Functional Characterization of Rubisco Activase Genes in *Kandelia candel* Under the Stress of Flooding and Salinity

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Article

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Abstract

Rubisco activase (RCA) is an ATP-dependent enzyme that plays a crucial role in plant stress responses by regulating the catalytic activity of Rubisco. However, the alternative splicing and functional characteristics of the *RCA* gene exhibit notable species-specific diversity. The variable splice forms and functions of the *RCA* gene in mangrove plants remain poorly understood. We herein cloned the *RCA* genes in the leaves of mangrove plant *Kandelia candel* (L.) in response to combined flooding and salinity stress, and performed systematic expression analysis and functional validation. Our results demonstrated that the *RCA* gene undergoes alternative splicing to produce two isoforms, designated as *KcRCAl* (GenBank accession: MG492021) and *KcRCAs* (GenBank accession: MG492022), respectively. The *KcRCAl* encodes a 440-amino acid protein (42.49 kDa) belonging to the β-isoforms, while *KcRCAs* encodes a 474-amino acid protein (46.10 kDa) classified as the α-isoforms. Moreover, protein structure analysis revealed that both isoforms contain phosphorylation and lysine acetylation modification sites. Phylogenetic analysis indicated that *KcRCA* shares the closest evolutionary relationship with *RCA* from *Cicer arietinum* (chickpea) and *Durio zibethinus* (durian). Furthermore, RT-qPCR analysis revealed that the expression levels of *KcRCAl* and *KcRCAs* were significantly upregulated in *K. Candel* leaves under the combined stress condition. The following functional validation studies in transgenic *Arabidopsis* demonstrated that overexpression of the *KcRCA* genes enhances the plant's tolerance to resist flooding and salinity stress while improving antioxidant capacity, increased RCA and Rubisco activities, thereby maintaining photosynthetic efficiency under combined flooding and salinity stress. Our study not only provides new experimental evidence for understanding the molecular mechanisms of plant flooding and salinity stress, but also offers theoretical foundations for breeding flooding-and salinity-tolerant crops.

Keywords: *Kandelia candel*; flooding and salinity; combined stress; Rubisco activase; gene cloning; functional analysis

1. Introduction

Flooding and salinity stress are critical abiotic factors that severely impair plant growth and agricultural productivity [14]. In recent years, driven by intensified climate change and soil

salinization, significant progress has been made in understanding plant responses to either flooding or salinity stress alone, including identifying key physiological adaptations and stress-related genes [2–4]. However, in natural environments (e.g., in coastal farmlands), these stresses often occur simultaneously, and their combined effects pose a more complex challenge [5]. How do plants integrate molecular and physiological responses to cope with the combined flooding and salinity stress, and what are the crucial antibiotic resistance genes (*ARGs*) involved in these adaptive mechanisms? Addressing this question is essential for understanding plant stress resilience and developing stress-resistant crop varieties.

As we know, two typical strategies for adaptation to flooding have been developed in plants over the course of long evolution including 'escape' and 'quiescence' [6]. The plants with the escape strategy respond to flooding stress by rapid elongation of stems and leaves, enabling plant organs to breach the water surface for access to oxygen and light. In contrast, the quiescence strategy allows plants to maintain essential life processes by reducing metabolic rates and optimizing carbon allocation [7]. Notably, the successful implementation of both strategies is heavily reliant on a continuous supply of energy [8].

Flooding stress often leads to multiple secondary stresses, especially reduced light intensity and impaired gas diffusion, which significantly diminish the efficiency of photosynthesis [9]. It has been reported that the certain aquatic plants have evolved special adaptive structures, such as the continuous gas film formed on the surface of rice leaves, which maintaining effective gas exchange under flooding stress [10].

Furthermore, as a terrestrial plant, maize exhibit a compensatory increase in photosynthetic rate during the initial stages of flooding (within 6 days) [11]. Our research on the mangrove species *K. candel* further reveals that this species can sustain high photosynthetic efficiency under effects of flooding stress through the synergistic action of protein phosphorylation modifications and transcriptional regulatory networks, continuously accumulating sucrose to provide sufficient carbon skeletons and energy for stress-resistant metabolism [12].

In response to salinity stress, plants also need to mobilize substantial energy for the regulation of ion homeostasis and the synthesis of osmoprotectants [13]. Based on the chloroplast proteome study of *K. candel*, it was found that salinity stress primarily induces the upregulation of photosystem-related proteins, while key enzymes of the Calvin cycle maintain stable expression. This differential regulation effectively mitigates the inhibition of net photosynthetic rate caused by salinity stress [14]. Therefore, maintaining an adequate energy supply is a crucial pathway for plants to withstand adverse stress conditions [15].

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO, EC 4.1.1.39), located in the chloroplast stroma, is the key rate-limiting enzyme in the photosynthetic carbon assimilation pathway, and its activity directly influences plant adaptability to environmental stress [16,17]. The catalytic function of this enzyme requires a specific activation process mediated by Rubisco activase (RCA) [18]. RCA provides energy through ATP hydrolysis, first dissociating ribulose-1,5-bisphosphate (RuBP) bound at the active site of RuBisco, and subsequently promoting the formation of a catalytically active ternary complex (ECM) between RuBisco, CO_2, and Mg^{2+} [19]. As a member of the AAA^+ superfamily, RCA exhibits typical ATPase activity [20]. In most higher plants, the *RCA* gene produces two main transcripts through alternative splicing: *RCA1* (long form) and *RCA2* (short form), which encode α (45-48 kDa) and β (41-43 kDa) isoforms of different molecular weights, respectively [21]. Phylogenetic analysis indicates that plants such as *Arabidopsis* [22], rice [23], and soybean [24] express both isoforms, while species like maize [25], tobacco [26], and cucumber [27] retain only the β isoform. It was recently reported that RCA functions as an important regulatory factor in plants in responses to temperature and drought stress [28]. Maintaining photosynthetic homeostasis is a key strategy for plant tolerance to flooding and salinity stress [29]. Our previous studies have confirmed that *K. Candel* can sustain normal photosynthesis to meet energy demands under individual flooding and salinity stress conditions [12,14]. Therefore, we hypothesize that RCA,

which regulates photosynthesis, may play a significant role in the plant's response to the combined flooding and salinity stress.

