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TYMV induced gene silencing of TrxG family gene *BrULT2* affects the leaf shape of chinese cabbage

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ABSTRACT

The SAND domain protein *ULTRAPETALA1 (ULT1)* and its paralog *ULT2* define a small family of closely related plant proteins, both of them physically associated to form heterodimers of trxG (Trithorax group) factors. Although trxG gene *ULT1* has well-defined roles in controlling gynoecium patterning and cell fate decisions of plants, relatively little is known about the specific functions of *ULT2*. In this study, bioinformation analysis revealed that the *BrULT2* gene of *Brassica rapa* encodes a protein of molecular mass of 26.37 KDa, an isoelectric point of 7.9. qRT-PCR was performed to detect the expression profiles of the *BrULT2* gene of Chinese cabbage, showing that the transcription level of BrULT2 gene was significantly down-regulated during the vegetative stage. We silenced the trxG family gene *BrULT2* with optimized TYMV induced gene silencing, the TYMV-derived vector pTY was recombinated to infect *Brassica rapa*. After twenty days of inoculation, the infected plants showed abnormal phenotypes: leaf rolling, smaller and increased number leaves, thin stem, branch outgrowth. We detected that the expression level of *BrULT2* gene of infected plants was down-regulated meanwhile the transcriptional level of *BrULT1* gene was up-regulated. Overall, our analysis introduce a novel mechanism that mediated *BrULT* family genes control the morphogenesis of *Brassica rapa*.

KEY WORDS: *BRULT1*, *BRULT2*, CHINESE CABBAGE, LEAF SHAPE, VIGS

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INTRODUCTION

The epigenetic factors Trithorax group (trxG) and Polycomb group (PcG) proteins act as antagonistic regulators to maintain gene in transcriptional repressed and active states through their histone lysine methyltransferase (HKMT) activity during plant developmental processes (Bernadett and Jürg, 2006, Maria et al., 2015). Historically, the two major and best-characterized PcG proteins are Polycomb repressive complex 1 (PRC1) and PRC2 (Zheng and Chen, 2011), several proteins with trxG-like functions also have been identified in plants: ARABIDOPSIS HOMOLOG OF TRITHORAX1 (Alvarez-Venegas et al., 2003), ATX2 (Saleh et al., 2008), ATXR3/SDG2 (Alexandre et al., 2010, Lin et al., 2010), ATXR7/SDG25 (Berr et al., 2009, Tamada et al., 2009), and the SAND-domain DNA binding proteins ULTRAPETALA1 (ULT1) and ULT2 (Carles et al., 2005). Previous reports have demonstrated that the *Arabidopsis* trxG gene *ULT1* can switch the key floral homeotic gene *AGAMOUS (AG)* locus from repressed state to active state through restricting the deposition of H3K27 methylation, which was mediated by *CURLY LEAF (CLF)* containing the PRC2 complexes (Carles et al., 2009, Goodrich et al., 1997). The *ULT1* gene also acts antagonistically to *KAN1* to pattern adaxial-abaxial polarity of gynoecium and rosette leaves (Pires et al., 2014, Pires et al., 2015). The trxG gene *ULT2* have a very similar amino acid sequence and mRNA expression pattern with *ULT1* (Carles et al., 2005), but there are little researchs focus on the specific biological function of *ULT2* gene. To gain insight into the molecular mechanism of *ULT2* activity in plant, the optimized VIGS technology was performed.

Virus-induced gene silencing (VIGS) is an RNA mediated specific degradation mechanism based on highly conserved nucleic acid levels. By using the viral vector carrying the cDNA fragment of the target gene to infect plant, VIGS can specifically induce the sequence degradation or methylation of the homologous gene along with the replication and transcription of the virus, that will generate changes in plant phenotype or physiological indicators in favour of identifing the functional gene (Burch-Smith et al., 2010, Vaghchhipawala et al., 2011). In 1995, Kumagai constructed a VIGS vector based on the tobacco mosaic virus (TMV) for the first time, then the VIGS was successfully performed to silencing the expression of phytoene desaturase gene *PDS* in tobacco (*Nicotiana benthamiana*). Nowadays, VIGS system has been successfully established not only on model plants such as *Arabidopsis* (Manhães et al., 2015), *Nicotiana benthamiana* and *Solanum lycopersicum* (Velásquez et al., 2009), but also on wheat (Bennypaul et al., 2012), cotton (Ye et al., 2014), rice (Kant et al., 2015). Turnip yellow mosaic virus (TYMV) is a higher plant virus with a positive RNA strand, which can infect many cruciferous plants (Martínezherrera et al., 1994). Recently improved VIGS system based on TYMV-derived vector has been exploited, which has been successfully performed by gun powder with synthesized 80-nt fragment identical to target gene (Yu et al., 2018).

