Genome-wide sequence identification and expression analysis of *N*^{6}-methyladenosine demethylase in sugar beet (*Beta vulgaris* L.) under salt stress

Jie Cui [Corresp., Equal first author, 1], **Junli Liu** [Equal first author, 1], **Junliang Li** [1], **Dayou Cheng** [1], **Cuihong Dai** [1]

[1] Harbin Institute of Technology, Harbin, Heilongjiang, China

Corresponding Author: Jie Cui
Email address: cuijie@hit.edu.cn

N^6-methyladenosine (m^{6}A) is the most abundant and highly conserved RNA modification in eukaryotes. m^{6}A demethylase can remove the m^{6}A marker and dynamically regulate the m^{6}A level in vivo, which plays an important role in plant growth, development and response to abiotic stress. The confirmed m^{6}A demethylases in *Arabidopsis thaliana* include ALKBH9B and ALKBH10B, both belonging to the ALKB family. In this study, ALKB family members were screened in sugar beet genome-wide database, and their conserved domain, gene structures, chromosome location, phylogeny and other factors were analyzed. The results showed that almost all BvALKB proteins contained the conserved domain of 2OG-Fe II-Oxy. All the proteins were classified into five groups, each of which had similar motifs and gene structures. Three *Arabidopsis* m^{6}A demethylase homologous proteins were of particular concern. Almost all genes were up-regulated or down-regulated to varying degrees under salt stress, especially the uxaj gene homologous to ALKBH10B, which was significantly up-regulated, suggesting that the genes were in response to salt stress. This study provides a theoretical basis for further screening of m^{6}A demethylase in sugar beet, and also lays a foundation for studying the role of ALKB family proteins in growth, development and response to salinity stress.

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Jie Cui[1], Junli Liu[1], Junliang Li[2], Dayou Cheng[3], Cuihong Dai[4]

Harbin Institute of Technology, Harbin, China

Corresponding Author:
Jie Cui
92 Xidazhi Street, Harbin, Heilongjiang, 150001, China
Email address: cuijie@hit.edu.cn

Abstract

N^6-methyladenosine (m^{6}A) is the most abundant and highly conserved RNA modification in eukaryotes. m^{6}A demethylase can remove the m^{6}A marker and dynamically regulate the m^{6}A level in vivo, which plays an important role in plant growth, development and response to abiotic stress. The confirmed m^{6}A demethylases in *Arabidopsis thaliana* include ALKBH9B and ALKBH10B, both belonging to the ALKB family. In this study, ALKB family members were screened in sugar beet genome-wide database, and their conserved domain, gene structures, chromosome location, phylogeny and other factors were analyzed. The results showed that almost all BvALKB proteins contained the conserved domain of 2OG-Fe II-Oxy. All the proteins were classified into five groups, each of which had similar motifs and gene structures. Three *Arabidopsis* m^{6}A demethylase homologous proteins were of particular concern. Almost all genes were up-regulated or down-regulated to varying degrees under salt stress, especially the uxaj gene homologous to ALKBH10B, which was significantly up-regulated, suggesting that the genes were in response to salt stress. This study provides a theoretical basis for further screening of m^{6}A demethylase in sugar beet, and also lays a foundation for studying the role of ALKB family proteins in growth, development and response to salinity stress.

Keywords: Sugar beet, *N*^{6}-methyladenosine , demethylase, ALKB , salt stress, bioinformation

Introduction

N^6-methyladenosine(m^{6}A) is the most abundant modification in mRNA among all higher eukaryotes , manifested as methylation at the sixth *N* of adenosine , which has been a hot spot of epigenomic studies in recent years(*Huang & Yin., 2018*). Previous studies have shown that m^{6}A, including methytransferase complex (METTL3, METTL14, WTAP, etc.), demethylase(FTO, ALKBH5, etc.) and RNA binding proteins (YTHDF1/2/3, YTHDC1/2, etc.) *(Desrosiers,*

Friderici K & Rottmanl, 1974; *Ortega et al., 2003*; *Jia et al., 2011)*, is a reversible and dynamic co-regulation process(*Miao et al., 2020*). In animals, genes encoding m^{6}A-related proteins have been identified and characterized(*Wei, Gershowitz & Moss, 1976*; *Levis & Penman, 1978*), and their important roles in animal development and in coping with different environments have been demonstrated, but the function of these proteins in plants is only now being revealed. m^{6}A is generally enriched near the stop codon and the 3 'UTR, as well as at the long introns and transcription start sites*(Meyer et al., 2012*), which are common in mammals. m^{6}A is found to be enriched near the start codon in *Arabidopsis thaliana*, which may play a role in the plant-specific pathway *(Luo et al., 2013*; *Wan et al., 2015*). A recent explosion of molecular studies centered on m^{6}A methylation has revealed its role in eukaryotic transcriptome regulation, RNA stability, and translation efficiency*(Niu et al., 2013*; *Pan, 2013*; *Yue et al., 2019*). Some proteins are involved in regulating the formation of plant cells and tissues(*Zhong et al., 2008*; *Shen et al., 2016*; *Bhat et al., 2020*; *Scutenaire et al., 2018*), while others regulate the expression of drought and high temperature signal related genes in plants(*Zhao X, 2014*; *Lu et al., 2020*), which play a significant role in plant stress resistance.

The reversibility of m^{6}A is achieved by demethylases, which was confirmed in the paper by He et al(*Jia et al., 2011*). Proteins identified as m^{6}A demethylases belong to the ALKB family and contain highly conserved synthase-like domains. m^{6}A demethylases found in mammals mainly include obesity-related genes FTO and ALKBH5 (*Jia, Fu & He, 2013*; *Liu & Jia, 2014*). The unique C-terminal long loop structure of FTO may determine its function of promoting protein-protein or protein-RNA interactions. Compared with FTO catalyzed m^{6}A to A through intermediates, ALKBH5 could directly catalyze m^{6}A to A(*Mauer et al., 2017*; *Wei et al., 2018*). Due to differences in tissue specificity and substrate, FTO and ALKBH5 play different roles in mRNA processing and metabolism. Studies have shown that FTO can regulate the binding of precursor RNA with splicing factor SRSF2 to affect its splicing maturation, and ALKBH5 is related to the nuclear transport mRNA (*Zhao et al., 2014*).

Bioinformatics analysis revealed that there were 13 ALKB homologous proteins in *Arabidopsis*, among which ALKBH9A, ALKBH9B, ALKBH9C, ALKBH10A and ALKBH10B had the most similar amino acid sequence to ALKBH5. Proteins that have been confirmed as m^{6}A demethylases include ALKBH9B and ALKBH10B. ALKBH10B is highly abundant in all tissues, especially in flowers. ALKBH10B only has a specific catalytic function on m^{6}A modified mRNA, and experiments have shown that it can mediate the early flowering transition by regulating the demethylation of *FT*, *SPL3* and *SPL9*(*Duan et al., 2017*). As the only ALKBH5 homologous protein in the cytoplasm, ALKBH9B was responsible for removing *N*^{6}-methyladenosine from ssRNA in vitro and participating in mRNA silencing or degradation. In addition, it also plays a role in plant protection against specific viral pathogens, and through interaction with viral cap protein, modulates the m^{6}A demethylation modification of the AMV genome to affect its life cycle and infection capacity (*Martínez-Pérez et al., 2017*), but has no effect on the activity of cucumber mosaic virus. m^{6}A demethylase has not been found in other plants.

Previous studies have demonstrated the role of some ALKBH members in plant growth and development. The stress response of plant demethylase was mainly studied in model plant *Arabidopsis*. ALKBH9A was highly expressed in roots under salt stress, and ALKBH10A was significantly down-regulated under heat stress(*Růžička et al., 2015*). Under drought, cold or ABA treatment, ALKBH1 levels were signicantly up-regulated, while ALKBH6, ALKBH8B and ALKBH10A expressions were decreased(*Hu, Manduzio & Kang, 2019*), indicating that ALKBH members may play an important role in abiotic stress. In recent studies, it was found that ALKBH6 could bind to m^{6}A marked mRNA and remove the mark n *Arabidopsis*, which may be a potential m^{6}A demethylase. Under drought or heat stress, the survival rate of the ALKBH6 mutant was lower than that of the wild type, but not under salt stress. In addition, ALKBH6 affected ABA response by regulating the expression of genes related to ABA signaling(*Huong, Ngoc & Kang, 2020*). These results suggest that RNA demethylation plays a crucial role in plant responses to abiotic stress.

