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Genome-Wide Analysis of the *Cinnamoyl-CoA Reductase (CCR)* Gene Family in *Rosa chinensis* and *Rosa × hybrida* and Drought Stress Response of Four *RhCCR* Genes

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ABSTRACT: The *cinnamoyl-CoA reductase (CCR)* gene family plays a pivotal role in lignin biosynthesis and plant stress adaptation by catalyzing the first committed step in the monolignol-specific branch of the phenylpropanoid pathway. However, a comprehensive and systematic analysis of CCRs in the economically important *Rosa* genus remains lacking. Here, we conducted a systematic genome-wide investigation of CCR genes in a diploid species, *Rosa chinensis*, and a tetraploid cultivar, *Rosa × hybrida*. We identified 15 and 36 non-redundant CCR genes in *R. chinensis* and *R. × hybrida*, respectively. From these, we selected 14 high-confidence orthologs of *RcCCR* in the *R. × hybrida* genome as a core set for in-depth evolutionary and functional analysis. Our genomic analysis revealed that the expansion of the *RhCCR* family is likely primarily driven by whole-genome and tandem duplication events, with the duplicated gene pairs undergoing strong purifying selection. Promoter analysis of these 14 *RhCCR* genes further revealed a significant enrichment of stress- and hormone-related cis-acting elements. Expression profiling via qRT-PCR uncovered distinct tissue-specific expression patterns among these core genes. Notably, four genes—*RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29*—were significantly upregulated under drought stress (simulated by PEG), methyl jasmonate (MeJA), and abscisic acid (ABA) treatments. Crucially, the induction of these genes by both PEG and MeJA was substantially suppressed by the ABA biosynthesis inhibitor fluridone (FLU). This finding suggests that ABA signaling may play a key role in the drought-responsive regulation of these *RhCCRs*. Furthermore, it raises the possibility that crosstalk between ABA and jasmonate pathways could be involved in modulating stress-responsive lignification, though this remains a hypothetical point. Our findings provide a genomic and functional framework for the CCR family in rose, providing valuable genetic resources and candidate targets for breeding programs aimed at optimizing lignin content and enhanced stress resilience.

KEYWORDS: CCR gene family; *Rosa*; abiotic stress; hormone crosstalk

1 Introduction

Lignin, one of the most abundant organic polymers in nature, serves as a critical long-term carbon sink and fulfills diverse roles in plant growth, development, and environmental adaptation [1,2]. As a key structural component of plant cell walls, it provides essential mechanical strength and rigidity, which are crucial for maintaining vascular tissue integrity, enabling upright growth, and facilitating the efficient transport of water and nutrients [3]. Beyond its structural role, lignin constitutes a vital defensive barrier against both biotic and abiotic stresses. Its biosynthesis proceeds through the oxidative polymerization of three primary monolignols—p-coumaryl, coniferyl, and sinapyl alcohols—catalyzed by a series of specific

enzymes [4]. Among these enzymes, cinnamoyl-CoA reductase (CCR; EC 1.2.1.44) catalyzes the first committed step in the monolignol-specific branch, thereby acting as a central regulator of carbon flux into lignin biosynthesis [5–7]. Belonging to the short-chain dehydrogenase/reductase (SDR) superfamily, CCR mediates the reduction of hydroxy cinnamoyl-CoA esters (e.g., p-coumaroyl-CoA, feruloyl-CoA) to their corresponding aldehydes [8,9]. Beyond its canonical role in lignification, *CCR* genes also participate in plant stress responses. Notably, functional specialization exists among *CCR* isoforms: in *Arabidopsis thaliana*, *AtCCR1* governs developmental lignification [10], while *AtCCR2* is pathogen-induced and contributes to defense by facilitating phenolic compound synthesis [11]. The involvement of *CCR* in biotic stress is further supported by the upregulation of *SbCCR2-2* in sorghum following aphid infestation [12]. Structurally, CCR proteins contain conserved functional motifs, including the KNWYCYGK motif (for NADP(H) binding) and the G-X-X-G-X-X-A/G motif (for substrate catalysis) [5,13]. Notably, the HXXK motif has recently been identified as a more accurate diagnostic feature to distinguish true CCRs from CCR-like proteins [14].

CCR genes typically form multigene families in angiosperms, with duplication events driving functional innovation and adaptive evolution [15,16]. This is reflected in the varying number of *CCR* homologs across species: 115 in *Triticum aestivum* [17], 10 in *Solanum tuberosum* [18], 30 in *Medicago sativa* [19], 11 in *Populus tomentosa* [13], and 10 in *Eucalyptus grandis* [20]. Economically important species like *Dalbergia odorifera* and *Linum usitatissimum* also possess multiple *CCR* homologs [21,22]. Functional divergence among isoforms is common, often manifesting as distinct substrate preferences. For instance, *MtCCR1* in *Medicago sativa* preferentially utilizes feruloyl-CoA, while *MtCCR2* favors caffeoyl-CoA and *p*-coumaroyl-CoA [23]. In flax (*Linum usitatissimum*), *CCR* activity directly influences fiber quality, as excessive lignin impedes the retting process and compromises textile properties, positioning *CCR* regulation as a strategic target for crop improvement [22]. Unlike these cash crops valued for fiber or timber, rose is a premier ornamental species where *CCR*-mediated lignification underpins key horticultural traits. These include the mechanical strength of cut flower stems, postharvest longevity of petals, and the stability of ornamental features under drought stress. Therefore, elucidating the *CCR* family in rose not only addresses evolutionary biology questions but also provides valuable genetic resources as potential targets for breeding programs focused on optimizing stress resilience while preserving ornamental quality.

The *CCR* gene family, with well-established roles in lignification and stress adaptation, represents a compelling yet uncharacterized research frontier in rose (*Rosa* spp.), a globally important ornamental genus. The genus exhibits significant ploidy variation, including diploid species such as *Rosa chinensis* and the widely cultivated allotetraploid hybrid *Rosa* × *hybrida* ($2n = 4x = 28$). This predominant cultivar arose from interspecific hybridization among several diploid species (including *R. chinensis*, *R. gallica*, and *R. moschata*) and subsequent allopolyploidization [24]. This genomic event drove both the diversification of key horticultural traits (e.g., flower size and color) and significant genome expansion with genetic redundancy, creating a unique system to study gene family evolution and functional divergence post-polyploidization [25,26]. The recent availability of high-quality genome sequences for these roses now provides an unprecedented opportunity to investigate the *CCR* family within this dynamic context. However, despite extensive characterization of *CCR* genes in other plant lineages, a systematic comparative analysis within the genus *Rosa*, particularly across its distinct ploidy levels, remains a conspicuous gap in the current literature.