Previous study have indicated that the trxG genes *ULT1* and *ULT2* have overlapping roles in regualting shoot and floral stem cell accumulation during the reproductive period, however, they have not been sufficiently characterized during the vegetative growth stage. Here, we characterized the role of *BrULT2* gene of *Brassica rapa*, optimized VIGS system was deployed to silenced the *BrULT2* gene, qRT-PCR was performed to decideted the expression level of *BrULT* gene of innoculated and wild type Chinese cabbage, and the morphology of *BrULT2*-silenced transgenic plants was investigated. The collective results of our study will provide some basic information related to the meshanism of leaf growth of Chinese cabbage.

MATERIALS AND METHODS

Plant Materials and VIGS Assay: *Brassica rapa* L.CV. heading type 'Bre' and non-heading type '49 caixin' were grown in soil (1:1:1 mixture of perlite:vermiculite: topsoil) and placed in artificial climate room at 22 with 16h/8h light/dark photoperiod cycle, 50% relative humidity. When the cotyledons and the first 3 true leaves have emerged, the empty vector pTY-S and recombined vector pTY-PDS and pTY-BrUL T2 were transformed into plants by applying 10μL 2mg cm^{-3} plasmid to leaves which was injured advanced by rubbing with quartz sand. Three weeks post-inoculation, leaf samples were collected directly from the control plant and *BrULT2*-silenced plant, it was frozen with liquid nitrogen and stored at -80 until used. All experiment were conducted with three biological replicates.

Plasmid Construction: The sequence of *BrULT2* gene was downloaded from the *Brassica* database (http://brassicadb.org/brad/), total RNA was extracted from the tissue of heading type 'Bre'. The open reading frame (ORF) of *BrULT2* gene was amplified by PCR, using first-strand cDNA as template and gene-specific primers which are listed in Table 1. The PCR conditions were: 95 for 5 min, followed by 35 cycles at 95 for 30 s, 55 for 30 s and 72 for 1 min, with a final extension at 72 for 10 min. The PCR products were detected by 1.2% agarose gel electrophoresis and recovered with AxyPrep DNA gel extraction kit (AxyPrep, China), then they were cloned into the pMD19-T vector (TaKaRa, Japan) and verified by sequencing. 40-nt fragment identical to *BrULT2* gene was selected for plasmid construction (5'-TTAGGGTTTTCTCAGATGGAGACCTCCAAATCACTT-

Table 1. Sequences of the specific Primers used in gene cloning.

gene name	Sense Primer	Anti-sense Primer
BrULT2	5'-ATGGAGAGAGAATGCGGGTCGA-3'	5'-TCAAATGGGTTTGGTGTTGG-3'

GCCA-3'), specific method has been described in Yu et al (2018). The fragment was self-hybridized and the empty vector pTY-S was digested with SnaBI, then the 80-nt inverted-repeat fragment of *BrULT2* was inserted into the linearized vector pTY-S at 3:1 ratio. Afterwards, the recombined vector BrULT2-pTY, pTY-PDS and the empty vector pTY-S were transformed into *Escherichia coli* strain DH5α cells and subjected to sequencing. The empty vector and recombinant vector were isolated using maxiprep-quality plasmid (Tiangen, China) and concentrated to 2mg cm $^{-3}$ for VIGS assay.

Bioinformatics Analysis of *ULT* family genes and Conserved Motif Analysis: A phylogenetic tree of the *ULT* family genes was generated using the MEGA (version 6.0) program with the neighbor-joining method based on multiple alignments of their protein amino acid sequences, the internal branch support was estimated using 1,000 bootstrap replicates. The *ULT* genes of *Arabidopsis thaliana* were obtained from the TAIR database (http://www.arabidopsis.org/), the *ZmULT1* gene was searched from NCBI database (https://www.ncbi.nlm.nih.gov), and the *BrULT* gene of *Brassica rapa* and *BolULT* gene of *Brassica oleracea* were downloaded from *Brassica* database (http://brassicadb.org/brad/), all accession numbers are listed in Table 2. Multiple sequences alignment of BrULT1 protein and BrULT2 protein was performed by using DNAMAN software (version 5.0). The EMBOSS Pepstats software was used to calculate statistics of protein properties. Conserved motif analysis used the Multiple Expectation Maximization for Motif Elicitation (MEME) (http://meme.nbcr.net/meme/) search tool. The protein secondary structure was predicted by predictprotein (https://www.predictprotein.org/), gene interaction model was analysised by SMART (Main page http://smart.embl-heidelberg.de/).

