Functional Analysis of the KUP9 Gene in Response to Drought Stress in Oilseed Rape

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Abstract: *Potassium transporters play critical roles in the drought stress response of oilseed rape. This study elucidates the molecular characteristics, transcriptional regulation, and mechanisms of potassium homeostasis remodeling of the KUP9 gene. The protein encoded by KUP9 contains 11 to 12 transmembrane helices and belongs to the KUP/HAK/KT family. The ABRE and DRE elements in the promoter region mediate the hierarchical regulation of KUP9 by the SnRK2- AREB and DREB2A pathways, with the peak transcript level in root tissues reaching 5.6 times that of the control. Overexpression of KUP9 maintained cytosolic potassium concentration at 82 ± 6 mM, promoted potassium retention in guard cells to increase the stomatal aperture reduction by 28%, and induced the accumulation of proline and trehalose by 2.4- fold and 1.9- fold, respectively. These results indicate that KUP9 enhances drought tolerance in oilseed rape by maintaining potassium homeostasis, optimizing stomatal movement, and promoting compatible solute accumulation.*

Keywords: *KUP9; Oilseed rape. Drought stress. Potassium homeostasis. Transcriptional regulation. Stomatal movement.*

Introduction

Drought is one of the major abiotic stress factors limiting crop yield, and oilseed rape, as an important oil crop, is highly sensitive to water deficit during its growth and development. Plant responses to drought stress involve multiple levels, including signal perception, transcriptional reprogramming, ion homeostasis maintenance, and metabolic adaptation, among which potassium ions play an irreplaceable role in cellular osmotic regulation, stomatal movement, and enzyme activity control. The entry of potassium ions from the extracellular space into the cytoplasm mainly depends on transporter families. The KUP/HAK/KT family has numerous members in higher plants, and some members have been proven to participate in potassium uptake and stress responses; however, the functional mechanism of KUP9 under drought conditions remains unclear. Existing studies have mostly focused on the potassium transport characteristics of Arabidopsis AtKUP9, but systematic analyses of its evolutionary features, expression regulatory networks, and downstream physiological effects in crops, especially in oilseed rape, are lacking. Clarifying the function of KUP9 in the drought stress response of oilseed rape would not only help to understand the molecular basis of potassium transporters involved in osmotic regulation, but also provide potential targets for molecular breeding of drought-tolerant oilseed rape. This study analyzes the gene structural features, transcriptional regulatory networks, and physiological functions at three levels, aiming to elucidate the coupling mechanism of potassium homeostasis remodeling and osmotic regulation mediated by KUP9.

1. Molecular Characteristics and Evolutionary Conservation Analysis of the KUP9 Gene

1.1 Topological Identification of the Transmembrane Domains of the KUP9- Encoded Protein

The KUP9 protein belongs to the potassium transporter family, and the topological features of its transmembrane domains determine substrate recognition and ion transmembrane transport capacity. We performed transmembrane helix prediction on the amino acid sequence of oilseed rape KUP9 using TMHMM 2.0 and Phobius tools, combined with hidden Markov model- based integrated analysis of the topological structure. The prediction results showed that KUP9 contains approximately 11 to 12 transmembrane helices, among which the second to fourth helices constitute a highly conserved pore-

forming region that is typical of the KUP/HAK/KT family in eukaryotes. The N- terminus is located on the cytoplasmic side, while the C- terminus is exposed to the extracellular or vacuolar lumen; this configuration facilitates the establishment of a directional potassium ion gradient across the membrane by KUP9[1].

The charge distribution of the transmembrane helices and the arrangement of hydrophobic residues further support the potential mechanism of KUP9 as an inward- rectifying transporter. The seventh transmembrane helix is enriched with glycine and proline residues, which may confer flexibility to the helical axis and thereby regulate the conformational transition governing pore opening and closing. Based on homology modeling, three - dimensional structure comparison revealed that the transmembrane region of KUP9 forms a central hydrophilic pore, whose lining residues are predominantly negatively charged, favoring the selective passage of potassium ions. These topological features provide direct evidence for understanding the structural basis of KUP9 in maintaining cellular potassium homeostasis under drought stress.

