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Review

Physiological Control and Genetic Basis of Leaf Curvature and Heading in *Brassica rapa* L

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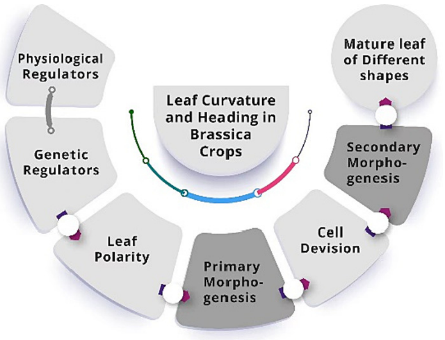
HIGHLIGHTS

- The heading leaves play an important role as nutrient storage organs, enhancing the high quality and commercial benefit of leafy heads.
- Primary and secondary morphogenesis determines leaf shape.
- Leaf incurvature is established by downregulation or knockdown of abaxial regulators and upregulation of adaxial regulators.
- In heading vegetables, leaf incurvature is prerequisite for the development of leaf heads.
- Understanding *Arabidopsis* leaf development enables us to predict the important regulators of leafy head formation in *Brassica* crops like Chinese cabbage and cabbage.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Heading is an important agronomic feature for Chinese cabbage, cabbage, and lettuce. The heading leaves function as nutrition storage organs, which contribute to the high quality and economic worth of leafy heads. Leaf development is crucial during the heading stage, most genes previously predicted to be involved in the heading process are based on *Arabidopsis* leaf development studies.

Aim of review: Till date, there is no published review article that demonstrated a complete layout of all the identified regulators of leaf curvature and heading. In this review, we have summarized all the identified physiological and genetic regulators that are directly or indirectly involved in leaf curvature and heading in *Brassica* crops. By integrating all identified regulators that provide a coherent logic of leaf incurvature and heading, we proposed a molecular mechanism in *Brassica* crops with graphical illustrations. This review adds value to future breeding of distinct heading kinds of cabbage and Chinese cabbage by providing unique insights into leaf development.

Key scientific concepts of review: Leaf curvature and heading are established by synergistic interactions among genes, transcription factors, microRNAs, phytohormones, and environmental stimuli that regulate

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primary and secondary morphogenesis. Various genes have been identified using transformation and genome editing that are responsible for the formation of leaf curvature and heading in *Brassica* crops. A range of leaf morphologies have been observed in *Brassica,* which are established because of the mutated determinants that are responsible for cell division and leaf polarity.

Contents

Introduction

Leaves are formed through primary and secondary morphogenesis [1]. Primary morphogenesis includes the development of the lamina, midrib, petiole, leaf base, leaflets, lobes, and serrations [2]. Secondary morphogenesis determines the ultimate shape of the leaf, which is regulated by precisely defined cell proliferation and expansion that is affected by cell number and cell size and is an irreversible, synchronized genetic process that can be altered by multiple factors. [3,4]. Out of all altered leaf shapes, leaf curvature (LC) and leaf heading (LH) are of particular research interest, because they are crucial in determining the texture, increasing the shelf life, and improving the ease of harvest in *Brassica* crops. Leaf curvature (LC) is inward or outward curling of leaves that reflects the potential of plant photosynthesis, storage and edible organ in commonly cultivated leafy vegetables, especially *Brassica* species, which include mustard greens (*Brassica juncea*), Chinese cabbage (*Brassica rapa* ssp. *pekinensis*), and Cabbage (*Brassica oleracea* var. *capitata*) [5]. LC can be categorized into two types [6]: positive Gaussian curvature, or upward/inward curling, and negative Gaussian curvature, or downward/outward curling [7]. Leaf incurvature (Positive Gaussian curvature), characterized by curling, crinkling and folding of leaves is an essential prerequisite for LH formation [8,9]. Incurvature (inward curling) begins at folding and is completed at the heading stage, at which point the leaves serve as storage organs in *Brassica* crops [10]. Changes in LC determine the angle of light absorption, efficiency of photosynthesis, and biomass yield in all leafy vegetables. The establishment of a spherical mass of edible leaves (LH), particularly in Chinese cabbage and cabbage, that is high in ascorbic acid, tocopherol, and vitamin A [11,12]. The final LH phenotype is determined by events that occur at four developmental stages: the seedling, rosette, folding, and heading stages. *Brassica* leaves typically show a flat shape at the seedling and rosette stages and are actively involved in photosynthesis and respiration [13,14]. LH shape is created by the overlapping of leaves [6] based on positive Gaussian curvature [7] and the folding of leaves around the shoot apexes to form compact and cylindrical heads or hearts in Chinese cabbage [15]. Many heading cultivars are high in nutrients and commercially valuable, such as Wakefield, Danish Ballhead, and Savoy [16].

Some *Brassica* species possess LC and LH e.g., *Brassica rapa* (*B. rapa*), and *Brassica oleracea* (*B. oleracea*), are polyploid, and their genome sequences have not only provided insight into evolution, domestication, and diversification but have also revealed the complexity of genetic LC and LH regulation [17]. *B. rapa* and *B. oleracea* both exhibit LH morphology, and their resequencing has provided evidence for their convergent domestication [18]. Studies on the regulation of leaf morphology have been reported more often in *Arabidopsis thaliana* than in *Brassica.* Identification of *A. thaliana* homologs in *Brassica* presents new challenges to researchers for finding leaf morphology regulators [19,20].

Leaf morphogenesis events are regulated by a complex network of genetic, physiological, and environmental factors that are regulated by different phytohormone signaling pathways. Phytohormones are chemical messengers that are generated in one region of the plant and then translocated to other parts of the plant to regulate plant growth and development [21,22]. A combined *B. rapa* and *B. oleracea* gene enrichment analysis revealed gibberellic acid (GA) biosynthesis as well as auxin-, cytokinin (CK), brassinosteriods (BR) and jasmonic acid (JA)-mediated signaling pathways. These pathways have been linked to the start and development of leaves [18]. These phytohormonal signaling pathways are also known to affect leaf polarity (primary morphogenesis) and cell division (secondary morphogenesis) that triggers LC and LH establishment [23,24].

