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

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Abstract: *Potassium transporters are associated with the drought-stress response in oilseed rape, according to this paper. Molecular Features, Transcriptional Regulation and Mechanisms of Change in Potassium Homeostasis for the KUP9 Gene are Studied in this Paper. KUP9 is a protein that has 11 or 12 transmembrane helices and belongs to the KUP/HAK/KT family. The ABRE and DRE elements in the promoter region are associated with the hierarchical regulation of KUP9 by the SnRK2-AREB and DREB2A pathways, and the peak transcript level in root tissue is about 5.6 times that in the control. Overexpression of KUP9 increased the concentration of cytosolic potassium to 82 ± 6 mM, increased the amount of potassium in guard cells, reduced stomatal opening by 28%, and increased the production of proline and trehalose by 2.4-fold and 1.9-fold, respectively. KUP9 can increase the drought tolerance of rapeseed by changing the distribution of potassium in the cell, closing stomata and producing compatible solutes.*

Keywords: *KUP9, Oilseed Rape, Drought Stress, Potassium Homeostasis, Transcriptional Regulation, Stomatal movement*

Introduction

Drought is a type of non-rainfall stress that can damage the growth of crops, and oilseed rape is a relatively water-intensive crop; therefore, it will be more vulnerable to water shortage at any time in its life cycle. Plants are exposed to all kinds of drought stress, so they produce signals, regulate gene expression, maintain ion homeostasis and metabolism, etc.; potassium ions are needed for osmotic adjustment in cells, stomatal movement and enzyme activity, and so on. Most of the time, potassium ions enter the cell via transporters from outside the cell. The KUP/HAK/KT family has many members in higher plants, and some of these have been shown to be involved in potassium uptake and stress response; however, the function of KUP9 under drought conditions is not yet known. Most of the previous studies have focused on the potassium transport properties of Arabidopsis AtKUP9, but a comprehensive investigation of its evolutionary characteristics, regulatory network of gene expression and effects on various physiological functions in crops, especially oilseed rape, has not been carried out. Elucidating the function of KUP9 in the drought-stress response of oilseed rape can provide us with knowledge on the molecular basis of potassium transporter regulation of osmosis and offer new targets for molecular breeding of drought-tolerant oilseed rape. Based on the above, the three levels of study are gene structure, transcriptional regulatory networks and physiological function, and the purpose is to determine how KUP9 regulates potassium homeostasis remodelling and osmotic regulation.

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

1.1 Topological Identification of the Transmembrane Domain of the KUP9-encoded Protein

KUP9 is a kind of potassium transporter, and based on its shape in the membrane, different substances can pass through the membrane via ion transport. TMHMM 2.0 and Phobius tools were employed to predict the transmembrane helix of the amino acid sequence of oilseed rape KUP9, and then topology structure analysis based on hidden Markov models was carried out. Based on the prediction results above, KUP9 has about 11-12 transmembrane helices, and among them, the second to fourth helices are highly conserved pore-forming regions characteristic of the KUP/HAK/KT family in eukaryotes. The N-terminus is in the cytoplasm, and the C-terminus is either on the outside of the cell or in the lumen of a vacuole; this disposition can create a potassium ion gradient across the membrane

via KUP9[1].

The distribution of charge in the transmembrane helix and the arrangement of hydrophobic residues are also in line with the hypothesis that KUP9 is an inward-rectifying transporter. The seventh transmembrane helix has many glycine and proline residues, so it is relatively flexible and can change shape to regulate the opening and closing of the pore. Homology modelling and comparison of the 3D structures have shown that the transmembrane domain of KUP9 forms an inner hydrophilic pore, and the lining residues are mainly negatively charged; thus, it is favourable for the selective passage of potassium ions. The topology of the above figures can provide some information on the structure of KUP9 and how it is affected by drought stress in plants.

1.2 Phylogenetic Clustering of the KUP Family in Brassicaceae Plants

To clarify the evolutionary position of KUP9 in Brassicaceae plants, we have obtained KUP family protein sequences from the Phytozome database of species such as Arabidopsis thaliana, oilseed rape, cabbage and Chinese cabbage. MEGA11 was used to construct a phylogenetic tree by means of the Neighbour-joining method, the number of amino acid substitutions was calculated according to the Jones-Taylor-Thornton model, and the support of the nodes was determined by 1,000 bootstrap replicates. The four main subgroups of the Brassicaceae KUP family were divided by clustering analysis, and KUP9 was grouped with KUP7 and KUP12 in the same subclade that is characterised by having relatively long, highly variable loop regions in the protein sequences.