This study presents the first comparative genome-wide analysis of the *CCR* gene family between a diploid rose (*Rosa chinensis*) and its derived allotetraploid cultivar (*R.* × *hybrida*). We identified 15 and 36 *CCR* members in *R. chinensis* and *R.* × *hybrida*, respectively, and systematically characterized their phylogeny, gene structures, conserved motifs, and *cis*-regulatory elements. Collinearity and evolutionary analyses further

elucidated gene duplication events and selective pressures. To move beyond descriptive characterization and directly test the functional divergence following polyploidization, we uniquely integrated evolutionary genomics with functional physiology. We focused on 14 high-confidence *RcCCR* orthologs in the *R. × hybrida* genome—a targeted strategy that mitigates confounding effects from lineage-specific duplications, thereby enabling a clearer reconstruction of evolutionary trajectories and a more interpretable analysis of expression dynamics. Expression profiling of these core orthologs via qRT-PCR revealed distinct tissue-specific patterns and dynamic responses to drought stress and phytohormone treatments. This integrated approach specifically tested the hypothesis that polyploidization facilitated the emergence of stress-responsive *CCR* subclades whose expression is co-modulated by ABA and jasmonate signaling under drought. Consequently, our work not only delineates the evolutionary trajectory of *CCRs* in a polyploid ornamental system but also uncovers novel regulatory crosstalk potentially governing lignin-mediated drought adaptation, offering crucial insights for future breeding strategies aimed at enhancing stress resilience and optimizing horticultural traits in rose.

2 Materials and Methods

2.1 Identification of *CCR* Gene Family Members in *Rosa chinensis* and *Rosa × hybrida*

The reference genome assembly and annotation files for *R. chinensis* ‘Old Blush’ and *R. × hybrida* ‘Samantha’ were obtained from the Ensembl Plants database and the figshare repository [27], respectively (Ensembl Plants; <https://figshare.com/>, both accessed on 10 June 2025). To comprehensively identify all putative *CCR* family members, we employed a multi-step strategy. First, a local protein database was constructed using 17 experimentally validated *CCR* sequences from diverse plant species (Table S1). This database was queried against the proteomes of both *Rosa* species using BLASTP in TBtools-II v2.136 with an E-value cutoff of $1e-5$. Second, to verify the initial hits, we performed a domain-based search using the hidden Markov model (HMM) profile of the NADB_Rossmann superfamily domain (PF01370) from the Pfam database (InterPro, accessed on 16 June 2025) via the “HMM-based Search” function in TBtools-II (v2.136). Finally, all candidate proteins were manually inspected to confirm the presence of characteristic *CCR* motifs, including NWYCY, G-X-X-G-X-X-A/G, and the catalytic H-X-X-K motif.

2.2 Chromosomal Localization

The genomic coordinates of all identified *RcCCR* and *R. × hybrida* ‘Samantha’ *RhCCR* genes were extracted from their respective GFF3 annotation files. These genes were then mapped to their chromosomal positions based on start and end coordinates, and their distribution and density were visualized using the ‘Advanced Circos’ function in TBtools-II (v2.136).

2.3 Phylogenetic Analysis

To elucidate the evolutionary relationships of *CCR* proteins, we performed a phylogenetic analysis using the full-length sequences of all identified *RcCCR* and *RhCCR* proteins, along with functionally characterized *CCRs* from 17 reference plant species. The sequences were aligned using ClustalW with default parameters, and a Neighbor-Joining (NJ) tree was constructed from the alignment in MEGA 11. The tree’s robustness was evaluated with 1000 bootstrap replicates. The final evolutionary tree was visualized and annotated using the Interactive Tree of Life (iTOL; iTOL: Interactive Tree Of Life, accessed on 20 June 2025). A core set of high-confidence *RcCCR*-*RhCCR* orthologs was established to enable clear evolutionary and functional comparisons. Orthologs were defined as gene pairs forming well-supported monophyletic

sister clades (bootstrap $\geq 70\%$) in the phylogenetic tree and further validated as reciprocal best BLAST hits (RBH). This stringent phylogeny-based criterion identified 14 core ortholog pairs.

2.4 Conserved Motifs and Domains Analysis

Conserved motifs in the full-length RhCCR protein sequences were predicted using the online MEME suite (MEME—Submission form; accessed on 2 July 2025) with the following parameters: site distribution set to “any number of repetitions,” motif width range set to 6–50 residues, and the maximum number of motifs to identify set to 10. In parallel, conserved functional domains were annotated using the Conserved Domain Database (CDD) and its associated CD-search tool on the NCBI website (Home—Conserved Domains—NCBI; accessed on 12 July 2025). The resulting phylogenetic tree, conserved motifs, and domain architectures were subsequently integrated and visualized using TBtools-II (v2.136).

2.5 Synteny Analysis

To identify collinear regions containing CCR genes between the *R. chinensis* and *R. × hybrida* genomes, we performed a synteny analysis using the “One-Step MCScanX” tool in TBtools-II (v2.136), with an all-versus-all BLASTP search ($E\text{-value} \leq 1 \times 10^{-5}$) as input. The selective pressure on the duplicated CCR gene pairs identified from this analysis was assessed by calculating their non-synonymous (K_a) and synonymous (K_s) substitution rates. For each pair, protein and coding sequences (CDS) were aligned, and K_a/K_s values were computed using the MA model in KaKs Calculator 2.0, with the ratio used to infer the mode of selection.

2.6 Promoter Cis-Acting Element Analysis

To investigate potential transcriptional regulatory mechanisms, the promoter sequences (defined as the 2.0 kb genomic region upstream of the translation start site, ATG) of the identified RhCCR genes—which are homologous to RcCCR genes—were extracted and analyzed for cis-acting regulatory elements using the PlantCARE database (<https://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 26 July 2025). Predicted elements were carefully filtered to exclude low-confidence annotations, and the final results were systematically visualized using TBtools-II (v2.136).

2.7 Plant Materials and Treatments

Tissue-cultured plantlets of *R. × hybrida* cv. ‘Samantha’ (plant materials provided by China Agricultural University) were grown in a controlled environment at 25°C with 40% relative humidity, a 16 h/8 h light/dark cycle, and a light intensity of $200 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. For tissue-specific expression profiling, samples were collected from the root tips (1–2 cm segments), stems, fully expanded functional leaves (harvested from the 3rd to 4th node), and petals of healthy, uniformly grown plants at the same developmental stage.

To investigate responses to abiotic stress, uniformly grown plantlets were divided into the following treatment groups: (1) a control group (CK), sprayed with distilled water; (2) drought stress simulated by spraying with 20% (w/v) polyethylene glycol 6000 (PEG-6000); (3) phytohormone treatments consisting of foliar sprays of 50 μM methyl jasmonate (MeJA) or 50 μM abscisic acid (ABA), and (4) combined treatments involving MeJA + PEG, FLU + PEG, ABA + PEG, and MeJA + FLU + PEG, where FLU denotes the ABA biosynthesis inhibitor fluridone (50 μM). For the combined treatments, plantlets were first sprayed with the respective hormone or inhibitor solution and, after a 12-h interval, were subjected to drought stress via root drenching with 20% PEG-6000. Tissue samples were collected at 0, 12, 24, and 48 h after the initiation

of PEG-6000 treatment (or at corresponding time points for non-PEG groups). Each treatment and time point included three independent biological replicates.