Nonetheless, an integrated understanding of the genetic, physiological, and environmental regulation of LC and LH is still needed. Therefore, this review provides a detailed summary of the developmental mechanisms by which the above-mentioned factors influence LC and LH. *Arabidopsis* regulators of leaf developmental stages, with a focus on orthologs genes in *Brassica* crops, provide the foundation for identifying the LC and LH regulators. We

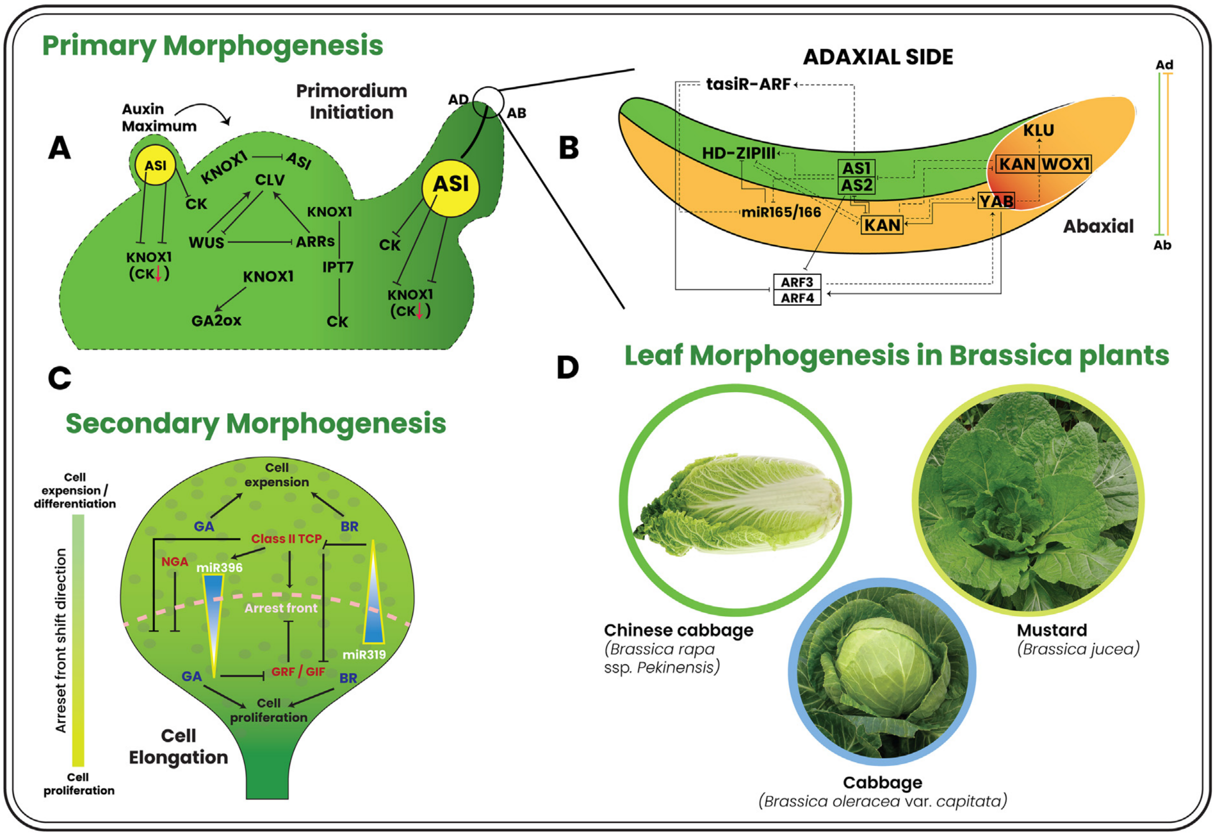

Fig. 1. . Primary and secondary leaf morphogenesis, together with LC and LH morphology, in *Brassica* plants The general mechanism of primary morphogenesis, from leaf initiation to lamina formation, is shown. **A**. The molecular mechanism of leaf primordium initiation. **B**. The adaxial/abaxial patterning mechanism. **C**. Secondary morphogenesis and leaf lamina formation by cell proliferation and expansion. **D**. *Brassica* plants showing LC and LH leaf morphologies.

describe recent advances in our understanding of leaf shape development (LC and LH), and we outline how genetic mechanisms regulate this process, alone or in combination with other factors. We discuss physiological regulation through the effects of hormones and provide a brief account of environmental influences on leaf shape. We include graphical schematic model to enable rapid understanding of these regulatory mechanisms. This detailed overview can suggest new research avenues for investigating the coordination among regulators of leaf curvature and heading and their corresponding pathways.

Leaf formation: Primary and secondary morphogenesis

Leaves show dynamic differences in thickness, size, and shape, which distinguishes the plant species [25]. Nonetheless, leaf morphology is not fully predetermined. Thus, primary and secondary morphogenesis play a major role in leaf development, influencing the final shape of leaves [1,2]. **Primary morphogenesis** consists of primordium initiation (Fig. 1-A) and the establishment of abaxial/adaxial (ad-ab) polarity (Fig. 1-B) [26]. The process involves several stages: auxin maxima activate the callus to initiate primordium formation; the second stage leads to an adaxial-abaxial polarity; and the third stage leads to a leaf blade that is detached from the petiole, which is the final step of the process [27,28]. The formation of ad-ab patterns requires precise coordination between hundreds of cells during primordium development. Adaxial-abaxial polarity is responsible for defining medial–lateral polarity and leaf blade production. The medial–lateral axis of the leaf blade is flattened through perpendicular cell division [20]. Leaf primordium begin to expand once they have established polarity and reached their final shape that is controlled by coordination of several transcription factors. The majority of these transcription factors such as *HD-ZIPIII*, *KANADI* (*KAN*), Auxin response factor (*ARFs*) and *YABBY* are classified into various classes and thus are activated in ways that are simultaneously conserved and sometimes redundant [20].

As a leaf blade develops, its cells must proliferate and expand in a balanced manner, coordinated by molecular networks. The process of cell division at the distal end of the leaf ceases as it grows, and instead of proliferating at the base, a process of expansion takes place. Eventually, older cells fall out of the proliferation zone due to displacement by the base. Additionally, the dynamic cell proliferation regions develop rapidly at the "arrest front" boundary between proliferation and expansion for several days before disappearing rapidly. In other words, the two main processes of cell proliferation and cell expansion determine the size of the leaf at maturity, and any changes in either process may affect leaf development [29]. At leaf lamina establishment, the timing of the transition from division to expansion is essential in determining final size, flatness, and shape [30]; this process of leaf blade formation is called **secondary morphogenesis** (Fig. 1-C). The *class II TCPs* are the key regulators of timing from division to expansion [31]. There are many genes that positively regulate and control the transition from proliferation to expansion, for example, AINTEGUMENTA (*ANT*) [32], *KLUH/CYP78A5* [33] and GROWTH REGULATING FACTORS (*GRFs*) [34], whereas the negative regulators include *CIN-TCPs* and DAI [33]. In secondary morphogenesis, mature leaves of different sizes and shapes (such as., flat, curved, folded, or headed) develop by cell expansion are regulated by a well-integrated set of genes [2,35]. Any perturbation in the genetic regulators of these morphogenesis events, which can be triggered by phytohormones or environmental regulators, has the potential to transform the flat leaf into a variety of leaf morphologies (Fig. 1-D).