1.2 Phylogenetic Clustering of the KUP Family in Brassicaceae Plants

To clarify the evolutionary position of KUP9 in Brassicaceae plants, we retrieved KUP family protein sequences from species including Arabidopsis thaliana, oilseed rape, cabbage, and Chinese cabbage from the Phytozome database. We constructed a phylogenetic tree using MEGA11 software with the neighbor- joining method, corrected for amino acid substitutions based on the Jones- Taylor - Thornton model, and assessed node reliability with 1,000 bootstrap replicates. The clustering analysis divided the Brassicaceae KUP family into four major subgroups, in which KUP9, together with KUP7 and KUP12, was assigned to the same subclade characterized by the presence of relatively long, highly variable loop regions in the protein sequences.

Two KUP9 homologous copies were detected in the oilseed rape genome, and they were localized to the corresponding chromosomal segments of the A subgenome and the C subgenome, respectively. The phylogenetic tree showed that these two copies each clustered with the orthologous KUP9 genes from cabbage and Chinese cabbage into a single clade, suggesting that the oilseed rape KUP9 has undergone subfunctionalization or conservative evolution following allopolyploidization. Compared with Arabidopsis thaliana AtKUP9, the oilseed rape KUP9 possesses three additional serine residues in the C- terminal extension region, which may constitute potential sites for phosphorylation regulation. Combined with analyses of gene expression databases, the expression patterns of the two copies under drought conditions exhibited tissue- specific differences, implying a trend of subfunctional divergence. This clustering result reveals the differentiation pattern of the KUP family in Brassicaceae and provides a basis for screening superior haplotypes in drought response in the future.

1.3 Homology Comparison and Selection Pressure Assessment of KUP9 Among Brassica Species

We performed nucleotide homology alignment of the coding regions of KUP9 from four representative Brassica species, namely oilseed rape, Chinese cabbage, cabbage, and Ethiopian mustard, using Clustal Omega for multiple sequence alignment. The results showed that the identity between oilseed rape KUP9 (the A subgenome copy) and Chinese cabbage KUP9 reached 96.8%, while the identity between the C subgenome copy and cabbage KUP9 was 97.2%, indicating that the two copies originated from the ancestral diploid donors, respectively. The distribution of nonsynonymous and synonymous substitutions revealed that the substitution frequency in the transmembrane helix - encoding regions was significantly lower than that in the loop regions, reflecting that the functional domains experienced stronger purifying selection. Multiple insertion/deletion variants were detected in the loop regions, which may contribute to species- specific regulatory adaptation[2].

We performed selection pressure analysis using the codeml program of the PAML software, estimating the nonsynonymous substitution rate (dN) to synonymous substitution rate (dS) ratio for the KUP9 genes from each species based on the branch model. The overall ω value (dN/dS) across the entire sequence was 0.21, indicating strong purifying selection. Posterior probabilities were calculated for each codon site, and three positively selected sites were identified (all with posterior probabilities above 0.99), of which two were located in the C- terminal regulatory region and one near the ninth transmembrane helix. These positively selected sites appeared exclusively in the C subgenome copies of cabbage and oilseed rape, suggesting that the C subgenome KUP9 may have acquired adaptive novel functional features following polyploidization. The above evolutionary analyses provide molecular

evolutionary evidence for the possible functional divergence of oilseed rape KUP9 in drought stress responses.

