Identification of *YUC* genes associated with leaf wrinkling trait in variety Tacai of Chinese cabbage

Xuelian Ye [1] , **Dandan Liu** [1, 2] , **Rui Liu** [1] , **Ji Sun** [3] , **Yuan Tian** [1] , **Jingwen Chen** [1] , **Xiangtan Yao** [4] , **Xinhua Quan** [4] , **Li Huang** [Corresp. 1, 2]

[1] College of Agriculture and Biotechnology, Zhejiang University, hangzhou, China

[2] Hainan Institute of Zhejiang University, Sanya, China

[3] College of Agriculture and Biotechnology, Wenzhou Vocational College of Science and Technology (Wenzhou Academy of Agricultural Sciences), wenzhou, China

[4] Jiaxing Academy of Agricultural Sciences, jiaxing, China

Corresponding Author: Li Huang
Email address: lihuang@zju.edu.cn

Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* (L.) Makino) stands as a widely cultivated leafy vegetable in China, with its leaf morphology significantly influencing both quality and yield. Despite its agricultural importance, the precise mechanisms governing leaf wrinkling development remain elusive. This investigation focuses on ‘Wutacai’, a representative cultivar of the Tacai variety (*Brassica campestris* L. ssp. *chinensis* var. *rosularis* Tsen et Lee), renowned for its distinct leaf wrinkling characteristics. Within the genome of ‘Wutacai’, we identified a total of 18 *YUCs,* designated as *BraWTC_YUCs*, revealing their conservation within the *Brassica* genus, and their close homology to *YUCs* in Arabidopsis. Expression profiling unveiled consistent expression of *BraWTC_YUC3* and *BraWTC_YUC6* across diverse organs of Chinese cabbage. Additionally, transcriptome sequencing data from flat leaves (the 1st true leaf) and wrinkled leaves (the 5th true leaf) of ‘Wutacai’ indicated relatively higher expression levels of *BraWTC_YUC3b* and *BraWTC_YUC6a/b/c* during the early stages of leaf morphogenesis. RT-qPCR validation demonstrated expression variations of both *BraWTC_YUC3* and *BraWTC_YUC6* across distinct leaf positions (the 1st, 3rd, 5th, 7th, and 9th leaves). Overexpression of *BrYUC6a* induced the formation of wrinkled leaves in an originally flat Chinese cabbage cultivar, underscoring the close association of *YUCs* with the leaf wrinkling trait in Chinese cabbage. In summary, this study successfully identified the *YUC* gene family in ‘Wutacai’ and elucidated the biological function of *BrYUCs*, providing valuable insights into the prospective molecular mechanisms that regulate leaf wrinkling in Chinese cabbage.

Identification of *YUC* genes associated with leaf wrinkling trait in Tacai variety of Chinese cabbage

Xuelian Ye[1] Dandan Liu[1,2] Rui Liu[1] Ji Sun[3] Yuan Tian[1] Jingwen Chen[1] Xiangtan Yao[4] Xinhua Quan[4] Li Huang[1,2]*

1 College of Agriculture and Biotechnology, Zhejiang University, Hangzhou, 310058, China.
2 Hainan Institute of Zhejiang University, Sanya 572024, China.
3 College of Agriculture and Biotechnology, Wenzhou Vocational College of Science and Technology (Wenzhou Academy of Agricultural Sciences), Wenzhou, 325006, China.
4 Jiaxing Academy of Agricultural Sciences, Jiaxing, 314016, China.

Corresponding Author:
Li Huang
Zhejiang University, Hangzhou, Zhejiang province, 310058, China
Email address: lihuang@zju.edu.cn

Abstract

Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* (L.) Makino) stands as a widely cultivated leafy vegetable in China, with its leaf morphology significantly influencing both quality and yield. Despite its agricultural importance, the precise mechanisms governing leaf wrinkling development remain elusive. This investigation focuses on ‘Wutacai’, a representative cultivar of the Tacai variety (*Brassica campestris* L. ssp. *chinensis* var. *rosularis* Tsen et Lee), renowned for its distinct leaf wrinkling characteristics. Within the genome of ‘Wutacai’, we identified a total of 18 *YUCs,* designated as *BraWTC_YUCs*, revealing their conservation within the *Brassica* genus, and their close homology to *YUCs* in Arabidopsis. Expression profiling unveiled consistent expression of *BraWTC_YUC3* and *BraWTC_YUC6* across diverse organs of Chinese cabbage. Additionally, transcriptome sequencing data from flat leaves (the 1st true leaf) and wrinkled leaves (the 5th true leaf) of ‘Wutacai’ indicated relatively higher expression levels of *BraWTC_YUC3b* and *BraWTC_YUC6a/b/c* during the early stages of leaf morphogenesis. RT-qPCR validation demonstrated expression variations of both *BraWTC_YUC3* and *BraWTC_YUC6* across distinct leaf positions (the 1st, 3rd, 5th, 7th, and 9th leaves). Overexpression of *BrYUC6a* induced the formation of wrinkled leaves in an originally flat Chinese cabbage cultivar, underscoring the close association of *YUCs* with the leaf wrinkling trait in Chinese cabbage. In summary, this study successfully identified the *YUC* gene family in ‘Wutacai’ and elucidated the biological function of *BrYUCs*, providing valuable insights into the prospective molecular mechanisms that regulate leaf wrinkling in Chinese cabbage.

1 Introduction

Chinese cabbage (*Brassica campestris* L. ssp. *chinensis* (L.) Makino), a member of the Cruciferae family, is indigenous to China and extensively cultivated across China and East Asia. As a prominent leafy vegetable crop, the attributes of its leaves hold significant importance for its economic value and agricultural breeding strategies. Chinese cabbage exhibits a diverse range of leaf traits, encompassing leaf shape, margin, and surface features. These variations not only profoundly influence the quality, yield, and resistance in cultivation, but also serve as morphological indicators for the classification of different subspecies and varieties within the species. The “Specification for Description of non-heading Chinese cabbage Germplasm Resources” classifies leaf wrinkling into four categories: flat, slightly wrinkled, wrinkled, and highly wrinkled. ‘Wutacai’, belonging to the Tacai variety (var. *rosularis* Tsen et Lee), exhibits obvious leaf wrinkling characterized by regular ridges ascending towards the leaf axis, thus classified as highly wrinkled. Investigation into the leaf wrinkling of Tacai offers the potential insights not only into the leaf wrinkling of Chinese cabbage but also into the molecular mechanisms regulating leaf development.