Primary morphogenesis establishes LC and LH

Leaf developmental mechanisms in *Arabidopsis* provides a foundation to predict the candidate regulators of LC and LH in *Brassica* crops such as Chinese cabbage and cabbage [9]. It is shown that the *B. rapa* and *B. oleracea* leaf head formation is linked to a stable genetic network established through precise lineage analysis of the adaxial and abaxial progenitors in *Arabidopsis* [18]. Chinese cabbage and *Arabidopsis* are member of Cruciferae, most genes involved in leaf ad-ab polarity establishment are concluded to be conserved between these two genomes [18]. Furthermore, 45 genes (Version 1.5) involved in the founding of leaf adaxial-abaxial polarity were observed in Chinese Cabbage [36]. Adaxial-abaxial leaf polarity (primary morphogenesis) is regulated by a complex regulatory network of genetic regulators, which is profoundly influenced by phytohormones.

Interplay of phytohormones develops LC and LH via leaf polarity

Plant hormones are produced in small amounts but have the ability to regulate a wide range of plant cellular activities. They serve as chemical messengers, connecting cellular processes and playing critical roles in harmonizing numerous signal transduction pathways, which ultimately impact leaf shape [22]. Auxin has an extensive role on plant development and establishment of organ morphology by regulating the signal transduction pathways. Auxin maxima points are established at shoot apical meristem (SAM) for organ initiation, PIN-FORMED1(PIN1) which is a polar auxin transporter, defines the positions of leaf primordia initiation and shapes leaf margins [35,37,38]. Auxin regulates the expression of *BrPIN* recognized as leaf patterning regulator that is involved in leaf heading in *B. rapa*. Auxin also regulates the expression of *BrLUX*, a leaf patterning regulator that is also identified as LH regulator in Chinese cabbage [39].

Auxin response factor (ARFs) that regulate the expression of auxin response genes, such as *AtARF2*, *AtARF3*, *AtARF4*, and MONOPTEROS (MP)/*ARF5*, have been found to influence leaf development in *Arabidopsis* (*AtARFs*) [40]. Double mutant plants *arf3arf4* showed narrow abaxial outgrowth with upward curved leaves in *Arabidopsis* [41] whereas in Chinese cabbage *BrARFs* are elucidated as a candidate regulator of LC formation [42–45]. Several investigations have found that variations in leaf polarity genes are strongly linked with the establishment of LH in *B. rapa* [14,18,46]. After primordium initiation, primary morphogenesis proceeds to develop a flat lamina through a juxtaposition of abaxial and adaxial tissues which is controlled by multiple genetic regulators that might be controlled via phytohormones signaling pathways [47,48].

In *Arabidopsis*, Auxin signaling pathway is involved into the determination of adaxial polarity that is regulated by the class III HD-ZIP family, including PHABULOSA (*PHB*), PHAVOLUTA (*PHV*), and REVOLUTA (*REV*). These adaxial regulated are also controlled with the activation of miR165 and miR166 [49–53]. All three mutated genes (*phb*, *phv*, and *rev*) culminated in radial abaxial leaves, a phenotype comparable to that of dominant flat leaf morphology [49]. Overexpression of *REV* in the *hyl1 Arabidopsis* mutant caused double-leaf curvature downward and upward (longitudinally and transversely) [54]. Moreover, downregulation of *BrpREV-1*, *BrpREV-2*, and *BrpPHB-1* produced downward leaf curling in *B. rapa* [55].

A polar auxin transporter named PIN affects the activity of adaxial regulator *KAN* to negatively regulate the expression of WOX1/ PRESSED FLOWER (*PRS*). Narrow leaves with unaffected lengths (leaf length same as of wild type leaf) were showed in *kan* mutants of *Arabidopsis* [56,57]. In *Brassica* heading crops, the ortholog of ARABIDOPSIS THALIANA HOMEOBOX 15 in *B. oleracea*; *BoKAN2.2* and in *B. rapa*; *BrKAN2.1* and *BrKAN2.3* are suggested as possible genes of adaxial-abaxial patterning establishing the in LC and LH [18,36].

In lettuce, *LsKN1* has been discovered as an LH regulator that represses the adaxial gene *LsAS1*, which is partially involved in the production of leafy heads [46]. Besides that, *LsKN1* inhibits the expression of SAWTOOTH 1 (*LsSAW1*), an *Arabidopsis* homolog. The functionally redundant SAWTOOTH (*SAW1*) and *SAW2* proteins promote leaf serrations, and *saw1saw2* mutants promote leaf serrations in *Arabidopsis* [58,59]. Transcriptomic results demonstrate that *LsSAW1* modulates leaf dorsiventrality and its silencing downregulates adaxial genes. By binding to the promoter region of the adaxial gene ASYMMETRIC LEAVES 1 (*LsAS1*), *LsSAW1* enhances the expression of *LsAS1*. Overexpression of *LsAS1*, decrease the impact of *Lssaw1* on heading. In contrast, *LsSAW1* binds to the promoter of YABBY 1 (*LsYAB1*) to inhibit its expression. *LsYAB1* overexpression caused curving of leaves in *LsSAW1* genotypes. *LsSAW1* interacts with *LsKN1*, which is requisite for the establishment of lettuce LH [60]. The role of *SAW1* in downregulating the abaxial gene and upregulating the adaxial gene for the development of LC and LH in *B. rapa* and *B. oleracea* also needs to be investigated.

Auxin together with adaxial polarity regulator ASYMMETRIC LEAVES1/2 (*AS1/2*) play an essential role to repress expression of other genes such as KNOX gene BREVIPEDICELLUS (BP) in the establishment of leaf morphology [61]. A mutation in *AS2* causes a petiole to curl upwards, resulting in a small leaflet on the petiole [62,63]. The overexpression of pak choi *BcAS2* in an *Arabidopsis* transgenic line causes the *AS2* to be engaged in the production of curved leaves [64,65]. Moreover, the *AS1-AS2* complex negatively regulates *YABBY*, *ARF*, and *KANADI* gene families (abaxial regulators) [66]. The AS1-AS2 complex also directly suppresses the production of miR166A and ETTIN (ETT) in the adaxial region, as well as binding the promoter of the ta-siRNA precursor TAS3A activation [66]. By suppressing the ARF family members (ARF2, ARF3, and ARF4), TAS3 tasiRNAs and miR390 expand a pathway that determines leaf patterning and developmental time [67–69]. The findings of the study provide the basis for the assumption that downregulation of abaxial regulators could be a cause of LC and LH.