Sugar beet is one of the most abundant sugar-producing crops, and its yield and quality are of great significance to agricultural production. However, the soil salinization in China is serious, and the saline-alkali land highly coincides with the sugar beet production area. In addition, although the sugar beet has a certain salt tolerance, the degree of salt tolerance is limited, and the seed germination and seedling growth are greatly affected, which is bound to severely damage the sugar industry. Therefore, the analysis of sugar beet m^{6}A will be helpful to understand its transcriptional modification and expression regulation, and will be of great benefit to reveal its salt-tolerant mechanism and to cultivate new stress resistant strains. m^{6}A demethylase is involved in the response of abiotic stress(*Hu et al., 2021*), so far there has been no specific analysis of sugar beet salt stress. In this study, bioinformatics analysis of m^{6}A demethylase was carried out based on the beet genome database, and the expression level of m^{6}A demethylase in different parts of beet under salt stress was analyzed to determine the genes related to salt treatment, so as to provide theoretical basis for breeding beet varieties.

Materials & Methods

Materials

The salt-tolerant strain ‘O68’ of beet was used as the experimental material in this experiment(*Shi et al., 2008*). The seeds were soaked under running water for 12 h, then disinfected with 75% ethanol and washed aseptic for 3 times. The seeds were sown into the wet sponge and cultured in the dark at 24 h for 2 days. After germination, it was transferred to a culture pot containing nutrient solution (light for 16 h, dark for 8 h). After the growth of three pairs of true leaves, 300 mM NaCl solution was used to replace the nutrient solution for 24 h, and the other conditions remained unchanged. The control group was set without salt treatment. After the salt stress, leaves and roots were sampled, and immediately precooled in liquid nitrogen and stored in a refrigerator at -80 ^{o}C until analysis.

Screening and identification of sugar beet m^{6}A demethylase

The whole genome database of sugar beet was published (http://bvseq.molgen.mpg.de/index.shtml). The seed sequence of the demethylase conserved domain 2OG-Fe II-oxy(PF13532) was downloaded from Pfam. The *e*-value < $1e^{-5}$ was set on HMMER(http://www.hmmer.org/), and the beet genome-wide database was searched. Pfam online tool was used to analyze the domain of candidate proteins, and the proteins with the conserved domain were screened out. DNAMAN7.0 was used to multiple sequence alignment of candidate proteins, and Weblogo was used for conservative domain identification (http://weblogo.berkeley.edu/logo.cgi).

Bioinformatics analysis of ALKB family

ExPASY (https://web.expasy.org/protparam/) was used to analyze physical and chemical properties of proteins, including the average molecular weight, isoelectric point, the average number of amino acids, etc(*Gasteiger et al., 2003*). Protein subcellular localization was predicted by CELLO (http://cello.life.nctu.edu.tw/). Mapchart was used to map the position of genes on chromosomes. MEME (http://meme-suite.org/tools/meme) was used to predict protein motifs(*Bailey et al., 2006*), and the number of searching motifs was set to 20, with other parameters for tacit recognition. Gene intron and exon structures were analysis in Splign(https://www.ncbi.nlm.nih.gov/sutils/splign/splign.cgi?textpage=online&level=form). A phylogenetic tree (1000 replicates) was constructed by neighbor-joining method using MEGA7 for protein sequence progression and multi-sequence alignment between *Arabidopsis* and sugar beet(*Kumar, Stecher & Tamura, 2016*).

Expression analysis of BvALKB under salt stress

Sugar beet samples collected after salt stress treatment were quickly frozen in liquid nitrogen with a mortar and pestle, and ground into a fine powder. Total RNA was extracted using Trizol reagent and the concentration of RNA was determined using the MicroDrop spectrophotometer. Total RNA was reverse transcribed into cDNA by using PrimeScript ™ II 1st Strand cDNA Synthesis Kit(TaKaRa, Japan). In order to detect the gene expression level, qRT-PCR was performed using the CFX96 real-time system and the iTaq^{TM} Universal SYBR Green Supermix Kit(BIO-RAD, USA). The primers were designed using Primer 5 and the sequences were listed in Table 1. UBQ5, PP2A and 25S RNA were used as internal controls. All experiments were repeated at least three times. Data analysis was calculated by $2^{-\triangle\triangle Ct}$ method, and the relative expression of each gene was expressed by mean±standard deviation.

Results

Screening and identification of sugar beet m^{6}A demethylase

The seed sequence of the conserved domain (PF13532) was downloaded from Pfam and searched in the beet genome database by HMMER. A total of 12 homologous proteins were screened. The *e*-value of all the other proteins was less than $1e^{-5}$ except uxaj, which was 0.016. Among them, kacr.t1/t2 and swwm.t1/t2 are two transcriptional variants of kacr and swwm,

respectively, their corresponding proteins are consistent, so they are not discussed separately. The domain markers of candidate proteins analyzed online by Pfam are shown in Fig. 1. Except uxaj, all the 9 candidate proteins have complete or partial 2OG-Fe II-Oxy domain, indicating that these proteins are highly conserved. In terms of domain distribution, the domains of eskg and njrf were located in the middle, the domains of sqec were located in the front, and the domains of other proteins were all located in the end. The RRM domain of pgse was related to mRNA and rRNA processing, RNA output and RNA stability by query. However, due to low sequence similarity, the *e*-value of uxaj in Pfam database comparison is 0.023, and it has a high possibility of possessing the 2OG-Fe II-Oxy domain, so it will be regarded as a member of this family for subsequent analysis.

As shown in Fig. 2, the alignment results of DNAMAN7.0 showed certain homology but low conservatism in the domain sequences. The homology was very high at sites 162, 212, 215, 222, 255, 259, etc, which might be related to the function of the domain and amino acids at these specific locations. Among the 10 candidate proteins, 6 proteins were confirmed to belong to ALKB family by BLAST comparison with NCBI, while ryeg, pkhc, njrf and uxaj were not described before and belonged to new ALKB family members. This is shown in Table 2.

Analysis of physicochemical properties of BvALKB proteins

The results of physical and chemical properties analysis ExPASY showed that the average length of the coding region of 10 genes was 1260 bp (783-1755 bp), the average number of amino acids encoding proteins was 416 (260-584), the average molecular weight was 46.41 kDa (28.91-64.97 kDa), and the average isoelectric point was 7.12 (5.11-9.02). This is shown in Table 3.

Chromosomal localization of genes

The suagr beet has nine pairs of chromosomes. As shown in Fig. 3, chromosome localization analysis showed that each gene tended to be dispersed, and members of this family were found on chromosomes 3 to 8, while ryeg, swwm and pkhc were concentrated on chromosome 7. uxaj has no specific location information and is only shown on chromosome 7, probably located in the gap region of fragments splicing from whole gene sequencing.

Phylogenetic relationships and gene structures analysis of BvALKB

Multiple sequence alignment was performed on 14 ALKB family proteins of *Arabidopsis* and 10 proteins of sugar beet using MEGA7, and the alignment diagram of protein local domain was shown in Fig. 4. For the convenience of observation, proteins with high sequence similarity were compared together, and it could be seen that the reason for the low homology of each domain might be that the domain similarity of different subclasses was not high, and they were the same only at some special sites.

Then a phylogenetic tree (1000 replicates) was constructed using neighbor-joining method to observe the evolutionary relationship between *Arabidopsis* and sugar beet, as shown in Fig. 5. It could be seen that most of the bootstrap values are greater than 70, indicating high reliability. All

the proteins were divided into five categories: Class I(AtALKBH9-like) includes njrf and eskg, which are similar to AtALKBH9; Class II(AtALKBH10-like) only contains uxaj, which is similar to AtALKBH10; Only one BvALKB protein belongs to Class III(AtALKBH2-like); Class IV(AtALKBH6/8-like) consists of sqec, swwm and pgse; Three members are assigned to Class V(AtALKBH1-like), including huzh, ryeg and pkhc. *Arabidopsis* ALKBH9B and ALKBH10B in the first two classes have been confirmed to be m^6A demethylases, so njrf, eskg and uxaj are likely to also have demethylation functions, which should be focused on.

The structure of each type of gene was labeled and plotted, as shown in Fig. 6. All genes contain introns and are broken genes. Generally speaking, genes belonging to the same protein also show similar intron and exon distribution in gene structure. The njrf and eskg of Class I have 6 exons, and the ryeg and pkhc of Class V have 4 exons, which are due to their sequence similarity. sqec and pgse in Class IV are similar in structure, although the number of exons is different. Other genes, such as swwm and huzh, are more or less different in structure from similar genes.