2.8 Determination of Lignin, Malondialdehyde, and Relative Water Content

Lignin content was quantified using a modified Klason method [28]. Briefly, 10 mg of dried sample powder was reacted with 1 mL of 25% (v/v) acetyl bromide in glacial acetic acid at 70°C for 30 min. After cooling, the reaction was terminated with 2 mL of 0.5 M hydroxylamine hydrochloride and brought to a final volume of 10 mL with glacial acetic acid. The absorbance of the supernatant was measured at 280 nm after centrifugation, and the lignin concentration was calculated using a standard curve derived from dealkalized lignin ($A = 19.576 \times C - 0.1475$, $R^2 = 0.9914$, Fig. S1).

Malondialdehyde (MDA) content was determined via a modified thiobarbituric acid (TBA) method [29]. A total of 0.4 g of leaf tissue was homogenized in 5 mL of 0.1% trichloroacetic acid (TCA), with the final volume adjusted to 10 mL before centrifugation (4°C, 4000 rpm, 15 min). Then, 3 mL of the supernatant was mixed with an equal volume of 0.5% TBA, heated in a boiling water bath for 15 min, and rapidly cooled. After centrifugation, the absorbance was measured at 450, 532, and 600 nm. MDA concentration was calculated as: $[6.45 \times (OD_{532} - OD_{600}) - 0.56 \times OD_{450}] \times (V_t / (V_s \times W))$, where V_t = 10 mL (total volume), V_s = 3 mL (supernatant volume), and W = 0.4 g (sample fresh weight).

Leaf relative water content (RWC) was measured following Barrs and Weatherley [30]. Briefly, leaf discs were first weighed to obtain fresh weight (FW). They were then fully hydrated by floating on distilled water at 4°C for 12 h in darkness, after which surface moisture was gently removed with filter paper and the turgid weight (TW) was recorded. Subsequently, the samples were oven-dried at 80°C for 24 h to a constant weight, yielding the dry weight (DW). The RWC was calculated using the standard formula: $[(FW - DW) / (TW - DW)] \times 100\%$. All measurements were performed with three independent biological replicates.

2.9 RNA Extraction and Quantitative Real-Time PCR Analysis

Total RNA was extracted from liquid nitrogen-ground tissues (roots, stems, leaves, and flowers) of *R. × hybrida*, as well as from leaves collected at 0, 12, 24, and 48 h post-treatment, using the SteadyPure Plant RNA Extraction Kit. RNA quality and concentration were assessed on a Pultton P100+ ultra-micro spectrophotometer. First-strand cDNA was synthesized from 1 µg of total RNA using the Evo M-MLV RT Premix for qPCR (37°C for 15 min, 85°C for 5 s). Gene-specific primers were designed with Primer Premier 5.0, and quantitative real-time PCR was performed on a LightCycler 480 system using SYBR Green Pro Taq HS Premix in a 20 µL reaction volume. The *RhACTIN* gene (GenBank: AB239794) was used as the reference, and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method with three biological replicates. The primers utilized in this work are detailed in Table S2.

2.10 Statistical Analysis

All experimental data were derived from three independent biological replicates. Statistical analyses were performed using SPSS software (version 22.0, IBM Corp., Armonk, NY, USA). Differences among treatment groups or across time points were evaluated by one-way analysis of variance (ANOVA), and significant differences between specific means were further compared using Duncan's multiple range test. A probability value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Genome-Wide Identification, Characterization, and Phylogenetic Analysis of the CCR Gene Family in *Rosa chinensis* and *Rosa × hybrida*

A combined BLAST and HMM search strategy, supplemented with screening for the conserved CCR signature motif (H-X-X-K), identified 15 and 36 CCR genes in the genomes of *R. chinensis* and *R. × hybrida*, respectively. All identified genes were systematically named according to their chromosomal locations (Fig. 1). Sequence alignment confirmed the universal presence of the conserved CCR substrate-binding motif (CCR-SBM, H202(X)₂K205) across all predicted RcCCR and RhCCR protein sequences (Fig. S2).

Chromosomal localization revealed that the RcCCR and RhCCR genes were predominantly distributed in the upper and middle regions of the chromosomes. In *R. chinensis*, the 15 RcCCR genes were unevenly localized to three chromosomes: Chr1 (11 genes), Chr7 (3 genes), and Chr2 (1 gene). In contrast, the 36 RhCCR genes in *R. × hybrida* were dispersed across ten chromosomes, with the majority (21 genes) clustered on Chr1B, Chr1C, and Chr1D. The remaining genes were distributed on Chr2A, Chr2C, and Chr2D (3 genes) and on Chr7A, Chr7B, Chr7C, and Chr7D (12 genes). This uneven, cluster-like distribution suggests that tandem duplication events may have contributed to the expansion of the CCR gene family.

Considerable variation was observed in the lengths of the encoded RcCCR and RhCCR proteins. Among the 15 core RcCCR proteins analyzed in detail, RcCCR12 was the longest (340 aa) and RcCCR7 was the shortest (190 aa), with an average length of 311 aa. For the 36 RhCCR proteins, RhCCR6 was the longest (611 aa) and RhCCR8 was the shortest (234 aa), yielding an average length of 322 amino acids (aa) (Table S3).

A Neighbor-Joining (NJ) phylogenetic tree was constructed using CCR protein sequences from *R. chinensis*, *R. × hybrida*, and 17 reference species (Fig. 2). The 68 CCR proteins were classified into three distinct subfamilies: Group I (30 members), Group II (23 members), and Group III (15 members). Phylogenetic analysis and BLAST searches identified 14 high-confidence orthologs of RcCCR in the *R. × hybrida* 'Samantha' genome (highlighted in red, Fig. 2). To minimize complexity from recent duplications in the tetraploid genome and to clarify the core biological functions of CCRs, subsequent analyses focused on this set of 14 orthologs.

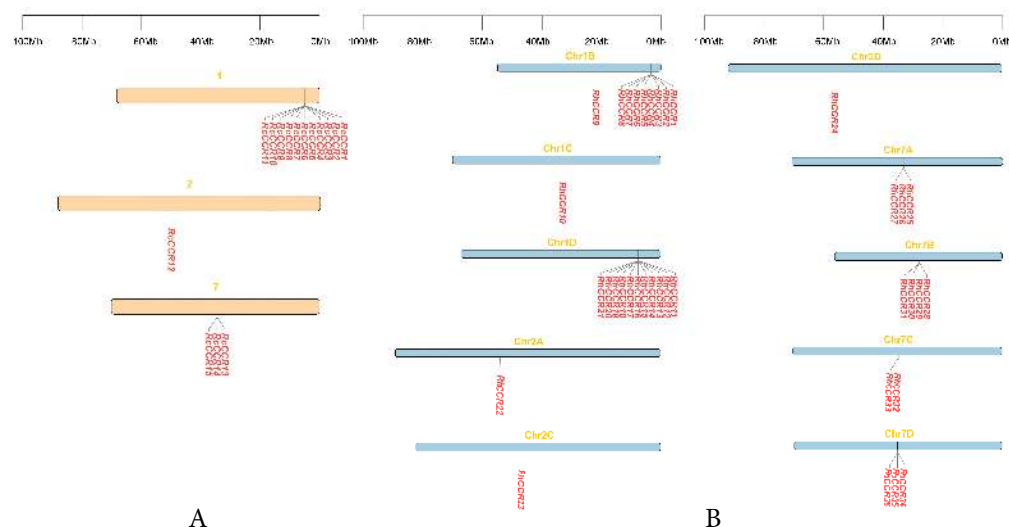


Figure 1: Chromosomal locations of cinnamoyl-CoA reductase (CCR) related genes in *Rosa chinensis* and *Rosa × hybrida*. (A) shows the chromosomal localization of CCR genes in *Rosa chinensis* (yellow), and (B) shows the chromosomal localization of CCR genes in *R. × hybrida* (blue). Chromosome numbers are indicated on the left, gene names are highlighted in red.