Auxin homeostasis is also affected by the WUS-related homeobox (*WOX*) genes (*WOX1*, *WOX3*) that is linked to adaxial/abaxial patterning and the formation of flat leaves [57,70,71]. Furthermore, studies suggest that auxin signaling acts upstream of *WOX* genes, demonstrating the feedback loops between auxin signaling and CK biosynthesis that assist to *WOX* function [40]. *BrWOX1.1* (BraA05g029540.3C), *BrWOX1.2* (BraA03g038230.3C), *BrWOX3.1* (BraA04g020450.3C), and *BrWOX3.2* (BraA03g024810.3C) were discovered as *AtWOX* orthologues in *B. rapa* LH study by specific SSR markers that are associated with LH establishment [36]. However, complete understanding of *BrWOXs* in LC and LH establishment has not been reported yet.

Exogenous auxin spray and transcriptomic analysis of the heading mutant flat growth-1 (*fg-1*) demonstrated that auxin is essential for the establishment of LH in Chinese cabbage [15,72]. These findings also suggest that auxin continues to play a conserved role in establishing leaf polarity. Six adaxial identity genes, *BrREV*, *BrHB8.1*, *BrHB8.2*, *BrHB9*, *BrHB14.1*, and *BrHB14.2*, and six abaxial identity genes, *BrKAN1*, *BrBOP2*, *BrYAB1.1*, *BrYAB1.2*, *BrYAB2*, and *BrYAB3* were significantly expressed in *fg-1* mutant plants, indicating the involvement of primary morphogenesis regulators in the LH development of Chinese cabbage. Moreover, the exogenous application of auxin spray to the dorsal side of *Brassica* leaves causes upregulation of *BcpLH/BrHYL*, leading to head formation

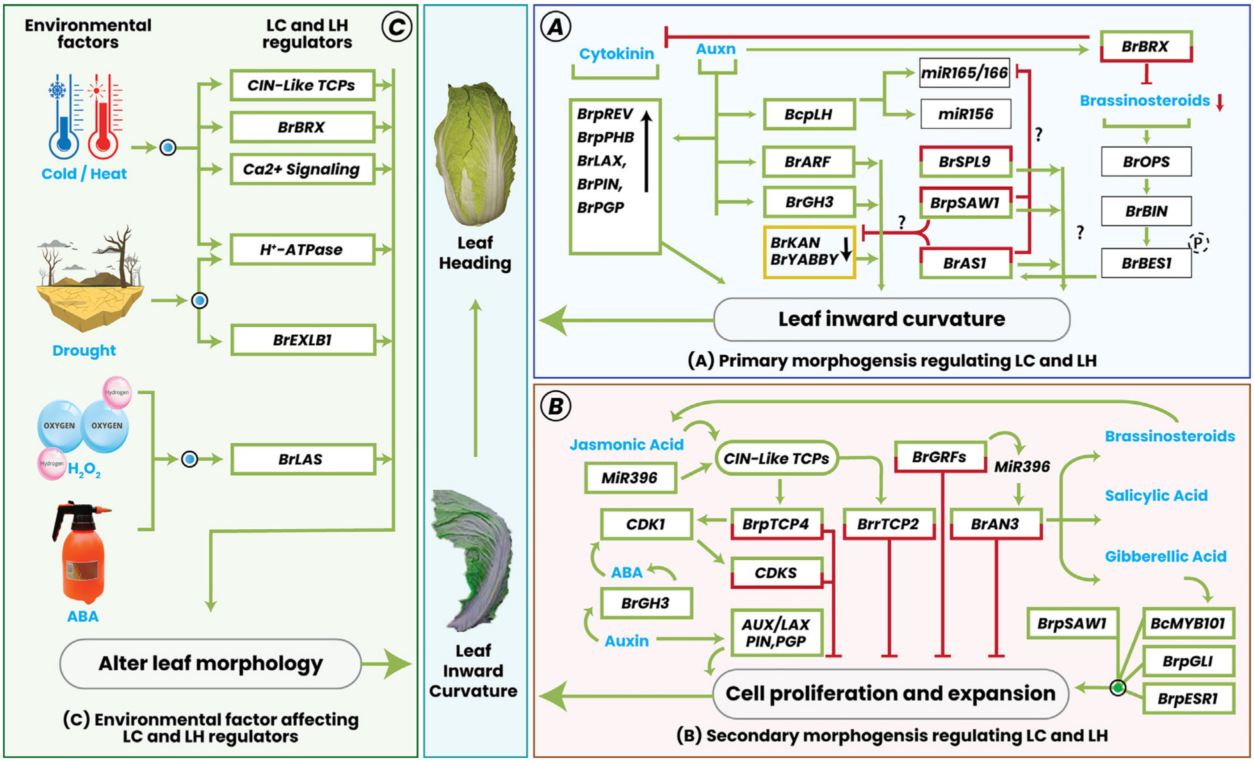

Fig. 2. . Genetic and environmental regulators of LC and LH morphology in *Brassica* plants. A. Expression pattern of primary morphogenesis regulators to induce LC and LH. Leaf abaxial-adaxial polarity regulators involved in leaf curvature and heading, the regulation of leaf curvature and heading and the influence of polarity mutations are shown. Adaxial and abaxial genes that are responsible for the regulation of leaf curvature and heading through their overexpression are indicated in green box (*BsAS2*, *BrpPHB1*, *BrpREV1*, *BrpREV2*, and *BrpSAW1*). Genes whose downregulation induces leaf curvature and heading are indicated in yellow box (*BrKan* and *BrYABBY*). Green arrows indicate enhancement, and red bars indicate inhibition. **B. Expression pattern of secondary morphogenesis regulators to induce LC and LH.** Cell division and proliferation regulators involved in the formation of leaf curvature and heading, *BrpTCP4*, *BrpTCP2* (regulated by *miR319*), *BrGRFs*, *BrAN3* (regulated by *miR396*), and *CDKs* (downregulated by *CDKI*) have been identified as candidate genes for leaf curvature and heading because of their negative effects (represented by red T lines) on cell division. *BrpGL1*, *BrpESR1*, and *BrpSAW1* have a positive effect on leaf curvature and heading (represented by green arrows) through the regulation of cell division and are considered to be candidate genes for these traits. **C. Environmental cues culminate LC and LH.** Temperature, drought, H_2O_2 and exogeneous phytohormonal spray affects the genetic regulators (*CIN-LIKE TCPs, BrBRX, AHA2, BrEXLB1,* and *BrLAS*) and signaling pathways (Ca^{+2} signaling) that cause the LC and LH.