In the context of global climate change, the genetic engineering of key photosynthetic enzymes has emerged as a significant strategy for enhancing crop yields [30]. As a direct regulatory factor for RuBisco activation, RCA is considered an ideal target for genetic improvement of photosynthesis [31]. Given the substantial differences in activity among the *RCAs* from various plant species, exploring efficient *RCA* gene resources from the plants in specialized habitats holds a considerable application potential [32]. However, the alternative splicing and functional characteristics of the *RCA* gene exhibit notable species-specific diversity [33,34]. The variable splice forms and functions of the *RCA* gene in mangrove plants that thrive in flooding and saline habitats within the intertidal zone remain poorly understood.

K. candel has evolved unique mechanisms for tolerance combined flooding and salinity stress during its long-term adaptation to intertidal environments, harboring a wealth of gene resources related to flooding and salinity stress [35,36]. Herein, we are the first to isolate the *RCA* gene from *K. candel*, identifying two variant splice variants of the *RCA* gene, designated *KcRCAl* and *KcRCAs*. Gene expression analysis and transgenic experiments showed that both *KcRCAl* and *KcRCAs* are the key genes involved in alleviating the adverse effects of the flooding and salinity stresses.

2. Materials and Methods

2.1. Materials and Treatments

Seedlings of *Kandelia candel* (L.) were collected from the Zhangjiangkou Mangrove National Nature Reserve in Zhangzhou, Fujian Province (23°55'N, 117°26'E). Healthy, mature, and similarly sized seedlings were selected and planted in nutrient pots (10 cm × 12 cm) filled with clean sand, with one seedling per pot. A total of 30 pots were arranged in a single plastic basin (42 cm × 30 cm × 14 cm) and irrigated with Hoagland nutrient solution. The seedlings were watered twice daily to replenish evaporated water, and the Hoagland nutrient solution was replaced every three days. After the seedlings developed two pair of true leaves, they were subjected to combined flooding and salinity stress treatments. Building on previous research regarding salinity stress in *K. candel*, we employed a treatment of 500 mmol•L^{-1} NaCl in this study [14]. Seedlings with relatively uniform heights (average height difference ± 3 cm) were selected and completely submerged in a Hoagland nutrient solution contenting 500 mmol•L^{-1} NaCl, with the solution level maintained 5 cm below the surface. Non-stressed seedlings serving as the control. All seedlings were grown in a greenhouse at an air temperature of 27–32 °C under natural sunlight. Leaf samples from both treantment and control groups were collected after 6, 12, 24, and 48 hours of treatment. The collected leaf samples were rapidly frozen in liquid nitrogen for 10 minutes and subsequently stored at -80 °C for RNA extraction. Each biological replicate consisted of 60 seedlings, with three biological replicates for each treatment.

2.2. Cloning of RCA Gene

Total RNA was extracted from the leaves of *K. candel* subjected to flooding stress using the TaKaRa Mini BEST Plant RNA Extraction Kit, following the manufacturer's instructions. The quality and integrity of the total RNA were assessed using 1% agarose gel electrophoresis and a NanoDrop ND-2000 spectrophotometer. cDNA synthesis and genomic DNA removal were performed using the TransScript® Uni One-Step gDNA Removal and cDNA Synthesis SuperMix, and the products were stored at -20°C. Based on the longest ORF sequence of *RCA* gene (Gene ID: CL9747Contig1, Supplementary table 1) established in the preliminary transcriptome database of *K. candel* [37], cloning primers were designed using Primer Premier 5.0 software (Supplementary Table 2). PCR amplification of cDNA was conducted, followed by purification. The PCR products were ligated into the cloning vector pMD18-T and transformed into competent *E. coli* Trans 5*α* cells. Positive clones were screened and sequenced.

2.3. Bioinformatics Analysis of RCA Gene

The relative molecular weight, isoelectric point, and other physicochemical properties of the proteins were analyzed using the ExPASy online tool. The TargetP 1.1 Server was utilized to analyze the transit peptide segments of the proteins. The InterProScan online tool was employed to analyze the domains and functional sites of the amino acid sequences. The NetPhos 3.1 Server was used to predict phosphorylation sites in the proteins. Homologous amino acid sequences were identified using the BLAST tool in NCBI. A phylogenetic tree was constructed using MEGA 6.06 software. The software and websites used for the bioinformatics analysis of *KcRCAl* and *KcRCAs* protein structures are listed in Supplementary Table 3.

2.4. Measurement of H_2O_2 and $O_2^{\bullet-}$ Contents and Expression Analysis of KcRCAl and KcRCAs in K. Candel under Combined Stress Condition

The contents of H_2O_2 and $O_2^{\bullet-}$ were measured at defferent time points for both treatment and control groups of *K. Candel* under combined flooding and salinity stress conditions. The $O_2^{\bullet-}$ content was measured using the hydroxylamine oxidation method [38], while H_2O_2 content was quantified using the Micro Hydrogen Peroxide (H_2O_2) Assay Kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Following the methodology described in Section 2.2. RNA was extracted from the leaves of *K. candel* for both treatment and control groups at various time points, and cDNA was subsequently synthesized. Primers were designed based on the sequenced *KcRCAl* and *KcRCAs* sequences, with the *Actin* gene of *K. Candel* serving as the internal control. The expression levels of *KcRCAl* and *KcRCAs* in the leaves under different treatments were analyzed using RT-qPCR with the SYBR® Premix ExTaq™ (Tli RNaseH Plus) kit, and relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences are provided in Supplementary Table 4.

2.5. Arabidopsis Transformation Assay

The Gateway technology was employed to construct the expression vector for *Arabidopsis*. Primers with BP adapters were designed (Supplementary Table 5), and the target genes *KcRCAl* and *KcRCAs* were ligated to the entry vector pDONR207 via BP reaction, resulting in the plasmids pDONR207-*KcRCAl* and pDONR207-*KcRCAs*. The target genes were then ligated to the expression vector pEarleyGate104 via LR reaction, yielding the expression vectors pEarleyGate104-*KcRCAl* and pEarleyGate104-*KcRCAs*, which were transformed into Agrobacterium tumefaciens EHA105 competent cells to obtain positive strains. The floral dip method was utilized for Agrobacterium-mediated transformation of *Arabidopsis*, resulting in T_0 generation seeds [39]. T_0 seeds were screened for resistance to Basta (glufosinate) across multiple generations to obtain T_2 generation transgenic plants, which were confirmed as positive by PCR. The transgenic plants were cultivated in a growth chamber maintained at 22 ℃and 16 h light/8 h dark photoperiodand. The expression levels of the *RCA genes* were assessed by RT-qPCR in T_2 transgenic plants and wild-type(WT) plants, with the *Actin* gene of *Arabidopsis* serving as the internal control, following the methodology outlined in section 2.4. Primers were designed based on the mRNA sequences of *Arabidopsis* RCA-α and RCA-β isoform available in the GeneBank database (AEC09714.1, AEC09716.1). The primer sequences are provided in Supplementary Table 4.

