



RESEARCH ARTICLE

Integrated hormone and transcriptome analyses of Chinese cabbage mutant *lic86* reveal the involvement of auxin in leaf curling

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Highlights

- Chinese cabbage leaf inward curling mutant, *lic86*, displayed markedly increased transverse leaf curvature.
- Exogenous IAA induced outward leaf curling, and treatment with the auxin transport inhibitor TIBA resulted in inward curling in both WT and *lic86* plants.
- VIGS of *BrLAX1* in WT induced inward curling, while silencing *BrAS1* in *lic86* flattened leaves, indicating that low expression of *BrLAX1* coupled with high expression of *BrAS1* promotes inward leaf curling in Chinese cabbage.

Abstract

Leaf curling is a key agronomic trait that promotes the formation of leafy heads in heading vegetable crops. However, the role of auxin in regulating leaf curling in Chinese cabbage remains largely unknown. In this study, we identified a Chinese cabbage mutant, *lic86*, which exhibited inward leaf curling and displayed significantly increased transverse curvature indices compared to the wild type. Additionally, among the measured hormones the concentration of indole-3-acetic acid (IAA) was significantly reduced in *lic86*. Exogenous application of IAA caused outward leaf curling, whereas applying the auxin transport inhibitor, TIBA, resulted in inward leaf curling in both wild type and *lic86* plants. Transcriptomic analysis revealed that the differentially expressed genes between WT and *lic86* were predominantly associated with hormone signal transduction pathways. Notably, the expression levels of two *Brassica rapa asymmetric leaves1* (*BrAS1*) homologs were significantly elevated in *lic86*, while the expression of *B. rapa LIKE-AUX1* (*BrLAX1*), the auxin influx carrier gene, was markedly downregulated compared to WT plants. Virus-induced gene silencing of *BrLAX1* in WT plants resulted in leaf inward curvature, whereas silencing of *BrAS1s* in *lic86* seedlings led to flattened leaf morphology. These findings offer insights into the molecular mechanism underlying leaf curling and leafy head development in Chinese cabbage.

Keywords: Chinese cabbage, mutant, leaf curling, hormone, auxin

1. Introduction

Brassica crops include some of the most important vegetable species worldwide. China is the leading producer of cabbages, with about one million hectares harvested and a total production of 97 million tons in 2022 (www.fao.org/faostat). As a key member of *Brassica* vegetables, Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) remains a long cultivation history with strong environmental adaptability and rich nutritional value. Leafy head of Chinese cabbage

serves as the primary agricultural and harvested organ for long-term storage, contributing to marketability and nutrient preservation, particularly during winter. Leaf polarity and curvature are essential biological processes underlying leafy head formation (Mao *et al.* 2014), directly regulating the morphology and compactness of vegetables.

Leaves are lateral organs that originate from the shoot apical meristem (SAM) (Barkoulas *et al.* 2007). Following the consecutive processes of cell proliferation, differentiation and expansion, leaves obtain their final size and shape

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(Donnelly et al. 1999; Reddy et al. 2004). Previous studies showed that axial polarity patterning contributes to leaf curling, wrinkling, and folding, which are regulated by multiple factors including transcription factors and small RNAs (Kidner and Timmermans 2006; Barkoulas et al. 2007; Du et al. 2018). For example, Class III homeodomain leucine zipper (HD-ZIP III) family proteins, including REVOLUTA (REV), PHAVOLUTA (PHV) and PHABULOSA (PHB), are involved in promoting adaxial identity (McConnell et al. 2001; Emery et al. 2003; Prigge et al. 2005). In addition, blocking microRNA165/166 (miR165/166) biosynthesis leads to HD-ZIP III-mediated adaxialisation of leaves (Grigg et al. 2005). Loss-of-function mutations in asymmetric leaves1 (*AS1*) and *AS2* in *Arabidopsis* results in a lotus-like leaf morphology (Xu et al. 2003; Machida et al. 2015). Mutations in the *YABBY* and *KANADI* (*KAN*) gene families result in abnormality in abaxial fate specification, indicating that these genes promote abaxial leaf growth (Siegfried et al. 1999; Kerstetter et al. 2001; Stahle et al. 2009; Sarojam et al. 2010; Du et al. 2018). The *AS1*–*AS2* complex represses the expression of *KNOX* (knotted-like homeobox) genes and facilitates adaxial fate by inhibiting abaxial-promoting genes, such as *YABBY5* (*YAB5*) and *KAN2*, and positively regulating HD-ZIP III levels on the adaxial side of the leaf (Lin et al. 2003; Li et al. 2005; Garcia et al. 2006; Iwakawa et al. 2007; Husbands et al. 2015).

Auxin activity gradients contribute to leaf axial patterning through precise spatiotemporal regulation of gene expression (Barkoulas et al. 2007). Auxin response factors, such as (ARF) *ARF3/ETT* and *ARF4*, modulate *KAN* activity to mediate abaxial fate (Pekker et al. 2005). The expression of abaxial fate-promoting genes *ARF3* and *ARF4* is regulated by ta-siRNAs that are derived from non-coding TAS3 transcripts in the adaxial domain (Allen et al. 2005; Adenot et al. 2006; Fahlgren et al. 2006; Garcia et al. 2006; Hunter et al. 2006). In addition to *ARF3* and *ARF4*, *ARF5/MONOPTEROS* (*MP*) also plays an important role in maintaining adaxial-abaxial polarity in leaves (Krogan and Berleth 2012). The antagonistic activities of *KAN1* and HD-ZIP III transcription factors regulate nearly all major hormone signaling pathways, including those of auxin, brassinosteroid, cytokinin and abscisic acid (*ABA*) (Brandt et al. 2012; Dello Iorio et al. 2012; Reinhart et al. 2013; Huang et al. 2014). Furthermore, auxin and brassinosteroid (*BR*) coordinately regulate leaf shape by activating the transcription of *DWARF4*, encoding a key *BR* biosynthetic enzyme, through the actions of *ARF6* and *ARF8* (Xiong et al. 2021). However, the role of phytohormones in regulating leaf morphogenesis remains largely unexplored in Chinese cabbage.