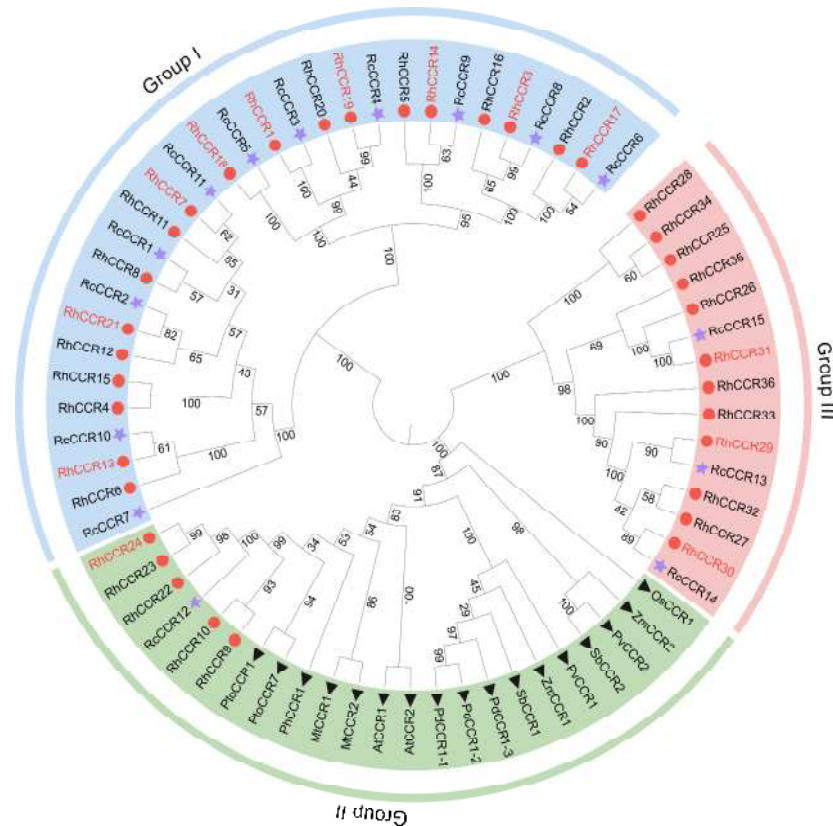


Figure 2: Subfamily classification of CCR proteins reveals three distinct clades. The phylogenetic tree, comprising CCRs from *Rosa chinensis* (purple stars), *Rosa × hybrida* (red circles), and other plants (black triangles), is divided into three subfamilies, as indicated by the colored backgrounds.

3.2 Conserved Motifs and Domains in the CCR Genes of *Rosa chinensis* and *Rosa × hybrida*

To elucidate the structural and evolutionary relationships of the CCR gene family in rose, we integrated phylogenetic analysis with conserved motif and domain architecture assessments (Fig. 3). The phylogenetic tree of all RcCCR and RhCCR proteins clearly separated them into three distinct clades (Fig. 3A), providing an evolutionary framework for structural comparison.

Conserved motif analysis identified ten motifs, with Motif 1, Motif 2, and Motif 3 present in all members (Fig. 3B). Notably, Motif 1 contains the G-X-X-G-X-X-G/A sequence, which contributes to the Rossmann fold for NADPH-binding—a signature feature essential for CCR reductase activity. The motif distribution strongly corroborates the phylogenetic classification, as proteins within the same clade exhibit highly similar motif compositions, underscoring the evolutionary conservation at the sequence level.

Domain architecture analysis identified four conserved superfamilies: NADB-Rossmann, PLN02662, FR-SDR-e, and PLN00198 (Fig. 3C). The variation in domain composition across phylogenetic clades suggests potential functional divergence among lineages. Collectively, the congruent patterns observed in phylogenetic grouping, motif architecture, and domain organization provide robust evidence delineating the evolutionary trajectory of the CCR family in *Rosa*. These findings indicate that while a core structural framework is conserved within each lineage, lineage-specific modular variations may underpin functional specialization.