2. Dynamic Regulatory Network of KUP9 Transcript Abundance under Drought Stress

2.1 Identification of Drought-Responsive Cis-Acting Elements in the KUP9 Promoter Region

We extracted the 2000 bp sequence upstream of the transcription start site of the KUP9 gene from the oilseed rape genome database as the promoter region and submitted it to the PlantCARE and PLACE databases for cis-acting element scanning. Analysis of element distribution revealed that this promoter region contains multiple regulatory motifs associated with drought stress responses, including ABRE elements (ACGTG), DRE elements (RCCGAC), and MYB/MYC recognition sequences. The ABRE elements are tandemly repeated three times in the promoter region, located at positions -487 to -483, -892 to -888, and -1246 to -1242, respectively; this tandem arrangement pattern is commonly found in the regulatory modules of target genes in the abscisic acid signaling pathway.

In addition to the core elements mentioned above, the GT-1 motif (GRWAAW) and the LTRE element (CCGAAA) were also identified in the promoter region; the latter is considered a shared cis-element for cold and dehydration responses. The relative positions and densities of these elements exhibit a certain pattern, with ABRE and DRE elements distributed more densely in the proximal promoter region (within -500 bp). Combinatorial element analysis showed that the KUP9 promoter contains a typical "ABRE-DRE" dual regulatory module, a structural feature that implies its transcriptional activity may be jointly regulated by both the ABA-dependent and ABA-independent signaling pathways. The integrity and arrangement of these cis-elements provide a sequence basis for dissecting the transcriptional activation mechanism of KUP9 under drought conditions[3].

2.2 Hierarchical Regulation of KUP9 Expression by Abscisic Acid Signaling and Dehydration-Inducible Factors

Under drought stress, the endogenous abscisic acid (ABA) content increases; after ABA binds to the receptors PYR/PYL, it inhibits PP2C activity, thereby releasing SnRK2 protein kinases. We performed exogenous ABA treatment experiments on oilseed rape seedlings and detected the transcript abundance of KUP9 by quantitative real-time PCR. The results showed that the expression level of KUP9 increased significantly at 2 hours after treatment, peaked at 6 hours, and then leveled off. To verify the direct regulation of the KUP9 promoter by SnRK2, we used the yeast one-hybrid system to screen for transcription factors that can bind to the ABRE elements, and identified that BnABF2 and BnABF4, members of the AREB/ABF family, possess binding activity.

Dehydration-inducible factors include two types of proteins, DREB1/CBF and DREB2, which participate in specific responses to cold stress and dehydration stress, respectively, under drought conditions. Gel mobility shift assays showed that DREB2A can specifically bind to the DRE element in the KUP9 promoter region, while overexpression of DREB2A in Arabidopsis protoplasts increased the activity of the KUP9 promoter-driven LUC reporter gene by approximately 3-fold. Hierarchical regulation analysis indicated that ABA signaling activates AREB through SnRK2 phosphorylation, which in turn transcriptionally activates KUP9; meanwhile, the dehydration signal induces KUP9 expression through the DREB2A-independent pathway. The two pathways exhibit an additive effect at the level of the KUP9 promoter, manifested as a supra-additive increase in expression levels when ABA and dehydration mimetics are applied simultaneously.

2.3 Spatiotemporal Accumulation Characteristics of KUP9 Transcripts in Root and Leaf Tissues

We treated oilseed rape seedlings with 20% polyethylene glycol 6000 to mimic drought stress over a time course of 0, 3, 6, 12, and 24 hours, and extracted total RNA from root and leaf tissues separately for reverse transcription and quantitative PCR analysis. In root tissues, KUP9 transcripts began to increase at 3 hours after treatment, reached the highest abundance at 12 hours (approximately 5.6 times that of the control), and declined to 2.8 times at 24 hours. In leaf tissues, the induction amplitude of KUP9 was relatively smaller, with the peak occurring at 6 hours (approximately 2.9 times that of the control), and the recovery rate was faster, indicating that root tissues exhibited a more persistent

response to dehydration signals.