Leaf curvature is a fundamental biological process that drives the formation of leafy head in vegetables, such as lettuce, cabbage and Chinese cabbage. Convergent subgenome parallel selection analysis revealed that genes involved in axial polarity patterning and phytohormone signaling play key roles in regulating leaf curvature and heading. In *B. rapa*, these genes include *BrARF3.1*, *BrARF4.1*, *BrKAN2.1*, *BrKAN2.3*, *BrBRX.1* and *BrBRX.2*, while in *B. oleracea*, *BoATHB15.2* (an HD-ZIP III gene), *BoKAN2.2* and *BoBRX.2* are implicated in similar processes (Cheng et al. 2016). In addition, introducing foreign auxin

synthesis-related genes into Chinese cabbage enhances early folding of expanding leaves (He et al. 2000). The transition from the rosette stage to the heading stage in Chinese cabbage is strongly impacted by ambient temperature and the ethylene signaling pathway (Zhang K et al. 2022). Previous studies identified that genes involved in the gibberellin (*GA*) biosynthetic pathway (*ent*-kaurenoic acid oxidase 2 and *ent*-kaurene synthase), as well as the *ANGUSTIFOLIA* gene, regulate inward leaf curling and folding during leafy head development in Chinese cabbage (Gao et al. 2020; Huang et al. 2022; Xin et al. 2022). In *Lactuca sativa*, *KNOTTED 1* (*LsKN1*) and *SAWTOOTH1* (*LsSAW1*) physically interact and play antagonistic roles in leaf dorsiventrality and leafy head formation by regulating the expression of abaxial and adaxial genes (Yu et al. 2020; An et al. 2022). *OCTOPUS* (*BrOPS*) positively regulates brassinosteroid (*BR*) signaling by antagonizing *BrBIN2*, thereby promoting leaf epinastic growth during the heading stage in Chinese cabbage (Zhang X M et al. 2022). Furthermore, miR319a-targeted *BrTCP* genes modulate head shape in Chinese cabbage by differentially arresting cell division in leaves (Mao et al. 2014). miR156-targeted *BrpSPL9* (*B. rapa* ssp. *pekinensis* *SQUAMOSA PROMOTER BINDING-LIKE 9*) controls the timing of leaf folding and head formation in Chinese cabbage (Wang et al. 2014). *BcpLH* (*B. rapa* ssp. *pekinensis* *LEAFY HEADS*) gene, a close homolog of *AtHYL1*, regulates leaf curvature and leafy head shape by promoting microRNA biogenesis through the precise processing of primary miRNAs (Ren et al. 2020). However, the role of auxin in regulating leaf curling and leafy head patterning in Chinese cabbage requires further investigation.

Previously, we developed an ethyl methanesulfonate (*EMS*)-mutagenized population of Chinese cabbage, which yielded diverse morphological mutants (Sun et al. 2022). In this study, a mutant, named *leaf inward curling 86* (*lic86*) was identified. Transcriptomic analysis revealed that hormone signal transduction pathways and two copies of *BrAS1* gene were involved in the leaf curling of *lic86*. Hormone measurement and exogenous application experiments showed that *IAA* was the dominant hormone regulating leaf curling in *lic86*. These findings demonstrate that auxin plays a key role in leaf curling of Chinese cabbage, providing new insights into the molecular mechanism underlying auxin-mediated leaf development in heading vegetables.

2. Materials and methods

2.1. Plant materials and growth conditions

A mutant library of Chinese cabbage was derived from *EMS*-treated seeds of the inbred line *B. rapa* L. ssp. *pekinensis* cv. A03 (Lu et al. 2016; Sun et al. 2022), from which a leaf inward curling mutant *lic86* was identified in the *M*₅ generation. In August 2022, seeds of both wild type A03 and *lic86* were sown in seedling trays. The seedlings were transplanted to the field at Hebei Agricultural University, Baoding, China in mid-September. At the rosette stage, the third young leaf (counted from the center outward) from both wild type and *lic86* plants was immediately frozen in liquid nitrogen and stored at −80°C for RNA and phytohormone analysis. The

seedlings treated with hormone were grown in the controlled climate room with 50% relative humidity and a 16 h light/8 h dark photoperiod at 22°C.

2.2. Morphological characteristics of *lic86*

At the rosette stage (45 days after sowing) and the heading stage (90 days after sowing), plant height and plant width were measured for both the wild type and *lic86*. At the rosette stage, the leaf width was measured at the widest point from the fifth to eighth leaves in the natural state (LW1) and fully flattened state (LW2). The curvature indices in the transverse axis (CIT) were calculated according to the formula, $CIT = (LW2 - LW1) / LW1$. At the heading stage, the leaf width of the first layer of heading leaves (three leaves) was used to measure the curvature indices in the transverse axis.

At the rosette stage, the adaxial and abaxial epidermis cells of the secondary leaf veins were observed using the glue-marking method (Appendix A). The glue was evenly coated on the leaf surface. After air-drying, the cells were gently removed using tweezers and mounted on a slide for imaging. Cell structures were examined using the optical microscope (Olympus CX41, Tokyo, Japan).

2.3. RNA sequencing and data analysis

Total RNA and cDNA libraries were prepared and sequenced as previously described (Li et al. 2019). The reference genome and gene model annotation files were obtained from the *B. rapa* database (*B. rapa* L. ssp. *pekinensis* cv. A03) (Sun et al. 2022). The fragments per kilobase of exon model per million mapped fragments (FPKM) was used to analyze the gene expression levels. The $|\log_2(\text{Fold Change})| > 1$ and corrected P -value < 0.05 were taken as the thresholds for significant difference in gene expression. The enriched pathways were annotated according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). Three biological replicates were conducted. The $2^{-\Delta\Delta Ct} \times 1,000$ method was used to calculate gene relative expression levels between wild type and *lic86* plants via RT-qPCR. Primers are listed in Appendix B. Each experiment was repeated three times.