However, the mechanisms underlying the formation and regulation of leaf wrinkling characteristics in plants remain inadequately understood. Previous studies have identified a wrinkled leaf mutant, *cin*, in *Kalanchoe daigremontiana*, attributed to a mutation in a TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTORS) protein, The *cin* mutant led to excessive growth in the leaf margin region, leading to leaf wrinkling (Nath et al., 2003). TCP4, another member of the TCP family, has also been implicated in leaf development. Transgenic Arabidopsis (*Arabidopsis thaliana*) plants overexpressing *TCP4::7mTCP4* exhibited upward bending of leaves. Furthermore, a miRNA-targeted gene loss-of-function mutant, *phb-6* (PHABULOSA, a member of the Class III HD-Zip family), displayed subtle bending phenotypes in leaves (Liu et al., 2010). The *GhBOP1* (Block of proliferation 1) gene, crucial in ribosome maturation and cell cycle progression, when silenced in upland cotton (*Gossypium hirsutum*) plants, resulted in the wrinkling phenotype of young leaves (Wang et al., 2022b). In Chinese cabbage, a stably inherited wrinkled leaf mutant named *lcm* was identified through EMS mutagenesis, with the candidate gene *BraA01g007510.3C* found to encode an H^+ATPase 2 (Zhang 2020). Functional investigations of these genes have provided valuable insights into the formation of leaf wrinkling characteristics. However, while leaf wrinkling characteristics in Chinese cabbage share similarities with those observed in the aforementioned mutants, they also exhibit distinct features. Unlike the partial bulges seen in those mutants, Chinese cabbage leaf wrinkling, particular in cultivar 'Wutacai', is characterized by highly wrinkled leaves, a trait that is heritable. The underlying mechanisms governing the formation and regulation of leaf wrinkling trait in Chinese cabbage necessitate further investigation.

Auxin, as a pivotal endogenous hormone, exerts a profound influence on plant growth and development. The TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA)/YUCCA (YUC) pathway is widely recognized as the most critical and well-defined pathway for auxin production (Korasick et al., 2013). Within this pathway, YUC proteins (YUCs) function as flavin monooxygenases (FMOs), catalyzing the rate-limiting and irreversible oxidation decarboxylation of indole-3-pyruvic acid (IPyA) to yield indole-3-acetic acid (IAA). YUCs represent the earliest-discovered FMO proteins in plants, exhibiting structural and functional properties similar to mammalian FMOs (Cashman 2002). Both bioinformatic and biochemical studies have revealed that plant FMO proteins typically contain six highly conserved motifs (or amino acid residues) (Schlaich 2007). Particularly noteworthy are the flavin adenine dinucleotide (FAD)-binding motif and the nicotinamide adenine dinucleotide phosphate (NADPH)-binding motif, which harbor highly conserved GxGxxG sequences recognized as integral components of the classical Rossman fold. Mutations affecting these motifs in maize (*Zea mays*) (Gallavotti et al., 2008) and Arabidopsis (Schlaich 2007) have led to the complete loss of *YUC* function, highlighting their crucial role as essential conserved motifs for *YUC* functionality (Cao et al., 2019).

The *YUC* genes (*YUCs*) were initially identified in Arabidopsis via activation tagging lines, which manifest developmental anomalies, such as downward curling leaves and semi-erect growth, attributed to an excess of auxin (Zhao et al., 2001). Compared to proteins involved in auxin transport and signal transduction, the gene family encoding YUCs is comparatively small. In recent years, comprehensive whole-genome phylogenetic analyses have unveiled the presence of *YUC* gene family in over 20 plant species, encompassing Arabidopsis, rice (*Oryza sativa*), maize, cucumber (*Cucumis sativus*), and wild strawberry (*Fragaria vesca*), with 11, 14, 14, 10, and 8 family members, respectively (Cao et al., 2019).

The local concentration of auxin intricately governs the normal morphogenesis of plant leaves. For instance, the polar differentiation of leaf primordia, delineating the proximal-distal axis, is orchestrated by the localized accumulation of auxin in leaf margin cells, a process modulated by multiple *YUCs* (Zgurski et al., 2005; Wang et al., 2011). Overexpression of *FaYUC1* results in narrow and downward curled leaves (Liu et al., 2012). Mutations in *YUC1/2/4/6* lead to diminished vein and vascular bundle numbers, with the severity of the phenotype being depended on the dosage of these four *YUCs* (Cheng et al., 2006). Transgenic potato (*Solanum tuberosum* L.) overexpressing *AtYUC6* displays a high-auxin phenotype, characterized by narrow, downward-curling leaves and increased plant height (Kim et al., 2013). These findings collectively underscore the involvement of *YUCs* in plant leaf morphogenesis, where their overexpression, along with an elevation in local auxin concentration in Arabidopsis and other plants, frequently results in leaf curling, reminiscent of the leaf wrinkling trait observed in Chinese cabbage.

This study aimed to investigate the potential involvement of the *YUC* gene family in regulating the leaf wrinkling trait in Chinese cabbage. Utilizing bioinformatics methodologies, 18 *YUCs* were identified based on the genome data of the Chinese cabbage variety, ‘Wutacai’, renowned for its distinctive leaf wrinkling trait. Subsequent structural and phylogenetic analyses of the *YUC* gene family in Chinese cabbage were conducted. Through gene expression data sourced from various organs available in the *Brassica* database BRAD, we pinpointed the dominant or stable expression of *YUCs* in leaves. We further investigated the expression patterns of *YUCs* in both flat and wrinkled leaves of Chinese cabbage, drawing insights from transcriptome sequencing data obtained from the 1st flat leaf and the 5th wrinkled leaf of ‘Wutacai’. Additionally, we validated and compared the expression patterns of the predominant *BrYUC3* and *BrYUC6* across leaves in different positions, employing ‘Wutacai’ and the flat-leaved representative variety ‘Youqing 49’ through RT-qPCR. Furthermore, functional verification of *BrYUC6a* was conducted to substantiate the role of *YUCs* in shaping leaf wrinkling characteristics. This investigation seeks to elucidate the potential association of the *YUC* gene family with the leaf wrinkling trait, thereby laying the groundwork for molecular breeding in Chinese cabbage and charting future research avenues pertaining to plant leaf development.

2 Materials and Methods

2.1 Identification of *YUCs* in ‘Wutacai’

The protein sequences of *YUCs* in ‘Wutacai’ were obtained from a previous investigation (Cai et al., 2021). Subsequently, YUC protein sequence for cucumber, maize, Arabidopsis, and rice were obtained by querying the National Center for Biotechnology Information (NCBI) website using the keyword “YUC”. These sequences were then individually aligned with the protein sequences from ‘Wutacai’ using TBtools (Chen et al., 2023) to conduct pairwise sequence alignment. The intersection of these four alignment results produced a set of 40 sequences. Following this, the 40 protein sequences from ‘Wutacai’ were subjected to the NCBI Conserved Domain Database (CDD) online platform (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) for gene structure domain prediction. Additionally, YUC protein sequence files of *Brassica oleracea* and *Brassica nigra* were acquired from the BRAD website (http://brassicadb.cn/#/Transcriptome/) utilizing the “Search-Syntenic Gene @ Subgenomes” function. This process involved inputting the gene ID of Arabidopsis to identify syntenic genes in those genomes. Specifically, the genome “Braol_JZS_V2.0 *Brassica oleracea* cv. JZS V2.0” was utilized for *B. oleracea*, while the genome “Brani_Ni100_V2 *Brassica nigra* cv. Ni100. V2.0” was employed for *B. nigra*.