2.6. Flooding and Salinity Tolerance Assay of Transgenic Arabidopsis

Thirty-day-old T_2 transgenic plants and wild-type(WT) plants were subjected to combined flooding and salinity stress treatment, with a salt concentration of 200 mmol·L^{-1} NaCl. The treatment method was consistent with that described for *K. Candel* in Section 2.1. Samples were collected at 0 h, 6 h, 12 h, 24 h, and 48 h, and RT-qPCR was employed to detect the expression levels of the *KcRCAl* and *KcRCAs* genes in transgenic plants, following the methodology and primer described in section 2.4. The number of surviving plants was counted separately for transgenic and wild-type plants after 3 and 7 days of treatment, and the survival rates were calculated. The cultivation conditions for both

the control and treatment groups were identical to those outlined in Section 2.5. Each biological replicate consisted of 45 seedlings, with three biological replicates for each treatment.

2.7. Determination of Physiological Indexes in Transgenic Arabidopsis under Combined Flooding and Salinity Tolerance

The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) in the leaves, as well as the contents of superoxide anion ($O_2^{\bullet-}$) , hydrogen peroxide (H_2O_2), and malondialdehyde (MDA), were measured. SOD activity was determined using the Hydroxylamine method SOD detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); POD activity was measured using the Guaiacol microplate POD detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) ; CAT activity was assessed using the Ammonium molybdate microplate CAT detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); The methods for measuring the contents of H_2O_2 and $O_2^{\bullet-}$ outlined in Section 2.4; and MDA content was determined using the thiobarbituric acid (TBA) method [40]. RCA activity was measured using a kit developed by GENMED, and Rubisco activity was assessed using a kit from Shanghai Enzyme-linked Biotechnology Co., Ltd.

2.8. Statistical Analysis of Data

Each measurement was performed with at least three biological replicates, and all data are presented as mean ± standard deviation. Statistical significance was determined using ANOVA or the Student's t-test via GraphPad Prism 8 software, and difference at $p < 0.05$ and $p < 0.01$ was used to determine statistical significance.

3. Results

3.1. Isolation and Characterization of Two Distinct RCA Gene Isoforms in Kandelia Candel Leaves

The *RCA* gene was cloned from the *K. candel* leaves after being subjected to combined flooding and salinity stress for 6 hours, followed by bioinformatics analysis was performed. Using cDNA from the leaves of *K. candel* as a template, a 1500 bp gene fragment was successfully amplified via RT-PCR. The PCR product underwent an A-tailing reaction and was ligated into the cloning vector pMD18-T, followed by transformation into *E. coli* Trans 5α (Supplementary Figure 1). Positive clones were screened and sequenced, revealing the presence of two distinct *RCA* gene isoforms in *K. candel*: a long-chain isoform (*KcRCAl*, GenBank accession: MG492021) and a short-chain isoform (*KcRCAs*, GenBank accession: MG492022). The cDNA fragment length of the *KcRCAl* gene was 1461 bp, while that of the *KcRCAs* gene was 1425 bp, indicating a difference of 36 nucleotides between them. Furthermore, 30 positive clones from each treatment group (Control and Treatment) were randomly selected for sequencing, revealing that the occurrence probability of *KcRCAl* was approximately 1:3 compared to *KcRCAs* (Supplementary Table 6), suggesting a higher abundance of *KcRCAs* in *K. candel*.

The cDNA sequences of *KcRCAl* and *KcRCAs* were analyzed using ORF Finder software, which revealed that *KcRCAl* contains a premature stop codon, terminating translation at the 441 st amino acid. In contrast, the cDNA sequence of *KcRCAs* encodes 474 amino acid residues, with 34 additional residues at the C-terminus compared to *KcRCAl*. The *KcRCAs* protein sequence contains 37 differing amino acid residues (Figure 1): QAAQQVNLPVPPGCTDPAATNYDPTARSDDGSC.
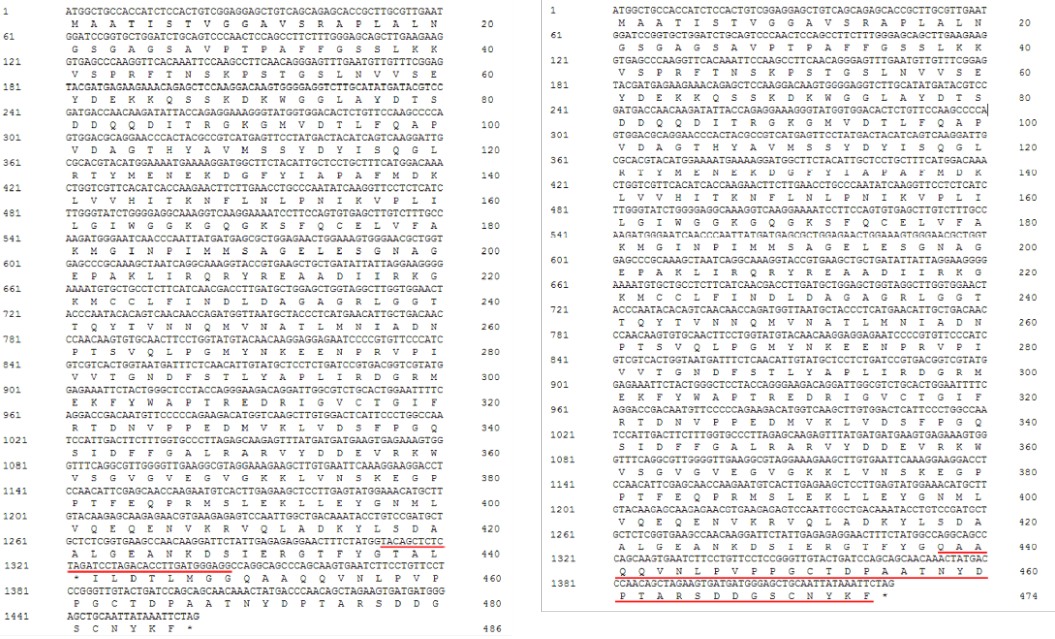
Figure 1. *KcRCA*'s cDNA sequence and its deduced amino acid sequence.