by controlling the abaxial miR165/166 [15]. The expression level of the adaxial identity gene *REV* was increased and the expression position was restricted on both sides of growing leaves near the margins in curved leaves of *hyl1* mutants, whereas expression of the miR165 gene was significantly reduced. These findings suggest that *HYL1* orthologue of *BcpLH* maintains growing leaf polarity by regulating the level of microRNA [54]. Auxin is also involved into the upregulation BREVIS RADIX (*BRX*) genes; *BrBRX.1*, *BrBRX.2* and *BrBRX.3* that are identified as LC and LH regulator in Chinese cabbage [73]. The functional analysis of *BrBRX* revealed their role in petiole length, leaf number, and leaf angle that are associated with leaf curvature and heading [73]. *BRX* orthologues, *AtBRX* showed epinastic leaf phenotype in *Arabidopsis brx* mutants [74]. *BRX* ortholog *BoBRX.2* was identified under selection in leaf-heading accessions of *B.oleracea* in crop domestication of leaf-heading morphotype [18]. Furthermore, *BRX* is involved in cross-talk between cytokinin signaling, brassinosteroid biosynthesis and auxin signaling that regulates cell proliferation and cell expansion in the leaf [75,76]. It will be interesting to determine if *BrBRXs* are involved in cross-talk between auxin and BR signaling pathways in Chinese cabbage LH development [73].

BR is also responsible for the transfer of OCTOPUS (*BrOPS*) to the nucleus and interacts with *BrBIN2* to suppress the phosphorylation of BRINSENSITIVE1 (*BRI1*)-EMS-SUPPRESSOR1 (*BrBES1*). This suppression leads to the downregulation of the leaf polarity transcription factor *BrAS1*, influencing LC and LH in Chinese cabbage [9]. Chinese cabbage inward curling mutant (*ic1*) boosted the phosphorylation of *BrBES1*, with the accumulation of *BrBIN2* in the nucleus in the absence of *BrOPS* when the level of BR is low. Phosphorylated *BrBES1* stimulates the repression of *BrAS1*, culminating the overexpression of the adaxial regulator *BrAS1*, which triggers leaf inward curling in Chinese cabbage *ic1* mutants [9]. All these finding strongly consistent with the statement that primary morphogenesis regulators highly influenced the formation of LC and LH in *Brassica* crops (Fig. 2).

Role of posttranscriptional regulators and DNA methylation

Gene expression is tightly controlled at both the transcriptional and post-transcriptional levels. Primary morphogenesis (adaxial-abaxial polarity) is also regulated by posttranscriptional mechanism [36]. Pre-messenger RNA (mRNA) processing (capping, splicing, and polyadenylation), mRNA stability, and mRNA translation are all instances of post-transcriptional gene expression control [77]. The production of microRNAs and tasiRNAs requires a number of proteins, which include ARGONAUTE1 (AGO1), ARGONAUTE7 (AGO7), ARGONAUTE10 (AGO10), SUPPRESSOR OF GENE SILENCING 3 (SGS3), RNADEPENDENT RNA POLYMERASE 6 (RDR6), DICERLIKE1 (DCL1), DICER-LIKE4 (DCL4) and SERRATE (SE) [78].

Orthologs of *Arabidopsis AGO1*, *AGO7*, *AGO10*, SGS3, *RDR6*, *DCL1*, *DCL4*, and *SE* that are associated with the leaf adaxial abaxial polarity, were determined in Chinese cabbage as *BrAGO1.1* (BraA05g020200.3C), *BrAGO7* (BraA07g030370.3C), *BrAGO10.1* (BraA06g043560.3C); *BrAGO10.2* (BraA09g019960.3C), *BrSGS*

(BraA06g031000.3C), *BrRDR6* (BraA01g023640.3C), *BrDCL1* (BraA10g000840.3C), *BrDCL4* (BraA10g020250.3C), and *BrSE1* (BraA07g018000.3C); *BrSE2* (BraA04g019460.3C) respectively. It is strongly hypothesized by previous study that these posttranscriptional polarity regulators are associated with the LC and LH formation in Chinese cabbage [56].

Hyponastic leaves 1 (*HYL1*) encodes a protein that regulates the leaf abaxial determinant miR166, and *Arabidopsis hyl1* mutants showed inward-curling leaves [79]. *BcpLH* (BraA06g006590.3C), a homolog of *Arabidopsis HYL1*, was isolated from Chinese cabbage and shown to influence the inward curvature of folding leaves [54]. leaf Ad–Ab patterning candidate genes and *BrHYL1.1* (also known as *BcpLH*) are also linked to head formation by regulating the expression of *miR156*, *miR165/6*, and *miR319* [80,81]. *BcpLH* and *BcpLH2* regulate the *BrpSPL9-2* (a homolog of *Arabidopsis* SQUAMOSA PROMOTER BINDING-LIKE (*SPL*), which control the timing (developmental stage of plant) of leaf incurvature and heading [82]. Tasi-RNAs have also been identified as the regulators of leaf polarity determinants such as *ARF3/4* abaxial genes is regulated by tasiR-ARF [83]. Genetic linkage map screening of polarity regulators determined that *BrARFs,* leaf abaxial regulator is associated with the LC and LH in Chinese cabbage [36]. These findings prompted us to hypothesize that tasiR-ARF induce any mutation in the abaxial polarity regulator *BrARFs* to enhance or repress the LC and LC.