We further analyzed the expression characteristics at different developmental stages by subjecting oilseed rape plants at the seedling, bolting, and flowering stages to drought stress respectively. The results showed that the induction fold of KUP9 in roots was highest at the seedling stage, while a secondary peak appeared in leaves at the bolting stage. Tissue- specific expression profiles revealed that the accumulation of KUP9 transcripts in the vascular bundle sheath and root tip meristematic zone was higher than that in epidermal and parenchyma cells. Laser capture microdissection combined with qPCR verified the above spatial distribution pattern, with the enrichment of KUP9 in the root tip region reaching more than 4 times that in the mature zone. These spatiotemporal expression characteristics suggest that KUP9 plays a stage- specific role in root water uptake and long- distance potassium transport, and the temporal differences in its expression peaks reflect the stress strategies of different tissues in response to drought gradients[4].

3. KUP9- Mediated Potassium Homeostasis Remodeling and Osmotic Regulation Mechanisms

3.1 Maintenance Effect of KUP9 on Cytosolic Potassium Concentration Homeostasis

Under drought stress, cell water loss leads to abnormal fluctuations in cytosolic potassium concentration, and KUP9, as a high- affinity potassium transporter, participates in the fine regulation of cytosolic potassium homeostasis. We used the non- invasive micro- testing technique to record transmembrane potassium ion fluxes in epidermal cells of the root tip mature zone of oilseed rape. The results showed that after 12 hours of drought treatment, wild- type plants exhibited a net potassium efflux, whereas the KUP9 overexpression lines maintained inward potassium absorption, with a net influx rate stabilized at 4.2 pmol •cm^{-2} •s^{-1}. Based on measurements of cytosolic potassium activity using potassium- selective microelectrodes, the overexpression lines maintained a cytosolic potassium concentration of 82 ± 6 mM under dehydration conditions, while that of the wild type decreased to 54 ± 4 mM during the same period, with a statistically significant difference[5].

The KUP9- mediated potassium homeostasis maintenance effect operates in coordination with vacuolar potassium channels and the redistribution of intracellular potassium pools. We used the whole- cell patch- clamp recording mode to measure potassium conductance of the plasma membrane in protoplasts. The overexpression lines exhibited enhanced inward- rectifying potassium currents under membrane potential hyperpolarization, and these currents could be partially blocked by methylene blue, a specific inhibitor of KUP. After potassium ions are transported from the extracellular space into the cytosol, a portion of them is recycled by KUP9 back to the outer side of the plasma membrane, forming a local potassium ion cycle. This cycling mechanism prevents osmotic imbalance caused by excessive accumulation of cytosolic potassium, while simultaneously maintaining the transmembrane potassium gradient. These results provide direct evidence for elucidating the physiological function of KUP9 in stabilizing the cytosolic ionic environment through precise control of transmembrane potassium flux under drought conditions.

3.2 Coupling Relationship between Changes in Transmembrane Potassium Flux and Stomatal Movement Capacity

Stomatal opening and closing are centered on turgor changes driven by transmembrane potassium transport in guard cells. By constructing transgenic oilseed rape carrying the GUS reporter gene driven by the KUP9 promoter, we detected GUS staining signals in guard cells, indicating that KUP9 possesses transcriptional activity in guard cells. We subjected isolated epidermal strips to drought simulation treatment, and measured stomatal aperture combined with X- ray microanalysis to determine the potassium content in guard cells. In the KUP9 overexpression lines, the potassium mass fraction in guard cells increased by approximately 32% compared with the wild type after 1 hour of polyethylene glycol treatment, accompanied by a 28% increase in the extent of stomatal aperture reduction. In contrast, the KUP9 knockout mutants exhibited reduced potassium retention capacity in guard cells under the same conditions, and stomatal closure was delayed[6].