Analysis of the physicochemical properties, including relative molecular weight and isoelectric point (Supplementary Table 7), indicated notable differences between *KcRCAl* and *KcRCAs*. The *KcRCAl* exhibited greater stability than *KcRCAs*, with *KcRCAl* classified as a stable protein and *KcRCAs* as an unstable protein.

Using the TargetP 1.1 Server, the amino acid sequences of *KcRCAl* and *KcRCAs* were analyzed, revealing that the first 58 amino acid residues (relative molecular weight of 5620.42 Da) are predicted to function as a chloroplast transit peptide. The mature *KcRCAl* protein is estimated to have a relative molecular weight of 42.49 kDa, suggesting it corresponds to the β isoforms of *RCA* (41-43 kDa), while the mature *KcRCAs* protein is estimated to have a relative molecular weight of 46.10 kDa, indicating it corresponds to the α isoforms of RCA (45-48 kDa). Thus, it can be inferred that the *RCA* gene in *K. candel* leaves encodes both α and β isoforms.

InterProScan analysis of the domains and functional sites of *KcRCAl* and *KcRCAs* revealed that the amino acid sequence from positions 138 to 332 contains a P-loop nucleoside triphosphate hydrolase functional domain, while the sequence from positions 163 to 304 corresponds to an ATPase, AAA-type, core functional domain, indicating ATP-binding biological function and belonging to the AAA⁺ superfamily of proteins (Supplementary Figure 2). The C-terminus of *KcRCAl* lacks conserved sequences; however, the C-terminus of *KcRCAs* contains a unique C-extension structure specific to the α isoforms: DPAATNYDPTARSDDGSC, which includes two cysteine residues (Cys) at positions 451 and 470 in the amino acid sequence, further confirming that *KcRCAs* is an α isoform and *KcRCAl* is a β isoform.

Online analysis using the NetPhos 3.1 Server predicted four phosphorylation sites in both *KcRCAl* and *KcRCAs* proteins, including one serine (Ser) phosphorylation site, one threonine (Thr) phosphorylation site, and two tyrosine (Tyr) phosphorylation sites. This finding is consistent with the phosphoproteomic data obtained from *K. candel* leaves [12].

NCBI BLAST sequence alignment analysis indicated that the sequences share over 80% similarity with 24 homologous amino acid sequences encoded by *RCA* genes. A phylogenetic tree constructed using MEGA 6.06 software, which included *RCA* genes from 24 plant species, revealed that *KcRCAl* (MG492021) and *KcRCAs* (MG492022) are most closely related to those of *Cicer arietinum* and *Durio zibethinus*, clustering with *Populus euphratica*, while showing more distant relationships

with the RCA-encoding amino acid sequences of 20 other species, indicating they belong to two different subfamilies (Figure 2).
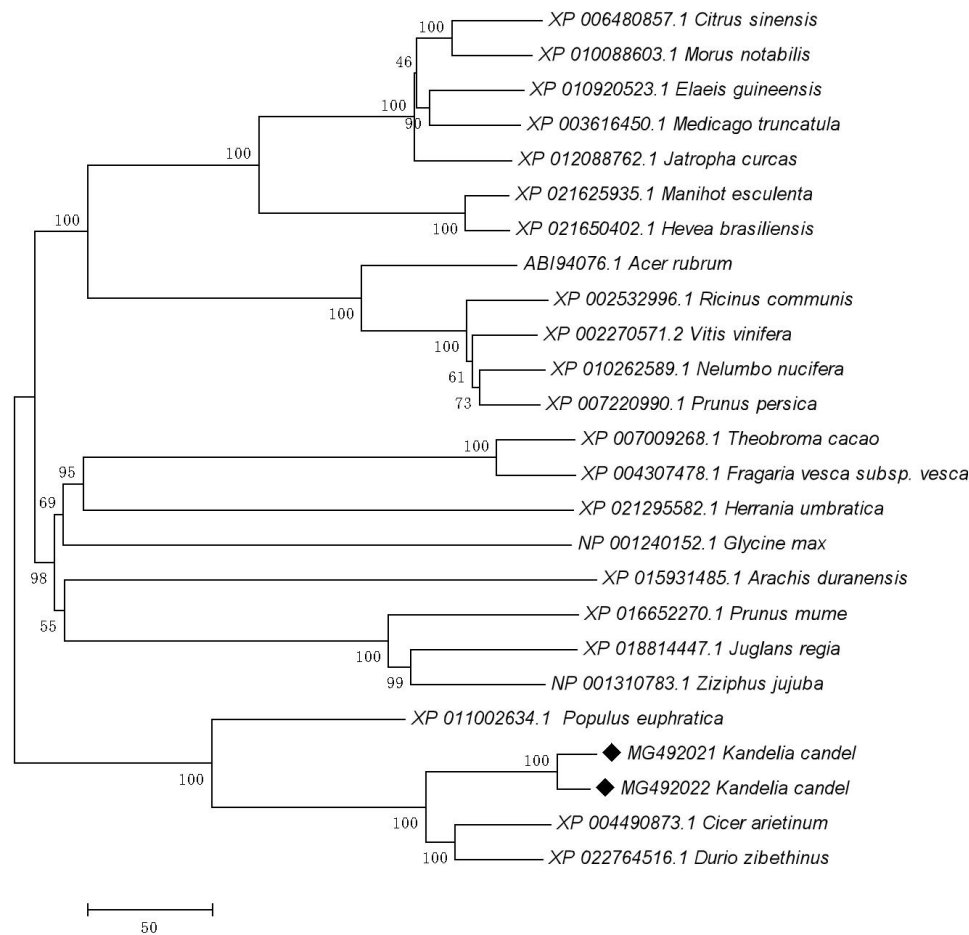

Figure 2. Phylogenetic analysis of *RCA* from *Kandelia candel* and other plant species.