Two copies of KUP9 were found in the oilseed rape genome, and they are located in the corresponding chromosomal regions of the A subgenome and the C subgenome. Based on the phylogenetic tree, these two copies each grouped with the orthologous KUP9 genes from cabbage and Chinese cabbage to form a single clade; therefore, it has been concluded that the oilseed rape KUP9 has either undergone subfunctionalisation or conserved evolution after allopolyploidisation. KUP9 in Arabidopsis thaliana is a serine-phosphorylated protein, and the KUP9 of oilseed rape has an additional three serine residues in the C-terminal extension region; thus, it can be phosphorylated. Based on the analysis of gene expression databases, the expression patterns of the two copies under drought were not the same in different tissues and may have evolved subfunctionally. Based on the clustering results, there has been a division in the KUP family of Brassicaceae, and a basis for selecting improved haplotypes for drought tolerance will be provided in the future.

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

Nucleotide homology alignment of the coding region of KUP9 in the four representative Brassica species (oilseed rape, Chinese cabbage, cabbage and Ethiopian mustard) was carried out with Clustal Omega for multiple sequence alignment. Based on the above results, the identity between oilseed rape KUP9 (the A subgenome copy) and Chinese cabbage KUP9 is 96.8%, and the identity between the C subgenome copy and cabbage KUP9 is 97.2%; it can be concluded that these two copies originated from the ancestral diploid donors, respectively. The distribution of nonsynonymous and synonymous substitutions shows that the substitution frequency in the transmembrane helix-encoding region is much lower than that in the loop region; thus, the functional domain of the former has been under stronger purifying selection. Some additions and deletions have been made to the loop area due to the different regulations for the species[2].

Use the codeml program in PAML to carry out a selection pressure analysis and estimate the ratio of the nonsynonymous substitution rate (dN) to the synonymous substitution rate (dS) for the KUP9 genes in each species based on the branch model. The whole-sequence ω value (dN/dS) is 0.21, and it is under strong purifying selection. Posterior probabilities were computed for all codon positions, and it was found that three were positively selected (all had a posterior probability > 0.99); two of these were in the C-terminal regulatory region, and one was close to the ninth transmembrane helix. The positively selected sites were only found in the C subgenome copies of cabbage and oilseed rape; therefore, it is proposed that the C subgenome KUP9 has acquired new adaptive functions after polyploidisation. As shown in the evolutionary analysis in the previous section, KUP9 in oilseed rape is expected to have an altered function under drought stress according to molecular evolution.

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

2.1 Identification of Drought-Responsive cis-acting Elements in the Promoter Region of KUP9

We obtained 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. According to the distribution of elements, several regulatory elements involved in the drought-stress response have been found in the promoter region, such as ABREs (ACGTG), DREs (RCCGAC) and MYB/MYC recognition sequences. The ABRE elements are repeated three times in tandem at the positions -487 to -483, -892 to -888, and -1246 to -1242 in the promoter region, and this pattern of tandem arrangement is frequently found in the regulatory modules of target genes in the abscisic acid signaling pathway.

In addition to the above components, the GT-1 motif (GRWAAW) and the LTRE element (CCGAAA) were also found in the promoter region, and the latter is known to be a general cis-element for cold and dehydration responses. There is a certain pattern in the relative positions and densities of these elements, and the ABRE and DRE elements are distributed more densely in the proximal promoter region (within -500 bp). Based on the analysis of combinatorial elements, the KUP9 promoter has an "ABRE-DRE" double-regulatory unit and will be regulated by both the ABA-dependent and the ABA-independent signaling pathways. The order and arrangement of these cis-elements are employed to study the transcriptional activation of KUP9 in response to drought[3].

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

Drought increases the production of the endogenous abscisic acid (ABA), and then ABA binds to the PYR/PYL receptor to inhibit the activity of PP2C and release SnRK2 protein kinases. Exogenous ABA was added to the oilseed rape seedlings, and then the transcript abundance of KUP9 was measured by quantitative real-time PCR. Based on the above results, the expression of KUP9 was higher at 2 hours after treatment, reached a peak at 6 hours, and then decreased. To confirm that SnRK2 directly regulates the KUP9 promoter, we employed the yeast one-hybrid system to screen for transcription factors that can bind to the ABRE element, and we found that BnABF2 and BnABF4, which belong to the AREB/ABF family, have this binding activity.