Motifs analysis and subcellular localization prediction of BvALKB proteins

Set the expected number of searching motifs as 20 on MEME, and the search results are sorted from small to large by *e*-value, as shown in Figs. 7 and 8. In general, almost all of the 10 proteins except uxaj have motifs 1, 2, 4, and 8, which are probably important components of the 2OG-Fe II-Oxy domain. Proteins belonging to the same Class had similar motif composition. The homologous proteins njrf, eskg and uxaj of ALKBH9B/10B in *Arabidopsis* were different from other protein motifs in that they were closely linked to motif 3 and 6, which was speculated to be related to demethylation function.

The scores of different locations of CELLO predicted proteins showed that most of the proteins were located in the nucleus and mainly exercised the function of demethylation in the nucleus. Individual proteins such as uxaj was located in the cytoplasm, and sqec was located in the cytoplasm and extracellular, indicating that they may perform other extracellular functions.

Quantitative analysis of BvALKB genes in sugar beet under salt stress

m^6A plays an important role in response to abiotic stresses. In order to understand the changes of potential m^6A demethylation genes in sugar beet under salt stress, we compared the expression level of the genes under normal condition and salt stress. The phenotypic changes of sugar beet cultured to three pairs of true leaves were observed by 300 mM salt stress, and the expression of each gene was analyzed by qRT-PCR.

The results are shown in Figs. 9 and 10. In leaves, all the other genes were up-regulated or down-regulated to varying degrees except huzh. pkhc, kacr and uxaj were up-regulated, especially uxaj was highly up-regulated. ryeg, pgse, eskg, sqec, njrf and swwm were down-regulated, and swwm was significantly down-regulated. In root, huzh, ryeg, eskg, njrf and swwm were up-regulated, while the other five genes were down-regulated. pkhc, kacr and pgse were

down-regulated significantly. Different expression levels in leaves and roots suggest that the expression of these genes is tissue-specific.

Discussion

Soil salinization has become a global problem. In China, saline-alkali land is mainly distributed in northwest, northeast and north China, and highly coincides with sugar beet production area, which puts forward higher requirements for sugar beet salt tolerance. Previous studies have shown that ALKB family proteins are involved in plant growth and development and abiotic stress processes, especially the proteins confirmed as m^6A demethylase. However, the ALKB family members in sugar beet have not been studied. Therefore, bioinformatics and quantitative methods were used to study the response of ALKB proteins in sugar beet under salt stress and the theoretical basis for screening m^6A demethylase in sugar beet was put forward.

Through the beet genome-wide analysis, we found 12 BvALKB family proteins that were transcribed by 10 genes. The number was similar to *Arabidopsis* (14) and rice (12), but far less than which of wheat (29) and quinoa (27) (*Yue et al.*, *2019*), which might be caused by different copy number during plant evolution.

Phylogenetic analysis can quickly identify protein homology. The phylogenetic tree of BvALKB proteins and AtALKB proteins was constructed by MEGA using neighbor-joining method. Proteins with high homology to AtALKBH9B/10B could be considered as potential m^6A demethylase. All the proteins were divided into five categories. The Class I included AtALKBH9A/9B/9C proteins, and two BvALKB proteins (njrf and eskg) belonged to this group. The Class II included AtALKBH10A/10B proteins, with only one beet protein (uxaj) belonging to it. Therefore, the transcriptional proteins of njrf, eskg and uxaj are likely to be potential m^6A demethylases, which play an important role in the response of sugar beet to salt stress. Only one BvALKB protein belongs to Class III and may be involved in protecting plants from DNA methylation damage(*Meza et al.*, *2012*). Three BvALKB proteins belong to Class IV, which may participate in tRNA modification and DNA repair(*Leihne et al., 2011*; *Zdżalik et al., 2014*). Three BvALKB proteins belong to Class V, associated with redox and tRNA modifications in cytoplasm and mitochondria (*Kawarada et al., 2017*). The exon and intron analysis of the genes showed that the number of exons in the same class of genes is basically the same and the distribution is similar, which suggesting their homology.

The subcellular localization of the protein was predicted, indicating that most proteins were located in the nucleus, while some proteins were located in the cytoplasm and extracellular, which might play different roles in transcriptional regulation. All the proteins have the 2OG-Fe II-Oxy domain, suggesting that m^6A is evolutionarily conservative.

The structure of a protein determines its function, and a specific structure has a specific biological function. Motif analysis of BvALKB proteins showed that motif 1, 2, 4 and 8 constituted the conserved domain, and the location of these motifs was consistent with that of the previously identified domain. Due to the conservatism of evolution, the composition of the motifs of ALKB proteins in a group is basically similar. Notably, the three homologous proteins

to AtALKBH9B /10B contained unique motif 3 and motif 6, suggesting that they may be involved in demethylation function.

The expression profiles of sugar beet leaves and roots under normal and salt stress conditions were analyzed. In leaves, all other genes except huzh were induced or inhibited by salt stress. In roots, five genes were up-regulated while five genes were down-regulated, and three genes were highly down-regulated. Except for pgse and sqec, the other eight genes showed opposite expression trends in leaves and roots, suggesting tissue specificity of gene regulation. We paid the most attention to the gene expression levels of three homologous proteins. njrf and eskg were down-regulated in leaves, while uxaj was significantly up-regulated, and the opposite trend was observed in roots. uxaj is homologous to AtALKBH10B, although the *e*-value was minimal in the initial HMMER search. The significant changes in uxaj expression level indicate our strong concern in subsequent experiments and provide a basis for the study of salt tolerance of sugar beet.

Conclusions

This study identified 12 sugar beet ALKB family proteins. We used bioinformatics method to analyze its gene structures, chromosome location, physical and chemical properties of protein, motifs, subcellular localization and the phylogenetic tree construction etc, and quantitatively comparing the expression of BvALKB under normal conditions and salt stress. In addition, homologous *Arabidopsis* m^6A demethylase proteins were screened and identified as potential sugar beet m^6A demethylase, which laid a foundation for further research on its function and provided ideas for the cultivation of new salt-tolerant strains.

References

Bailey TL, Williams N, Misleh C, Li WW. 2006. MEME: discovering and analyzing DNA and protein sequence motifs. *Nucleic Acids Research* **34**:W369-W373 DOI 10.1093/nar/gkl198.

Bhat SS, Bielewicz D, Gulanicz T, Bodi Z, Yu X, Anderson SJ, Szewc L, Bajczyk M, Dolata J, Grzelak N, Smolinski DJ, Gregory BD, Fray RG, Jarmolowski A, Szweykowska-Kulinska Z. 2020. mRNA adenosine methylase (MTA) deposits m^6A on pri-miRNAs to modulate miRNA biogenesis in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences* **117**:21785-21795 DOI 10.1073/pnas.2003733117.

Desrosiers R, Friderici K, Rottman F. 1974. Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells. *Proceedings of the National Academy of Sciences* **71**:3971-3975 DOI 10.1073/pnas.71.10.3971.

Duan HC, Wei LH, Zhang C, Wang Y, Chen L, Lu Z, Chen PR, He C, Jia G. 2017. ALKBH10B is an RNA N^6-methyladenosine demethylase affecting *Arabidopsis* floral transition. *Plant Cell* **29**:2995-3011 DOI 10.1105/tpc.16.00912.

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research* **31**:3784-3788 DOI 10.1093/nar/gkg563.

Jia G, Fu Y, He C. 2013. Reversible RNA adenosine methylation in biological regulation. *Trends in Genetics* **29**:108-115 DOI 10.1016/j.tig.2012.11.003.

Jia G, Fu Y, Zhao X, Dai Q, Zheng G, Yang Y, Yi C, Lindahl T, Pan T, Yang YG. 2011. *N*^{6}-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. *Nature Chemical Biology* **7**:885-887 DOI 10.1038/nchembio.687.

Huang J, Yin P. 2018. Structural Insights into *N*^{6}-methyladenosine (m^{6}A) modification in the transcriptome. *Genomics, Proteomics and Bioinformatics* **16**:85-98 DOI 10.1016/j.gpb.2018.03.001.

Hu J, Cai J, Park SJ, Lee K, Li Y, Chen Y, Yun JY, Xu T, Kang H. 2021. *N*^{6} - Methyladenosine mRNA methylation is important for salt stress tolerance in *Arabidopsis*. *The Plant Journal* DOI 10.1111/tpj.15270.

Hu J, Manduzio S, Kang H. 2019. Epitranscriptomic RNA methylation in plant development and abiotic stress responses. *Frontiers in Plant Science* **10**:500 DOI 10.3389/fpls.2019.00500.