2.2 Construction of the phylogenetic tree

In order to explore the evolutionary relationships of the *YUCs* in ‘Wutacai’, all identified YUC protein amino acid sequences from ‘Wutacai’, *B. oleracea*, *B. nigra*, Arabidopsis, cucumber, rice, and maize were aligned using the “One Step Build a ML Tree” feature within the TBtools software. The maximum likelihood (ML) method was utilized to construct the phylogenetic tree with default parameters.

2.3 Bioinformatic analysis of protein sequences

The protein sequences of *YUCs* in ‘Wutacai’ were subjected to analysis using online tools to assess protein physicochemical properties and predict subcellular localization. Specifically, we utilized the ProtParam (https://web.expasy.org/protparam/) and PSORT (https://wollpsort.hgc.jp/) tools for this purpose.

2.4 Conservation motif and gene structure analysis

The Simple MEME Wrapper tool within the TBtools software was employed with default parameters to identify the conserved motifs within the 'Wutacai' YUC protein sequences. Additionally, gene structure domains were predicted using the online analysis website NCBI CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). Finally, TBtools was utilized to visualize both the conserved motifs and gene structure patterns.

2.5 Prediction of cis-acting elements of promoters

The upstream 2,000 bp sequences of the coding regions of the 'Wutacai' *YUCs* were extracted using the Gtf/Gff3 sequences Extract tool in TBtools. Then the data were submitted to PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002) for predicting cis-acting elements. The results were visualized using the BioSequence viewer tool in TBtools.

2.6 Expression analysis

The expression data of *YUCs* in various organs of Chinese cabbage were acquired from the BRAD database, specifically from the "GSE43245" library. Transcriptome sequencing was conducted on two early stages of leaf morphogenesis in the 1st and 5th leaves of 'Wutacai'. The data were subsequently deposited in the National Center for Biotechnology Information, U.S. National Library of Medicine (NCBI; https://www.ncbi.nlm.nih.gov/) under the accession number PRJNA1077639. Visualization of the data was achieved using the HeatMap tool within TBtools.

2.7 Vector construction and plant transformation

The full-length coding sequence of *BrYUC6a* was amplified from cDNA of 'Youqing 49' leaves using gene-specific primers (Table S1). The PCR product was cloned into the pCAMBIA1300 vector, driven by the CaMV 35S promoter. The constructed vector was introduced into *Agrobacterium tumefaciens* strain GV3101 (WeiDi, Shanghai, China) before being transformed into Chinese cabbage plants through the floral dip method (Hu et al., 2019). Transgenic plants were identified and validated by PCR.

2.8 RT-qPCR experiment

Total RNA was extracted by Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) and reverse transcribed into the first strand of cDNA using HiScript II RT SuperMix for qPCR (+gDNA wiper) (Vazyme, Nanjing, China). With the reference gene *BrUBC10*, RT-qPCR reactions were performed using the SYBR Green Premix Pro Taq HS qPCR Kit (AG, Changsha, China) in a CFX96 Real-Time System (Bio-Rad, California, USA). Primers used in the experiment were shown in Table S1. The results were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

3 Results

3.1 Identification and phylogenetic analysis of the *YUC* gene family

A total of 18 *YUCs* named *BraWTC_YUCs* were identified in the 'Wutacai' genome, while *B. oleracea*, *B.*

nigra, Arabidopsis, cucumber, rice, and maize genomes possess 18, 18, 11, 10, 14 and 14 *YUCs* respectively. To investigate the evolutionary relationships within the *YUC* gene family, phylogenetic trees were constructed utilizing the protein sequences of the *YUCs* in these species (Fig. 1). The results revealed that the 'Wutacai' *YUCs* were most closely related to those of *B. oleracea* and *B. nigra*, both of which belong to the same genus as 'Wutacai'. Secondly, a close relationship was observed with those of Arabidopsis. However, the evolutionary relationship between 'Wutacai' and rice was notably distinct, as was the case with 'Wutacai' and maize.

3.2 Analyses of physicochemical properties, conserved motifs, and subcellular localization

The results of primary structure prediction unveiled that the *BraWTC_YUCs* encoded amino acids within the range of 262 to 607, with corresponding molecular weights spanning from 29.66 to 68.24 kDa. The isoelectric points fell within the range of 5.99 to 9.46, and the protein instability index ranged from 27.82 to 50.55 (Table 1). According to the established criteria, proteins with an instability index of less than 40 are considered stable, those with an isoelectric point below seven are categorized as acidic, and proteins with a hydrophilicity index less than zero are classified as hydrophilic. Therefore, it can be inferred that BraWTC_YUC5a/b, BraWTC_YUC6b, BraWTC_YUC8a/b, and BraWTC_YUC9a/b were stable proteins. BraWTC_YUC5b and BraWTC_YUC10 were acidic proteins, while the remaining members were classified as alkaline proteins. All 18 members were identified as hydrophilic proteins. Analysis of conserved motifs revealed that BraWTC_YUCs encompassed five to eight conserved motifs, including motif 1, motif 2, motif 5, motif 7, and motif 8. With the exception of BraWTC_YUC10, the other members exhibited motif 3, and motif 4 was shared by the remaining members, excluding BraWTC_YUC10 and BraWTC_YUC1b. Additionally, except for BraWTC_YUC1b/5a/6c/10, motif 6 was prevalent in the others (Fig. 2). This observation suggests a certain level of conservation in the YUC protein motifs within 'Wutacai'. Subcellular localization analysis demonstrated that BraWTC_YUC6b was localized in the nucleus, whereas the remaining members were localized in the cytoplasmic membrane (Table 1).

3.3 Analysis of the gene structure and prediction of cis-regulatory elements

The 2,000 bp upstream sequences of *BraWTC_YUCs* were extracted to serve as promoter regions for the identification of cis-regulatory elements. The outcomes revealed that these sequences encompassed diverse types of cis-regulatory elements, primarily categorized into three groups: plant hormone-related elements, growth and development-related elements, and stress response-related elements (Fig. 3A). The plant hormone-related elements comprised response elements for auxin, salicylic acid, abscisic acid, methyl jasmonate, and gibberellin. Meanwhile, the growth and development-related elements included light response, meristem expression, epidermal cell differentiation, physiological control, endosperm expression, and others. Lastly, the stress response-related elements encompassed defense and stress response, anaerobic induction, low-temperature response, and drought induction. The types of cis-regulatory elements within *BraWTC_YUCs* exhibited variability. Specifically, *BraWTC_YUC6a* displayed the fewest response elements, comprising only four types, whereas *BraWTC_YUC8b* featured the most response elements, encompassing ten types. Within the plant hormone response elements, *BraWTC_YUC10* and *BraWTC_YUC5b* each possessed only one type of plant hormone response element, specifically auxin response and gibberellin response, respectively. *BraWTC_YUC5a* contained four response elements excluding methyl jasmonate, and *BraWTC_YUC8b* featured four response elements excluding auxin. All members were found to contain a substantial number of light response elements, with *BraWTC_YUC8b* featuring as many as 22 types of light response elements, while *BraWTC_YUC10* exhibited the fewest (four types). Regulatory elements for meristem expression were present in *BraWTC_YUC1a*, *BraWTC_YUC3b/c*, *BraWTC_YUC5a/b*, *BraWTC_YUC6c*, and *BraWTC_YUC10*. Only a select few members featured elements related to flavonoid biosynthesis gene regulation, physiological control, and zein protein metabolism regulation. Specifically, *BraWTC_YUC7* and *BraWTC_YUC10* contained response elements for epidermal cell differentiation and endosperm expression, respectively, which were absent in other members. Gene structure analysis showed that *BraWTC_YUCs* had 2~4 CDS (Fig. 3B).