3.2. Analysis of H_2O_2 and $O_2{}^{\bullet-}$ Contents and KcRCA Gene Expression Levels in Kandelia candel Leaves Under Combined Stress of Flooding and Salinity

To assess the effects of combined flooding and salinity stress on the growth of *K. candel*, the contents of hydrogen peroxide (H_2O_2) and superoxide anions ($O_2{}^{\bullet-}$) in the leaves were measured at 6, 12, 24, and 48 hours post-treatment, and morphological changes of the plants were observed and photographed after 48 hours. The results indicated that 6 hours after the stress treatment, the contents of H_2O_2 and $O_2{}^{\bullet-}$ in the leaves of *K. candel* were significantly higher than those in the control group, and remaining elevated thereafter (Figure 3, A, B). Notably, the seedlings of *K. candel* exhibited significant stress responses within 6 hours of combined flooding and salinity stress. After 48 hours, the leaves of the treatment group displayed slight wilting, although no obvious color changes were observed. Upon excavation, it was noted that the roots of the treatment group had turned black, with some fine roots exhibiting signs of rot (Figure 3, C, D). This indicates that the combined stress on *K. candel* for 48 hours resulted in significant phenotypic changes.

To verify the expression of the two splice variants of the *RCA* gene under stress conditions, this study analyzed the expression levels of *KcRCAl* and *KcRCAs* in the leaves of *K. candel* at various stress time points in both the treatment and control groups using RT-qPCR. Under flooding and salinity stress treatment, the expression levels of *KcRCA* in the leaves of *K. candel* demonstrated that combined stress significantly promoted the overall expression levels of both *KcRCAl* and *KcRCAs* (Figure 3, E, F). Notably, short-term flooding (6 hours) resulted in a significant enhancement of *KcRCAl* and

$KcRCA_s$ expression levels in the leaves, which is advantageous for the plant's adaptation to flooded and saline environments.
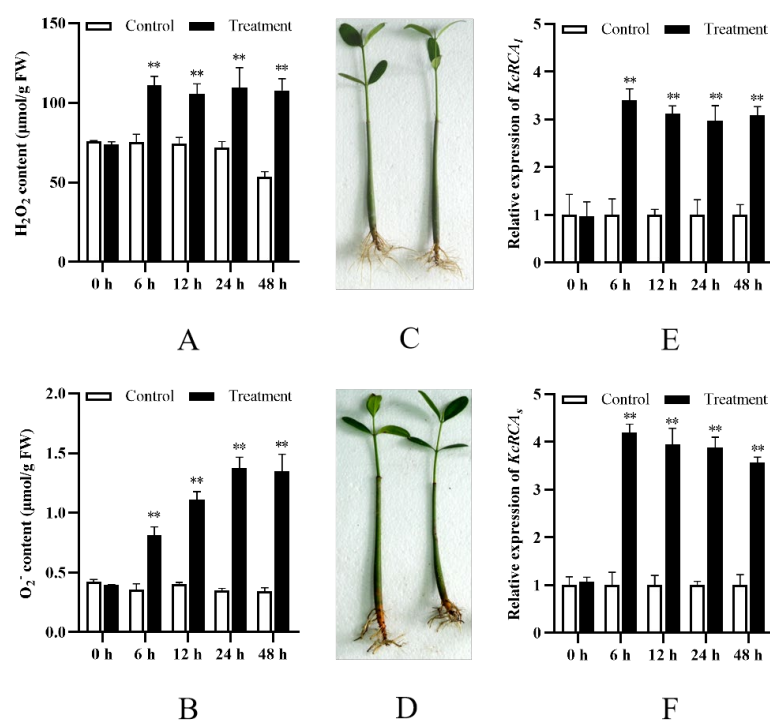

Figure 3. Relative expression levels of *KcRCA* from *K. candel* leave under combined   flooding and salinity stress. (A) and (B) H_2O_2 and $O_2^{\bullet-}$ contents in *K. candel* leave at various time points under combined stress condition. (C) and (D) Images of *K. Candel* seedling from the control and treatment groups after 48 h of combined stress condition. (E) and (F) Relative expression levels of $KcRCA_l$ and $KcRCA_s$ from *K. candel* leave under combined stress condition. .

3.3. Establishment and Molecular Confirmation of Arabidopsis Transgenic Plants

In this study, the *RCA* gene was transferred into *Arabidopsis* for functional verification, as no established genetic transformation system currently exists for *K. candel*. Transgenic *Arabidopsis* was successfully obtained through herbicide resistance and PCR identification. The results indicated that transgenic plants expressing $KcRCA_l$ exhibited a specific band of approximately 1500 bp (Supplementary Figure 3), while transgenic plants expressing $KcRCA_s$ also displayed a specific band of about 1500 bp (Supplementary Figure 4). No corresponding bands were amplified in wild-type *Arabidopsis*. Additionally, a specific band of approximately 1500 bp was cloned using the expression vector plasmid as a template. The PCR products from positive plants were sent for sequencing, and the results were consistent with the PCR detection, confirming that the exogenous genes $KcRCA_l$ and $KcRCA_s$ have been successfully integrated into the transgenic *Arabidopsis*. The results of vector construction (Supplementary Figure 5, 6) and Agrobacterium PCR detection (Supplementary Figure 7) are presented in the supplementary materials.

Through generation resistance screening, T_2 generation transgenic *Arabidopsis* seedlings (Supplementary Figure 8, Supplementary Figure 9) were obtained and utilized for resistance verification of the transgenic lines. We analyzed the phenotypic characteristics and morphology of the transgenic plants. No significant differences in seedling were observed between the wild-type and transgenic plants (Figure 4, A-C). The RT-qPCR data indicated that the expression levels of the $KcRCA_l$ and $KcRCA_s$ genes in the transgenic plants were significantly higher than those in the wild-type plants (Figure 4, D, E). These results confirm the successful generation of transgenic *Arabidopsis* plants that overexpress the $KcRCA_l$ and $KcRCA_s$ genes.
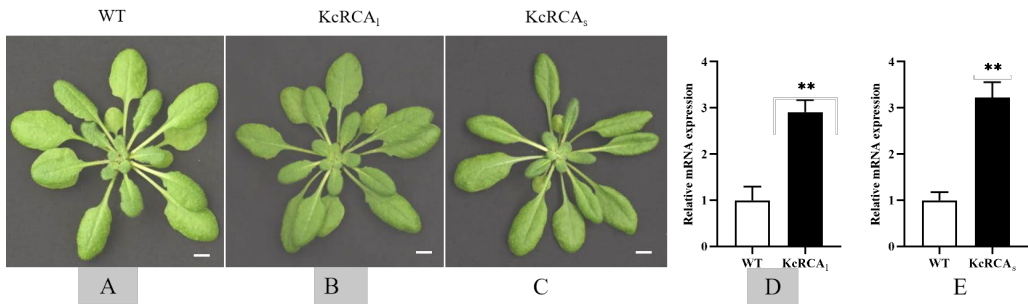
Figure 4. Establishment of *Arabidopsis* transgenic plants overexpressing *KcRCAl and KcRCAs* in a wild-type (WT) background. (A) Wild type(WT). (B) T$_2$ transgenic seedlings with the *KcRCAl* gene. (C) T$_2$ transgenic seedlings with the *KcRCAs* gene. (D) Relative expression of *KcRCAl* gene in the transgenic plants. (E) Relative expression of *KcRCAs* gene in the transgenic plants. Bar = 0.5 cm.