Huong TT, Ngoc LNT, Kang H. 2020. Functional characterization of a putative RNA demethylase ALKBH6 in *Arabidopsis* growth and abiotic stress responses. *International Journal of Molecular Sciences* **21**:6707 DOI 10.3390/ijms21186707.

Kawarada L, Suzuki T, Ohira T, Hirata S, Miyauchi K, Suzuki T. 2017. ALKBH1 is an RNA dioxygenase responsible for cytoplasmic and mitochondrial tRNA modifications. Nucleic Acids Research. **45**:7401-7415 DOI 10.1093/nar/gkx354.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**:1870-1874 DOI 10.1093/molbev/msw054.

Leihne V, Kirpekar F, Vågbø CB, van den Born E, Krokan HE, Grini PE, Meza TJ, Falnes PØ. 2011. Roles of Trm9- and ALKBH8-like proteins in the formation of modified wobble uridines in *Arabidopsis* tRNA. *Nucleic Acids Research* **39**:7688-7701 DOI 10.1093/nar/gkr406.

Levis R, Penman S. 1978. 5'-terminal structures of poly(A)+ cytoplasmic messenger RNA and of poly(A)+ and poly(A)- heterogeneous nuclear RNA of cells of the dipteran Drosophila melanogaster. *Journal of Molecular Biology* **120**:487-515 DOI 10.1016/0022-2836(78)90350-9.

Liu J, Jia G. 2014. Methylation modifications in eukaryotic messenger RNA. *Journal of Genetics and Genomics* **41**:21-33 DOI 10.1016/j.jgg.2013.10.002.

Lu L, Zhang Y, He Q, Qi Z, Zhang G, Xu W, Yi T, Wu G, Li R. 2020. MTA, an RNA m^{6}A methyltransferase, enhances drought tolerance by regulating the development of trichomes and roots in poplar. *International Journal of Molecular Sciences* **21**:2462 DOI 10.3390/ijms21072462.

Luo GZ, Macqueen A, Zheng G, Duan H, Dore LC, Lu Z, Liu J, Chen K, Jia G, Bergelson J. 2013. Unique features of the m^{6}A methylome in Arabidopsis thaliana. *Nature Communications* **5**:5630 DOI 10.1038/ncomms6630.

Martínez-Pérez M, Aparicio F, López-Gresa MP, Bellés JM, Sánchez-Navarro JA, Pallás V. 2017. *Arabidopsis* m^{6}A demethylase activity modulates viral infection of a plant virus and the m^{6}A abundance in its genomic RNAs. *Proceedings of the National Academy of Sciences* **114**:10755-10760. DOI 10.1073/pnas.1703139114.

Mauer J, Luo X, Blanjoie A, Jiao X, Grozhik AV, Patil DP, Linder B, Pickering BF, Vasseur JJ, Chen Q, Gross SS, Elemento O, Debart F, Kiledjian M, Jaffrey SR. 2017. Reversible methylation of m^{6}A$_m$ in the 5'cap controls mRNA stability. *Nature* **541**:371-375 DOI 10.1038/nature21022.

Merret R, Nagarajan VK, Carpentier MC, Park S, Favory JJ, Descombin J, Picart C, Charng YY, Green PJ, Deragon JM, Bousquet-Antonelli C. 2015. Heat-induced ribosome pausing triggers mRNA co-translational decay in *Arabidopsis thaliana*. *Nucleic Acids Research* **43**:4121-4132 DOI 10.1093/nar/gkv234.

Meyer KD, Saletore Y, Zumbo P, Elemento O, Mason CE, Jaffrey SR. 2012. Comprehensive analysis of mRNA methylation reveals enrichment in 3'UTRs and near stop codons. *Cell* **149**:1635-1646 DOI 10.1016/j.cell.2012.05.003.

Meza TJ, Moen MN, Vågbø CB, Krokan HE, Klungland A, Grini PE, Falnes PØ. 2012. The DNA dioxygenase ALKBH2 protects *Arabidopsis thaliana* against methylation damage. *Nucleic Acids Research* **40**:6620-6631 DOI 10.1093/nar/gks327.

Miao Z, Zhang T, Qi Y, Song J, Han Z, Ma C. 2020. Evolution of the RNA *N*^{6}-methyladenosine methylome mediated by genomic duplication. *Plant Physiology* **182**:345-360 DOI 10.1104/pp.19.00323.

Niu Y, Zhao X, Wu YS, Li MM, Wang XJ, Yang YG. 2013. *N*^{6}-methyl-adenosine (m^{6}A) in RNA: an old modification with a novel epigenetic function. *Genomics Proteomics Bioinformatics* **11**:8-17 DOI 10.1016/j.gpb.2012.12.002.

Ortega A, Niksic M, Bachi A, Wilm M, Sánchez L, Hastie N, Valcárcel J. 2003. Biochemical function of female-lethal (2)D/Wilms' tumor suppressor-1-associated proteins in alternative pre-mRNA splicing. *Journal of Biological Chemistry* **278**:3040-3047 DOI 10.1074/jbc.M210737200.

Pan T. 2013. *N*^{6}-methyl-adenosine modification in messenger and long non-coding RNA. *Trends In Biochemical Sciences* **38**:204-209 DOI 10.1016/j.tibs.2012.12.006.

Růžička K, Zhang M, Campilho A, Bodi Z, Kashif M, Saleh M, Eeckhout D, El-Showk S, Li H, Zhong S, De Jaeger G, Mongan NP, Hejátko J, Helariutta Y, Fray RG. 2017. Identification of factors required for m^{6}A mRNA methylation in *Arabidopsis* reveals a role for the conserved E3 ubiquitin ligase HAKAI. *New Phytologist* **215**:157-172 DOI 10.1111/nph.14586.

Scutenaire J, Deragon JM, Jean V, Benhamed M, Raynaud C, Favory JJ, Merret R, Bousquet-Antonelli C. 2018. The YTH domain protein ECT2 is an m^{6}A reader required for normal trichome branching in *Arabidopsis*. *Plant Cell* **30**:986-1005 DOI 10.1105/tpc.17.00854.

Shen L, Liang Z, Gu X, Chen Y, Teo ZW, Hou X, Cai WM, Dedon PC, Liu L, Yu H. 2016. *N*^{6}-methyladenosine RNA modification regulates shoot stem cell fate in *Arabidopsis*. *Developmetal Cell* **38**:186-200 DOI 10.1016/j.devcel.2016.06.008.

Shi SZ, Cui J, Lu ZX, Cheng DY, Luo CF. 2008. Salt tolerance screening of sugarbeet germplasm resources. *China Beet and Sugar* **4**:7–9

Wan Y, Tang K, Zhang D, Xie S, Zhu X, Wang Z, Lang Z. 2015. Transcriptome-wide high-throughput deep m^6A-seq reveals unique differential m^6A methylation patterns between three organs in *Arabidopsis thaliana*. *Genome Biology* **16**:272 DOI 10.1186/s13059-015-0839-2.

Wei CM, Gershowitz A, Moss B. 1976. 5'-Terminal and internal methylated nucleotide sequences in HeLa cell mRNA. *Biochemistry* **15**:397-401 DOI 10.1021/bi00647a024.

Wei J, Liu F, Lu Z, Fei Q, Ai Y, He PC, Shi H, Cui X, Su R, Klungland A, Jia G, Chen J, He C . 2018. Differential m^6A, m^6A_m, and m^1A demethylation dediated by FTO in the cell nucleus and cytoplasm. *Molecular Cell* **71**:973-985 DOI 10.1016/j.molcel.2018.08.011.

Yue H, Nie X, Yan Z, Weining S. 2019. *N^6*-methyladenosine regulatory machinery in plants: composition, function and evolution. *Plant Biotechnology Journal* **17**:1194-1208 DOI 10.1111/pbi.13149.

Zdżalik D, Vågbø CB, Kirpekar F, Davydova E, Puścian A, Maciejewska AM, Krokan HE, Klungland A, Tudek B, van den Born E, Falnes PØ. 2014. Protozoan ALKBH8 oxygenases display both DNA repair and tRNA modification activities. *Plos One* **9**:e98729 DOI 10.1371/journal.pone.0098729.

Zhao X, Yang Y, Sun BF, Shi Y, Yang X, Xiao W, Hao YJ, Ping XL, Chen YS, Wang WJ, Jin KX, Wang X, Huang CM, Fu Y, Ge XM, Song SH, Jeong HS, Yanagisawa H, Niu Y, Jia GF, Wu W, Tong WM, Okamoto A, He C, Rendtlew Danielsen JM, Wang XJ, Yang YG. 2014. FTO-dependent demethylation of *N^6*-methyladenosine regulates mRNA splicing and is required for adipogenesis. *Cell Research* **24**:1403-1419 DOI 10.1038/cr.2014.151.