3.4 Expression analysis of *BraWTC_YUCs*

Based on the expression data of *YUCs* in Chinese cabbage extracted by the BRAD database, it is evident that the expression profiles of the *YUCs* vary significantly across different organs. Notably, in roots, *BraWTC_YUC5/6/8* showed higher expression levels, while in stems, *BraWTC_YUC4/6* exhibited dominant expression. In flowers and siliques, *BraWTC_YUC6/8/10* displayed higher expression levels compared to other members. Expression levels were relatively low in callus tissue, whereas in leaves, overall expression levels of *YUCs* at the sampling period (7 weeks after sowing) were relatively low. However, *BraWTC_YUC6* showed stable expression in leaves compared to other members. Additionally, distinct copies of the same gene, such as *BraWTC_YUC3/4/5/8*, exhibited expression pattern variations. Specifically, *BraWTC_YUC3a* was not expressed in leaves, *BraWTC_YUC3c* showed low expression, while *BraWTC_YUC3b* exhibited relatively high expression, being the highest among all detected *YUCs*. Additionally, *BraWTC_YUC1/7/10* showed no expression in leaves (Fig. 4E).

According to the expression data, *BraWTC_YUC3/6* showed higher expression levels in the leaves compared to other members. Therefore, RNA was extracted from mature leaves (1st, 3rd, 5th, 7th, 9th leaves) of the flowering Chinese cabbage ‘Youqing 49’ and ‘Wutacai’ to further analyze the expression levels of *BraWTC_YUC3/6*. Our preliminary observations revealed that the leaf surfaces of the 1st to 3rd true leaves of ‘Wutacai’ were flat, resembling flat-leaved varieties such as ‘Suzhouqing’ and ‘Youqing 49’ (Fig. 4B), without any wrinkling. However, the 4th true leaf exhibited localized wrinkling during the heart leaf stage, which either became flat or retained localized wrinkling upon maturation. Leaves from the 5th to 8th positions showed localized wrinkling during the juvenile stage, which persisted after maturation. Starting from the 9th leaf, the leaves exhibited a higher degree of wrinkling, irrespective of their maturity (Fig. 4A and C). RT-qPCR results showed variations in the expression levels of *BraWTC_YUC3/6* among different leaf positions, with a more pronounced difference observed in ‘Wutacai’ (Fig. 4G and H). Additionally, the expression difference of *BraWTC_YUC6* between ‘Youqing 49’ and ‘Wutacai’ was more significant compared to *BraWTC_YUC3*.

Considering the potential occurrence of the wrinkling characteristic during early leaf development stages, we conducted an in-depth investigation into the involvement of *YUCs* in the formation of leaf wrinkling in ‘Wutacai’. Transcriptome sequencing was conducted to evaluate the expression patterns of *YUCs* during the juvenile stages of the 1st and 5th leaves of ‘Wutacai’ (Fig. 4D). The analysis revealed that *BraWTC_YUC3a/5b/10* were undetectable in either the 1st or 5th leaves during the juvenile stage, and they remained unexpressed in the mature stage. Conversely, *BraWTC_YUC3b/6* exhibited relatively higher expression levels compared to other family members in both the 1st and 5th leaves at both developmental stages (Fig. 4F). Particularly, *BraWTC_YUC3b* displayed significant upregulation in the mature stage of the 5th leaf in comparison to that of the 1st leaf.

3.5 Overexpression of *BrYUC6a* leads to the formation of wrinkled leaves in unwrinkled variety

To further elucidate the role of *BrYUC6a* in the leaf wrinkling trait, we conducted an overexpression experiment of *BrYUC6a* in 'Youqing 49', a variety characterized by flat leaves, and subsequently observed the phenotypes of the transgenic plants. Three independent transgenic lines, namely *BrYUC6a*^{OE}*#3*, *BrYUC6a*^{OE}*#9*, and *BrYUC6a*^{OE}*#17*, were selected, all of which exhibited significantly higher expression levels of *BrYUC6a* compared to the wild-type (WT) plants (Fig. 5A). In contrast to the flat leaves observed in WT plants, all *BrYUC6a*^{OE} lines initially displayed flat leaves in the first one or two true leaves. However, subsequent true leaves exhibited partial wrinkling (Fig. 5B), resembling the leaf wrinkling trait observed in 'Wutacai'.

4 Discussion

Auxin plays an important regulatory role in establishing polarity and flattening during leaf development (Merelo et al., 2017; Wang et al., 2022a). Transcriptome sequencing analysis conducted on the wrinkled and flat parts of mature leaves in 'Wutacai' revealed that the differentially expressed genes between these regions were predominantly enriched in the auxin signaling pathway (Hou et al., 2023), indicating the involvement of auxin in the formation or maintenance of leaf wrinkling trait in Chinese cabbage. In their study, the researchers also measured the content of endogenous hormones in two different forms (flat and wrinkled) of the same leaf. The results indicated differential levels of six types of auxins, which might contribute to the leaf wrinkling of Tacai. However, the study mainly focused on the role of auxin signaling transduction in leaf development, leaving the relationship between local auxin synthesis and leaf wrinkling still unexplored.

In recent years, an increasing number of studies have shown that local synthesis of auxin is closely related to leaf morphogenesis. The TAA/YUC pathway is the main endogenous biosynthetic pathway for auxin (Gomes and Scortecci 2021). Leaf primordia originate from the differentiation of meristematic tissues, and leaf polarity is established through proximal-distal, medio-lateral, and adaxial-abaxial axes, which then elongate to form leaves (Byrne 2012). The establishment of proximal-distal polarity involves localized auxin accumulation in leaf margin cells mediated by multiple *YUCs* (Zgurski et al., 2005; Wang et al., 2011). Studies have indicated that the elimination of multiple *YUCs* leads to narrow leaf phenotypes due to auxin deficiency (Cheng et al., 2007), while excessive auxin leads to leaf curling, resembling the leaf wrinkling in Chinese cabbage. Based on this, it can be speculated that the *YUC* gene family may be involved in the formation of leaf wrinkling trait. Therefore, conducting a comprehensive investigation of the *YUC* gene family in Chinese cabbage is necessary to reveal the genetic regulatory mechanisms of leaf wrinkling trait.