Isolation of RNA and cDNA Synthesis: Total RNA was extracted from the leaves of BrULT2-pTY and pTY-S inoculated plant by using the RNAeasymini kit (TIANGEN, China). Subsequently, the first-strand cDNA was synthesized with 1μg of total RNA according to the instructions of the manufacturer's PrimeScript ™1 st Strand cDNA Synthesis Kit (TaKaRa, Japan).

Gene Expression Analysis by qRT-PCR: The Gene-specific primers were designed based on the gene sequences of *BrULT1* and *BrULT2* using the Becon Designer v. 7.9, that are listed in Table 3. The analysis was performed on 7500 Fast Real-Time PCR System (Applied Biosystems, USA) following the instruction manual for the SYBR Green (TaKaRa, Japan). The thermocycling conditions

Table 2. ULT family genes identified in *Brassica rapa*, Arabidopsis thaliana, *Brassica oleracea*, *Zea mays* and the sequence characteristics.

Gene name	Locus ID	Chromosome	Start	Stop	Isoelectric point	Molecular weight (KDa)
BrULT1	Bra026276	A01	10038908	10040319	7.0849	27397.08
BolULT1-1	Bol020015	Scaffold000127	1378942	1380353	6.8382	26993.52
BolULT1-2	Bol042381	C06	44651418	44652931	7.7838	26673.57
AtULT1	AT4G28190	Chr4	13985178	13987442	7.2871	26744.24
ZmULT1	NC_024466.2	Chr8	177291861	177295258	7.8795	26486.92
BrULT2	Bra036504	A07	33825	34786	7.9079	26375.91
BolULT2-1	Bol001467	Scaffold000442	29537268	29540037	6.9431	8982.30
BolULT2-2	Bol001263	Scaffold000457	120188	121156	7.6502	26378.92
AtULT2	AT2G20825	Chr2	8966867	8969756	7.5056	26097.69

Table 3. Sequences of the specific primers used in quantitative real-time PCR.

gene name	Sense Primer	Anti-sense Primer
CP	TCCACCCTCACCACCTTC	GGGACAGACCTCGCTAACT
BrULT1	ATGTGACCAAGACAAGTT	TTCTCCTCCAACGATAAC
BrULT2	GTGTTCATTGAGGGAGAC	CCGTTGGAGTTATTCTTCA
BrActin	TTGCTATTCAGGCTGTTCT	CACCATCACCAGAGTCAA

were as follows: initial polymerase activation at 94 °C for 30s; followed by 45 cycles at 94 °C for 10s , at 60 °C for 30s; and finally a melting curve was performed (60 cycles at 65 for 10 s). Each reaction was performed in triplicate. The *Actin* gene *(Bra028615*) was used as endogenous control (Dheda et al. 2004), and the relative expression levels of *BrULT1* and *BrULT2* gene were calculated using the $2^{-\Delta\Delta CT}$ method as described by Schmittgen and Livak (2008).

RESULTS AND DISCUSSION

Expression of BrULT2 during the Vegetative Stage of Chinese Cabbage

In *Arabidopsis thaliana*, the *ULT1* and *ULT2* gene are expressed coordinately in shoot and floral meristems, developing stamens, carpels and ovules, the *ULT1* transcripts could also be detected in vegetative meristems and leaf primordia during the vegetative stage, while the *ULT2* expression is specific to the reproductive developmental stage, which can be detected only in inflorescences, pollen and siliques (Carles et al., 2005). In this study, qRT-PCR was performed to determine the distribution of *BrULT2* mRNA transcripts in *Brassica rapa* L.CV. heading type 'Bre', the specific primer pairs are shown in Table 3. The analysis results showed that the *BrULT2* mRNA transcripts can be detected in the wild type leaves during vegetative stage, and it decreased significantly from the second leaf stage to fourth leaf stage with more than 80% drop at the middle and basal region of the leaf, 50% drop at apical portion (Fig. 1).