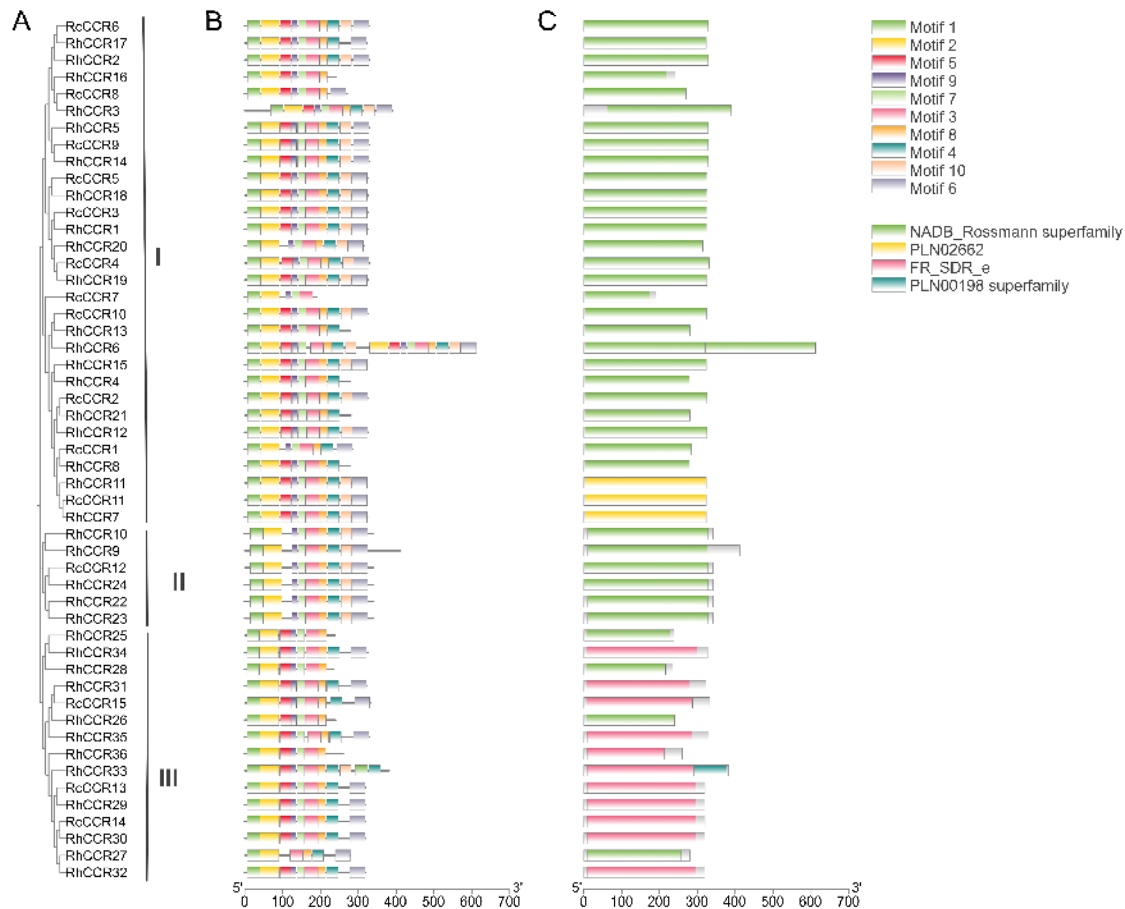


Figure 3: Phylogenetic tree, motifs, and domains of RcCCR and RhCCR proteins. **(A)** Phylogenetic tree of RcCCR and RhCCR proteins. **(B)** Motif composition of RcCCR and RhCCR proteins. **(C)** Domain architecture of RcCCR and RhCCR proteins.

3.3 Synteny Analysis of *Rosa chinensis* and *Rosa × hybrida*

Synteny analysis revealed extensive conserved collinear blocks between the *R. chinensis* and *R. × hybrida* genomes (Fig. 4), within which 12 putative collinear CCR gene pairs were identified. Notably, in two of these pairs, the orthologous genes in *R. × hybrida* were not originally annotated as CCRs, likely due to mutations within key conserved motifs essential for enzymatic function. This finding suggests that following duplication, some paralogs may have undergone neofunctionalization or pseudogenization.

Among the remaining 10 high-confidence CCR ortholog pairs, three RcCCR genes were found to correspond to multiple paralogs in *R. × hybrida*, indicating lineage-specific gene expansions driven by duplication events. To assess the evolutionary pressures acting on these genes, the nonsynonymous (Ka) and synonymous (Ks) substitution rates were calculated for all 12 duplicated pairs (Table S4). The resulting Ka/Ks ratios were significantly less than 1 for all pairs, demonstrating that the CCR gene family in *Rosa* is predominantly under strong purifying selection, which acts to maintain functional stability.

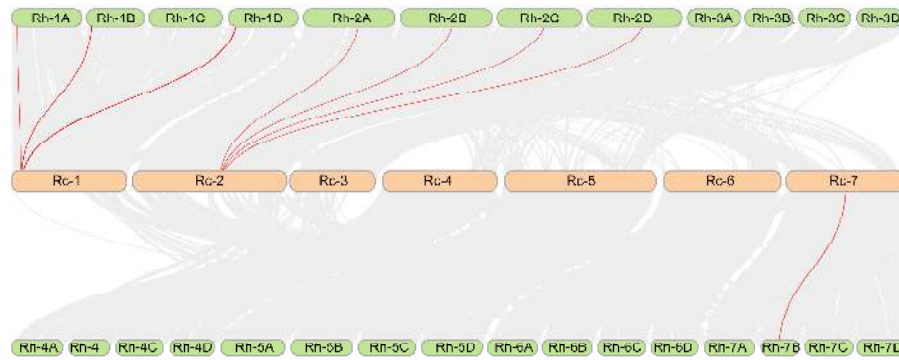


Figure 4: Synteny Analysis of CCR Genes between *Rosa chinensis* and *Rosa × hybrida*. Green represents *R. chinensis* chromosomes, yellow represents *R. × hybrida* chromosomes. The gray lines in the background depict syntenic blocks between *R. chinensis* and *R. × hybrida*, while the red highlighted lines indicate syntenic CCR gene pairs.

3.4 Analysis of Cis-Acting Elements in the Promoters of RhCCR Genes (Orthologs of RcCCR)

Analysis of the 2.0 kb promoter regions of the 14 *RcCCR*-homologous *RhCCR* genes reveals a strong enrichment of cis-acting elements associated with stress and hormone responses (Fig. 5). Hormone-responsive elements were predominant, with MeJA-responsive CGTCA/TGACG-motifs (25 of each) being the most abundant, present in 11 genes. ABREs (18 total) for ABA response were found in 8 genes, suggesting potential crosstalk between JA and ABA signaling. Stress-related elements, including ARE (31 instances in 10 genes) and the drought-inducible MBS (12 instances in 8 genes), were also frequently detected. Notably, four genes—*RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29*—exhibited pronounced co-enrichment of ABRE, CGTCA/TGACG-motif, and ARE or MBS elements, highlighting them as key candidates for integrated stress and hormone signaling. In contrast, the other 10 core *RcCCR* orthologs contained relatively low abundances of these stress- and hormone-related elements, or were only enriched for one or two types. These results suggest a regulatory network in which specific *RhCCR* genes respond to drought stress via ABA and JA pathways, a premise subsequently validated by our expression analyses.

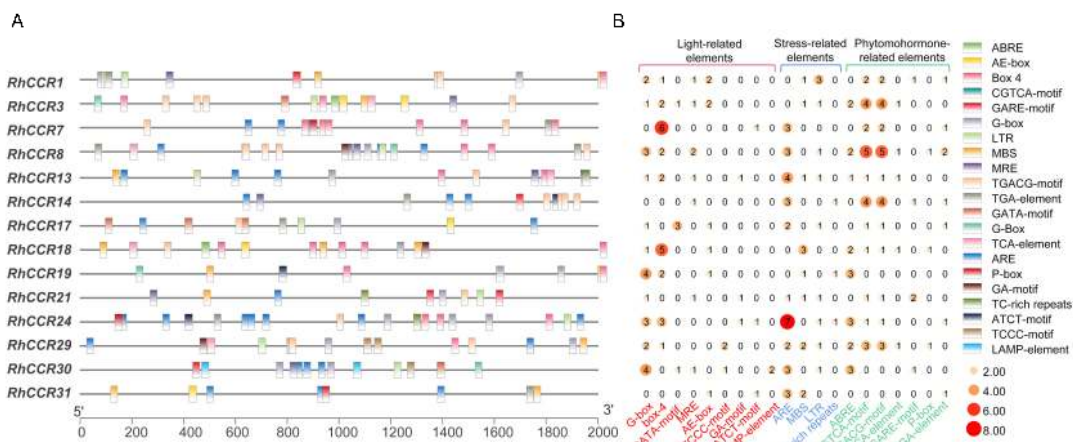


Figure 5: Analysis of cis-acting elements in the promoters of *RhCCR* genes (orthologs of *RcCCR*). (A) Distribution of cis-acting elements in the promoter regions of *RhCCR* genes homologous to *RcCCR*. (B) Heatmap depicting the number of cis-acting elements in the promoter sequences. The color scale from white to red represents the count of elements, ranging from 0 to 8. From left to right, the three distinct types of cis-acting elements are shown: Light-responsive (red), Stress-responsive (blue), and Hormone-responsive (green).