2.4. Measurement of different phytohormones

Samples (1 g) were ground into a fine powder and extracted with acetonitrile at 4°C. The concentrations of six classical phytohormones (IAA, ABA, GA₃, SA, JA and CK (zeatin and *trans*-zeatin riboside)) were analyzed using a high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) system. Three technical replicates and three biological replicates were conducted.

2.5. Exogenous hormone application on seedlings

The IAA and 2,3,5-triiodobenzoic acid (TIBA, auxin transport inhibitor) were sprayed on the surface of wild-type and *lic86* seedlings when two true leaves fully expanded. Two concentrations of IAA (20 and 50 mg L⁻¹) and two concentrations of TIBA (50 and 80 mg L⁻¹) were used to spray on the seedlings every three days. The leaf curling

morphology was assessed after the fifth application.

2.6. Virus-induced gene silencing (VIGS) in Chinese cabbage

VIGS was conducted as previously described (Yu et al. 2018). Briefly, the 40-nucleotide fragments of *BrLAX1* and *BrAS1* coding sequences (CDS) were selected for hairpin RNA construction using the vector pTY. A total of 5 µg each plasmid DNA, including pTY as a negative control, pTY-*PDS* as a positive control, pTY-*LAX1* and pTY-*AS1*, was transformed into the seeds without coat using particle bombardment, respectively. The seeds were then transplanted into growth substrate, and incubated in the dark for 24 h. The seedlings were grown in an illuminating incubator with 50% humidity and a 16 h light (10,000 lux)/8 h dark cycle at 22°C. On the 31st day after planting, photobleaching appeared in plants inoculated with pTY-*PDS*, and obvious differences in leaf curling phenotype were observed. The newly emerged leaves were collected for gene expression analysis. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative gene expression levels between wild type and *lic86* by RT-qPCR. Primers are listed in Appendix B.

3. Results

3.1. Morphological characterization of *lic86*

A mutant, *lic86*, with inward-curling leaves, was identified from EMS-induced mutant libraries of Chinese cabbage inbred line A03. The leaves of *lic86* exhibited pronounced inward curvature, particularly along the medio-lateral axis, ultimately forming an inwardly curled shape without developing an overlapping leafy head (Fig. 1-A). In contrast, the leaves of wild type (WT) plants displayed a slight outward curvature during the rosette stage, developing a leafy head with an outwardly curled shape at the heading stage (Fig. 1-A). There was no significant difference in plant height and plant width between WT and *lic86*; however, the curvature indices in the transverse axis (CIT) were significantly higher in *lic86* than in WT at both the rosette and heading stages (Fig. 1-B). At the heading stage, both plant weight and leafy head weight of *lic86* were significantly lower than those of WT (Appendix C). In addition, *lic86* exhibited the significantly increased ratio of abaxial to adaxial epidermal cell number (Ab/Ad) in leaf veins (Fig. 1-C and D).

3.2. The IAA concentration was significantly reduced in *lic86*

To further assess which phytohormone is involved in the formation of the curling leaf, six classical phytohormones, including IAA, ABA, GA₃, SA, JA and CK (zeatin and *trans*-zeatin riboside), were quantified using a high performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) system. As shown in Fig. 2, only IAA displayed the significant differences between *lic86* and WT at the rosette stage, as evidenced by the significantly lower IAA concentration in *lic86* (7.79 ng g⁻¹ fresh weight, FW) compared to WT (9.44 ng g⁻¹ FW) (Fig. 2). In contrast, the concentrations of the other five phytohormones showed no significant differences between *lic86* and WT.