Furthermore, DNA methylation appears to be a key regulator of the mechanisms underlying the formation and maintenance of the LC and LH. DNA methylation is essential for epigenetic control of the genome and gene expression [84]. DNA methylation, in particular with regard to histone modifications (acetylation and methylation), causes genetically inherited and/or transient alterations in chromatin structure and gene expression that do not make a significant difference in nucleotide sequence, and leads to genomic imprinting [85] and gene silence [86]. DNA methylation-regulated auxin pathways contribute to the establishment of LC and LH by suppressing DNA methylation in promoters, which might also regulate the expression of genes involved in LC and LH [87]. Auxin signaling pathway was negatively affected with the methylated promoters of *BrPIN1* and *BrARFs* and also these genes were downregulated in the detorted LH phenotype of Chinese cabbage [87]. Furthermore, a DNA methylation approach known as methylationsensitive amplification polymorphism (MSAP) indicated that *B. oleracea's* (heading crop) methylation polymorphism is the cause of morphological polymorphism as well as substantial structural genomic rearrangements in its related species [88]. Nonetheless, the mechanisms behind these impacts are unknown.

These alterations in gene expression most likely altered the adaxial-abaxial polarity of leaves, inducing inward leaf curling. Adaxial gene downregulation or loss of function, as well as abaxial gene overexpression, can indeed stimulate LH growth [60].

Secondary morphogenesis establishes LC and LH

In secondary morphogenesis, genes carry crucial instructions that determine leaf shape by specifying cellular growth patterns, cell cycling, cell differentiation, cell enlargement, and hormonal transport, via complex regulatory systems [34]. Any abnormal behavior of cell proliferation and expansion regulators may have a direct effect on LC and LH [80]. It has been speculated that genetic factors involved in cell division and expansion may influence the cabbage leafy head phenotype [89]. QTL mapping has been used for the efficient identification of genes responsible for different leaf traits in multiple crops (tomato, maize, grape, and *Arabidopsis*) [90–92]. In Chinese cabbage, three genes (*BrpGL1*, *BrpESR1*, and *BrpSAW1*) were identified as LH candidate genes (represented with green arrows in Fig. 2), and they also influence the development of trichomes, petioles, and leaf serration through their functions in the regulation of cell division [14].

Interplay of phytohormones develops LC and LH via cell proliferation and expansion

The auxin signal is essential for the formation of vascular system and the intake of nutrients for leaf cells. It might be difficult to discern the direct effect of auxin on cell proliferation/expansion from its indirect effect on cellular metabolism [20].

In the *megaintegumenta* (*mnt*) that are *Arabidopsis* mutant plants developed by creating mutations in auxin-inducible gene *AUXIN RESPONSE FACTOR 2* (*ARF2*). Extra cell proliferation characteristics in the vegetative and floral organs of *mnt* indicated that *ARF2* could function as a fundamental repressor of cell division in leaves [93]. These data suggested that the balance of auxin-inducible genes *ARF2* is required for proper auxin function in cell proliferation and cell expansion [94], its orthologue *BrARFs* also speculated as LC and LH regulators in *Brassica* crop [36].

An exogenous application of GA_3 to leaves of Chinese cabbage *nhm* non-heading mutants restored the heading phenotype, demonstrating the crucial role of GA in *B. rapa* heading [95]. The high level of GA (gibberellin) treatment, elevated the *BcMYB101* regulated by miR159 expression in pak choi. Overexpression of *BcMYB101* accelerated leaf quantity and induced downward curling of leaves. Silencing of GAMYB transcription factor from pak choi *BcMYB101*, a of an *Arabidopsis MYB* gene (first identified in barley), in pak choi (*Brassica rapa* ssp. *chinensis*) produced upward leaf curling [96]. MYB genes involved into the leaf development by controlling the cell cycle [97], that represents the secondary morphogenesis contribution into the LH establishment.

In leaf development, cytokinin influences the balance of differentiation and expansion. cytokinin degradation promotes cell expansion and early termination of cell proliferation in *Arabidopsis* leaf primordia, suggesting that cytokinin delays the initiation of cell differentiation [98]. The *Arabidopsis* Class I KNOTTED1-like homeobox (*KNOXI*) gene is overexpressed in lettuce (*Lactuca sativa*) leaves, resulting in unpredictable growth and cytokinin deposition [99]. *CIN-TCPs*, on the contrary hand, enhance leaf maturation in *Arabidopsis* by decreasing leaf sensitivity to cytokinin. As a corollary, the antagonistic functions of *KNOXI* and *TCPs* on leaf blade formation are dependent on GA/cytokinin balance regulation [32,100]. In *cin* mutants, the leaf margin is rumpled as a result of the slower arrest of cell divisions at margins relative to the middle of the leaf as highlighted by mutations in CINCINNATA (*CIN*) in *Antirrhinum* [101]. *BrpTCP4* regulates the head shape of Chinese cabbage by miR319a *BrTCP1*c of CYC/TB1 was apparently upregulated. *CIN-like TCPs*, named *BrrTCP2* in turnip, *BrpTCP4* in Chinese cabbage, and *LsTCP4* in lettuce, are responsible for the formation of wrinkles, bulges, serrations, and heading leaf morphologies by affecting on cell division [102–106]. The *cin* and triple *tcp2/4/10* mutations alter leaf shape from flat to unevenly curved because of excessive cell proliferation at the leaf margins, and overexpression of *BrrTCP2* restores the wild-type leaf morphology in these *Arabidopsis* mutants [107,108]. Furthermore, Mao et al. (2014) discovered that rosette leaf shape and leafy head shape in Chinese cabbage are associated, and *BrpTCP4* mutation at the miR319a recognition region altered cell division and proliferation, resulting in different rosette leaf and head morphologies [102].

CIN-Like TCPs is negatively regulated by *CYCLIN-DEPENDENT KINASE INHIBITOR (ICK*I)/ KIP RELATED PROTEIN1 (KRP1), which is involved in the regulation of organ shape through the control of cell division (secondary morphogenesis) [109]. A large number of cyclin-dependent kinases (CDKs) are involved in heading formation. Two *CDKs*, one mitogen–activated protein kinase *(MAPK)*, four MAPKKs, three MAPKKKs, eight receptor-like kinases (RLKs), and one calcium-dependent protein kinase (CDPK) participate in protein phosphorylation events in the rosette and folding leaves of Chinese cabbage [10], CDKs as key regulators for an accurate cell cycle progression, to guarantee that plants develop with the correct form, these kinases also participate in the modulation of cell divisions to confer plasticity to leaf development [110,111]. CDKs are also involved in regulation of the cell cycle and cell proliferation. Similarly, ICK1/KIP-Related Protein (KRP1) mutations repress cell proliferation and alter leaf morphology in *Arabidopsis* [112,113]. Moreover, plants overexpressing ICK2/KRP2 also showed an altered root system and modifications in organs morphology [114] that are induced with the ABA treatments [115]. Based on previous findings CDKs and CDKI can be hypothesized to play a crucial role in any undetermined state (such as reduced cell number or cell expansion) of secondary morphogenesis that establish LC and LH in *Brassica* crops.