3.5 Systematic Expression Profiling of *RhCCR* Genes (*RcCCR* Orthologs) by qRT-PCR

We profiled the expression of the 14 core *RhCCR* orthologs across four major tissues (root, stem, leaf, flower) and at three defined leaf developmental stages (S1: pre-flowering, S2: flowering, S3: post-flowering) via qRT-PCR (Fig. 6). These core orthologs display distinct tissue-specific expression patterns, indicating substantial functional diversification within the gene family.

Among these genes, *RhCCR8*, *RhCCR14*, *RhCCR17*, *RhCCR18*, *RhCCR19*, and *RhCCR30* were preferentially expressed in roots, suggesting key roles in root lignification. In contrast, *RhCCR13*, *RhCCR24*, and *RhCCR31* exhibited peak expression in stems, supporting their predominant role in secondary cell wall formation. Furthermore, *RhCCR21* and *RhCCR29* were the dominant isoforms in leaves, whereas *RhCCR1*, *RhCCR3*, and *RhCCR7* showed the highest expression in floral organs, implying involvement in the synthesis of specialized secondary metabolites. Collectively, the pronounced expression of these core *RhCCR* genes in root and stem vascular tissues strongly aligns with their canonical function in lignin biosynthesis.

Dynamic expression analysis across leaf development revealed that most core *RhCCR* genes were significantly modulated by the flowering process. Their transcript levels increased progressively from the pre-flowering (S1) to post-flowering (S3) stage, peaking during senescence—a trend potentially linked to enhanced lignification. Notably, *RhCCR14*, *RhCCR18*, *RhCCR19*, and *RhCCR29* were already strongly induced at full flowering (S2), indicating heightened sensitivity to flowering-phase physiology. In contrast, *RhCCR7* maintained low, stable expression across all stages, suggesting its functions are largely independent of these developmental cues.

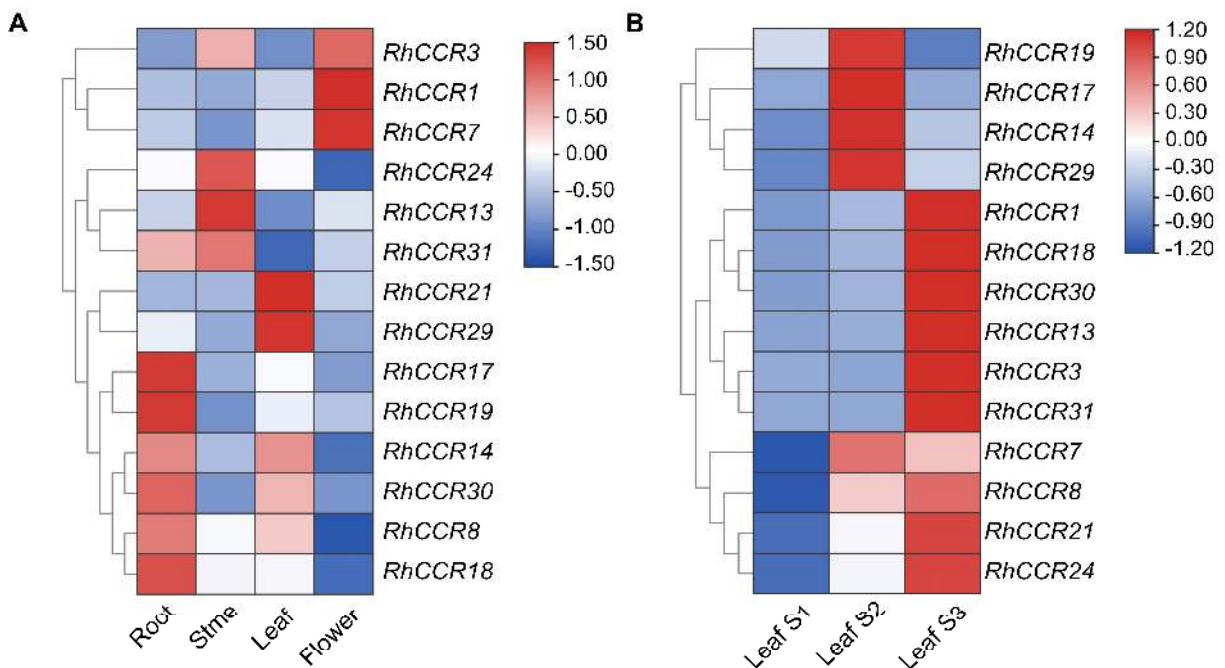


Figure 6: Tissue-specific and developmental expression profiles of *RcCCR* Orthologs in *Rosa × hybrida*. The color gradient from blue (low expression) to red (high expression) represents relative expression levels normalized by Z-score. (A) Expression levels of *RhCCR* genes in root, stem, leaf, and flower tissues. (B) Expression dynamics of *RhCCR* genes in leaves at three developmental stages: S1 (pre-flowering), S2 (flowering), and S3 (post-flowering).

3.6 qRT-PCR Analysis of *RhCCR* Genes (*RcCCR* Orthologs) Expression in Response to Abiotic Stress and Hormones

Based on promoter cis-acting element analysis, we selected four genes—*RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29*—for further investigation. Their promoters are enriched with abiotic stress and hormone-responsive elements (ABRE, MBS, and CGTCA-motif), implying potential roles in stress responses. To elucidate their transcription regulation under stress, *R. × hybrida* ‘Samantha’ seedlings were subjected to eight treatments: Control, ABA, MeJA, PEG, FLU + PEG, ABA + PEG, MeJA + PEG, and FLU + MeJA + PEG.

qRT-PCR analysis reveals broadly consistent induction patterns for the four *RhCCR* genes under stress (Fig. 7). All were significantly upregulated by ABA, MeJA, and PEG treatments relative to the control. However, the ABA biosynthesis inhibitor FLU substantially suppressed this induction by both PEG and MeJA. Among them, *RhCCR3* and *RhCCR24* exhibited the strongest synergistic induction by ABA and drought (PEG), reaching ~7-fold higher levels than the control at 48 h. The suppressive effect of FLU was most pronounced during early stress (12–24 h). These results demonstrate that *RhCCR* genes are integrated into a stress-responsive transcriptional network co-modulated by ABA and jasmonic acid signaling, and that MeJA-induced upregulation is potentially linked to ABA biosynthesis.