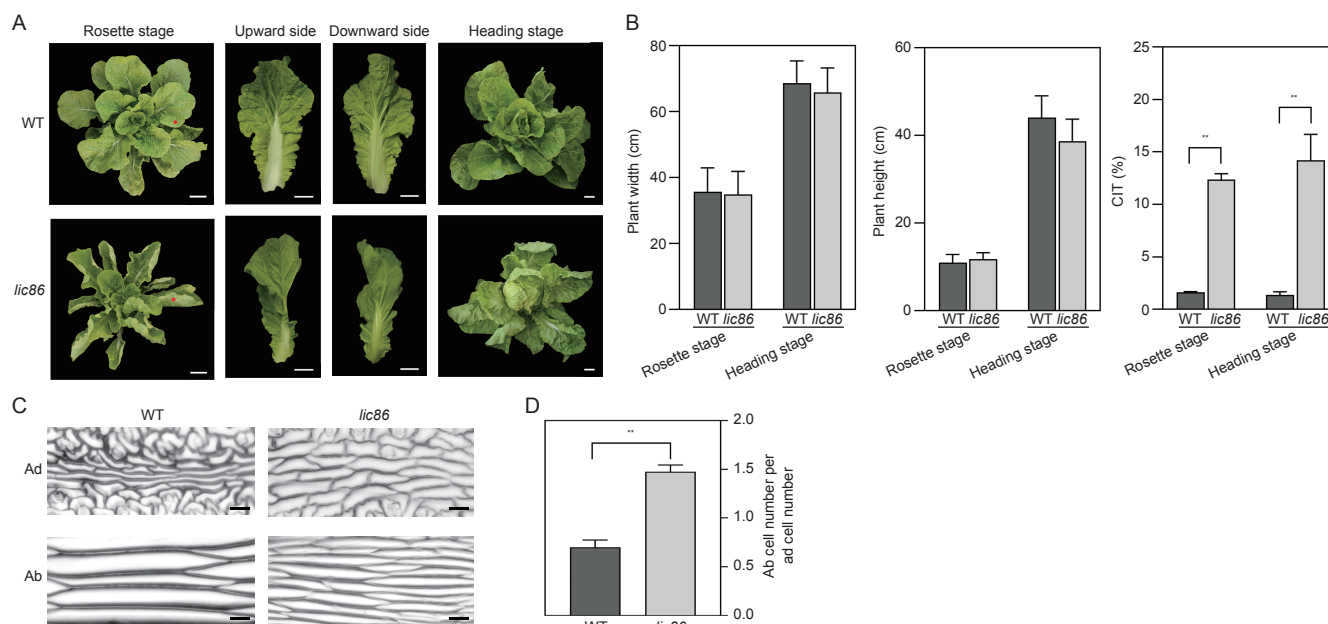


Fig. 1 Morphological differences between WT and *lic86* plants. A, phenotypes of WT and *lic86* plants at the rosette and heading stages. Leaves marked with the red dot showed the difference in curvature on both the upward and the downward sides. Bar, 5 cm. B, statistical analysis of plant height, plant width, and the curvature indices in the transverse axis (CIT) of WT and *lic86* at rosette and heading stages, respectively. Error bars are SD ($n=5$). **, $P<0.01$ (Student's *t*-test). C, microscopic observation of adaxial (Ad) and abaxial (Ab) epidermal cells in the secondary leaf veins of WT and *lic86* plants. Bar, 100 μ m. D, statistical analysis of the abaxial-to-adaxial epidermal cell number ratio in leaf veins of WT and *lic86* plants. Error bars are SD ($n=3$). **, $P<0.01$ (Student's *t*-test).

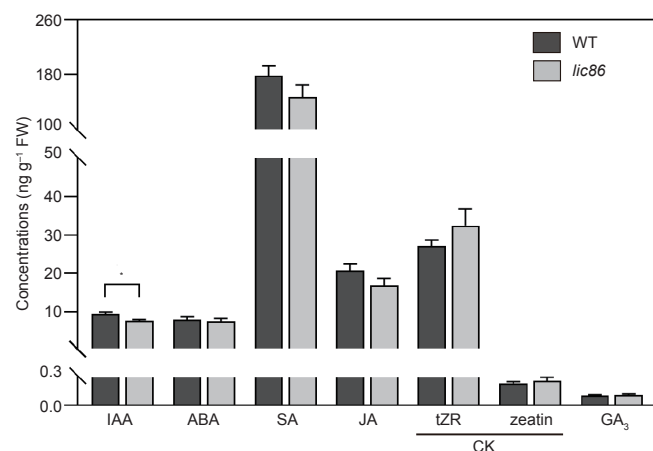


Fig. 2 Measurement of IAA, ABA, SA, JA, tZR, zeatin and GA₃ concentrations in *lic86* and WT plants. Error bars are SD ($n=3$). *, $P<0.05$ (Student's *t*-test).

These findings strongly indicate that IAA is a key factor regulating the leaf curling in *lic86*.

3.3. Exogenous application of IAA rescued the inward leaf curling phenotype in *lic86*

To further confirm the role of IAA in leaf curling, we applied exogenous IAA and IAA transport inhibitors (TIBA) to the seedlings of WT and *lic86* plants, respectively. Under normal growth conditions, the leaves of WT plants were flat, while the leaves of *lic86* exhibited slight inward curvature along

the edges (Fig. 3-A). After spraying IAA, the leaves of both WT and *lic86* displayed outward curling (Fig. 3-A). Notably, the curling degree of treated leaves increased following the increased concentrations of IAA (Fig. 3-B). However, the difference in leaf curvature between *lic86* and WT was limited after IAA treatment (Fig. 3-B). In contrast, TIBA application caused inward curling and surface wrinkling in the leaves of both WT and *lic86* without observed difference (Fig. 3-A). TIBA treatment inhibited seedling growth, resulting in delayed leaf development, with more potent inhibition effects at higher TIBA concentrations (Fig. 3-A). In addition, the expression levels of the three axial polarity genes, including *B. rapa* LITTLE ZIPPER 1 (*BrZPR1*), *BrAS1.1* and *BrAS1.2*, were significantly reduced in both WT and *lic86* in response to IAA treatment (Fig. 3-C). Taken together, these findings demonstrate that exogenous IAA application can rescue the inward curling phenotype in *lic86*, indicating the key role of IAA in regulating leaf curling of Chinese cabbage.

3.4. Transcriptomic analysis of *lic86* mutant revealed that genes associated with hormone signaling, and leaf axial polarity were significantly downregulated

To further understand the regulatory mechanisms underlying leaf curling, transcriptome sequencing was performed on *lic86* and WT plants. Following data quality assessment, high-quality and clean sequencing data were obtained for further analysis. A total of 527 differentially expressed genes (DEGs) were identified in *lic86* compared to WT, including 262 up-regulated and 265 down-regulated genes (Fig. 4-A). Among these, 108 DEGs were significantly enriched in 76 KEGG

pathways. Notably, of the top ten significantly enriched pathways including 47 genes, the plant hormone signal transduction pathway accounted for the highest proportion, comprising 30% (14 genes) (Fig. 4-B). Further analysis of the 14 DEGs involved in various hormone signaling pathways uncovered that six genes were associated with the auxin pathway, three with cytokinin signaling, two each with gibberellin and brassinosteroid signaling, and one with the abscisic acid pathway (Fig. 4-C; Appendix D). Notably, transcript levels of all six auxin signaling-related genes were significantly downregulated in *lic86* (Fig. 4-C; Appendix E). Moreover, three transcription factor genes, including *BrAS1.1*,

BrAS1.2 and *BrZPR1* that are involved in axial polarity and patterning, were significantly upregulated in *lic86* (Fig. 4-C and D).