3.4. Transgenic Arabidopsis Plants Exhibited Enhanced Tolerance to Flooding and Salinity Stress

The performance of wild-type and transgenic *Arabidopsis* seedlings were submerged in 200 mmol·L⁻¹ NaCl solution for flooding and salinity trentment. On Day 3 of stress, wild-type *Arabidopsis* leaves exhibited significant purpling and wilting, whereas the transgenic lines thrived(Figure 5, A). By monitoring the survival rates of the plants subjected to flooding and salinity stress treatments over several days, it was observed that, for the same duration of treatment, the survival rate of the transgenic plants was significantly higher than that of the wild-type plants. Furthermore, no significant difference was found between the two transgenic lines.(Figure 5,B, C). These phenotypic results indicate that the transgenic plants possess a higher tolerance to combined flooding and salinity stress compared to the wild-type plants.
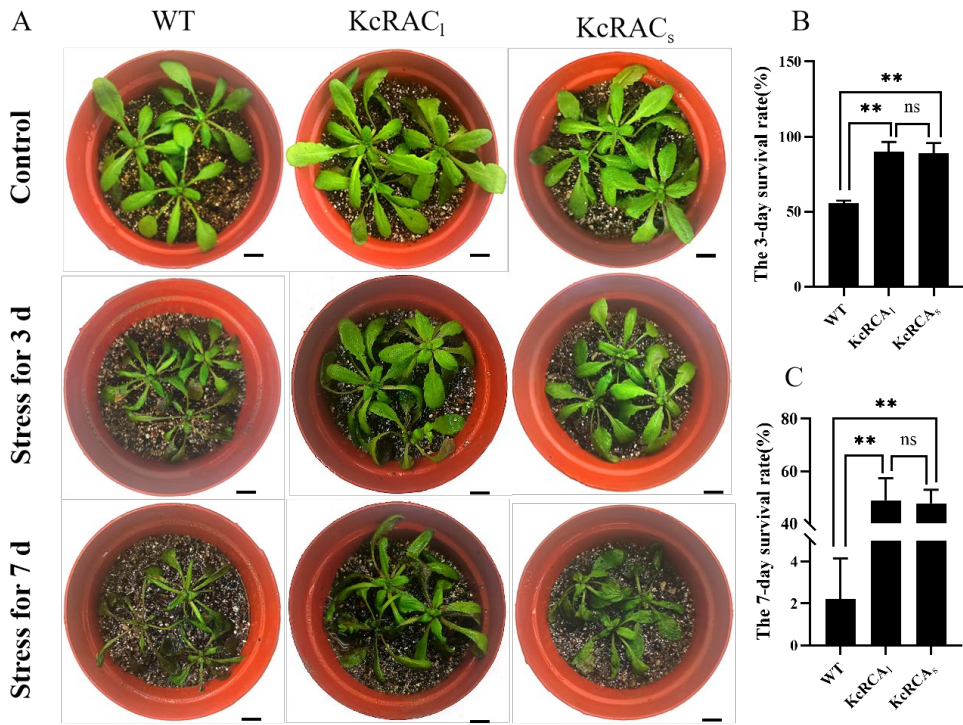
Figure 5. Flooding and salinity stress tests and phenotypic analysis of transgenic plants and wild-type (WT) plants. (A) Phenotypic images of plants undergoing combined stress condition for 3 and 7 days, respectively. (B) Statistical graph depicting the survival rates of WT and transgenic plants. Bar = 1 cm.

In this study, RT-qPCR technology was employed to analyze the expression levels of the *KcRCAl* and *KcRCAs* genes in transgenic plants under combined flooding and salinity stress at various time points. The results indicated that the expression level of *KcRCAl* and *KcRCAs* genes were upregulate in the transgenic plants after 6 hours of treatment, remaining significantly higher than those in the control group. Although expression level decreased after 48 hours of treatment, they remained elevated compared to the control group.(Figure 6). The upregulation of *RCA* gene expression may be associated with the enhanced tolerance to combined flooding and salinity stress observed in the transgenic plants.
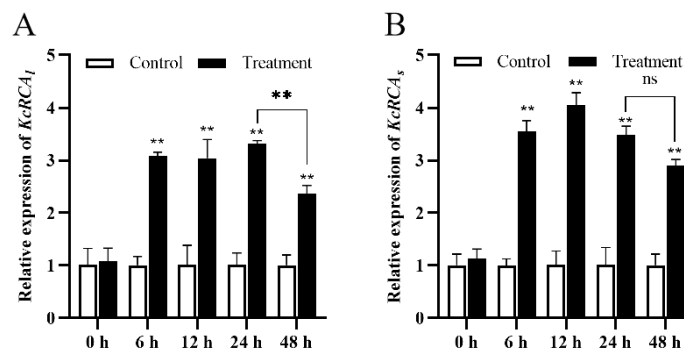
Figure 6. Relative expression levels of the *KcRCAl* and *KcRCAs* genes in transgenic plants under combined flooding and salinity stress treatments at various time points. (A) *KcRCAl* Transgenetic plant. (B) *KcRCAs* Transgenetic plant.