JA and BR signaling pathways are positively regulated by *CIN-like TCP* which are significant transcription factors for the cell division [116]. Through cell proliferation regulation, the miR319-*TCP* and miR396-*GRF* modules interact together to balance marginal and overall leaf growth [20]. *TCP4* represses the expression of *GROWTH-REGULATING FACTORs* (*GRF*) genes and *GIF1* that are not direct targets of miR396, via unknown mechanisms [117]. Thus, *GRFs* are negatively regulated by *miR319* and *miR396* [38,55], which physically interact with *GIF1/AN3*. Silencing of *BrAN3* produced leaf serration, curling, and heading in *B. rapa* through the repression of cell proliferation and striking of cell cycle [23]. Similarly, *AtAN3/AtGIF1* has been shown to regulate cell division, and silencing of *AtAN3* leads to the formation of narrow, serrated, and curling leaves in transgenic *gif1 Arabidopsis* and to smaller leaves than wild-type *Arabidopsis* because of reduced cell numbers [118]. GA, BR, and SA signaling pathways may be induced by the expression of *BrAN3*, an important heading candidate gene in Chinese cabbage, but these signaling pathways function negatively in heading formation [23] (Fig. 2). Gibberellins (GAs) and brassinosteroids (BRs) promote leaf growth by increasing cell proliferation and expansion [119]. *BcMYB101* identified as LC gene speculated as modulators of BR signaling pathway to regulate leaf shape development in *Brassica* crops [120]. However, how secondary morphogenesis is coordinated to regulate LC and LH morphology remains to be elucidated in *Brassica* crops.

Environmental cues culminate LC and LH

Apart from the morphogenesis regulators within plant tissues, plant organs also experience physical restrictions from the outside, such as neighboring organs and more significantly by external environments which includes biotic and abiotic stresses [34]. Abiotic stress which includes; temperature [121], drought, and salinity all have a substantial impact on organ growth and development, as well as overall plant architecture [122]. Leaves are also sensitive to a wide range of internal and external stimuli, each of which initiates a specific signaling pathway that controls leaf size and leaf shape. Leaf morphological patterns vary markedly in response to temperature [123], light, and other external stresses [124,125] and these effects have been documented for crops that exhibit LH and LC (Fig. 1-C). Primary and secondary morphogenesis can highly be affected with the influence of environmental stresses on phytohormonal signaling pathways. Modulation of phytohormones sets off a chain of events that trigger physiological processes in leaf architecture that aid in leaf development under inadequate growth conditions [126].

Temperature is a key factor that regulates leaf shape by controlling the ratio of midrib length to leaf width [24]. Low temperature activates the regulatory network that promotes head formation in Chinese cabbage [127]. *CIN-Like TCPs* are controlled by temperatures dynamics in *Arabidopsis*, leading to morphological changes in leaves [112]. The *AtBRX* homologs in *B. rapa* (*BrBRX.1*, *BrBRX.2*, and *BrBRX.3*) and in *B. oleracea* (*BoBRX.2*) influence epinastic leaf growth in which environmental factors are often involved [73]. *BRX* regulates leaf cell proliferation and epidermal cell size via modulating the crosstalk between cytokinin signaling, brassinosteroid production, and auxin signaling [76]. All these factors are linked with the leaf morphogenesis that further stimulates the establishment of LC and LH in *Brassica* crops.

A recent study of *Brassica* (Chinese heading cabbage and non-heading cabbage) revealed that a cluster of transcripts initiates the transition to the heading stage and suggested that temperature may be a conserved signal mediator or inducer of the leaf heading transition [128]. Lettuce is a low-temperature crop, stable head formation can be induced by complex factors such as light quality, soil fertility, and temperature [125]. Head weight is positively related to solar radiation and negatively related to mean temperature, indicating a temperature-weight association and solar radiation that affects not only head weight but also head shape and head density [129].

Levels of Ca^{2+} (a ubiquitous second messenger) is altered by external stimuli such as light or other stress conditions, leading to changes in plant growth and development [130]. Calmodulins (CaMs), CDPKs, and calcineurin B-like proteins are calcium sensor proteins, and calcium signaling has an important role in protein phosphorylation [131]. This phosphorylation affects head development by upregulating *CDPK* [132].

The H^+-ATPase encoded by H^+-ATPase 2 (*AHA2, BraA01g007510.3C*) that is hypothesized as a candidate gene for flat-leaf morphology and this gene is affected by environmental stress resulted into the leaf crinkled mutant *(Bralcm)* of *B. rapa* [133]. H + -ATPase is a transmembrane transporter enzyme and autoregulatory triggers the genes to alter their expression under any environmental stress which cause the different leaf morphologies [134]. Previous studies have shown that proton dynamics generated by H + -ATPase directly affect cell expansion [135]. During leaf morphogenesis, the GAs and BRs hormones promote both cell proliferation and expansion [30] that are associated with LC and LH.

Drought stress is a major environmental stress that influences the morphology of all *Brassicaceae* plants [136]. Under drought stress, the pleiotropic effects of *B. rapa* expansin-like B1 (*BrEXLB1*) on leaf were irregular and unstable [122]. *BrEXLB1* under its specific promoters may participate in the regulation of leaf and plant growth and that it responds to hormone availability, light quality, dark periods, developmental stages, and drought conditions [137]. Similarly, exogenous application of ABA and abiotic stresses; polyethylene glycol (PEG), NaCl and H_2O_2 upregulated the *B. rapa* LATERAL SUPPRESSOR(*BrLAS*) expression and produced altered leaf morphology [138]. With the application of salt treatments on *B. oleracea* genotypes showed significant changes in its plant growth, leaf weight and leaf area [139]. That predict salt stress effect on the leaf development of *Brassica* crops [140], that might link with the establishment of LC and LH.

Conclusions and future perspectives

The leaf shape is regulated by leaf polarity (primary morphogenesis) as well as by cell proliferation and cell expansion (secondary morphogenesis). The genes and pathways involved in LC and LH establishment may be conserved among evolutionarily

Table 1
Leaf curvature and heading regulators identified in *Brassica* crops.

