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RESEARCH ARTICLE

Developmental- and stress-mediated expression analysis of cinnamoyl-CoA reductase 1 (CCR1) from *Hibiscus cannabinus*

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Cinnamoyl-CoA reductase (CCR, EC 1.2.1.44) is an important enzyme responsible for lignin biosynthesis in plants that belongs to the family of oxidoreductases. We analyzed developmental, tissue specific, and stress-mediated expression of the *HcCCR1* (HM151381) gene from *Hibiscus cannabinus*. Gene expression analysis revealed that *HcCCR1* was highly upregulated in mature leaves of 16-week-old plants. The maximum downregulation and upregulation of *HcCCR1* was caused by cold and MeJA treatment, respectively. Sequence analysis demonstrated that *HcCCR1* protein (ADK24219) contains a conserved NWYCYGK catalytic domain, while bioinformatics prediction indicated the presence of a palmitoylation site in the *HcCCR1* protein. Phylogenetic analysis showed that *HcCCR1* is more closely related to *HcCCR2* (AGJ84130) and *AtCCR* proteins than CCR-like proteins. Comparative sequence analysis showed presence of significant differences between *HcCCR1* and *HcCCR2*, which are homologs of *H. cannabinus*. Expression analysis demonstrated that the *HcCCR1* gene is modulated by different external stresses.

Keywords: cinnamoyl-CoA reductase; catalytic domain; palmitoylation; abiotic stress

Abbreviations: *HcCCR*, *Hibiscus cannabinus* cinnamoyl-CoA reductase; CCR, cinnamoyl-CoA reductase; pI, isoelectric point; MeJA, methyl jasmonate; SA, salicylic acid; ABA, abscisic acid; H₂O₂, hydrogen peroxide; TMHMM, transmembrane domain prediction by hidden Markov model; PAL, phenylalanine ammonia-lyase; 4CL, 4-hydroxycinnamoyl CoA ligase; C3H, *P*-coumarate 3-hydroxylase; CCoAOMT, caffeoyl CoA O-methyltransferase; F5H, ferulate 5-hydroxylase; CAD, cinnamyl alcohol dehydrogenase.

Introduction

Lignin is the second most abundant biopolymer after cellulose and an important constituent of plant secondary cell walls that provides rigidity, hydrophobicity, and mechanical support to plant cells (Boerjan et al. 2003; Raes et al. 2003). Lignin creates a strong mechanical barrier against pathogen invasion and plays important roles in protecting against wounding and other abiotic stresses (Raes et al. 2003; Moura et al. 2010). Lignin creates large problems during pulp and biofuel production and hampers forage digestibility by cattle and other ruminants (Baucher et al. 2003; Weng et al. 2008). There have been many efforts to decrease lignin contents or change its composition by developing genetically modified plants. Lignin is produced by the phenylpropanoid pathway (Mohanta et al. 2012; Mohanta, 2012), which is very complex and involves role of multiple enzymes. Lignin biosynthesis primarily consists of two major steps, monolignol biosynthesis and cross-linking of the monolignols by peroxidases and laccases (Xu et al. 2009). Cinnamoyl-CoA reductase (CCR) is an important enzyme for lignin biosynthesis that catalyzes the first committed step in monolignol biosynthesis (Zhou et al. 2010). Hence, it can be a very good target for genetically

modified plants with reduced lignin contents. Genes related to lignin biosynthesis belong to diverse multigene families that produce several multifunctional enzymes. This diversity is found from lower plants to higher plants. A recent study showed that CCR is the most expanded gene family (Xu et al. 2009), and all CCR can be classified as CCR and CCR-like genes, which are diverse in their function, spatiotemporal expression, and biochemical properties (Raes et al. 2003; Barakat et al. 2011). The preferences for different substrates of various CCR isoenzymes diversify its functions. *Arabidopsis AtCCR1* is involved in developmental lignification, whereas *AtCCR2* is associated with stress responses (Raes et al. 2003). The *AtCCR2* expression level is generally lower in wild type, although its higher expression was observed in *ccr1* knockout lines and function of *AtCCR1* was partly compensated (Mir Derikvand et al. 2008). Transposon insertion in *Medicago CCR1* showed highly reduced lignin contents and growth, whereas *ccr2* knockouts exhibited no significant changes in growth (Zhou et al. 2010). Taken together, these results suggest that only a few CCR homologs function as the actual gene, whereas the others are backups. *Hibiscus cannabinus* is an annual dicotyledonous plant recognized as a potential source for future biomass production (Dempsey 1975). The broad

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ecological adaptability, high growth rate and quality of fiber (inner core fiber: 75–60%, short fiber, and outer bast fiber: 25–40%, long fiber) of this plant makes it an inexpensive raw material for biomass production (Ghosh et al. 2012). Here, we attempted to characterize tissue specific and stress-mediated expression of *HcCCR1* (HM151381) gene.