3.5. Physiological Analysis of Transgenic Arabidopsis Under the Combined Flooding and Salinity Stress

Physiological assessments of the transgenic plants' resistance for combined flooding and salinity tress and key photosynthetic enzyme activities revealed that, compared to wild-type plants, the activities of superoxide dismutase (SOD) and peroxidase (POD) in transgenic plants were significantly increased (Figures 7 A, B), while catalase (CAT) activity did not exhibit significant changes (Figure 7 C). The content of hydrogen peroxide (H_2O_2) did not change significantly (Figure 7 D), whereas superoxide anion ($O_2^{•-}$) content significantly increased (Figure 7 E) Meanwhile, malondialdehyde (MDA) content significantly decreased (Figure 7 F). These results indicate that the antioxidant capacity of plants expressing *KcRCAl* or *KcRCAs* has been enhanced to some extent. Furthermore, compared to wild-type plants, RCA activity in transgenic plants significantly increased, with Rubisco activity enhanced by 1.09-fold and 2.75-fold in *KcRCAl* and *KcRCAs* transgenic plants, respectively (Figure 7 G,H). This suggests that *KcRCAl* or *KcRCAs* contributes to the improvement of photosynthetic efficiency in transgenic plants.
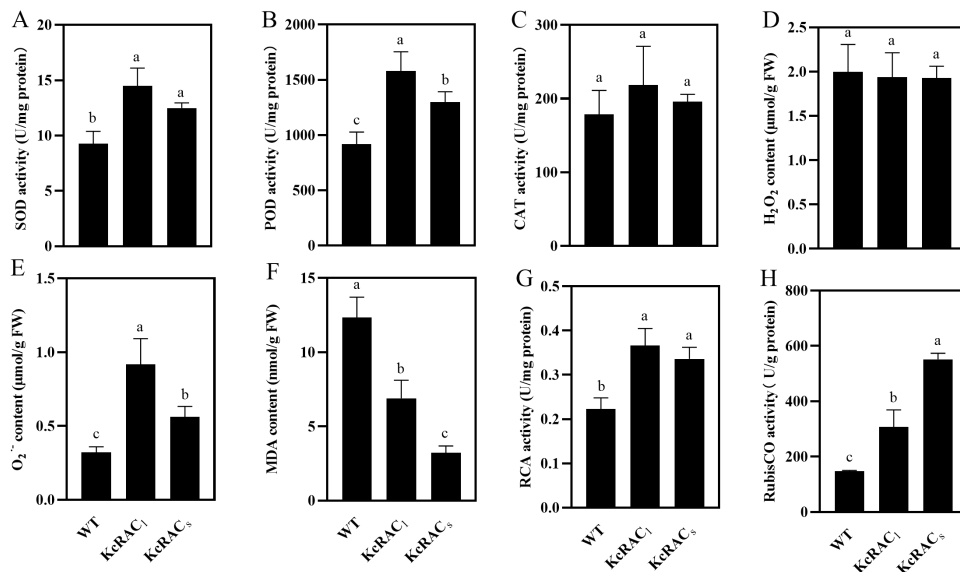
Figure 7. Physiological analysis of transgenic plants under combined flooding and salinity stress for 48 h. WT: Wild-type *Arabidopsis*; *KcRCAl*: *Arabidopsis* transformed with the *KcRCAl* gene; *KcRCAs*: *A. thaliana* transformed with the *KcRCAs* gene. (A) Superoxide dismutase (SOD) activity. (B) Peroxidase (POD) activity. (C) Catalase (CAT) activity. (D) Superoxide radical ($O_2^{\bullet-}$) content. (E) Hydrogen peroxide (H_2O_2) content. (F) Malondialdehyde (MDA) content. (G) Ribulose-1,5-bisphosphate carboxylase/oxygenase activase (RCA) activity. (H) Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity. Different lowercase letters indicate significant differences among the sample time or plant tissue ($P < 0.05$).

4. Discussion

4.1. Successfully Cloned Two Variable Splice Variants of RCA Gene from a Mangrove Plant

Combined stress presents a significant challenge to successful crop production, and the gene resources that enable plants to respond to such stresses are often found in species that thrive in specialized or extreme environments[41]. For instance, *K. candel* grows in coastal wetlands and has long been subjected to high levels of flooding and salinity stress. Consequently, it has developed a comprehensive suite of adaptive mechanisms to cope with the combined challenges of flooding and salinity, harboring a wealth of stress-resistant genes [42]. Photosynthesis, as the central process of plant energy metabolism, plays a crucial role in crop adaptation to environmental stress [43]. Previous studies have demonstrated that *K. candel* can sustain high photosynthetic efficiency by upregulating the expression of photosynthesis-related proteins while stabilizing the expression of key enzymes in the Calvin cycle, including Rubisco and RCA, in response to moderate salinity stress (100-200 mM NaCl) and flooding stress [10,31]. In this context, Rubisco serves as the key rate-limiting enzyme that catalyzes the carboxylation reaction of CO_2 with ribulose-1,5-bisphosphate (RuBP), and its activation is critically dependent on ATP hydrolysis mediated by Rubisco activase (RCA) [44,45]. This study successfully cloned two variable splice variants of RCA, *KcRCAl* and *KcRCAs*, from the leaves of *K. candel* for the first time. Bioinformatics analysis revealed that *KcRCAl* encodes 440 amino acids (42.49 kDa) and *KcRCAs* encodes 474 amino acids (46.10 kDa), both containing typical ATPase functional domains (P-loop NTP hydrolase domain and AAA+ ATPase core domain) [46]. Phylogenetic analysis indicated that the *RCA* of *K. candel* is most similar to that of *Cicer arietinum* and *Durio zibethinus* (>85%), clustering separately from *Populus euphratica*, while showing lower similarity (<70%) with the *RCA* genes of 20 other terrestrial plants. This confirms the conservation of the *RCA* gene and reveals the unique evolutionary characteristics of mangrove plants' *RCA* in intertidal

habitats. As the first *RCA* gene cloned from a mangrove plant, this study provides important molecular targets for elucidating the mechanisms of salt and flood tolerance in plants.

4.2. Multifunctional Regulation of RCA in the Flooding and Salinity Tolerance Physiology of Kandelia Candel

Recent studies have shown that RCA not only maintains photosynthetic carbon assimilation efficiency by activating Rubisco but also plays a central regulatory role in plant responses to various environmental stresses [47,48]. For instance, in response to temperature stress, heat-tolerant cucumber (*Cucumis sativus*) significantly upregulated *RCA* gene expression and enhanced RCA enzyme activity, effectively maintaining Rubisco's catalytic efficiency and allowing net photosynthetic rates to remain above 80% of the control group under high-temperature conditions [27]. Under low-light stress, although the photosynthetic rate of cucumber significantly decreased, the transcription level of *CsRCA* exhibited a compensatory increase, significantly alleviating the reduction in Rubisco activity [49]. Additionally, in maize (*Zea mays*) under ozone stress, the mRNA levels of *RCA* in senescent leaves significantly increased in response to the stress [25]. While *RCA* genes have been shown to play roles in plant resistance to high temperatures, ozone, and low light, their functions in response to flooding and salinity stress have not been previously reported. Generally, environmental stress inhibits Rubisco activity, leading to a significant decrease in photosynthetic rates; however, RCA can enhance and maintain Rubisco's catalytic activity to regulate photosynthesis under stress [50,51]. This study found that the expression levels of *KcRCA* in the leaves of *K. candel* significantly increased under flooding and high salinity stress, suggesting that *K. candel* may compensate for the adverse effects of stress on Rubisco activity by increasing RCA expression to maintain normal photosynthesis under environmental stress. These results indicate that *KcRCA* in the leaves of *K. candel* plays a crucial role in resisting flooding and salinity stress, a finding that has been confirmed for the first time in salt-tolerant and flood-tolerant mangrove plants.