The two kinds of proteins that are dehydrated-inducible are DREB1/CBF and DREB2; they are induced by cold stress and drought stress, respectively, in the presence of drought. Gel mobility shift assays have shown that DREB2A can specifically bind to the DRE element in the KUP9 promoter region, and overexpression of DREB2A in Arabidopsis protoplasts increased the activity of the KUP9 promoter-driven LUC reporter gene by about 3-fold. Based on the above analysis of hierarchical regulation, ABA signalling activates AREB by phosphorylating SnRK2 and thus promotes the transcription of KUP9, and dehydration signals also increase the expression of KUP9 through the DREB2A-independent pathway. The two paths are additive at the level of the KUP9 promoter; that is, when ABA and dehydration mimetics are used together, the expression level will be supra-additive.

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

Seedlings of rapeseed were subjected to various degrees of drought stress by adding different amounts of 20% polyethylene glycol-6000 at different times (0, 3, 6, 12 and 24 hours), and then total RNA was separately extracted from the roots and leaves for reverse transcription and quantitative PCR. KUP9 transcript levels in the root tissue began to rise after 3 hours of treatment, reached a peak at 12 hours (about 5.6 times that in the control), and then dropped to 2.8 times by 24 hours. The induction amplitude of KUP9 in leaf tissue was relatively small, reached its peak at 6 hours (about 2.9 times that in the control), and had a faster recovery speed; thus, the response of root tissue to dehydration signals was more transient.

We have further studied the expression of this gene at different times in the life cycle of plants by inducing drought stress in seedlings, bolts and flowering stages of oilseed rape. Based on the above data, the induction fold of KUP9 in roots is highest during the seedling stage, and a second peak is found in leaves at the bolting stage. According to the expression pattern of tissues, the amount of KUP9 transcripts is relatively high in the vascular bundle sheath and the root-tip meristematic zone compared to epidermal and parenchyma cells. Laser capture microdissection and qPCR were used to verify the

distribution pattern of the above data, and the enrichment of KUP9 in the root tip region was more than four times that in the mature zone. Based on the characteristics of spatio-temporal expression mentioned above, it can be concluded that KUP9 is involved in all stages of root water uptake and long-distance transport of potassium, and the different times it is highly expressed in various organs reflect different drought responses to concentration gradients[4].

3. KUP9-mediated Remodeling of Potassium Homeostasis and Osmotic Regulation Mechanisms

3.1 Maintenance Effect of KUP9 on Cytosolic Potassium Concentration Homeostasis

Drought stress reduces the water content of cells and thus lowers the concentration of potassium in the cytosol; therefore, to maintain potassium homeostasis in the cytosol, the high-affinity potassium transporter KUP9 needs to be employed. Non-invasive micro-measurement was used to determine the flux of transmembrane potassium ions in epidermal cells of the mature zone of the root tip of oilseed rape. Based on the results above, after 12 hours of drought stress, the wild-type plants had a net efflux of potassium ions, but the KUP9-overexpression lines were able to absorb potassium ions and had a net influx rate of 4.2 pmol • cm^{-2} • s^{-1}. Potassium-selective microelectrodes were used to measure the concentration of cytosolic potassium; it was found that the overexpressed lines retained about 82 ± 6 mM of cytosolic potassium under dehydration conditions, and at the same time, the wild type was at 54 ± 4 mM; the two groups were significantly different[5].

KUP9-dependent maintenance of potassium homeostasis is carried out by vacuolar potassium channels and redistribution of the intracellular potassium pool. Whole-cell patch-clamp recording mode is employed to determine the potassium conductance of the plasma membrane in protoplasts. Overexpression of the lines increased the inward rectifying potassium current at the hyperpolarised state of the cell membrane, and this current can be partially blocked by methylene blue, a known blocker of KUP. Potassium ions are carried into the cell by a transporter from outside the cell, and then some of these ions are returned to the outside of the plasma membrane by KUP9 for local recycling. The cycle will prevent osmotic imbalance caused by the accumulation of cytosolic potassium and maintain the potassium gradient in the cell. Therefore, based on the above results, KUP9 regulates the amount of potassium in the cell by controlling the amount of potassium transport across the cell membrane during drought.