Phylogenetic analysis of the *YUC* gene family in 'Wutacai', *B. oleracea*, *B. nigra*, Arabidopsis, cucumber, rice, and maize revealed relatively distant evolutionary relationships as well as between 'Wutacai' and rice. This distinction may be attributed to the monocotyledonous nature of maize and rice, while 'Wutacai' is a dicotyledonous plant. *YUCs* in 'Wutacai' (AA, 2n=20) demonstrated a close evolutionary relationship with those of *B. oleracea* (CC, 2n=18) and *B. nigra* (BB, 2n=16), likely due to the inherent relationships within the *Brassica* genus. In the 'Wutacai' genome, a total of 18 *YUCs* were identified, indicating the expansion of the *YUC* gene family in 'Wutacai' concurrent with whole genome duplication events. This phenomenon also happened in *B. oleracea* and *B. nigra*. As the fact that compared to Arabidopsis, the *Brassica* genus experienced a whole genome triplication event around 5.4 to 9 million years ago (Cheng et al., 2014).

YUCs encode a flavin-containing monooxygenase, FMO, which was first discovered in plants. They exhibit a typical structural and functional characteristic similar to mammalian FMOs, typically comprising six highly conserved motifs (Schlaich, 2007). Protein structure analysis revealed that BraWTC_YUCs contain 5-8 conserved motifs, all of which encompass FAD and NADPH binding motifs. Most of these proteins exhibited alkaline properties and displayed hydrophilic characteristics. Subcellular localization results indicated that, apart from BraWTC_YUC6b, which localized to the nucleus, others were localized to cytoplasmic membrane. Promoter analysis predicted the presence of various cis-acting elements associated with plant hormones, growth, and development, as well as stress responses in the promoter regions of 'Wutacai' *YUCs*. This suggests that *YUCs* may be involved in a broad spectrum of biological activities.

In Arabidopsis, the biological functions of the *YUCs* have been extensively studied, including their involvement in root development, leaf development, reproductive development, and response to abiotic stress (Cao et al., 2019). However, a previous study identifying the *YUC* gene family in Chinese cabbage (Qi et al., 2019) did not explore their expression and function. Therefore, investigating the function of *YUCs* in Chinese cabbage represents an intriguing avenue for research.

In accordance with previous studies, the occurrence of curly leaves is associated with failure or damage to leaf polarity establishment during leaf primordium development. The direction and degree of leaf bending are correlated with the expression ratio of proximal-distal genes. *HYL1* (*HYPONASTIC LEAVES1*) regulates the balance between proximal and distal axes, controlling leaf flatness by averting leaf curling, wavy margins, and downward bending (Liu et al., 2011). Thus, gene regulation takes place earlier than the completion of leaf morphogenesis during leaf development. Intriguingly, our study unveiled, for the first time, an association between the leaf wrinkling trait and leaf position in 'Wutacai'. Hence, we utilized leaf samples from the 1st and 5th leaves, which exhibited significant differences during both the juvenile and mature stages, to establish a comprehensive expression profile of *BrYUCs* during leaf wrinkling formation in Chinese cabbage. Focusing on early leaf morphogenesis rather than the mature leaf stages for transcriptome sequencing analysis allow us to better understand the relationship of *YUCs* to leaf wrinkling, leading to the identification of several candidate genes such as *BraWTC_YUC3* and *BraWTC_YUC6*. As anticipated, the expression levels of *BraWTC_YUCs* varied at different time points within the same leaf position, indicating dynamic changes in *YUCs* expression during leaf development. Through the construction of transgenic plants overexpressing *BrYUC6a*, we further confirmed the significant role of *YUCs* in leaf wrinkling formation in Chinese cabbage. It is noteworthy that the wrinkling characteristics in the transgenic plants also exhibited a leaf position-dependent pattern. Therefore, we propose that the *YUC* gene family is implicated in the intricate biological process of the leaf wrinkling trait in Chinese cabbage. Nevertheless, further investigations are required to elucidate the specific roles they play in this process.

Conclusion

In this study, we focused on the distinctive cultivar 'Wutacai' of the Tacai variety, renowned for its prominent leaf wrinkling trait. A total of 18 *YUCs* were identified in the Chinese cabbage genome. Protein structure and evolutionary relationship analysis revealed the conservation of *YUCs* within the genus *Brassica*. Through the analysis of *YUCs* expression during the initiation of leaf wrinkling and gene functional characterization, we established a comprehensive expression profile of *BrYUCs* and unveiled the potential involvement of *YUCs* in leaf wrinkling formation. These findings serve as a theoretical reference for further exploration of the regulatory mechanisms underlying the leaf wrinkling phenotype in Chinese cabbage and other plants.

Reference

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Figure 1

Evolutionary tree of *YUC* family in ‘Wutacai’, *B. oleracea*, *B. nigra*, Arabidopsis, cucumber, rice, and maize.