4.3. Molecular Characteristics and Functional Roles of Alternatively Spliced Isoforms of RCA in Kandelia Candel

The variable splicing of plant *RCA* genes exhibits significant species diversity. Phylogenetic analysis indicates that different plant groups have evolved unique regulatory mechanisms for *RCA* expression: in most higher plants (such as *Arabidopsis*, rice, spinach, and barley) [21,23,52,53], variable splicing of a single gene produces two isoforms of 41 kDa and 45 kDa; some species (such as tobacco and cucumber) retain only the expression of the smaller isoform [26,27]; while maize has evolved a unique 3' UTR variable splicing mechanism that generates different isoforms through post-translational modifications [54]. Additionally, plants like cotton and soybean have formed two independent *RCA* genes through gene duplication events [47,55]. This study reveals for the first time the variable splicing mechanism of *RCA* in the mangrove plant *K. candel*, identifying two transcripts, *KcRCAl* and *KcRCAs*, produced through alternative splicing. *KcRCAl* forms a smaller β isoform (42.49 kDa) via a premature stop codon (UAA), while *KcRCAs* encodes a complete α isoform (46.10 kDa). Notably, the α isoform contains two highly conserved cysteine residues (Cys247 and Cys392), suggesting its potential involvement in redox signaling. In response to environmental stress, *K. candel* can precisely maintain an α:β ratio of approximately 3:1(Supplymentary Table 6), a regulatory pattern that aligns with the reported functional division mechanism of RCA in rice [23], where the α isoform primarily activates Rubisco while the β isoform is responsible for maintaining its active state. These findings not only expand our understanding of the functional diversity of plant RCA but also provide new theoretical foundations for elucidating the unique adaptation strategies of intertidal plants. However, the specific molecular functions and regulatory networks of the two isoforms require further investigation.

4.4. Biotechnological Potential of RCA Gene Derived from Kandelia Candel for Enhancing Flooding and Salinity Tolerance in Crops

In recent years, *RCA* gene engineering has emerged as a significant strategy for enhancing crop photosynthetic efficiency and stress resistance. Multiple studies have demonstrated that exogenous overexpression of *RCA* genes can significantly improve the photosynthetic performance of transgenic plants [56]. For instance, overexpression of endogenous *RCA* genes in *Arabidopsis* and rice has been shown to enhance photosynthetic efficiency and improve adaptability to temperature stress and low-light environments [57–59]. This study is the first to clone the *RCA* gene from the highly salt- and flood-tolerant mangrove plant *K. candel* and validate its function through a heterologous expression system in *Arabidopsis*, successfully achieving *RCA* gene overexpression in *Arabidopsis* (Figure 4, D, E). The phenotypic analysis demonstrated that the transgenic plants exhibited significantly higher resistance to flooding and salinity stress compared to the wild type (Figure 5, A). Under the same stress duration, the survival rate of the transgenic lines was significantly higher than that of the wild type (Figure 5, B, C). These results indicate that overexpression of the *KcRCA* gene can enhance the tolerance of *Arabidopsis* to flooding and salinity stress. Meanwhile, this study also indicated that transgenic plants exhibited significantly enhanced antioxidant capacity, improved RCA enzyme activity, and better Rubisco activation status, thereby maintaining higher photosynthetic efficiency under stress conditions. These findings provide the first evidence that the *RCA* gene from mangrove plants possesses superior stress response characteristics, offering new high-quality gene resources for crop stress resistance breeding and pioneering a novel approach to crop improvement using genes from plants in unique habitats. Future research could validate the function of *KcRCA* in major crops, elucidate its molecular regulatory network, and develop molecular marker-assisted breeding technologies based on *RCA*. This study provides an important theoretical and technical foundation for utilizing specialized plant gene resources for crop improvement.

5. Conclusions

In the present study, we successfully cloned the *RCA* gene from the mangrove plant *K. candel* and identified two variable splice variants, *KcRCA$_l$* and *KcRCA$_s$*, which encode a β isoform of 440 amino acids and an α isoform of 474 amino acids, respectively. Bioinformatics analysis revealed that both isoforms contain multiple phosphorylation sites and lysine acetylation sites, suggesting potential regulation by post-translational modifications. Phylogenetic analysis indicated that *KcRCA* is most closely related to the *RCA* genes of chickpeas and durians (similarity > 85%). Expression analysis demonstrated that combined flooding and salinity stress significantly induced the upregulation of *KcRCA$_l$* and *KcRCA$_s$* expression. Through a heterologous expression system in *Arabidopsis*, it was confirmed that plants expressing the *KcRCA* gene exhibited significantly enhanced antioxidant capacity, with RCA and Rubisco activities markedly increased, thereby maintaining photosynthetic efficiency under flooding and salinity stress. Our study firstly clarified the molecular mechanism at the molecular level by which mangrove plants adapt to the unique intertidal habitat through the regulation of *RCA* gene expression. It not only expanded the molecular theoretical basis for plant responses to cope with combined flooding and salinity stress, but also provided gene resources of significant application value for the genetic improvement of crops against stress.

structure of KcRAC; Table S4: Primers used for RT-qPCR; Table S5: Primers used for Gateway Cloning (BP reaction); Table S6: The probability of *KcRCAl* and *KcRCAs* in Control and Flooding groups; Table S7: Physicochemical property of *KcRCAl* and *KcRCAs* proteins.

Author Contributions: Y.Z., J.Z. and J.X. conceived and designed the experiments; J.X. performed the gene cloning, transformation experiments, physiological assay, and wrote the manuscript; D.P. collected the plant materials, performed real-time PCR; C.L. analyzed the data; W.C. and S.Y. revised the manuscript critically. All authors contributed to the article and approved the submitted version.

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