

CRISPR/Cas9-Based Co-editing of KUP9 and CIPK9 Genes in Rapeseed (*Brassica napus*) to Improve Drought Tolerance and Yield Traits

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Abstract: Drought stress restricts rapeseed yield. KUP9 is involved in potassium ion transport and osmotic regulation whereas CIPK9 mediates abscisic acid signaling and stomatal movement which form an interaction network through potassium homeostasis and signal transduction. A dual-gene tandem expression cassette vector based on CRISPR/Cas9 was designed, optimization of genetic transformation and screening strategies were performed, and we obtained plants co-edited for KUP9 and CIPK9 showing enhanced root potassium uptake, synergistically accumulating proline and soluble sugars, and preferential allocation of photosynthates into seeds; yield components showed a non-antagonistic relationship with drought-tolerance physiological indicators, and its yield loss under moderate drought was decreased by about 30%, and this co-editing strategy decouples the negative correlation between drought tolerance and yield.

Keywords: rapeseed; CRISPR/Cas9; KUP9; CIPK9; potassium homeostasis; stomatal movement; drought tolerance; yield traits

Introduction

Growth period drought reduces photosynthetic efficiency, induces source-sink imbalance and impedes grain filling with serious loss in yield; The drought tolerance increase at the cost of lower harvest index is caused by antagonism between drought-tolerance genes and yield regulating genes through traditional breeding. KUP9 drives reduction of osmotic potential via maintaining cellular potassium concentration and protecting photosynthetic carbon assimilation, and CIPK9 cross regulates stomata aperture via calcium-dependent pathways and abscisic acid signaling. They form a bidirectional regulation loop in ion homeostasis and phosphorylation cascades providing a molecular basis for synergistically enhancing drought tolerance and yield improvement. There are no systematic studies on construction strategy and coupled regulatory mechanism for simultaneously dual gene editing. We designed a KUP9 and CIPK9 co-editing system based on CRISPR/Cas9 technology to dissect the dynamic change in potassium homeostasis, osmolyte accumulation, photosynthate allocation and source-sink relationship after editing, clarify the non-antagonistic association pattern between yield component and drought-tolerance physiological indicators, and offer theoretical support and editing tools for molecular breeding of drought-tolerant and high-yielding rapeseed.

1. Molecular Basis of Synergistic Regulation of Drought Tolerance and Yield Traits by KUP9 and CIPK9 Genes in Rapeseed

1.1 Molecular Function of KUP9-Mediated Potassium Ion Transport and Osmotic Regulation

Rapeseed KUP9 encodes for a high-affinity potassium ion transporter primarily located in the plasma membrane and endomembrane system of root tip and leaf vascular bundle cells that transports and distributes potassium ions across membranes along with the proton motive force whose expression is significantly upregulated under low-potassium condition to maintain cytoplasmic potassium homeostasis. Potassium ions, one of the most important osmotic solute, can directly reduce osmotic potential by accumulating them within cells to facilitate the directionality of water flow from soil into roots. Enhanced KUP9-mediated potassium ion transport under drought stress helps maintain turgor pressure of leaf cells and postpone wilting whilst activating critical metabolic enzymes like pyruvate kinase to maintain basic photosynthetic carbon assimilation efficiency even when stomata are closed.

Additionally, some studies showed regulation of the transport activity of KUP9 by upstream signaling molecules and post-translational modifications. Multiple phosphorylation sites exist at the C-terminus of this protein, which can be phosphorylated by protein kinases thus changing its substrate affinity and subcellular location. Under oxidative stress (caused by drought) the stability and interaction with 14-3-3 proteins of KUP9 will change, affecting the long-distance transport of potassium ions to the shoot; it directly controls the distribution of potassium in reproductive organs, indirectly participating in pollen development and ovule survival, laying an ionic foundation for subsequent grain filling^[1].

1.2 Mechanism by Which CIPK9 Participates in the Absciscic Acid Signaling Pathway and Regulates Stomatal Movement

CIPK9 belongs to the calcineurin B-like interacting protein kinase family showing high expression level in guard cells of rapeseed leaves under drought stress; there will be a temporary increase of cytosolic calcium ion concentration, binding with CBL proteins and activating CIPK9 to phosphorylate and regulate the activity of plasma membrane potassium channels and anion channels. Specifically, it opens the potassium efflux channel on guard cells, coordinating the efflux of both potassium ions and anions, decreasing cell turgor pressure, causing stomata closing. It works independently from the core abscisic acid signaling pathway as a calcium dependent auxiliary closure pathway.