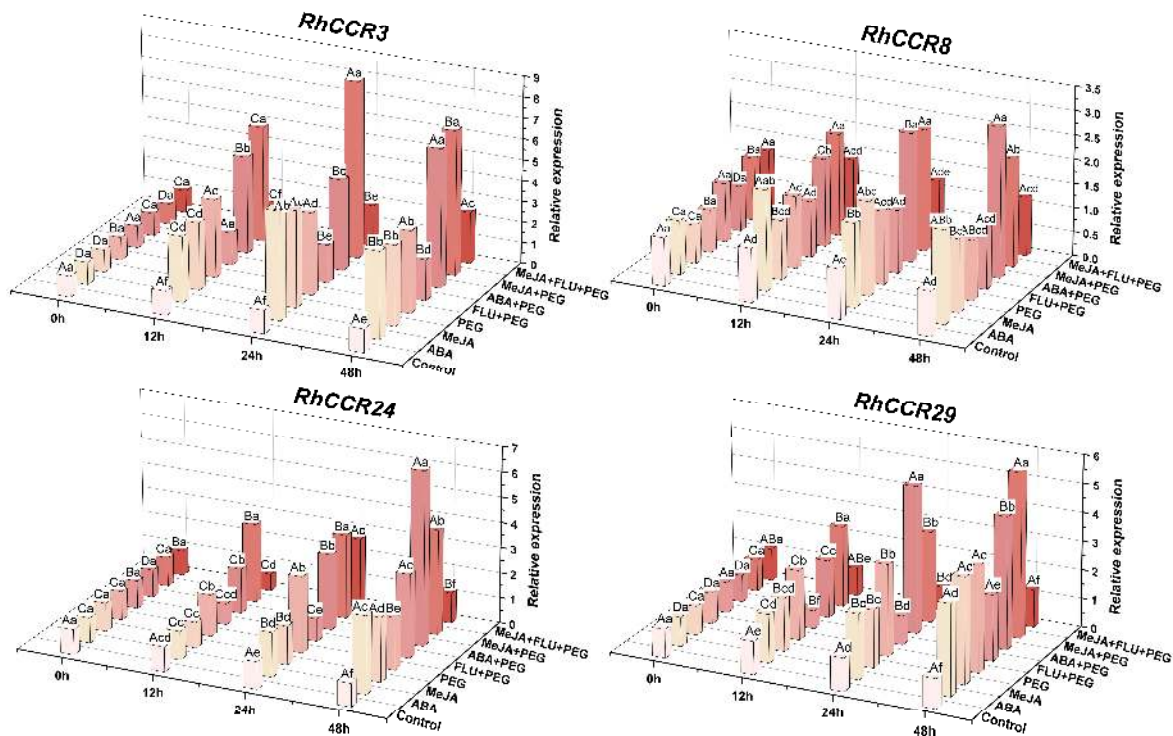


Figure 7: Expression dynamics of *RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29* in response to drought and hormone treatments. The three-dimensional bar plot shows relative expression levels (z-axis, normalized by the $2^{-\Delta\Delta CT}$ method) across different treatments (y-axis) and time points (x-axis). Treatments included a control, abscisic acid (ABA), methyl jasmonate (MeJA), polyethylene glycol (PEG; drought simulator), and their combinations with the ABA biosynthesis inhibitor fluridone (FLU). Uppercase letters indicate significant differences among time points within the same treatment, while lowercase letters denote significant differences among different treatments at the same time point. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test ($p < 0.05$).

3.7 ABA and Jasmonate Signaling Enhance Drought Tolerance through Lignin Deposition and Oxidative Stress Alleviation in *Rosa* × *hybrida*

Physiological responses of *R. × hybrida* ‘Samantha’ to abiotic stress and hormone treatments were assessed by quantifying lignin content, MDA levels, and RWC at 48 h post-treatment across eight experimental conditions (Table 1). Compared to the control, individual treatments with ABA or PEG-6000 significantly induced lignin accumulation. This induction was further enhanced under combined ABA + PEG and MeJA + PEG treatments but was markedly suppressed when the ABA biosynthesis inhibitor fluridone (FLU) was co-applied with PEG (i.e., in FLU + PEG and FLU + MeJA + PEG groups). Concurrently, PEG-induced drought stress increased MDA content, indicating oxidative membrane damage, and decreased RWC, reflecting cellular water deficit. Exogenous application of ABA or MeJA effectively mitigated these adverse effects, leading to significantly lower MDA accumulation and higher RWC. In contrast, FLU application exacerbated the PEG-induced damage, resulting in elevated MDA and reduced RWC. Collectively, our results demonstrate that ABA and MeJA treatments are associated with improved physiological parameters under drought stress, including increased lignin content, reduced MDA levels, and maintained relative water content. These changes coincide with the upregulation of key *RhCCR* genes. This correlation is consistent with a potential role for *CCR*-mediated lignin biosynthesis in the observed physiological responses, although further functional studies are needed to determine whether this pathway contributes to drought adaptation.

Table 1: Effects of plant hormones and drought stress on lignin content, MDA level, and RWC in *Rosa* × *hybrida* ‘Samantha’ leaves.

Treatment	Lignin Content (mg/g dry biomass)	MDA ($\mu\text{mol}\cdot\text{g}^{-1}$ FW)	RWC (%)
Control	230.19 $\pm$ 2.35d	28.33 $\pm$ 0.23e	80.39 $\pm$ 0.51ab
ABA	246.85 $\pm$ 2.43c	26.45 $\pm$ 0.02f	82.84 $\pm$ 0.55ab
MeJA	233.06 $\pm$ 3.18d	28.38 $\pm$ 0.17e	80.67 $\pm$ 0.47ab
PEG	251.12 $\pm$ 5.79c	33.43 $\pm$ 0.55c	77.86 $\pm$ 0.72b
FLU + PEG	211.02 $\pm$ 7.09e	44.15 $\pm$ 0.37a	67.84 $\pm$ 3.31c
ABA + PEG	265.97 $\pm$ 1.25b	30.98 $\pm$ 0.40d	79.15 $\pm$ 1.06ab
MeJA + PEG	287.46 $\pm$ 1.45a	28.73 $\pm$ 0.37e	79.05 $\pm$ 0.59ab
MeJA + FLU + PEG	230.47 $\pm$ 7.64d	37.95 $\pm$ 0.22b	67.33 $\pm$ 1.38c

Data are mean $\pm$ standard deviation. Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Duncan’s multiple range test ($p < 0.05$). Lowercase letters within the same column denote significant differences between treatments.

4 Discussion

The *CCR* gene family plays a central role in lignin biosynthesis and stress adaptation across diverse plant species [5,31]. However, despite the global economic and ornamental significance of the genus *Rosa*, comprehensive analyses of its *CCR* family—particularly across different ploidy levels—remain conspicuously limited. To address this gap, we conducted the first genome-wide comparative analysis of *CCR* genes in diploid *R. chinensis* and its allotetraploid derivative, *R. × hybrida*.