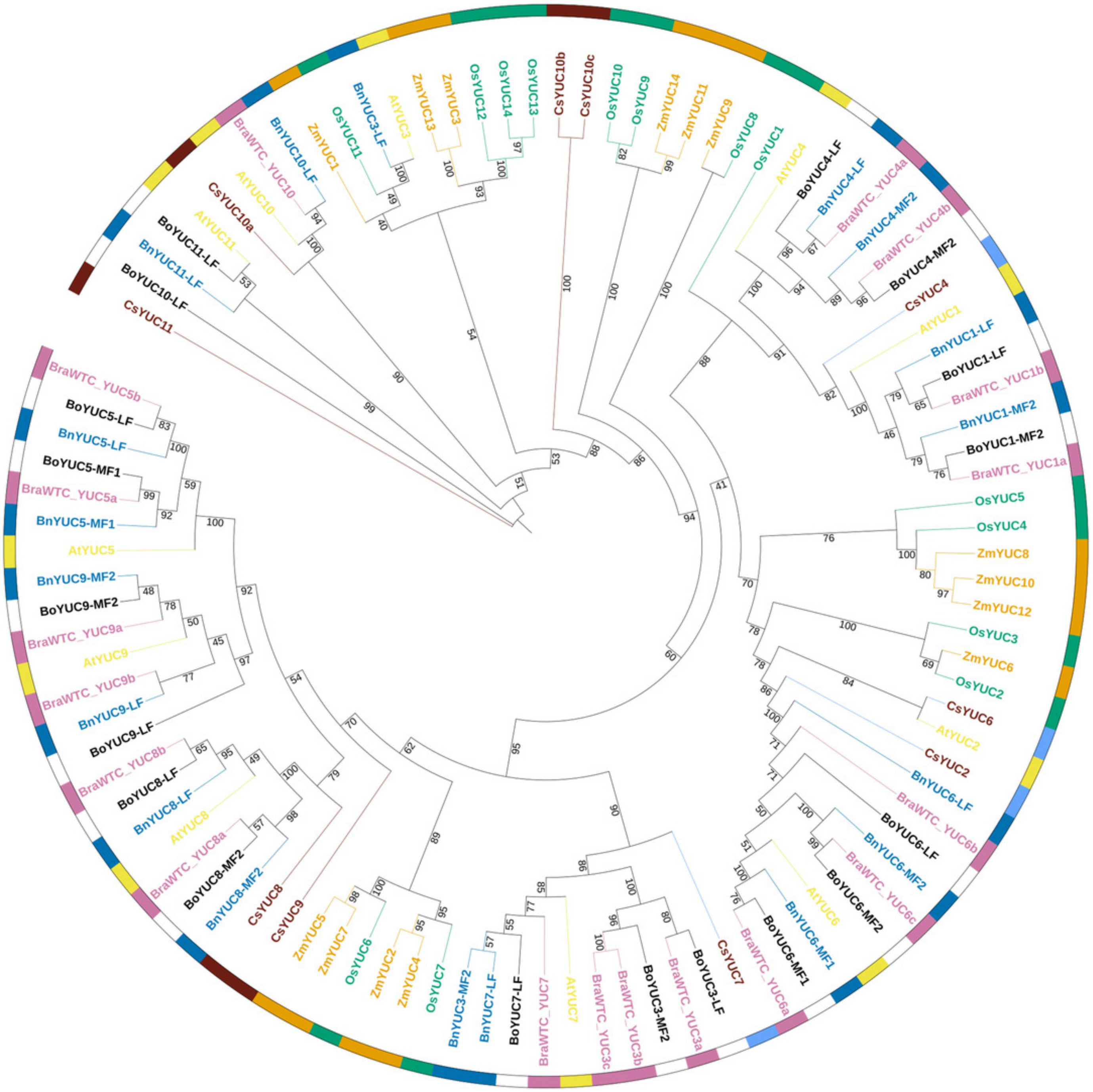


Figure 2

Conservative motif analysis of the YUC family.

Different color boxes and numbers indicate different conserved motifs, and black lines indicate amino acid sequences.

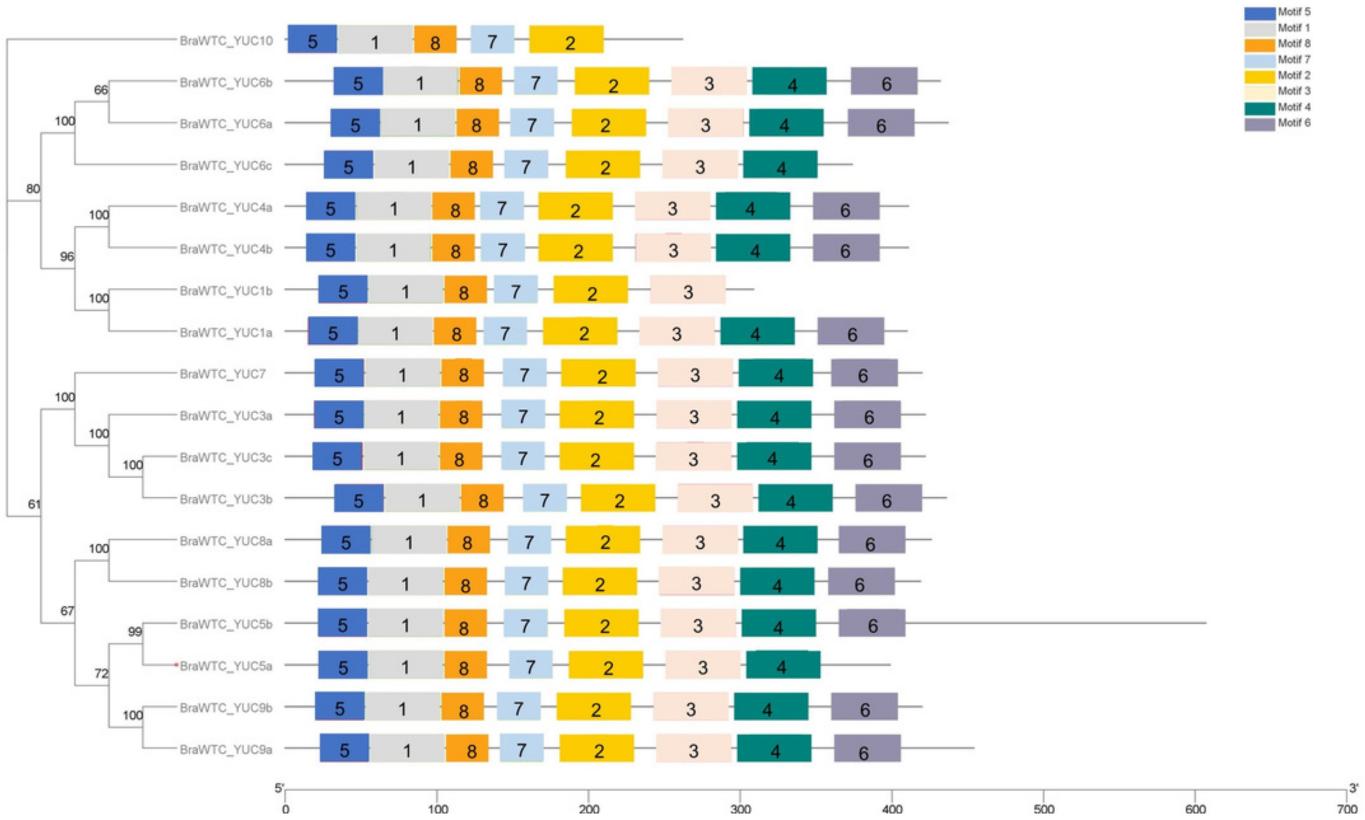


Figure 3

Analysis of gene structure and prediction of cis-acting elements.

A Prediction of cis-acting elements of *YUC* gene family of ‘Wutacai’. Different color boxes represent different types of cis-acting elements, and gray lines represent gene sequences. 1, Auxin response; 2, Defense and stress response; 3, Anaerobic induction; 4, Light response; 5, Meristem expression; 6, Palisade mesophyll cell differentiation; 7, Low temperature response; 8, Salicylic acid response; 9, Abscisic acid response; 10, Methyl jasmonate response; 11, Drought induction; 12, Gibberellin response; 13, Physiological control; 14, Endosperm expression; 15, Regulation of zein metabolism; 16, Gene regulation of flavonoid biosynthesis; 17, Down-regulated expression of photochrome. B Analysis of gene structure.