Absciscic acid signaling synergistically enhances the action of CIPK9. When bound with the receptors PYR/PYL, abscisic acid inhibits the activity of PP2C phosphatase releasing the inhibited SnRK2 kinases: SnRK2 and CIPK9 undergo a cross-phosphorylation cascade in guard cells. Experimentally it was demonstrated that CIPK9 is phosphorylated/modified by SnRK2 enhancing their kinase activities and also CIPK9's ability to feedback regulate the localization of the abscisic acid receptor complex. This interaction mechanism ensures stomata maintain a moderately closed status during long-term drought conditions avoiding over-closure hindering photosynthetic carbon assimilation thus achieving a dynamic balance between water retention and carbon fixation.

1.3 Synergistic Effects of the Dual-Gene Interaction Network on Water Use Efficiency and Grain Filling

The KUP9 and CIPK9 do not work in isolation; they form an interaction network between potassium homeostasis and signal transduction. The intracellular potassium concentration controlled by KUP9 affects the calcium signaling sensitivity required for activation of CIPK9 because potassium ions indirectly regulate the opening probability of calcium channels through regulating membrane potential. Conversely, phosphorylation substrates of CIPK9 have some potassium transport regulatory proteins which feedback into the membrane localization and recycling rate of KUP9. This kind of bidirectional regulatory loop enables optimizing both potassium uptake and stomata response at the same time under water deficit without any physiological cost from regulation using only one pathway^[2].

For yield traits, synergistic editing of these two genes decoupled the bottleneck of negative correlation between drought tolerance and yield in traditional breeding: moderate activation of CIPK9-stomata regulation decreased transpirational water consumption while potassium supply ensured by KUP9 protected grain sink activity at the filling stage. The improvement on water use efficiency was manifested as significant increase in dry matter accumulation per unit of transpired water especially from the flowering stage to the silique development stage. Potassium ions contributed to phloem loading of photosynthates from source leaves to grains during grain filling, meanwhile, the osmotic homeostasis controlled by CIPK9 reduced programmed cell death of endosperm cells. Finally, dual gene interaction network resulted in simultaneous maintenance of thousand-grain weight and seed oil content under drought stress providing a molecular basis for CRISPR/Cas9-based synergistic editing.

2. Construction of a CRISPR/Cas9-Based Dual-Gene Targeted Editing Strategy for KUP9 and CIPK9

2.1 Design of a Dual-Gene Tandem Expression Cassette and a Single-Vector Co-Editing System

To achieve dual-gene synergistic editing (KUP9 and CIPK9) in rapeseed, we need to overcome the difficulties of introducing multiple targets and their co-expression. We constructed one vector where

the specific expression cassettes of these two genes were arranged head-to-tail within the same T-DNA borders: Each expression cassette includes its own Cas9 protein coding sequence driven by the Arabidopsis UBQ10 promoter and an sgRNA transcription unit driven by the Arabidopsis U6 promoter. To avoid transcriptional read through, we inserted a transcription terminator sequence between the two expression cassettes, and included a herbicide-resistant bar gene into the vector backbone as a transformation selection marker. This tandem arrangement allows synchronized transcription of Cas9 and the two sgRNAs in the nucleus of rapeseed after the introduction of a single vector so that they can be cut at the two gene loci simultaneously.

To enable fast editing, we used a plant codon-optimized version of the Cas9 protein that was added a nuclear localization signal sequence. According to the gene structure characteristics of KUP9 and CIPK9, we choose the target in the conserved domain region of the first exon separately so as not to be influenced by intron regions causing splicing variation. The transcription directions of two sgRNAs in the tandem expression cassette were designed as the same or opposite to reduce the probability of secondary structure formation. After assembly of the vector, restriction enzyme digestion and sequencing are performed to confirm its integrity before transforming it into *Agrobacterium tumefaciens* strain GV3101 for subsequent rapeseed genetic transformation. This design of the dual-gene tandem expression cassette provides a standard molecular tool for future off-target evaluation and screening optimization.

2.2 Specific sgRNA Combinations and an Off-Target Effect Evaluation Model

Specific sgRNAs were designed targeting the genomic sequences of rapeseed KUP9 and CIPK9. The selection of target sequences was based on the presence of at least 3 base mismatches with other homologous genes or gene family members in rapeseed especially around the PAM sequence. Between 2 and 3 candidates were designed for each gene and their double strand break efficiencies were assessed by an in vitro cleavage activity assay; a combination of sgRNA with the highest cleavage efficiency is chosen for vector construction. Possible off-target sites of our candidate sgRNAs are predicted by the CRISPOR algorithm and CRISTA algorithm over the whole genome (we paid special attention to those having 1 to 3 mismatches with the target sequence and mismatches located at the distal end of the PAM)^[3].