The *CCR* family expanded in tetraploid *R. × hybrida* (36 members) compared to diploid *R. chinensis* (15 members), likely driven by whole-genome duplication (WGD) and tandem duplication (TD) [24,32,33]. Synteny analysis supports this finding, with multiple *RhCCR* genes corresponding to single *RcCCR* orthologs—a signature of allopolyploid species [34]. Most duplicated pairs are under strong purifying selection ($K_a/K_s < 1$), indicating functional conservation [35,36]. Structurally, all identified *RcCCR* and *RhCCR* proteins contain the

SDR superfamily hallmarks, including the NADPH-binding motif (G-X-X-G-X-X-A/G) [5,13,37]. Importantly, we applied the phylogenetically conserved CCR substrate-binding motif CCR-SBM [14], confirming their identity as bona fide CCRs. Phylogenetic analysis clustered rose CCRs into three distinct subfamilies. The conserved motif architecture shared within each subfamily suggests underlying functional conservation, a pattern consistent with observations in *Populus* [13], *Medicago* [23], and *Dalbergia odorifera* [21].

The tissue-specific expression patterns of *RhCCR* genes reflect their functional diversification (Fig. 6). Elevated expression of multiple *RhCCR* members in roots and stems aligns with their canonical role in developmental lignification, a process essential for providing mechanical support and facilitating long-distance water transport [5,38]. Conversely, the specific expression of other *RhCCR* genes in floral organs suggests neofunctionalization, potentially contributing to the synthesis of specialized phenolic compounds related to pigmentation or defense [7]. The dynamic upregulation of most *RhCCR* genes during leaf senescence (S1 to S3 stages) likely reflects programmed lignification associated with organ maturation, orchestrated by NAC/MYB transcription factors as previously reported in Arabidopsis [39,40]. Beyond development, *RhCCR* genes contribute to drought adaptation by regulating stress-induced lignin deposition and antioxidant capacity modulation (Table 1). Drought stress induced increased lignin deposition and altered antioxidant capacity—processes that enhance cell wall hydrophobicity and mitigate oxidative damage to improve stress tolerance [41,42]—a pattern consistent with the hypothesis that *RhCCR* genes contribute to these physiological adjustments critical for preserving rose ornamental quality under water deficit.

Consistent with findings in *Medicago sativa* [19] and *Dalbergia odorifera* [21], promoter analysis revealed that the promoter regions of the 14 high-confidence *RhCCR* orthologs are enriched with cis-acting elements associated with stress and hormone signaling. This bioinformatic prediction was strongly validated by qRT-PCR analysis under PEG-simulated drought stress, which identified four key genes—*RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29*—that were significantly upregulated in response to drought, ABA, and MeJA treatments. This co-induction pattern indicates that these *RhCCR* genes are integrated into both abiotic stress and phytohormone signaling pathways. The concomitant increase in lignin content under these treatments further supports enhanced lignification as a key physiological response associated with drought adaptation in rose, likely reinforcing cell walls and reducing transpirational water loss. A key insight from hormone perturbation experiments is that ABA signaling is critically involved in *RhCCR*-mediated lignification under both drought and JA treatments. The application of the ABA biosynthesis inhibitor FLU substantially suppressed both drought- and MeJA-induced upregulation of *RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29* (Fig. 7), as well as the concomitant increase in lignin content (Table 1). This indicates that an intact ABA biosynthetic pathway is necessary for the full transcriptional activation of these genes in response to both drought and MeJA. Based on these observations, we propose a hypothetical model in which MYB transcription factors [43,44] integrate ABA and JA signals to activate *RhCCR* expression. Moreover, although the one-way ANOVA was effective in detecting overall differences among treatment groups in this study, it cannot reveal the complex interactive effects among ABA, MeJA, and FLU. This analytical limitation means our results primarily characterize the main effects of individual treatments on *RhCCR* expression and associated physiological traits, rather than quantifying their potential synergistic or antagonistic interactions. Future studies employing factorial experimental designs analyzed by two-way ANOVA will be necessary to fully dissect these complex regulatory networks.

This study experimentally demonstrates that the CCR gene family in rose has undergone significant expansion, primarily driven by polyploidization and subsequent duplication events, leading to considerable functional diversification. Individual members have evolved specialized roles in both developmental lignification and stress-responsive pathways. Our study reveals that ABA and JA signaling coordinately

regulate *RhCCR* genes under drought stress and MeJA treatment. This finding points to a potential crosstalk between these two pathways in modulating stress-responsive lignification in ornamental plants, although this proposed interaction requires further validation. This integrated perspective advances our understanding of *CCR* family evolution in a polyploid context and pinpoints specific *RhCCR* genes as prime candidates for molecular breeding. Future efforts can build upon these findings to enhance drought resilience—an increasingly critical objective in light of global climate change [45,46]—and to precisely fine-tune lignin content for the improvement of key horticultural traits in rose cultivars [47].

5 Conclusion

This study presents the first comprehensive genomic and functional analysis of the *CCR* gene family in diploid (*R. chinensis*) and tetraploid (*R. × hybrida*) roses, elucidating its evolutionary history and functional diversification. Comparative genomic analyses revealed a marked expansion of the *CCR* family in the tetraploid genome, primarily driven by whole-genome and tandem duplication events following polyploidization. While the family is under strong purifying selection to conserve its core enzymatic function, the resultant genetic redundancy has facilitated functional divergence, as reflected in the distinct, tissue-specific expression patterns of individual *RhCCR* genes. Notably, we identified key members—notably *RhCCR3*, *RhCCR8*, *RhCCR24*, and *RhCCR29*—whose expression is closely linked to abiotic stress responses. A pivotal mechanistic insight from the current study is the delineation of a potential hormone-regulated network associated with stress-responsive lignification: Our data demonstrate that the drought- and MeJA-induced upregulation of these critical *RhCCR* genes is likely dependent on ABA signaling, as their induction was effectively suppressed by the ABA biosynthesis inhibitor FLU. These results suggest that ABA-JA signaling pathways interact to integrate environmental cues for lignin biosynthesis under stress conditions. This interaction may represent a sophisticated regulatory module involving crosstalk between the two pathways, a hypothesis that requires direct functional validation. Collectively, our findings advance the understanding of gene family evolution in polyploid ornamentals and establish a molecular framework for the targeted genetic improvement of roses. The identified *RhCCR* genes, especially those coregulated by ABA and JA, represent prime candidates for developing novel rose cultivars with enhanced drought resilience and optimized lignin properties, thereby improving horticultural performance and sustainability.

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Author Contributions: Weibiao Liao and Hua Fang conceived and designed the experiments. Cuifang Chang, Zhongfeng Yao and Qi Wang performed the experiments. Cuifang Chang and Caicai Ma analyzed the data. Xinfang Chen, Yali Zhu, and Weibiao Liao provided relevant professional knowledge. Cuifang Chang and Weibiao Liao wrote the paper. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: All data included in this work are available upon request by contact with the corresponding author.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest.

Supplementary Materials: The supplementary material is available online at <https://www.techscience.com/doi/10.32604/phyton.2026.077290/s1>. Figure S1: A standard curve of acetylated lignin solution. Figure S2: Alignment of

RcCCR and *RhCCR* proteins. Table S1: The sequence used in query sequence. Table S2: Primers for RT-qPCR. Table S3: Members of the *RcCCR* and *RhCCR* families. Table S4: Ka and Ks values for homologous gene pairs of the *CCR* gene family in *Rosa chinensis* and *Rosa × hybrida*.

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