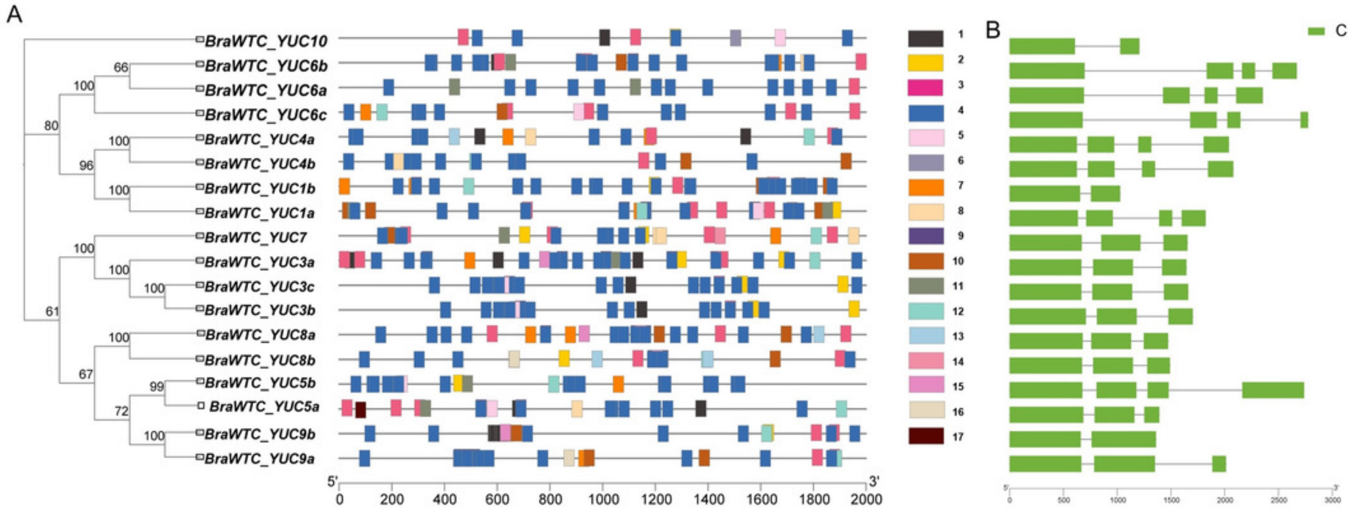
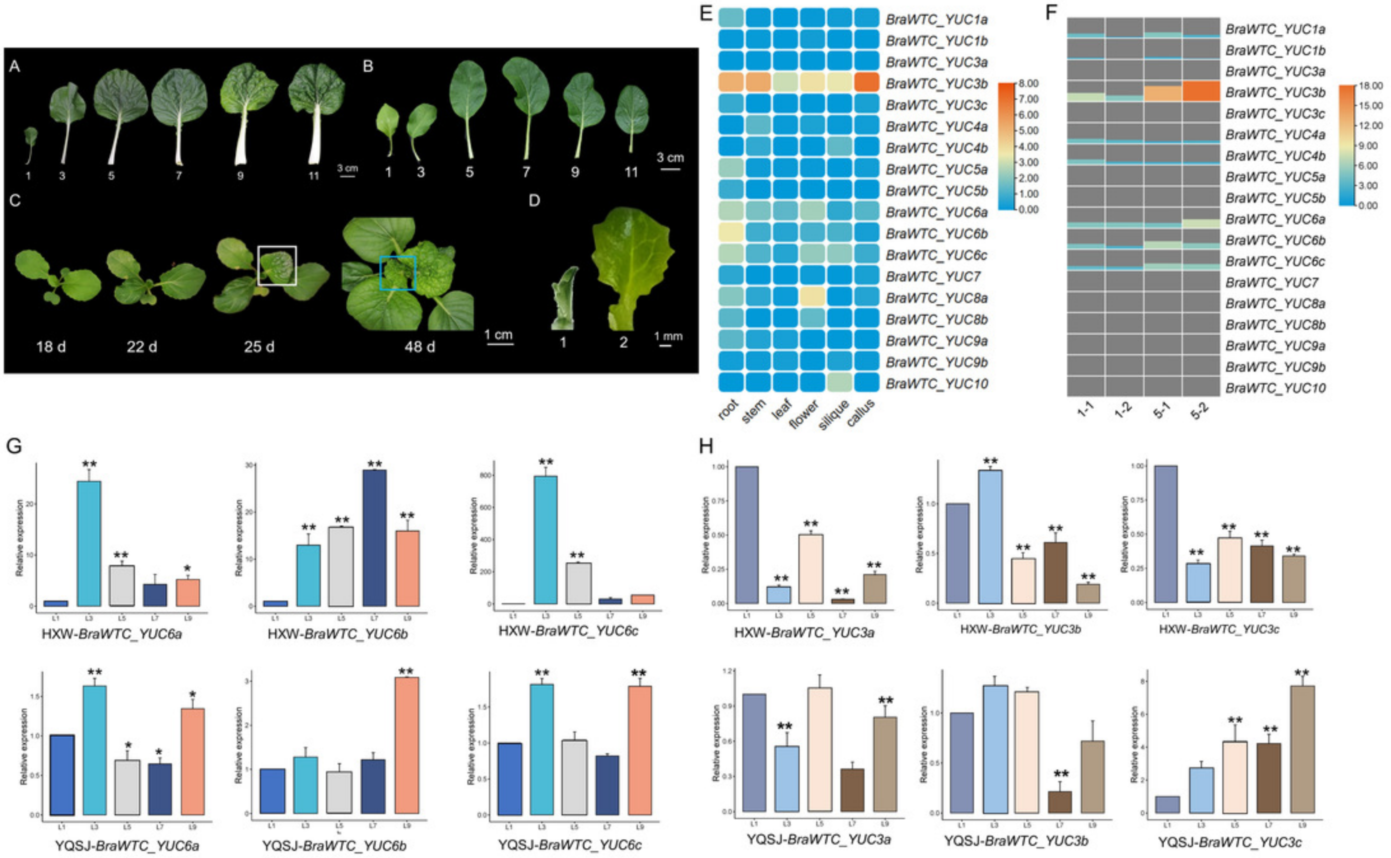


Figure 4

Leaf development of Chinese cabbage and the expression profile of *BraWTC_YUC*s.

A 1st, 3rd, 5th, 7th, 9th and 11th mature leaves of 'Wutacai' at 71 Days after sowing (DAS). B 1st, 3rd, 5th, 7th, 9th and 11th mature leaves of 'Youqing 49' at 35DAS. C 'Wutacai' on 18, 22, 25, and 48 DAS, the white boxes showed local wrinkling in the 4th true leaf at juvenile stage. The blue boxes showed total wrinkling in the 9th true leaf at juvenile stage, respectively. D Two periods of sampling for transcriptome sequencing. E Expression of *YUC* gene in different organs of Chinse cabbage (data from BRAD Database). The value in the figure is the TPM value (Transcripts Per Kilobase of exon model per Million mapped reads). F The expression of *YUC* gene at 1st and 5th leaf of 'Wutacai' in transcriptome sequencing results. 1-1 and 1-2 represent two periods at 1st leaf, and 5-1 and 5-2 represent two periods at 5th leaf, respectively. Value in the figure represents the FPKM value (Fragments Per Kilobase of exon model per Million mapped fragments). G RT-qPCR analysis of *BraWTC_YUC6a/b/c* expression in the 1st, 3rd, 5th, 7th and 9th mature leaves of 'Youqing 49' and 'Wutacai'; L1/3/5/7/9 represents the 1st, 3rd, 5th, 7th, 9th mature leaves respectively; YQSJ represents 'Youqing 49', HXW represents 'Wutacai'. H RT-qPCR analysis of *BraWTC_YUC3a/b/c* expression level in the 1st, 3rd, 5th, 7th and 9th mature leaves of 'Youqing 49' and 'Wutacai'.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BraWTC_YUC3b
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HXW-BraWTC_YUC6c
YQSJ-BraWTC_YUC6a
YQSJ-BraWTC_YUC6b
YQSJ-BraWTC_YUC6c
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HXW-BraWTC_YUC3a
HXW-BraWTC_YUC3b
HXW-BraWTC_YUC3c
YQSJ-BraWTC_YUC3a
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YQSJ-BraWTC_YUC3c
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Figure 5

Construction of the transgenic plants overexpressing *BrYUC6a* in Chinese cabbage.