Zhong S, Li H, Bodi Z, Button J, Vespa L, Herzog M, Fray RG. 2008. MTA is an *Arabidopsis* messenger RNA adenosine methylase and interacts with a homolog of a sex-specific splicing factor. *Plant Cell* **20**:1278-1288 DOI 10.1105/tpc.108.058883.

Figure 1

Conservative domain analysis of BvALKB proteins.

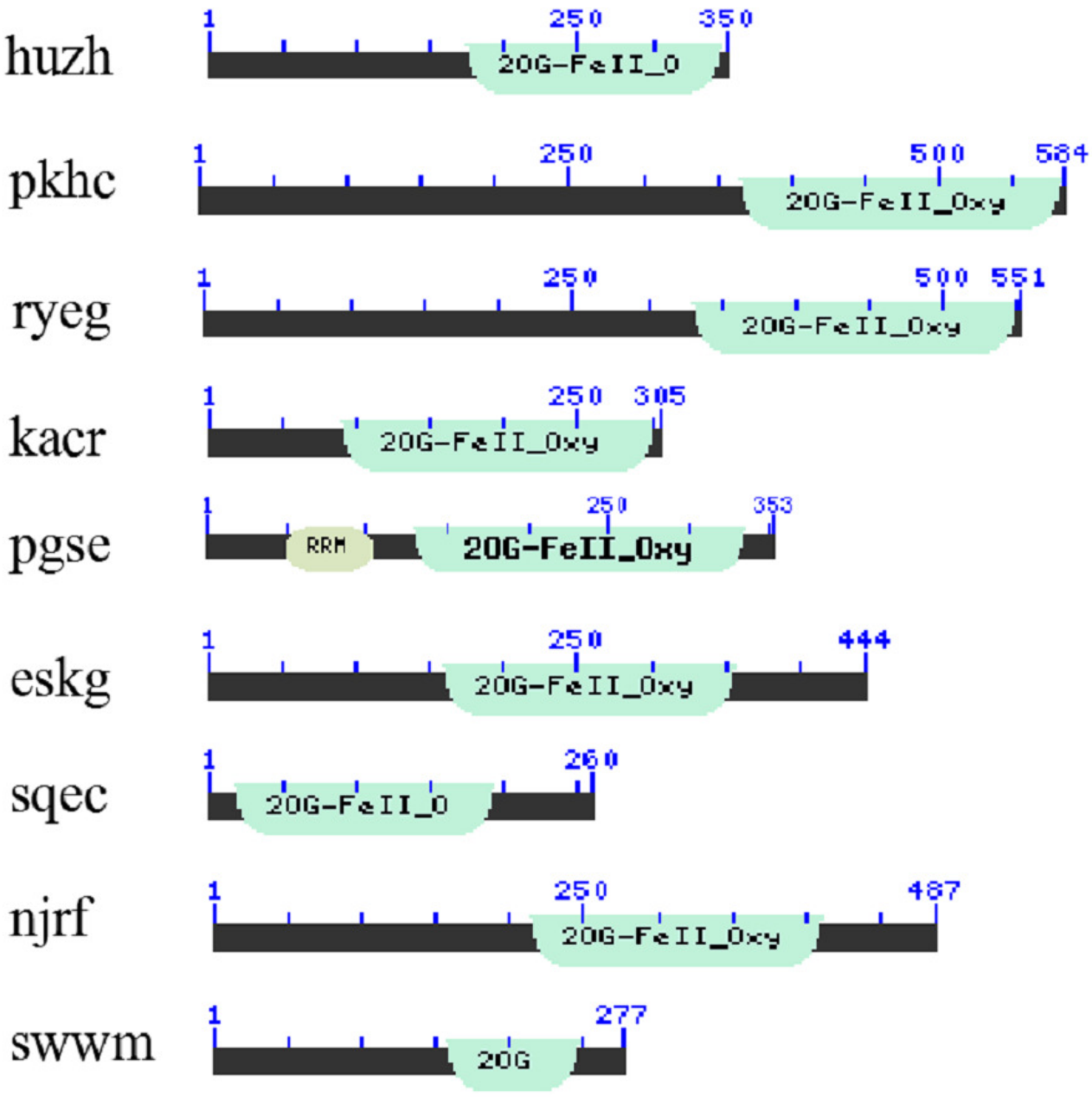

Figure 2

Conserved domain of BvALKB proteins.

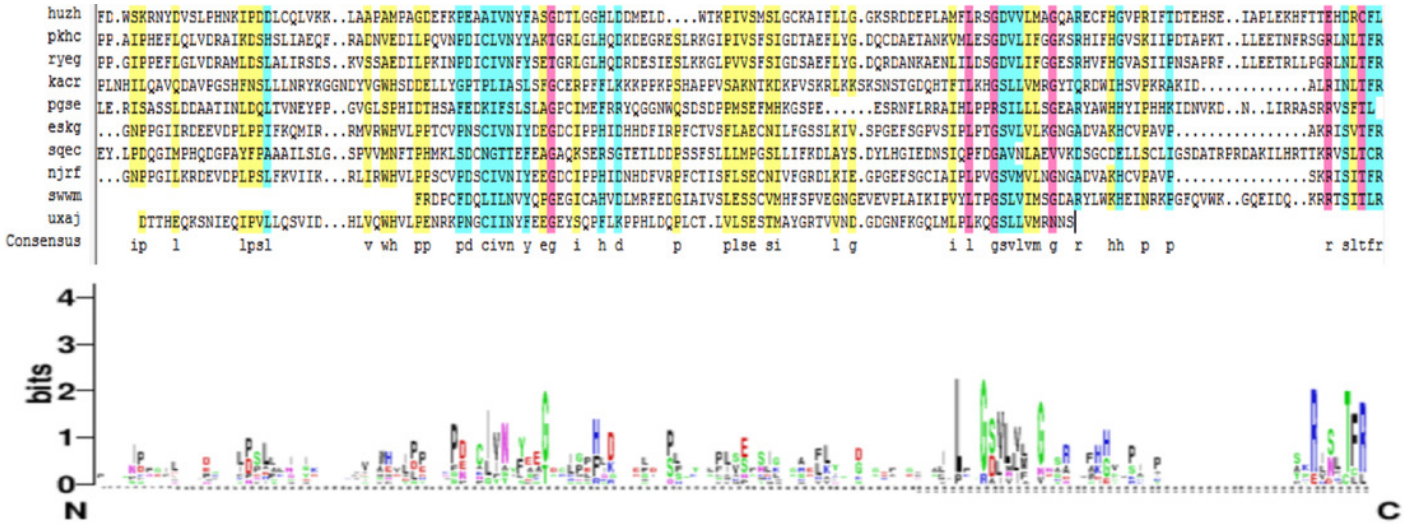

Figure 3

Chromosomal localization of BvALKB genes.

The unit of gene position is Mb.