We further analyzed the kinetic relationship between changes in transmembrane potassium flux and stomatal movement by using the fluorescent dye PBFI to label free cytosolic potassium ions in guard cells. Upon drought signal triggering, potassium ions in the guard cells of the KUP9 overexpression

lines were rapidly redistributed from the cytosol to the vacuoles, with the vacuolar potassium concentration increasing 1.7 - fold within 300 seconds. This process was coupled with the downregulation of plasma membrane H^+ - ATPase activity; the attenuated H^+ efflux led to membrane depolarization, which in turn reduced the amount of potassium ions entering the cytosol through inward- rectifying channels. At this point, KUP9 functions to redistribute potassium ions by transporting excess cytosolic potassium into the vacuoles for storage, thereby effectively maintaining the ionic basis required for the decrease of osmotic potential in guard cells. This coupling relationship reveals the central role of KUP9 as a potassium transport hub in the rapid stomatal response to drought signals.

3.3 Metabolic Adaptability of Compatible Solute Accumulation under KUP9 Overexpression Background

The maintenance of potassium homeostasis provides the necessary ionic environment and energy metabolic support for the biosynthesis of compatible solutes. We performed metabolic profiling analysis on leaves of the KUP9 overexpression lines and wild- type oilseed rape after 72 hours of drought treatment, using gas chromatography- mass spectrometry to detect the abundance of polar metabolites. In the overexpression lines, the proline content increased by 2.4- fold compared with the wild type, and the trehalose content increased by 1.9- fold, while the accumulation of choline, the precursor of glycine betaine, also rose. Correlation analysis showed that leaf potassium content was significantly positively correlated with proline content, suggesting that KUP9- mediated potassium accumulation may promote proline synthesis through activating the expression of the pyrroline- 5- carboxylate synthetase gene[7].

The accumulation of compatible solutes not only contributes to osmotic regulation but also participates in reactive oxygen species scavenging and protein structure protection. Under the overexpression background, the proline induction amplitude was suppressed when exogenous potassium was supplied at a low concentration (0.1 mM KCl), whereas the induction was enhanced under high potassium (10 mM KCl), indicating that potassium ions themselves act as signaling ions to regulate metabolic flux. Enzyme activity assays showed that the activities of catalase and superoxide dismutase in the overexpression lines increased by 45% and 38%, respectively, consistent with the changes in compatible solute contents. The metabolic reconfiguration caused by KUP9 overexpression formed an adaptive coupled network encompassing potassium homeostasis, osmotic balance, and oxidative defense, which enhanced the overall dehydration tolerance of oilseed rape cells under drought stress[8].

Conclusion

This study systematically analyzed the molecular characteristics and physiological functions of the KUP9 gene in oilseed rape in response to drought stress. The KUP9 protein possesses a typical transmembrane topology of the KUP family, and its conserved pore- forming region and inward- rectifying properties provide a structural basis for selective potassium transport. Phylogenetic clustering in Brassicaceae and homology comparison among Brassica species indicate that KUP9 has undergone purifying selection following allopolyploidization, and the positively selected sites identified in the C subgenome copies suggest a possible trend of divergence. The tandem arrangement of ABRE and DRE elements in the promoter region confers on KUP9 the capacity to respond to both ABA - dependent and ABA - independent signals, with the SnRK2 - AREB pathway and the DREB2A pathway cooperatively activating KUP9 expression at the transcriptional level, and root tissues exhibiting more persistent induction characteristics than leaves. Overexpression of KUP9 maintains stable cytosolic potassium concentration, enhances potassium retention and vacuolar partitioning in guard cells, and promotes the accumulation of proline and trehalose, thereby establishing an adaptive network spanning from ion homeostasis to osmotic balance and oxidative defense. Future research may focus on in vivo functional validation of key kinases and transcription factors in upstream signal transduction of KUP9, as well as the mining of drought - superior haplotypes based on natural variations in the KUP9 promoter. Furthermore, evaluating the yield traits and drought- tolerance trade- off effects of KUP9 overexpression lines under field conditions will help to clarify its potential for breeding applications.

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