3.5. Silencing of *BrLAX1* and *BrAS1s* resulted in altered leaf polarity in WT and *lic86* seedlings

To confirm the functions of specific genes in regulating leaf axial polarity, we employed turnip yellow mosaic virus (TYMV)-induced gene silencing (VIGS) to silence the expression of *BrLAX1*, the auxin influx carrier, and two axial polarity-related paralogs genes, *BrAS1.1* and *BrAS1.2*, in WT and *lic86* plants, respectively (Fig. 5; Appendices F

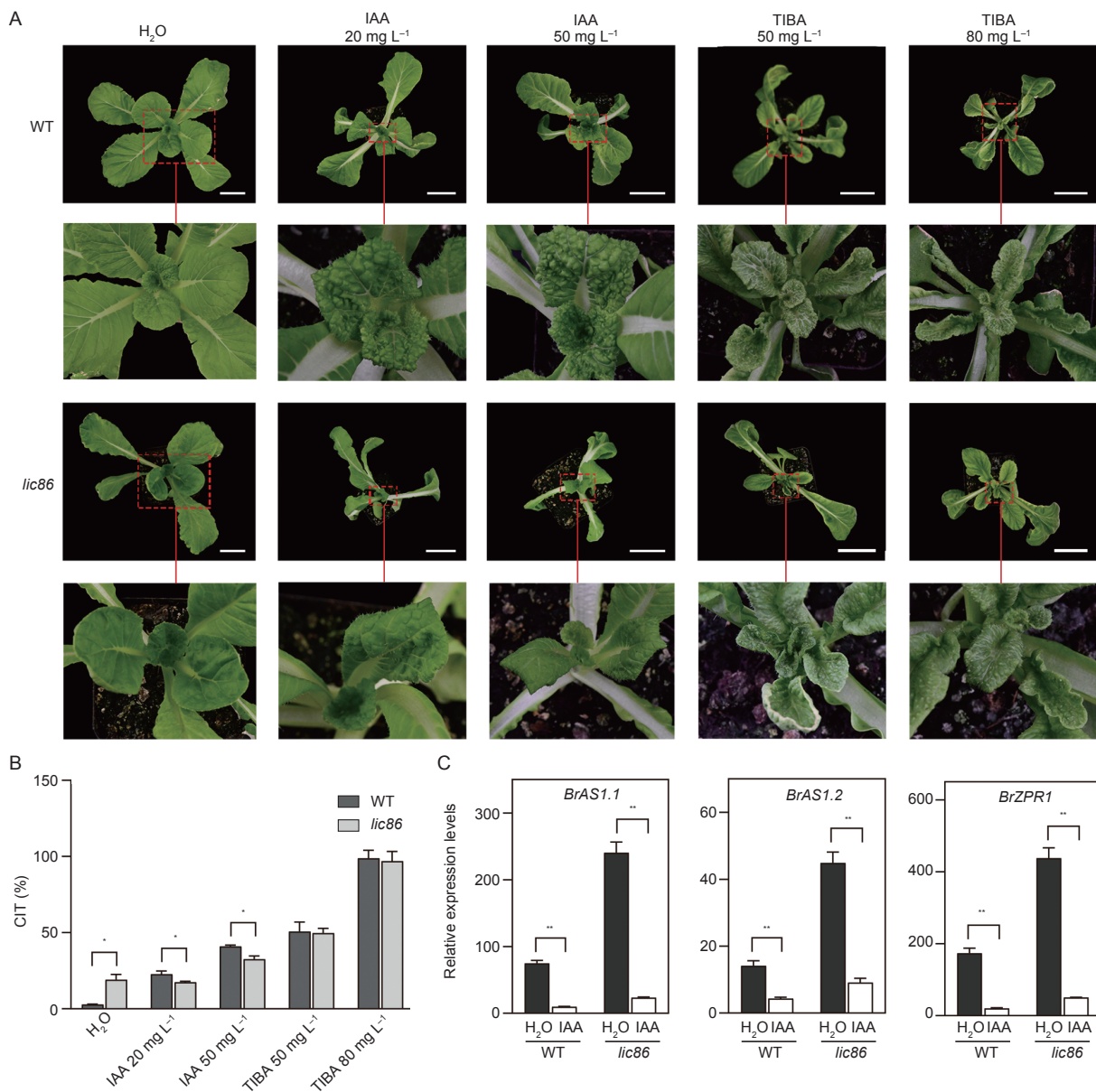


Fig. 3 Exogenous IAA rescues the inward curling phenotype in *lic86*. A, phenotypic observations following application of two concentrations of IAA (20 and 50 mg L⁻¹), which induced outward curling of leaves in both WT and *lic86*. Treatment with two concentrations of TIBA (50 and 80 mg L⁻¹) caused the leaf surface wrinkling and inward curling in both WT and *lic86*. Bar, 5 cm. B, statistical analysis of the curvature index in the transverse axis (CIT) of WT and *lic86* following IAA and TIBA applications. Error bars are SD ($n=5$). *, $P<0.05$ (Student's *t*-test). C, RT-qPCR analysis showing the expression levels of the three axial polarity genes, including *BrAS1.1*, *BrAS1.2* and *BrZPR1*, in both WT and *lic86* plants following IAA treatment. Error bars are SD ($n=3$). **, $P<0.01$ (Student's *t*-test).

and G). In WT plants bombarded with the silencing vector pTY-*LAX1*, *BrLAX1* expression was significantly reduced (Fig. 5-C), resulting in an inward leaf curling phenotype (Fig. 5-A; Appendix H), while leaves of plants bombarded with the vector control (pTY) remained normal flat morphology (Fig. 5-A; Appendix H). In contrast, the leaves of *BrLAX1*-silenced *lic86* plants displayed inward curling similar to the vector control plants (Appendices G and H). The expression levels of genes *BrAS1.1* and *BrAS1.2* showed no significant changes after VIGS of *BrLAX1* in either WT or *lic86* plants (Appendix I). Additionally, *BrAS1*-silenced *lic86* plants showed significantly reduced expression of both *BrAS1.1* and *BrAS1.2* (Fig. 5-B and C). The leaves of these plants displayed a restored flat growth compared to the pronounced inward curling leaves observed in pTY-silenced *lic86* plants (Fig. 5-B; Appendix H). In WT plants, silencing of *BrAS1.1* and *BrAS1.2* did not alter the flat leaf phenotype, resembling the vector control plants (Appendices G and H). The expression level of *BrLAX1* remained unaffected following the silencing of *BrAS1s* in both WT and *lic86* plants (Appendix I). Taken together, these findings suggest that *BrLAX1* and *BrAS1s* genes play opposing roles in regulating leaf polarity and curvature in Chinese cabbage.