A Expression level of *BrYUC6a* in the wild-type (WT) and transgenic plants. B The phenotypes of the wild-type and three independent transgenic lines at 30 and 37 DAS. The white arrow indicates the site of leaf wrinkling in transgenic plants.

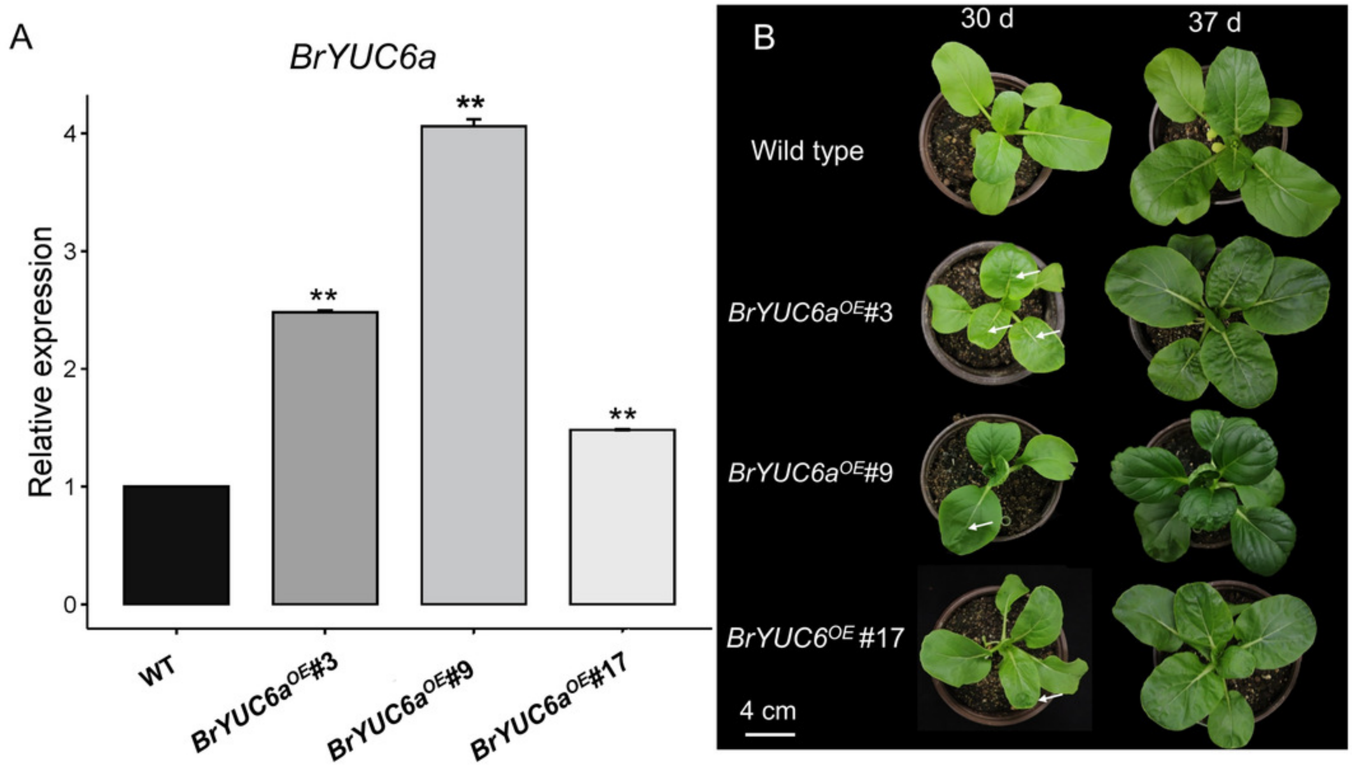

Table 1(on next page)

Table1 Physicochemical properties of YUC family proteins of ‘Wutacai’.

Gene	Gene ID	Number of amino acids	Molecular weight (kD)	Theoretical pI	Instability index	Grand average of hydropathicity (GRAVY)	subcellular localization (Localization Scores)
BraWTC_YUC1a	Ann023190.1_BraWTC	410	45.23	9.46	39.17	-0.151	Cytoplasmic membrane
BraWTC_YUC1b	Ann404350.1_BraWTC	309	34.16	9.46	39.26	-0.052	Cytoplasmic membrane
BraWTC_YUC3a	Ann445240.1_BraWTC	422	47.11	9.02	35.91	-0.201	Cytoplasmic membrane
BraWTC_YUC3b	Ann370100.1_BraWTC	436	48.65	9.04	36.85	-0.238	Cytoplasmic membrane
BraWTC_YUC3c	Ann315510.1_BraWTC	422	47.00	8.82	35.22	-0.199	Cytoplasmic membrane
BraWTC_YUC4a	Ann246750.1_BraWTC	411	45.62	9.35	34.72	-0.055	Cytoplasmic membrane
BraWTC_YUC4b	Ann040630.1_BraWTC	411	45.58	9.21	33.92	-0.079	Cytoplasmic membrane
BraWTC_YUC5a	Ann125900.1_BraWTC	399	44.27	8.76	45.89	-0.125	Cytoplasmic membrane
BraWTC_YUC5b	Ann300290.1_BraWTC	607	68.24	6.48	49.90	-0.231	Cytoplasmic membrane
BraWTC_YUC6a	Ann173900.1_BraWTC	437	48.68	8.92	39.64	-0.160	Cytoplasmic membrane
BraWTC_YUC6b	Ann075860.1_BraWTC	432	48.36	8.99	42.79	-0.216	Nucleus
BraWTC_YUC6c	Ann469230.1_BraWTC	374	41.77	8.67	33.30	-0.197	Cytoplasmic membrane
BraWTC_YUC7	Ann432590.1_BraWTC	420	47.03	8.87	38.95	-0.196	Cytoplasmic membrane
BraWTC_YUC8a	Ann021460.1_BraWTC	426	47.84	8.87	47.39	-0.208	Cytoplasmic membrane
BraWTC_YUC8b	Ann407190.1_BraWTC	419	46.95	8.87	49.78	-0.208	Cytoplasmic membrane
BraWTC_YUC9a	Ann315300.1_BraWTC	454	50.46	9.07	49.11	-0.152	Cytoplasmic membrane
BraWTC_YUC9b	Ann444810.1_BraWTC	420	46.88	9.37	50.55	-0.165	Cytoplasmic membrane
BraWTC_YUC10	Ann146080.1_BraWTC	262	29.66	5.99	27.82	-0.324	Cytoplasmic membrane