-month-old lettuce leaves in triplicate. Approximately 100 mg of leaves were ground in liquid nitrogen, incubated in 10 mL of 95% ethanol in the dark for 24 h at room temperature and centrifuged for 10 min at 13 523 *g*. The absorbance of the supernatant was measured at 470 nm, 649 nm and 665 nm using spectrophotometry. The concentration of photosynthetic pigments was calculated as described previously (Lichtenthaler and Wellburn, 1983).

Total lignin assay

The tissues of lamina joints from 2-month-old S23, S34 and *LsNRL4*-OX plants were used for lignin measurements. Total lignin, including acid-soluble and acid-insoluble lignin, was measured using the Laboratory Analytical Procedure of the National Renewable Energy Laboratory (Wu *et al.,* 2013). All analyses were performed from independent experiments performed in triplicate.

Measurement of photosynthetic parameters

For the chlorophyll fluorescence measurements, firstly, 2-month-old lettuce leaves from S23, S34, *LsNRL4*-OX and *Lsnrl4*-KO plants were kept in dark for 30 min. A chlorophyll fluorescence system (Imaging-PAM, Walz, Germany) was used to measure the chlorophyll fluorescence parameters, namely YII, qP and ETR, in leaves. The Pn was measured between 9:00 and 11:00 on a sunny day. Nine individuals were randomly selected from each genotype and the third, fully unfolded lettuce leaves from the top of the plants were used for measurements. The data were measured and recorded using a portable photosynthesis system (LI-6400XT, LI-COR, USA).

Analysis of chloroplasts by microscopy

Leaf sections were fixed with glutaraldehyde. Fixed mesophyll cells were released from the leaf sections and were visualized using differential interference contrast microscopy as previously described (Pyke and Leech, 1991). The chloroplast number per cell plan area, chloroplast plan area and total chloroplast plan area/cell plan area (i.e. chloroplast coverage) were quantified as described previously (Pyke and Leech, 1991).

Tissue section and *in situ* hybridization

For tissue sections, the tissues from lamina joints were excised and immersed in 70% FAA buffer (70% ethanol, 5% acetic acid and 3.7% formaldehyde). After the application of a vacuum for 15 min, samples were dehydrated using a series of ethanol solutions (70%, 85%, 95% and 100%). The ethanol was gradually replaced with xylene and further immersed in an increasing concentration of paraffin and finally embedded in absolute paraffin. Paraffin sections were prepared using a rotary microtome (Leica). Images were taken using a bright-field microscope (ZEISS).

The *in situ* hybridization and immunological signal detection were performed as described previously (Samach *et al.*, 1997). The probes were amplified using gene-specific primers. The PCR fragment was inserted into the SpeI/ScaII linearized pGEM-T and the probes were transcribed *in vitro* from either the T7 or SP6 promoter for sense and antisense probe synthesis using the Digoxigenin RNA labelling kit (Roche). The primers used in the *in situ* hybridization are shown in Table S10.

Accession numbers

The datasets and the gene sequence are available in the National Center for Biotechnology Information under the accession number PRJNA794278 and OM156463.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

G.A. performed the molecular experiments; Y.Q. assisted with the subcellular localization and vector construction; W.Z. provided assistance with bioinformatics; H.G. helped with lignin analysis; J.Q. helped with the chloroplast analysis; H.K. and J.C. designed the experiment; G.A. wrote the manuscript with the help from H.K., J.C. and R.M.L.

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Supporting information

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

Figure S1 Phenotypes in the F_2 segregating population.
Figure S2 *LsNRL4* gene was lost in the pale-green parent S23.
Figure S3 Phylogenetic analysis of the NRL proteins from lettuce and Arabidopsis.
Figure S4 Evolution of NRLs in land plants.
Figure S5 Frequency of *LsNRL4* and *Lsnrl4* genotypes in *Lactuca* populations.
Figure S6 Variations in leaf color and leaf angle in a T_1 population co-segregating with the vector insertion.
Table S1 The source data of the average of (SNP-index) in BSR-seq.
Table S2 The 181 SNPs in the region with average of (SNP-index) > 0.6.
Table S3 The 268 genes in the region with average of (SNP-index) > 0.6.
Table S4 The phenotype and genotype of 94 individuals used for primary mapping.
Table S5 Nine open reading frames in the candidate interval.
Table S6 Genes co-expressed with *LsNRL4*.
Table S7 GO terms enriched in the co-expressed genes.
Table S8 The 1421 DEGs in *LsNRL4* relative to *Lsnrl4*.
Table S9 GO terms enriched in the DEGs.
Table S10 Primers used in this research.