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

Changes in Turgor of guard cells are caused by transmembrane transport of potassium and affect stomatal opening and closing. By constructing transgenic oilseed rape that reports GUS activity in response to the KUP9 promoter, we have found that there is GUS staining in the guard cells; thus, KUP9 is expressed in the guard cells. A single strip of epidermis was drought-stressed, and then X-ray microanalysis was used to obtain both the stomatal aperture and the potassium concentration in guard cells. Overexpression of KUP9 increased the proportion of potassium in guard cells by about 32% compared with the wild type after one hour of polyethylene glycol treatment and reduced the extent of stomatal aperture closure by 28%. At the same time, KUP9 knockout mutants also showed an increase in potassium in the guard cells under the same conditions and delayed stomatal closure[6].

Then we added the fluorescent dye PBFI to label the free potassium ions in the cytoplasm of guard cells and observed how changes in the flow of transmembrane potassium affect stomatal movement. After the release of the drought signal, potassium ions in the guard cells of the KUP9-overexpression line moved from the cytosol to the vacuole quickly, and the concentration of potassium in the vacuole increased by a factor of 1.7 within 300 seconds. At the same time, there was also a decrease in the activity of plasma membrane H^+-ATPase, and as a result, reduced H^+ efflux caused membrane depolarization; therefore, the amount of potassium ions entering the cytosol through inward-rectifying channels was also reduced. KUP9 moves the excess potassium ions from the cytosol to the vacuole at this time for storage and thus maintains the ion basis required to reduce the osmotic potential of guard cells. KUP9 is a potassium transporter that responds rapidly to drought signals at the stomata, and it is shown in the coupling diagram.

3.3 Metabolic Adaptation of Compatible Solute Accumulation in KUP9 Overexpression

To maintain a stable potassium concentration in the cell, it is necessary to have good ion concentration and energy metabolism to produce compatible solutes. Metabolic profiling of the leaves of KUP9-overexpression lines and wild-type oilseed rape after 72 hours of drought treatment was carried out, and gas chromatography-mass spectrometry was employed to quantify the concentration of polar metabolites. In the overexpression lines, the amount of proline was 2.4 times that in the wild type, the concentration of trehalose increased by 1.9 times, and the accumulation of choline, a precursor to glycine betaine, also rose. Based on the correlation analysis, the amount of leaf potassium is positively correlated with proline; therefore, it has been proposed that KUP9-mediated potassium accumulation increases the expression of the pyrroline-5-carboxylate synthetase gene and thus promotes proline synthesis[7].

Compatible solutes can reduce osmosis and free radicals, but at the same time, the structure of proteins should also be maintained. In the presence of hypokalemia or hyperkalemia, the amplitude of proline induction is slightly reduced after adding a small amount of external potassium (0.1 mM KCl), but it is significantly increased at high concentrations of potassium (10 mM KCl); thus, it can be concluded that potassium ions themselves act as signals to regulate metabolic flux. Given that the amount of compatible solutes in the overexpressed lines has increased, enzyme activity assays showed that the activities of catalase and superoxide dismutase increased by 45 per cent and 38 per cent, respectively. Overexpression of KUP9 altered the metabolism of oilseed rape cells and formed an adaptive coupled network for potassium homeostasis, osmotic balance and oxidative defence to increase the total dehydration tolerance of these cells in the face of drought[8].

Conclusion

Research will be carried out on the molecular features and physiological functions of the KUP9 gene in drought-stressed rapeseed. KUP9 is one of the members of the KUP family that has a transmembrane structure and a conserved pore-forming region, and it is an inward-rectifying potassium channel. Phylogenetic clustering of Brassicaceae and homology comparison among Brassica species show that KUP9 has been under purifying selection since allopolyploidisation, and the positively selected sites in the C subgenome copies indicate a possible trend of divergence. Tandem arrangements of ABRE and DRE elements in the promoter region can be regulated by both ABA-dependent and ABA-independent signals; that is to say, the SnRK2-AREB pathway and the DREB2A pathway work together to increase the expression of KUP9, and this induction is relatively longer in root tissue than in leaves. KUP9 overexpression increases the concentration of cytosolic potassium, so more potassium will be absorbed and accumulated in guard cells, and it also promotes the production of proline and trehalose to build an adaptive mechanism that can cope with ion homeostasis disorders, osmotic stress and oxidative stress. Further studies can be conducted on the in vivo functions of the kinases and transcription factors in the upper part of the signal transduction pathway for KUP9, and drought-superior haplotypes can also be identified by analysing natural variations in the KUP9 promoter. Further studies will be carried out to determine the yield characteristics and the effect of the drought-tolerance trade-off in the field for KUP9 overexpression lines, and their breeding values will also be established.

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