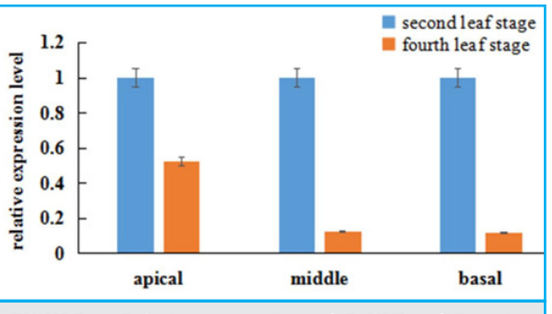

FIGURE 1. Relative expression of *BrULT2* of *Brassica rapa* L.CV. heading type 'Bre' under normal condtions, in different apart of leaf, at the different time point.

Sequences Analysis of BrULT genes

Bioinformatics analysis of *ULT* family genes showed that the predicted molecular weights (MW) of the ULT1 and ULT2 proteins ranged from 27.39 kDa to 8.98 kDa, and the theoretical isoelectric points (pIs) varied from 7.90 to 6.83 (Table 2). The cDNA sequence of *BrULT2* gene was 639bp in length, the calculated molecular mass of the protein encoded by *BrULT2* was 26.37 kDa with theoretical pI of 7.90. Multiple sequence alignment showed that the sequences of BrULT1 protein and BrULT2 protein were basically the same (Fig. 2a). To better understand the similarities and differences of *ULT* family genes between *Brassica rapa* and other plants, an unrooted phylogenetic tree was generated, the analysis result showed that the *ULT* family genes were highly conserved among the development of *Arabidopsis thaliana, Zea mays*, *Brassica rapa, Brassica oleracea* (Fig. 3a), and the *BrULT2* (*Brassica rapa*) and *BolULT2-2* (*Brassica oleracea*) clustered on the same branch. A total of 8 motifs were identified by analysis amino acid conserved domains of the *ULT* genes, showing that the conserved domain structure and positions were homogeneous (Fig. 3b). The prediction of secondary structure of *BrULT2* gene-encoding protein showed that BrULT2 protein has multiple RNA and protein binding sites (Fig. 2b), which

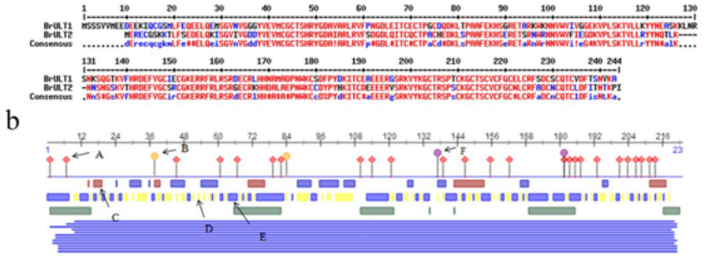

FIGURE 2. Protein analysis of BrULT1 and BrULT2. (a) Multiple sequence alignment of the BrULT1 and BrULT2 protein of *Brassica rapa*. (b) The predicted secondary structure of BrULT2protein in Chinese cabbage. A: protein binding region; B: DNA-binding region; C: α-helix; D:buried proteins; E: exposed proteins; F: RNA-binding region.

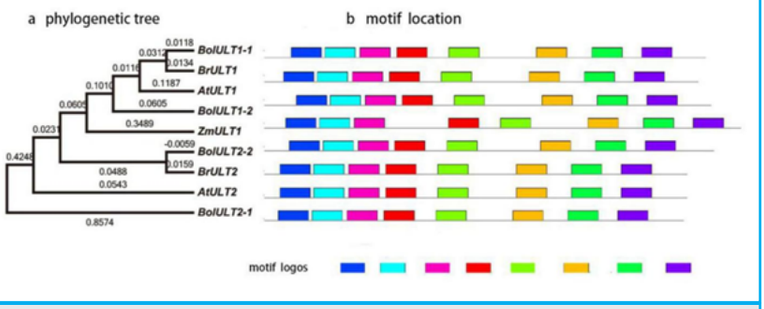

FIGURE 3. Phylogenetic tree and conserved motif analysis of ULT proteins. (a) Alignments are based on amino acid sequences of ULT proteins ofchinese cabbage and other plant species. The phylogenetic tree was constructed by the neighbor-joining method using MEGA 6.0 software with 1,000 bootstrap replicates. (b) Motif analysis of ULT proteins of *Brassica rapa*, *Arabidopsis thaliana*, *Brassica oleracea*, *Zea mays*. Motifs of the ULT proteins were elucidated by MEME. The box length corresponds to motif length. Different motif types are represented by different color blocks. The same color in different proteins indicates the same group or motif.

may facilitate its interaction with multiple transcription factors and contribute to its close regulation of a variety of growth and development-related genes.