Species	Gene name	Gene ID	Function	Reference
B. rapa L. ssp. *pekinensis*	*BrpSPL9-2*	BraA05g002720.3C	Interacts with *BcpLH1/2*, *BrpREV*, and *TCP4*; controls timing of leaf curvature	[82]
B. rapa L. ssp. *pekinensis*	*BcpLH*	BraA06g006590.3C	Interacts with other microRNAs, leading to the formation of leaf heading	[81]
B. rapa L. ssp. *pekinensis*	*BrpTCP4*	BraA05g032060.3C	Excessive cell proliferation, uneven organ curvature, curved leaves	[105]
B. rapa L. ssp. *rapa*	*BrrTCP2*	BraA02g012600.3C	Alters leaf morphology, recovers wavy leaf phenotype of *Arabidopsis*	[106]
B. rapa L. ssp. *pekinensis*	*BrpREV-* *BrpREV-2*	BraA10g018460.3C BraA02g010200.3C	Controls leaf curvature	[49]
B. rapa L. ssp. *pekinensis*	*BrWOX1.1* *BrWOX1.2*	BraA05g029540.3C BraA03g038230.3C	Role in leaf curling; affects leaf heading through leaf polarity	[57]
B. rapa L. ssp. *pekinensis*	*BrWOX3.1* *BrWOX3.2*	BraA04g020450.3C BraA03g024810.3C	Role in leaf curling; affects leaf heading through leaf polarity	[57]
B. rapa L. ssp. *pekinensis*	*BrAN3*	BraA09g004620.3C	Effects head formation in Chinese cabbage	[23]
B. rapa ssp. *chinensis*	*BcMYB101*	BraA05g012430.3C	Mutations cause upward leaf curling in pak choi	[120]
B. rapa ssp. *chinensis*	*BcAS2*	BraA02g016770.3C	Mutations cause leaf curling in pak choi	[64]
B. rapa ssp. *chinensis*	*BcYAB3*	BraA01g037320.3C	Involved in leaf morphology and responsible for leaf curling	[142]
B. rapa L. ssp. *pekinensis*	*BrLAX3*	BraA02g000160.3C	Responsible for uneven auxin distribution leading to leafy head formation in Chinese cabbage	[15]
B. rapa	*Br-ARF3.1* *Br-ARF3.2*	BraA05g011080.3C BraA04g024390.3C	Leaf abaxial determinants involved in leaf curling	[45]
B. rapa	*Br-ARF4.1*	BraA10g018230.3C	Leaf abaxial determinants involved in leaf curling	[43]
B. rapa L. ssp. *pekinensis*	*BrOPS*	BraA01004411	Inward curling and heading of leaves	[9]
B. rapa L.ssp. *pekinensis*	*AHA2*	BraA01g007510.3C	flat-leaf morphology	[133]
B. rapa	*BrEXLB1*	BraA03g047290.3C	Affect leaf growth and development under drought stress condition	[137]
B. rapa	*BrLAS*	BraA06g000510.3C	Involved in leaf morphology	[138]
B. rapa L. ssp. *pekinensis*	*miR319a*	GenBank ID KJ130320	Wavy and bulging leaves	[143]
B. rapa	*MiR156a*	GenBank ID NR_128632.1	Involved in leaf morphology	[144]

diverse crops, but specific mutations have led to variable heading phenotypes [18]. Although many regulators of LC and LH have been identified in *Brassica* crops, their identification alone is insufficient to explain their underlying mechanisms. Regulators of leaf curvature have been identified in *Arabidopsis*, but the number of corresponding *Brassica* is limited (Table 1).

Although transformation and functional verification of some candidate regulators have been performed in *Arabidopsis* transgenic lines and *Brassica* species, in most cases, the effects of gene functional loss and overexpression are still unknown. Based on Chang et al. (2016), leaf heading is a convergent morphotype in *B. rapa* and *B. oleracea*, confirming that these genes are involved in the regulation of cell division and leaf polarity during the formation of LC and LH [18]. An integrated understanding of the genetic, physiological, and environmental regulation of LC and LH is still needed to build an efficient and concise regulatory network of leaf incurvature and heading in *Brassica* crops. In particular, identification of the factors that affect *BrTCPs*, *BrAN3*, and *BrGRFs* and ad-ab regulators that alter leaf morphology is needed in order to construct the full molecular network that controls LC and LH.

In our study, we have found that leaf morphogenesis regulators might be adversely affect due to climatic changes such as heat, cold, light, humid, and nutrient level. Analysis of hormone levels, and inhibition treatments might confirm the leaf morphogenesis regulators that affect heading phenotype. Moreover, analysis of regulators utilizing functional genomics approaches and merging them with epigenetic technologies will thus be critical in identifying novel candidate genes for significantly improving *Brassica* crops [141]. Identifying leaf heading regulators and their interactions by using modern molecular techniques such as GWAS, MutMap, combined QTL and BSA analysis, Y2H and metabolomics can help to improve optimal leaf heading phenotypes as well as innovative vegetable varieties of *Brassica* crops. Moreover, different genome editing tools (ZFN, TALEN, and CRISPR/Cas system) to improve leaf heading trait in *Brassica* can lay foundation in the development of *Brassica* crops. All modern strategies would open up new avenues for manipulating leaf head shape in Chinese cabbage, cabbage and eventually other heading vegetables.

Compliance with ethics requirements

This review article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Javaria Tabusam: Conceptualization, Writing – original draft. **Mengyang Liu:** Conceptualization, Writing – original draft. **Lei Luo:** Writing – original draft. **Sumer Zulfiqar:** Writing – review & editing. **Shuxing Shen:** Supervision. **Wei Ma:** Conceptualization, Supervision. **Jianjun Zhao:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Zhao Jianjun is a professor of Hebei Agriculture University, "Changjiang Scholars" distinguished professor. To unravel the genetic regulation of domestication traits in Chinese cabbage, segregating populations and core collections are developed, genetic maps are constructed, and large number of Chinese cabbage genotypes representing the different morphotypes have been re-sequenced. These data, combined with resequencing data generated of *B. rapa* core collections by Chinese partners, made it possible to detect signals of domestication for Chinese cabbage leafy head, flowering time and other agronomic traits formation. The focus of my research is to study these domestication events and understand the role of candidate genes. In addition, using the whole genome sequence, we developed a large number of gene-specific SSR and SNP markers and created a Chinese cabbage mutant library to obtain different types of mutants for Chinese cabbage breeding. Now, our research moves towards breeding crops that are better adjusted to sustainable agricultural systems, ranging from strip cropping to organic agriculture. To date, he has published more than 50 SCI papers, including PNAS, Molecular Plant, Cell Reports.