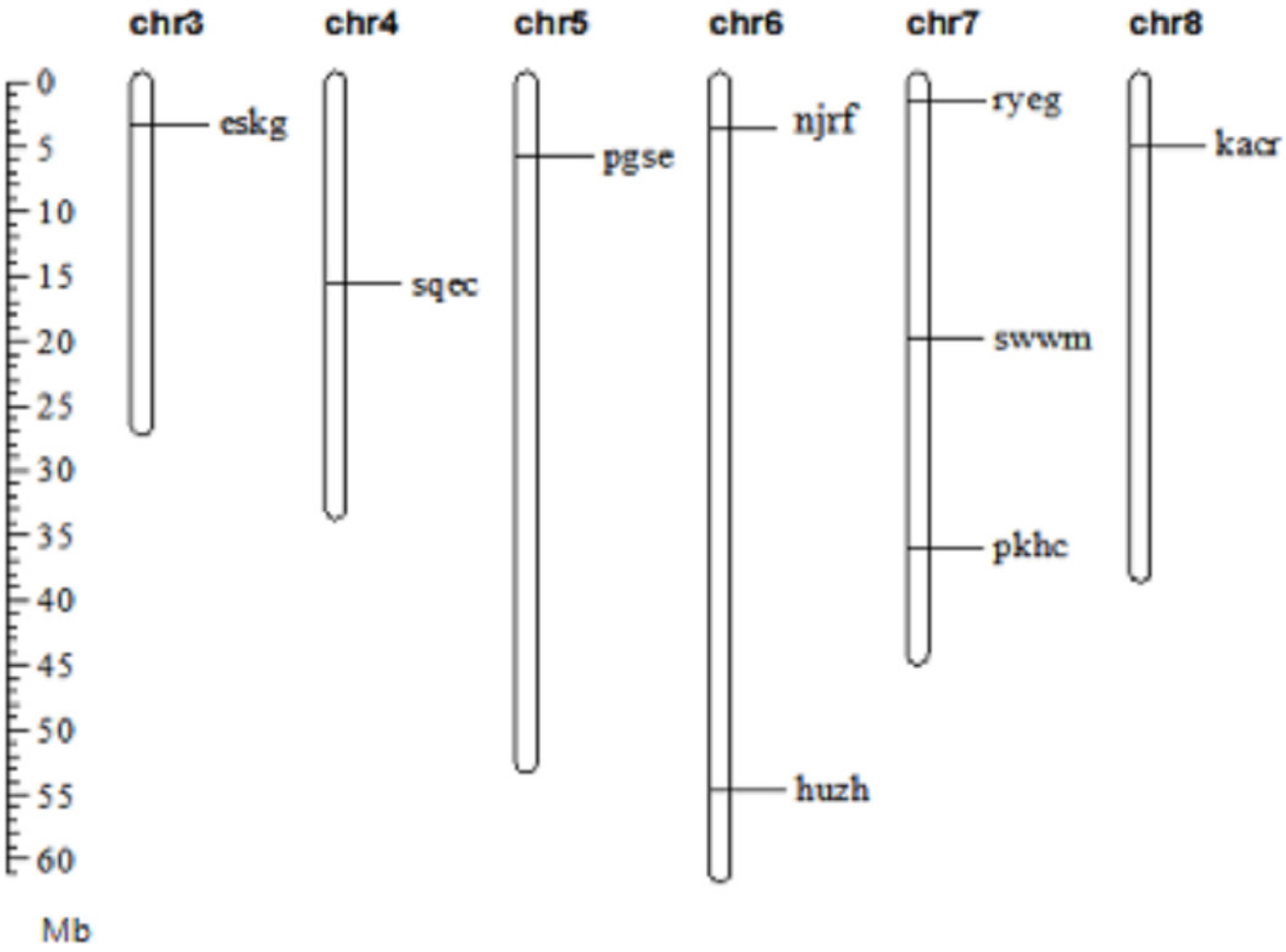

Figure 4

Multiple sequemce alignment between BvALKB and AtALKB proteins.

Different colors represent residues with different characteristics.

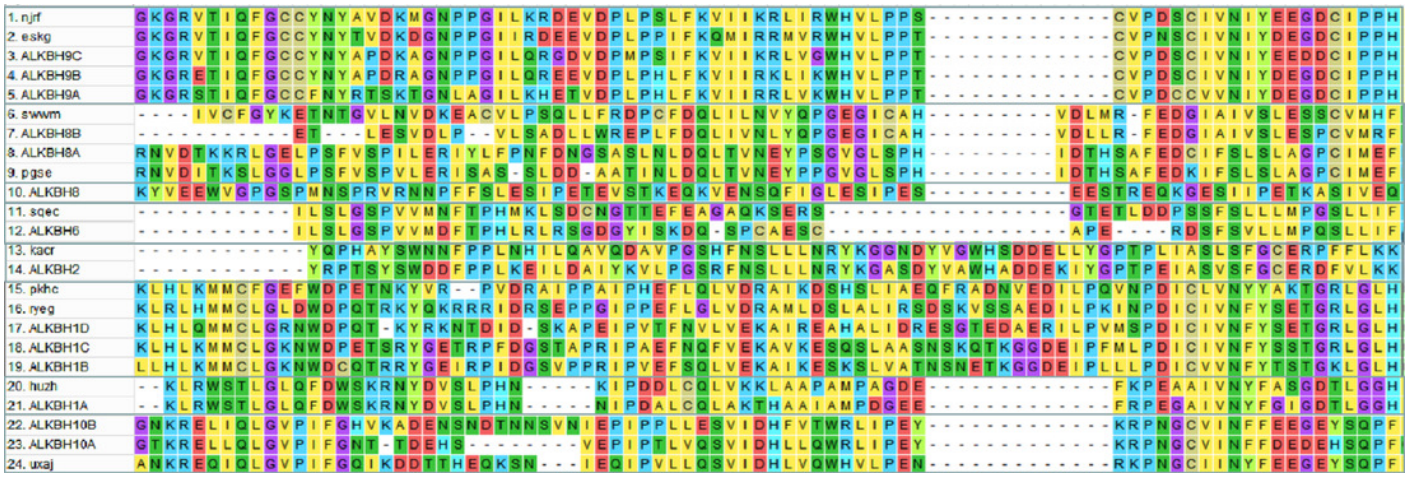

Figure 5

Phylogenetic relationships of BvALKB and AtALKB proteins.

The number on the node represents the confidence value of the branch.The gene class is represented in a different color on the right side of the rootless tree.

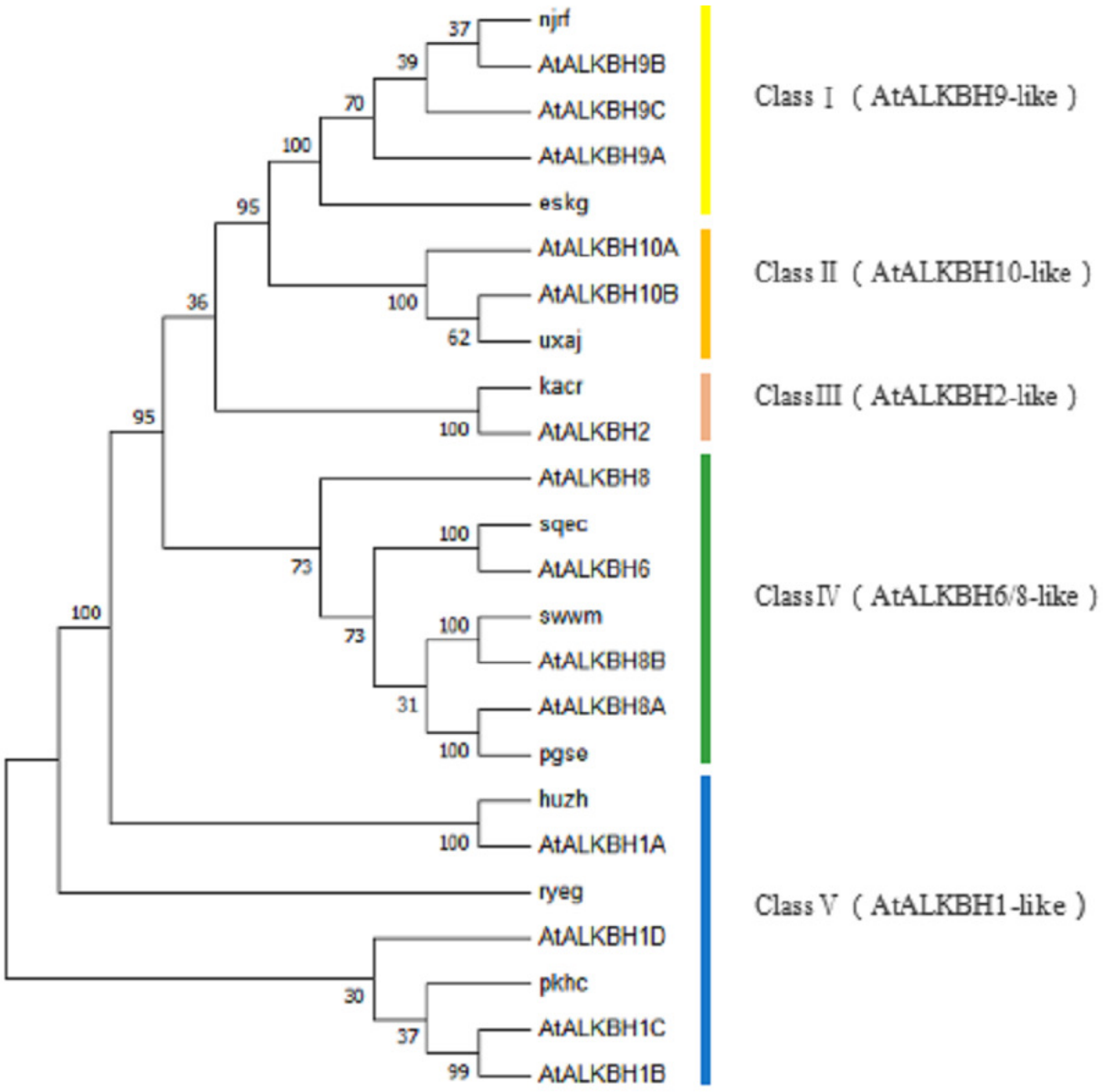

Figure 6

Gene structures of BvALKB genes.