4. Discussion

Leaves play important roles for photosynthesis in plants, and serve as the commodity organ in leafy vegetables, including cabbage, lettuce, Chinese cabbage. The leaf morphology is closely related to the yield of these leafy vegetables. The leaf curling affects the plant shape, and more importantly, is a necessary biological process for leafy head formation in Chinese cabbage (Mao *et al.* 2014; Zhang X M *et al.* 2022). In our study, hormone profiling and transcriptome analyses further revealed that auxin is involved in the leaf curling in Chinese cabbage (Fig. 6). In WT plants, the high concentration of IAA activates the auxin signal transduction pathway, resulting in the upregulation of the auxin influx carrier gene *BrLAX1* and five *B. rapa* SMALL AUXIN UP RNA genes (*BrSAURs*), and the suppression of *BrAS1s*, thereby causing the flat leaf development (Fig. 6). In contrast, the *lic86* mutant exhibits reduced levels of IAA, which repressed auxin signaling, thereby downregulating the expression of *BrLAX1* and five *BrSAURs*, while activating the expression of *BrAS1s*. This altered gene expression profile promotes inward leaf curling in *lic86* (Fig. 6).

Cell division along the adaxial-abaxial axis affects leaf

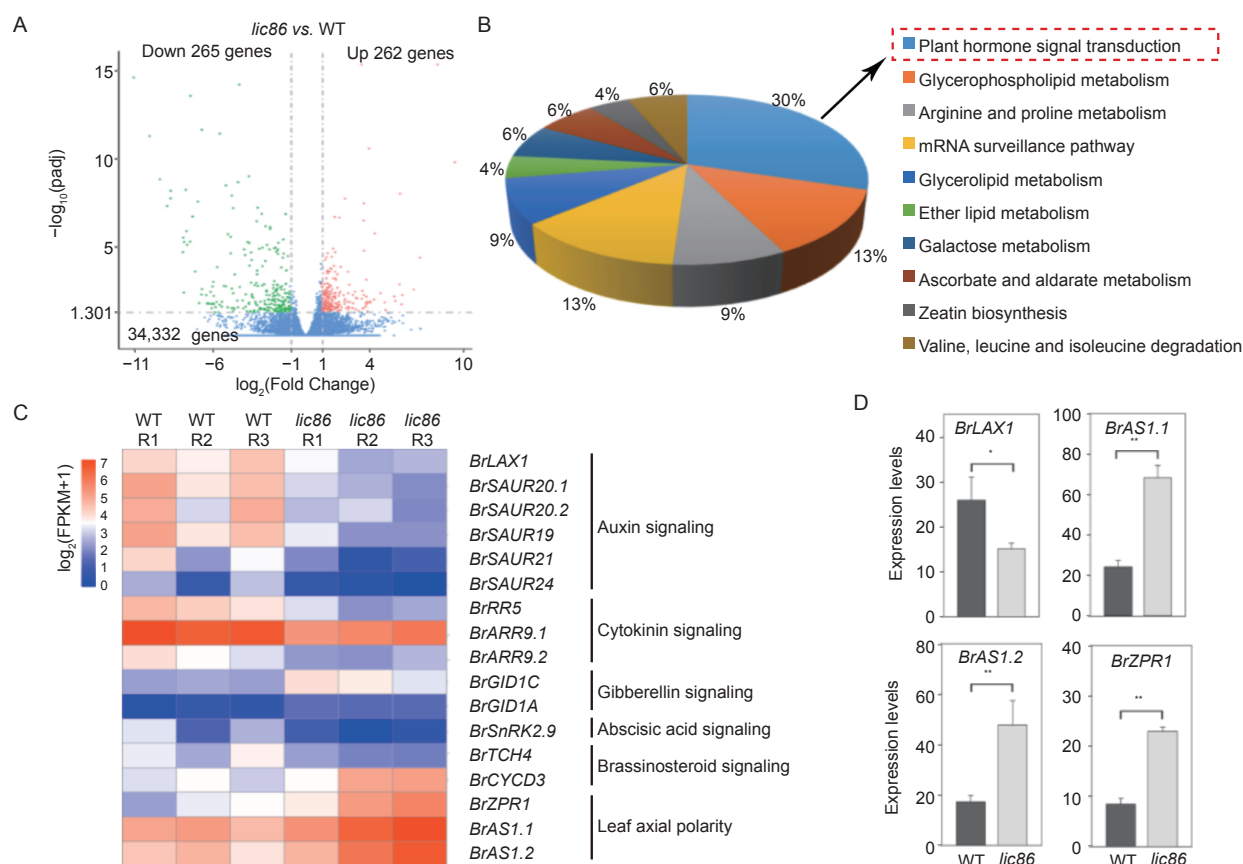


Fig. 4 Identification of differentially expressed genes (DEGs) and KEGG and heatmap analyses from RNA-seq data of the *lic86* mutant. A, number of DEGs in *lic86* compared to WT. B, top ten KEGG pathways enriched for DEGs in *lic86*. Different colors represent distinct pathways. The number of genes involved in each pathway is labeled in parentheses. Pathways in the rectangular boxes are involved in plant hormone signal transduction. C, heatmap showing the expression profiles of genes related to hormone signaling, and leaf axial polarity. The bar on the left side represents relative expression values, with red indicating high expression levels and deep blue indicating relatively low expression levels. D, RT-qPCR analysis showing the relative expression levels of four genes (*BrLAX1*, *BrAS1.1*, *BrAS1.2*, and *BrZPR1*). Error bars are SD ($n=3$). *, $P<0.05$; **, $P<0.01$ (Student's *t*-test).