VIGS of *BrULT2* was Successfully Performed in *Brassica rapa*

Comparing with genetic research methods such as transgene, gene knockout, and antisense inhibition, VIGS hardly require genetic transformation and mutant acquisition with a short research cycle, according to its outstanding merits: low cost, simple to operate, which has become one of the most attractive technological approaches in functional genomics research (Becker and Lange 2010). In this study, the optimized VIGS was performed to gain insight into the specific function of *BrULT2* gene.

The Phytoene desaturase (PDS) protein is a key enzyme in the carotenoid synthesis pathway, plants will lose the photoprotective effect of carotenoids and thus exhibit a whitening effect when the expression of *PDS* was blocked (Velásquez et al., 2009). In present study, the specific 40np of *BrULT2* and *BrPDS* CDS of the *Brassica rapa* was selected to construct intronic hair pin silencing vector to targeting the native *BrULT2* and *BrPDS* transcripts, the antisense construct generated dsRNA hairpins as a key elicitor for gene silencing (Christophe et al., 2003), the recombinate vector pTY-BrPDS was transformed into plants to confirming the visual success of

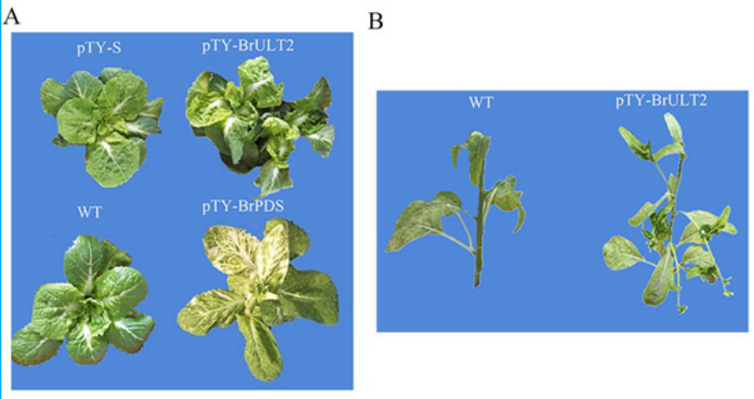

FIGURE 4. VIGS of *BrULT2* affects the leaf growth of *Brassica rapa*. (A) Growth performance of *Brassica rapa* L.CV. heading type 'Bre' inoculated with pTY-S and pTY-BrULT2, pTY-BrPDS. (B) The Phenotypes of *Brassica rapa* L.CV. non-heading type '49caixin' inoculated with pTY-s and pTY-BrULT2.

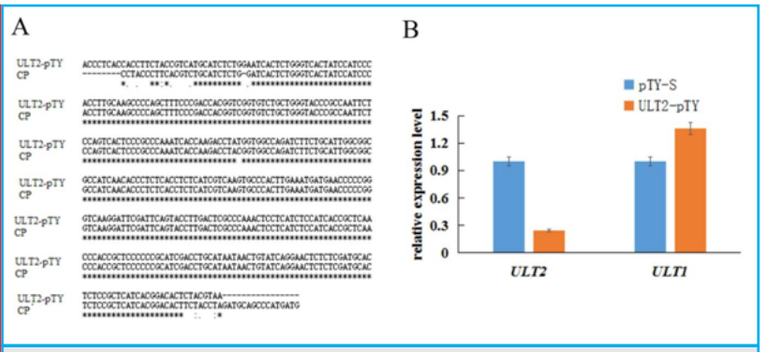

FIGURE 5. (A) Sequence alignment of PCR product amplified with specific primer of virus CP gene from inoculated plant leaves. (B) Relative expression level of *BrULT2* and *BrULT1* gene in the leaves of *Brassica rapa* L.CV. heading type 'Bre' inoculated with pTY-BrULT2.

VIGS. After three weeks of inoculation, photo-bleaching was observed in the leaves of pTY-BrPDS plant (Fig. 4A). Total RNA was extracted from the diseased 'Bre' leaves and reverse-transcribed into cDNA, which was used for the template of PCR with specific primers of virus coat protein *(CP)* gene. By sequencing, we verified that the *CP* mRNA was significantly abundant in the diseased 'Bre' plants (Fig. 5A); which implicated that TYMV virus had successfully infected Chinese cabbage.