Exon/intron structures of the BvALKB genes are represented in diffenrent ways. Exons and introns are represented by yellow box and black lines, respectively.

Figure 7

Motifs in BvALKB proteins.

The motifs were arranged according to the e-value from small to large, the letters in each motif were amino abbreviation. The size of the letter represented the saliency of the amino acid in the motif. The larger the letter, the higher the saliency, which is, the higher the frequency at which the amino acid appears in the same position in the same motif in different sequences.

Discovered Motifs

Figure 8

Analysis of BvALK proteins motif.

The different color blocks correspond to different motifs. The width of the color block is the length of the motif. The height of the color block represents the saliency of the motifs in the sequence. The higher the saliency, the more able to match the predicted motifs.

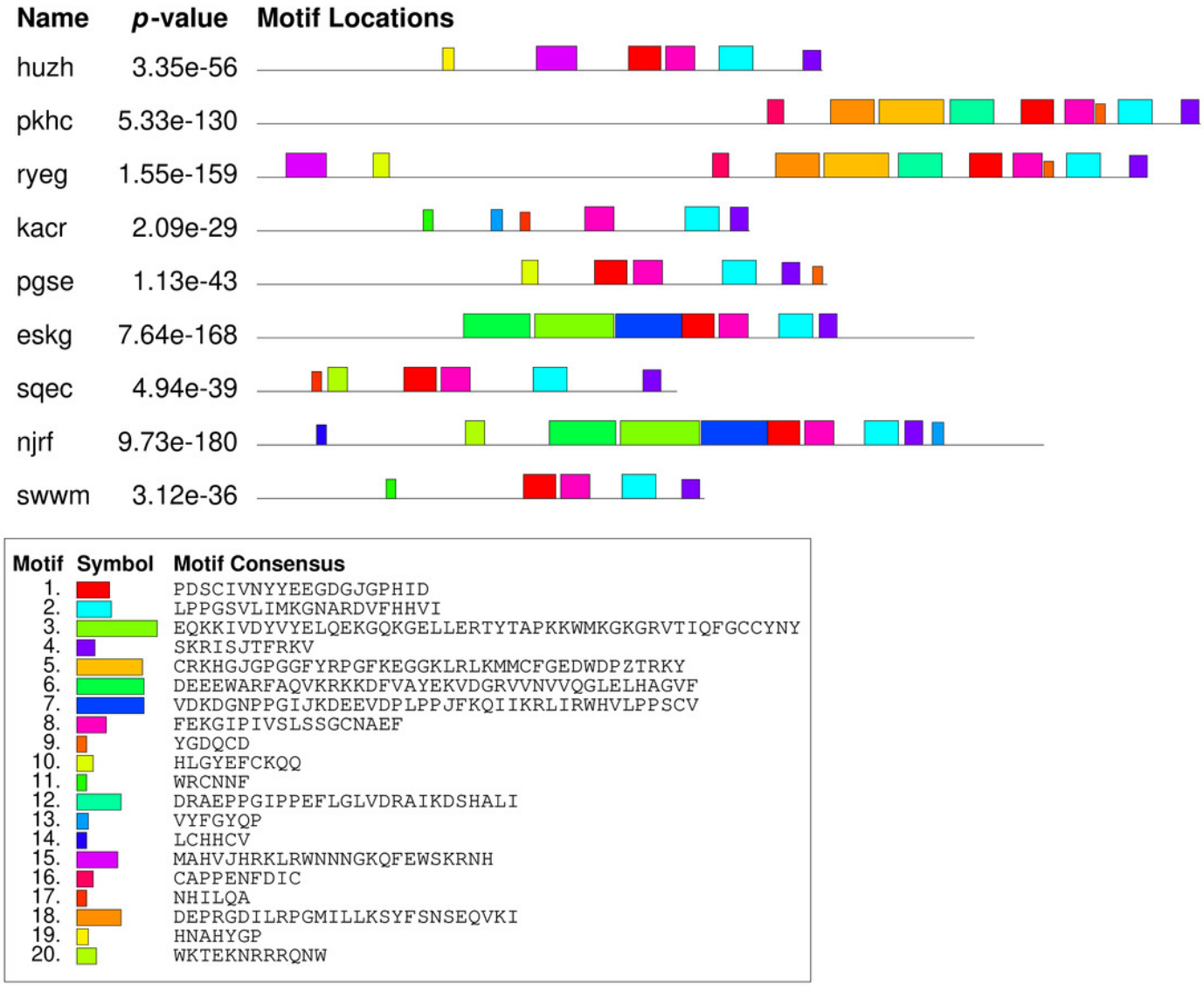

Figure 9

Expression analysis of BvALKB genes in leaf in response to salinity stress.

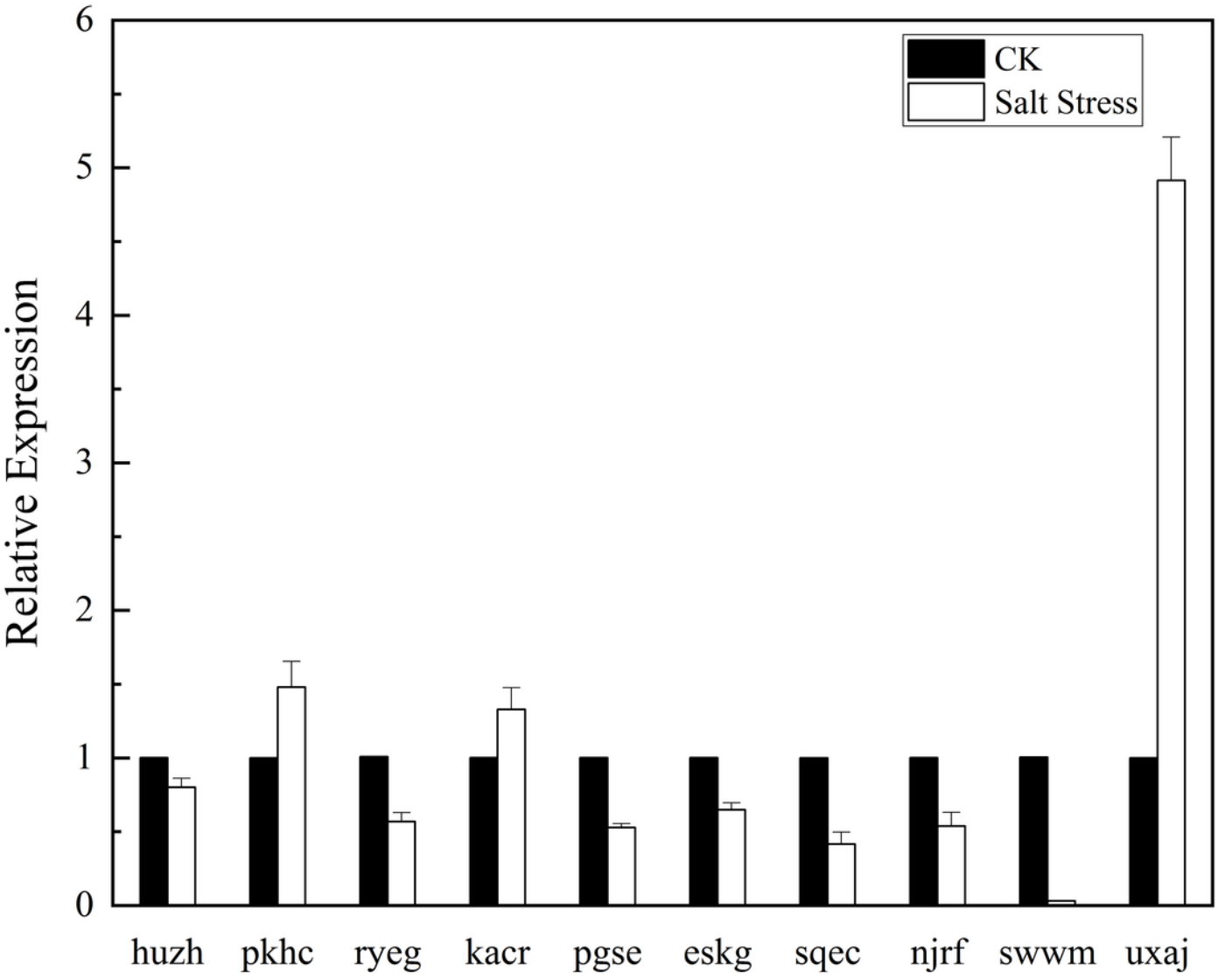

Figure 10

Expression analysis of BvALKB genes in root in response to salinity stress.