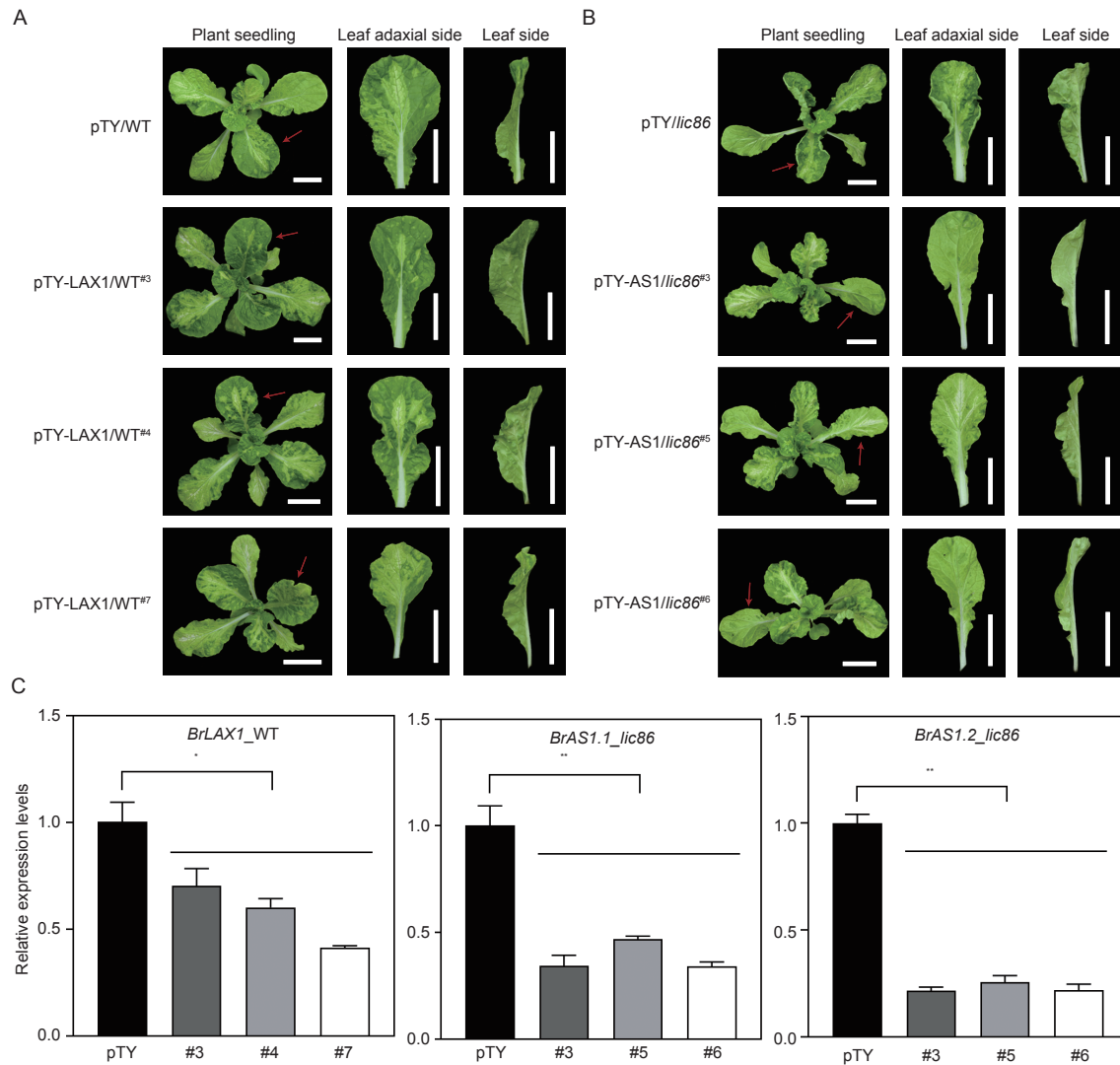


Fig. 5 Turnip yellow mosaic virus (TYMV)-induced gene silencing of *BrLAX1* and *BrAS1s* alters leaf polarity in WT and *lic86* seedlings of Chinese cabbage. A, the leaves of *BrLAX1*-silenced WT displayed inward curling phenotypes and the leaves of plants bombarded with empty vector control pTY remained flat in growth. Representative leaf blades are indicated by red arrows. Bar, 3 cm. B, in *lic86* mutant plants, simultaneous silencing of *BrAS1.1* and *BrAS1.2* restored flat leaf growth, while the leaves of the vector pTY-silenced plants remained pronounced inward curling. Red arrows indicate representative leaf blades. Bar, 3 cm. C, RT-qPCR analysis showed that the expression levels of *BrLAX1*, *BrAS1.1* and *BrAS1.2* were significantly reduced in plants bombarded with the silencing vectors pTY-LAX1 and pTY-AS1, respectively. Error bars are SD ($n=3$). *, $P<0.05$; **, $P<0.01$ (Student's *t*-test).

curling. Accelerated division of leaf adaxial epidermal cells reduces cell size, thereby promoting outward leaf curling in Chinese cabbage (Mao *et al.* 2014). In contrast, the increased ratio of abaxial to adaxial epidermal cell number (Ab/Ad) in leaf veins was found to be associated with inward curling leaves (Yue *et al.* 2022). Similarly, our studies found that the epidermal cells of leaf vein showed an Ab/Ad growing pattern (Fig. 1-C and D), facilitating leaves inward curling in *lic86* (Fig. 1-A). Leaf curling further affected the leafy head formation in Chinese cabbage. During heading, the inward curvature of leaves results in the formation of an inwardly curled leafy head (Fig. 1-A).