Twenty days post-inoculation, the heading type 'Bre' infected with pTY-BrULT2 exhibited an obviously irregulate growth state with an increased number leaves comparing with control plant (pTY-S), showing a distinctive phenotype with smaller and curled rosette leaves (Fig. 4A). The non-heading type '49 caixin' infected with pTY-BrULT2 virus also showed abnormal phenotypes along with slender and irregularly curved stem, additionally the leaves number increased significantly with an increased branch outgrowths (Fig. 4A). To confirm the silencing efficiency at molecular level, the *BrULT2* gene transcripts in the leaves of heading type 'Bre' was investigated by reverse transcription qPCR. The analysis result showed that the transcription level of *BrULT2* gene dropped by 75% approximately, which indicated that the *BrULT2* gene has been silenced successfully(Fig. 5B). Those novel phenotypes observed in the inoculated plant indicating that the ectopic *BrULT2* expression alters the plant growth state leading to the modification of leaf shape of Chinese cabbage, additionally it also functions to regulate the stem and branch growth.

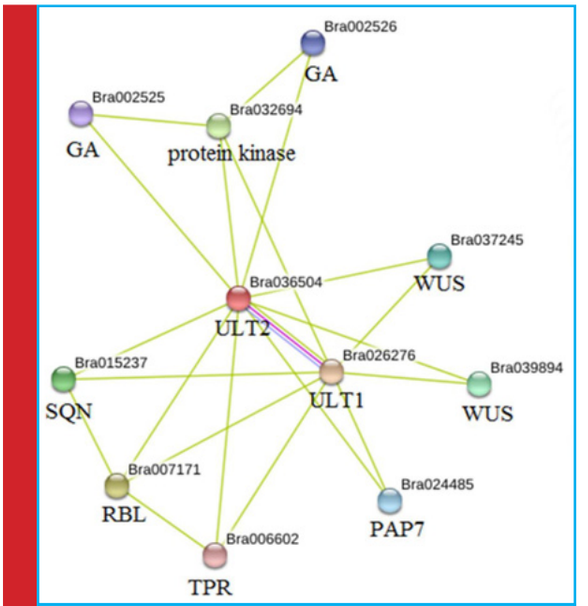

FIGURE 6. Predicted interaction networks of BrULT proteins.

In present study, we also detected that the expression level of *BrULT1* was up regulated 35% simultaneously when the *BrULT2* gene of heading type 'Bre' was silenced (Fig. 5B). An interaction network predicated by using SMART software, as shown in Fig. 6, the BrULT2 protein was predicted to interact with BrULT1 and other proteins: GA, WUS, PAP7, SQN, RBL, TPR. So we hypothesis that the *BrULT1* and *BrULT2* gene interact genetically during the vegetative growth stage of Chinese cabbage, and the *BrULT1* gene have similarly function with *BrULT2* to regulate the rosette leaf development, with the pTY-BrULT2 mutants display abnormal phenotypes: curly leaves and ectopic branch outgrowth. The results were in line with many studies. For example, Monfared et al. (2013) reported that the *ULT1* and *ULT2* genes function redundantly to specify the apical-basal polarity axis of the gynoecium, indicating that they may function in differentiating tissues, and in a study

by Monfared and Fletcher (2014) showed that the *ULT2* gene play a minor but overlapping role with *ULT1* in the regulation of shoot and floral stem cell accumulation.

Plant leaf shape is closely related to the photosynthetic efficiency and lodging resistance, exploring the leaf-shaped genes will contribute to the genetic improvement of excellent traits: disease resistance, stress resistance, high photosynthetic efficiency, leafy head formation. Previous report have demonstrated that class 1 *KNOTTED1-LIKE HOMEOBOX (KNOX)* genes are the common targets of *ULT1* and *ULT2* (Monfared et al. 2013), its ectopic expression may sculpt leaf morphogenesis with an auxin-directed mechanism controlling (Hay and Tsiantis, 2006). Nonetheless, future work will be required to exploring the hormone-related pathway of dramatic change in leaf shape for understanding the details of *BrULT* genes regulation.

CONCLUSION

In this study, we demonstrated that the optimized VIGS can be effectively performed to study specific gene function of *Brassica rapa*, and we firstly reveal that the *BrULT2* gene act as an important epigenetic factor to regulating the leaf shape development of *Brasssica rapa*. Meanwhile, we hypothesis that the closely related *BrULT1* and *BrULT2* gene may play overlapping roles in the regulation of leaf curl status and other vegetative phenotypes. Our study provide some important information for exploring the specific functions of *ULT* family genes during vegetative stage of plants.

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