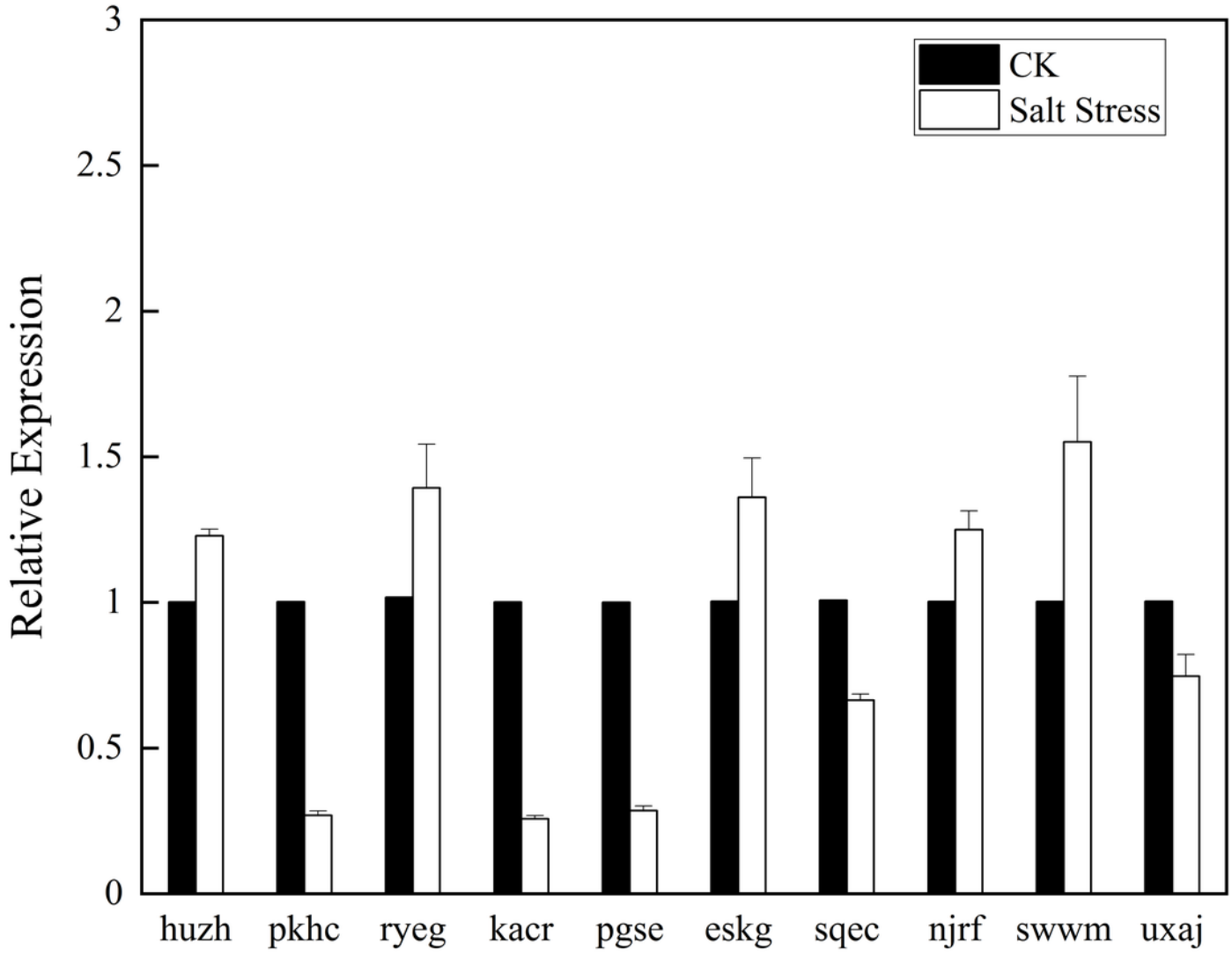

Table 1(on next page)

Primer sequences of BvALKB genes

1 Table 1 **Primer sequences of BvALKB genes**

Gene	Forward primer(5'-3')	Reverse primer(5'-3')
UBQ5	TCTGCTGGAAGAGCCTTTGG	TTGTCGCCGCTCTTTACACT
25S RNA	AGACAAGAAGGGGCAACGAG	CACATTGGACGGGGCTTTTC
BvPP2A	TCGTGTCCAAGAAGTGCCTC	CACAACGGTCATCAGGGTCA
huzh	AGGGAATGCTTTCATGGGGT	CTCGAACCAAGCTATCCGGG
ryeg	GTACTTCCAATAAAACGTCACCGT	GTTTTCAGATGAATCACATGTGCCA
pkhc	TAGCTCGGAACAGGCGAAAA	TGTGGAATTGCCGGTGGTAT
kacr	CATATTCTCCAGGCGGTCCA	GGCGTTCACAACCAAAGGAA
pgse	AGTCCGGAGGAGTCCAGAAA	AGGTCCTGTTCTGACCTTGC
eskg	AAACGGCAGCTTATGGAACG	ATGGGAGGCAAGGGATCAAC
sqec	GGCTTTACAGTCGGCTCTGT	GTCAGCCAAGGAGGCAAGTC
njrf	TTCCCTTGCCTGTTGGATCG	GCAAAATACACAGGCCGCTT
swwm	TACCAGCCAGGTGAGGGTAT	CGAGCATCGCCTGACATGAT
uxaj	GGTGGGAAACAAGGGAGGAG	CCTCATGTGAGCCTGTGTCA

2

Table 2(on next page)

Basic information of BvALKB.

Table 2 **Basic information of BvALKB.**

BvALKB name	NCBI Reference Sequence	Gene ID	Description
Bv6_150770_huzh.t1	XM_010684461.2	104897561	PREDICTED: Beta vulgaris subsp. vulgaris alpha-ketoglutarate-dependent dioxygenase alkB (LOC104897561)
Bv7_157650_ryeg.t1	XM_010686965.2	104899719	PREDICTED: Beta vulgaris subsp. vulgaris hypothetical protein
Bv7_169620_pkhc.t1	XM_010685256.2	104898211	PREDICTED: Beta vulgaris subsp. vulgaris uncharacterized LOC104898211
Bv8_184320_kacr.t1/t2	XM_010688312.2	104900793	PREDICTED: Beta vulgaris subsp. vulgaris DNA oxidative demethylase ALKBH2
Bv5_102160_pgse.t1	XM_010678383.2	104892444	PREDICTED: Beta vulgaris subsp. vulgaris alkylated DNA repair protein alkB homolog 8
Bv3_051230_eskg.t1	XM_010673069.2	104888178	PREDICTED: Beta vulgaris subsp. vulgaris RNA dementhylase ALKBH5
Bv4_083160_sqec.t1	XM_010676670.2	104891030	PREDICTED: Beta vulgaris subsp. vulgaris alpha-ketoglutarate-dependent dioxygenase alkB homolog 6
Bv6_130050_njrf.t1	XM_010681565.2	104895138	PREDICTED: Beta vulgaris subsp. vulgaris uncharacterized LOC104895138
Bv7_164580_swwm.t1/t2	XM_010686203.2	104899068	PREDICTED: Beta vulgaris subsp. vulgaris alkylated DNA repair protein alkB homolog 8
Bv7_179400_uxaj.t1	XM_010698038.2	104908870	PREDICTED: Beta vulgaris subsp. vulgaris hypothetical protein

Table 3(on next page)

Physical and chemical properties analysis of BvALKB proteins

Table 3 **Physical and chemical properties analysis of BvALKB proteins**

BvALKB name	ORF(bp)	Amino acid	Molecular weight(Da)	PI
huzh	1053	350	39477.03	7.13
ryeg	1755	584	64923.52	7.15
pkhc	1656	551	60969.22	8.74
kacr	1018	305	34594.96	9.02
pgse	1062	353	39620.72	6.53
eskg	1335	444	49776.81	8.86
sqec	783	260	28912.06	5.70
njrf	1464	487	54949.39	6.62
swwm	834	277	30792.26	5.11
uxaj	1641	546	60084.61	6.30