In *Arabidopsis*, AUXIN1/LIKE-AUX1 (AUX1/LAX) family members function as the major auxin influx, including four members AUX1, LAX1, LAX2, and LAX3 (Swarup and

Bhosale 2019). The *Arabidopsis aux1 lax1* double mutant showed downward bending of the leaf blade (Legris *et al.* 2021). Interestingly, the mutation in the *SILAX1* gene caused severe upward leaf curling (hyponastic) phenotype in tomato (Pulungan *et al.* 2018). *BrLAX1* was significantly downregulated in the inward-curling leaf mutant *lic86* (Fig. 1-A, Fig. 4-C and D), and transient silencing of *BrLAX1* in WT caused similar inward leaf curling (Fig. 5-A and C). This suggests that the low expression of *BrLAX1* contributes to the inward leaf curvature phenotype in Chinese cabbage. In WT Chinese cabbage, the relatively high concentration of IAA activates the auxin signal transduction pathway, upregulating the auxin influx carrier gene, *BrLAX1* and five *BrSAURs* within the auxin-responsive gene family (Fig. 4-C and D, Appendix E). Conversely, in *lic86*, the lower

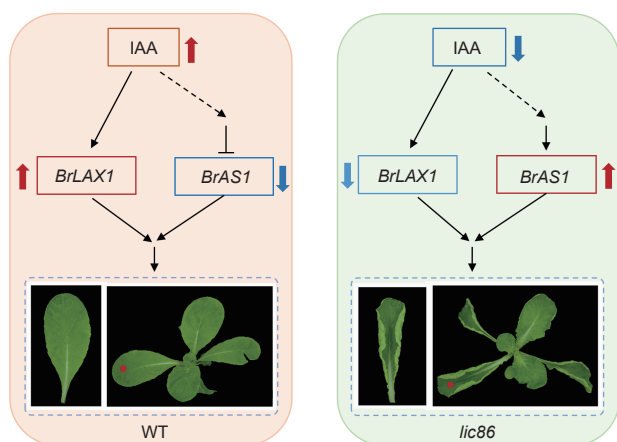


Fig. 6 Proposed model of auxin-mediated regulation of leaf polarity in WT (left) and *lic86* (right) plants. In WT plants, elevated concentrations of IAA activate the auxin signal transduction pathway, which results in the upregulation of the auxin influx carrier gene *BrLAX1*, and the suppression of *BrAS1*, thereby causing the flat leaf development in WT plants. In contrast, the *lic86* mutant exhibits reduced levels of IAA, which represses auxin signaling, thereby downregulating the expression of *BrLAX1*, while activating the expression of *BrAS1*. This altered gene expression profile promotes inward leaf curling in *lic86*.

concentration of IAA suppresses auxin signaling, leading to the downregulation of *BrLAX1* and five *BrSAURs* (Fig. 4-C and D), which might promote the inward leaf curling in *lic86* (Fig. 2). In addition, genes involved in other phytohormone signaling, including cytokinin, gibberellin, brassinosteroid, are differentially expressed in *lic86* (Fig. 4-C), suggesting the crosstalk of auxin and other hormones in regulating leaf curvature (Li *et al.* 2009; Cheng *et al.* 2016; Xiong *et al.* 2021). A previous study showed that high expression levels of auxin signaling genes contribute to the formation of a wrinkled leaf in *B. rapa* var. *tai-tsai* (Zhang *et al.* 2025). However, in our study, auxin-induced leaf crinkling was stronger in the wild type than in *lic86* (Fig. 3), suggesting that reduced expression of *BrLAX1* may impair auxin signaling (Fig. 4-C and D).

AS1/AS2 plays an important role in establishing leaf adaxial–abaxial polarity. Outward leaf curling is observed in *Arabidopsis as1* mutants (Hay *et al.* 2006). In *Brassica napus*, increased expression of *BnaC02.LBD6*, a homolog of *AtAS2*, causes inwardly curled leaves (Gao *et al.* 2024). In our study, the transcript levels of *BrAS1.1* and *BrAS1.2* were significantly upregulated in *lic86* compared with that in WT (Fig. 4-C and D). Transient silencing of *BrAS1.1* and *BrAS1.2* in *lic86* resulted in the growth of flat leaves (Fig. 5-B and C), while silencing these genes in WT plants did not alter leaf morphology (Appendices G and H). In addition, increased expression of *BrAS1* genes appeared to contribute to the formation of inwardly curled leaf without an overlapping leafy head in Chinese cabbage (Zhang X M *et al.* 2022). *BrZPR1* belongs to a gene family encoding small leucine zipper-containing proteins (LITTLE ZIPPER [ZPR] proteins), with a leucine zipper domain similar to those found in REVOLUTA, PHABULOSA, and PHAVOLUTA proteins

(Wenkel *et al.* 2007). The expression level of *BrZPR1* was significantly higher in *lic86* than in WT (Fig. 4-C and D). However, overexpression of *ZPR1* in *Arabidopsis* resulted in downward leaf curling (Wenkel *et al.* 2007). Therefore, we speculate that the inward curling phenotype observed in the *lic86* mutant is unlikely to be caused by increased expression of *BrZPR1*.

5. Conclusion

In this study, a Chinese cabbage mutant *lic86* exhibiting inwardly curled leaves was identified and the molecular mechanisms underlying its leaf curling phenotype were characterized. Comparative transcriptomic analysis, virus-induced gene silencing, and hormone spraying assays between WT and *lic86* collectively demonstrated that IAA, together with the auxin influx carrier gene *BrLAX1* and the leaf axial polarity gene *BrAS1*, played an essential role in the regulation of leaf curling in Chinese cabbage.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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