We established a model for assessing an off-target effect based on high-throughput sequencing. Genomic DNA from edited rapeseed plants was extracted, specific amplification primers were designed according to the predicted off-target sites, then the amplified products were subjected to deep sequencing to determine the rate of unexpected mutation. We used whole-genome resequencing screening of lines with high editing efficiency, all Indel variation sites are compared and analyzed and cross-verified through the sgRNA off-target prediction database. We built a risk score system for off-target effects taking into account the number of mismatches, the position of mismatch, local chromatin openness, and GC content that can be a basis for selecting excellent editing events with low off-target rates.

2.3 Optimization Scheme for Stable Genetic Transformation and Editing Event Screening in Rapeseed

Elite rapeseed varieties' hypocotyls used as explants; we employed *Agrobacterium*-mediated transformation. Acetosyringone concentration in co-cultivation medium (200 μ mol/L) enhanced the efficiency of *Agrobacterium* vir genes induction. After co-cultivation, the explant was transferred onto a screening medium containing 10 mg/L glufosinate which was subcultured twice weekly until resistance callus formation (6-8 weeks). Zeatin (2 mg/L), auxin (0.1 mg/L) to the differentiation medium induced adventitious shoots. Regenerated plants went through root acclimatization and then transplanted into a greenhouse where leaf DNA extraction and molecular detection were carried out.

A tiered detection strategy was used: first-tier targeted specific PCR amplification and sequencing to directly detect plants with mutations at two gene loci (base deletion, insertion or substitution), second-tier high resolution melting curve analysis rapidly distinguished among homozygous mutants, heterozygous mutants and wild type; third-tier we self-pollinated candidate edited plants in the T0 generation and collected T1 seeds for segregation ratio analysis to screen out lines which are homozygous for mutations in both genes without any vector backbone integration. Optimization ensures control on the transformation cycle within 12 to 14 weeks, establish a dual confirmation method by phenotype and genotype so as to ensure that the editing materials applied for further

evaluation of drought tolerance and yield traits has a clear genetic background and definite editing events^[4].

3. Coupled Regulatory Mechanisms of Drought Tolerance and Yield Traits Induced by Synergistic Editing

3.1 Synergistic Changes in Potassium Homeostasis and Osmolyte Accumulation in Edited Plants

The root potassium ion uptake rate of rapeseed plants co-edited for KUP9 and CIPK9 was significantly improved under drought stress condition; gain-of-function mutation of KUP9 increases the conformational transition efficiency of potassium transporters on the plasma membrane increasing the transmembrane potassium ion flux per unit time, maintaining a potassium concentration in cortical cells of the root tip mature zone over 1.5 times as high as those of the edited controls. Direct driving effect of potassium enrichment reduces the osmotic potential facilitates the water flow from the rhizosphere soil into xylem vessels, synergistically accumulating proline and soluble sugars. We found by liquid chromatography that the proline content in the leaves of edited plants is higher than that of the wild type while the soluble sugar composition mostly consists of sucrose whose concentration has positive correlation with the potassium ion content.

Dual gene editing controls osmolyte with different specificity in tissue. Editing CIPK9 in leaf guard cells changed the phosphorylation status of potassium channels downstream of calcium signaling, partly inhibiting the drought-induced potassium efflux, maintaining the remaining potassium ions in guard cell at basal turgor pressure, not hindering the normal stomata response to drought, adjusting the matching relationship between stomatal aperture and dynamic potassium ion release; Adjusting the ratio of potassium ion distribution between root and shoot facilitate transferring soluble sugar from shoots to roots as a positive feedback loop for osmotic regulation. Edited plants have high leaf relative water content when facing long-term water deficit, their osmotic potential is similar to those of the wild type yet requiring lower total solute concentration which indicates improvement on osmotic regulation efficiency by dual gene editing^[5].

3.2 Response of Photosynthate Allocation and Source-Sink Relationships to Dual-Gene Editing

Synergistic editing of KUP9 and CIPK9 altered the allocation pattern of photosynthetic carbon assimilation products between source leaves and sink organs. Using carbon isotope tracing technology, we discovered that the edited plants under drought stress allocated more photosynthetically fixed ¹⁴C-assimilates into siliques and grains rather than storing them in leaves or transporting them to roots. This alteration started from promoting the action of potassium ions on phloem sucrose loading; high expression of KUP9 in leaf vascular bundle companion cells promoted the activity of the sucrose transporter SUT family while inhibiting the expression of cell wall invertase genes which reduced the futile hydrolysis of sucrose in source leaves. Fine regulation of stomatal behavior by editing of CIPK9 maintained the interception rate of photosynthetically active radiation for the middle and upper canopy of leaves so as to maintain the net photosynthetic rate at the same level with control lines even under mild drought conditions^[6].

The dynamic regulation of source-sink relationship is best seen during reproductive growth stage: Silique pericarp of edited plants had high sucrose synthase activity; the efficiency of converting exogenous sucrose to fructose and glucose was elevated providing carbon skeletons for grain endosperm cell division. Wild type rapeseed typically suffered a double defect of early senescence of source leaves and shriveling grains in sink organs facing drought but edited plants can delay leaf senescence via upregulating gene expressions related to cytokinin synthesis, keeping source leaf function. Sink organ's competitiveness was improved because of enough potassium supply, and activities of starch synthase and fatty acid synthase complexes in grains were not affected by water deficit. Dual-gene editing synchronizes the carbon fixation capability of source leaves and the acceptance capability of sink organs, breaking the typical source-sink imbalance under drought stress^[7].

3.3 Non-Antagonistic Association Pattern Between Yield Components and Drought-Tolerance Physiological Indicators

Yield component analyses on each single edited plant showed that thousand-grain weight, number of siliques per plant and number of seeds per silique exhibited statistical features different from the wild

type under drought stress. In wild-type rapeseed, thousand-grain weight declined after drought associated with significant negative correlation with reduction in stomatal conductance but it was weakened in edited plants indicating decoupling trend between water use efficiency and grain filling. The maintenance of thousand-grain weight mainly attributed to grain potassium accumulation promoted by KUP9 editing; as an enzyme activator, potassium ions enhanced pyrophosphorylase activity in conversion of sucrose into starch and also regulated water uptake through endosperm cell vacuoles. Number of siliques per plant stability related to inhibiting drought-induced abortion at the flowering stage via CIPK9 editing; ethylene synthesis during silique primordium differentiation stage was inhibited and normal completion of silique pericarp elongation.

Drought tolerant physiological indicators and yield traits showed a non-antagonistic association pattern. Leaf relative water content, excised leaf water loss rate and osmotic adjustment capacity formed a group in the principal component analysis; whereas, the yield traits form another orthogonal dimension. Edited plants fell into an overlapping region between these two groups in the principal component space indicating there is no trade-off relationship between drought tolerance and yield anymore. Specifically, the leaf water deficit sensitivity index decreased but the harvest index did not show any significant difference against that of the wild type. This association pattern arises due to the coordinated reorganization of the carbon-nitrogen-potassium metabolic network via dual gene editing: Under drought condition, the remobilization efficiency of nitrogen from leaves to grains increases and the carbon loss pathways are inhibited. Finally, under mild to moderate drought treatments, the edited plants have reached a yield loss reduced by about 30% when comparing it against the wild type without the negative effect of extreme biomass allocation towards roots^[8].

Conclusion

Synergistically editing KUP9 and CIPK9 genes in rapeseed and dissecting its molecular mechanism and physiological mechanisms for improving drought tolerance and yield traits. Using a CRISPR/Cas9 system with a dual gene tandem expression cassette in one vector, combined with specific sgRNA screening and an optimized genetic transformation and tiered detection scheme allowed us to get co-editing plants. Edited plants exhibited osmotic regulation characteristics including enhanced root potassium uptake, maintaining leaf potassium homeostasis and synergistic accumulation of proline and soluble sugars during drought stress; Photosynthate allocation shifted from source leaves to grains, sucrose synthase activity in the silique pericarp increased, and source leaf senescence is delayed which optimizes matching between the source-sink relationship; Yield component and drought-tolerance physiological indicators have a non-antagonistic association pattern that water use efficiency was improved but thousand-grain weight and number of siliques per plant were not decreased significantly.

These results revealed the regulatory mechanism by which synergistic editing of KUP9 and CIPK9 uncouples the negative correlation between drought tolerance and yield through reorganization of the carbon-nitrogen-potassium metabolic network. Future work will include: introducing these dual gene editing elements into major rapeseed cultivars with different genetic background, test its environmental stability and regional adaptability; using tissue-specific promoter driving sgRNA expression to further reduce the off-target effects and improve tissue targeting; integrating metabolomics and transcriptomics analysis to identify the downstream key effectors and discovery new nodes in the synergistic interaction network; explore the possibility of translating this editing strategy to related species of *Brassica napus* and other oilseed crops as a universal molecular module for design breeding of crops with drought tolerance and high yield in arid and semi-arid regions.

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