Materials and methods

Plant materials, treatments, and RNA isolation

Hibiscus cannabinus L. C-9 plants were cultivated in a growth room (16-h light/8-h dark photoperiod, 22°C, and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity) for 4 weeks, after that they were transplanted and grown under greenhouse conditions with natural sunlight for up to 20 weeks. Various tissue samples (roots, petioles, leaves, and flowers) were collected from 16-week-old kenaf plants. Leaves were categorized as follows: (1) young leaf (YL, <2 cm long); (2) immature leaf (IL, 3–5 cm long); and (3) mature leaf (ML, >9 cm long). Similarly, flowers were categorized as follows: (1) young flower (YF, unopened green flower, <2 cm long with green sepal); (2) immature flower (IF, unopened white flower, >3 cm long with green sepal); and (3) mature flower (open white flower). Eight different types of stress were applied to 3-week-old kenaf seedlings. The stresses were; MeJA (100 μM), cold (10°C), H_2O_2 (10 mM), SA (5 mM), ABA (100 μM), wounding, NaCl (200 mM), and drought. For RNA isolation, a combination of the CTAB method and the Qiagen RNeasy Mini kit protocol was followed. To remove the DNA, isolated RNA was treated with RNase free DNase (Roche). cDNA was synthesized using 2 μg of RNA and Superscript® III First-strand synthesis supermix (Invitrogen) according to the manufacturer's instructions. Detailed descriptions of plant growth, stress treatments and RNA isolation were carried out according to (Ghosh et al. 2012).

qRT-PCR analysis

CDS of *H. cannabinus* *HcCCR1* was obtained from NCBI (HM151381) and qRT-PCR analysis was performed as previously described (Bae et al. 2008) using the Mx3000P qRT-PCR System (Agilent, Santa Clara, CA, USA) with SYBR Green qRT-PCR master mix (Agilent). Primers specific for the *HcCCR1* gene were designed using the Primer 3 software of Biology Workbench (<http://workbench.sdsc.edu/>). The forward and reverse primers of the *HcCCR1* homologs were as follows: forward primer, 5'-AGTATCCCGTCCCTACCAAGTGT-3'; reverse primer, 5'-TAAGATTGAATACGGAGCACTGGC-3'. *ACTIN* (DQ866836), a housekeeping gene, was used as an internal control using the following primer sequences: forward primer, 5'-ATGGACAAGTCATTACTATTGGAGC-3'; reverse primer, 5'-AGTGATTTCCTTGCTCATACGGT-3'. Normalization and data analysis were performed using the $2^{-\Delta\text{CT}}$ method (Schmittgen & Livak 2008).

Data analysis

To understand the sequence similarity between *HcCCR1* [HM151381(CDS), ADK24219 (protein)] and *HcCCR2* [JX524276 (CDS), AGJ84130 (protein)], pairwise sequence alignment was conducted using Biology Workbench employing the ClustalW programme with default parameters (Subramaniam 1998; Larkin et al. 2007). The palmitoylation site of the *HcCCR1* (ADK24219) and *HcCCR2* (AGJ84130) protein was predicted using the CSS-Palm 2.0 software (Ren et al. 2008). The transmembrane domain analysis was carried out with the TMHMM server v.2.0 (Kahsay et al. 2005). The ProtParam tool was used to predict different parameters of *HcCCR1* protein (Wilkins et al. 1999). The secondary structures of *HcCCR1* and the *HcCCR2* proteins were predicted using the Jpred 3 server (Cole et al. 2008), while the tertiary structure was modeled using the Phyre² server (Kelley & Sternberg 2009). The two structures were superimposed using BioSuper (<http://ablab.ucsd.edu/BioSuper/>). Phylogenetic analysis of the *HcCCR1* protein with *HcCCR2*, CCR, and CCR-like protein of *Arabidopsis thaliana* and switchgrass (*Panicum virgatum*) was carried out using the MEGA5 software (Tamura et al. 2011). Different statistical parameters were used to construct the phylogenetic tree were; statistical method: neighbor-joining, test of phylogeny: bootstrap method, no. of bootstrap replicates: 1000, substitution type: amino acids, model/method: poisson method, rates among sites: uniform and gaps/missing data treatment: complete deletion.

Results

Comparative bioinformatics analysis of *HcCCR1* and *HcCCR2*

Comparative evaluation of the nucleotide and amino acid sequences of *HcCCR1* and *HcCCR2* revealed very close similarity (Supplementary table). Specifically, *HcCCR1* and *HcCCR2* showed 91.1% and 94.1% homology among coding sequences (CDS) and protein levels, respectively. Amino acid substitutions between *HcCCR1* and *HcCCR2* were observed at the N- and C-terminal ends of their protein sequences. The CDS and protein sequence of *HcCCR1* are three nucleotides and one amino acid shorter than *HcCCR2*. Although their isoelectric points are identical (pI 6.27), their molecular weights differed slightly, being 37.10 and 37.37 kDa for *HcCCR1* and *HcCCR2*, respectively. Pair-wise alignment revealed the presence of a conserved NAD(P) binding domain (VTGAGGFIASWMVKLLLEKGY) and a catalytic (NWYCYGK) domain at the N-terminal region of the *HcCCR1* protein (Figure 1a). The Jpred 3 results showed that both had similar secondary structures (Figure 1a). The sequence (TVCVTGAGGFIASWMVKLLLEKGYIVKGTV) produces a sheet-helix-sheet ($\beta\alpha\beta$) structure (Figure 1a). However, the tertiary structures of *HcCCR1* and *HcCCR2* differed (Figure 1b). CSS-Palm analysis showed the presence of an N-terminal palmitoylation site (SSNGMTVCVTGAGGF) in *HcCCR1* (Figure 1a).

(a) MPVDSSCSNGTTVCVTGAGGFIASWWMVKLLLEKGYIVKGTVRNPDDPKNS CCR2
 MPDTPSSNGMTVCVTGAGGFIASWWMVKLLLEKGYSVKGTVRNPDDPKNS CCR1
 .*:.* ***** ***** ***** ***** ***** ***** ***** *****
 HLRELEGANERLSLHRADLLDYQSLKEAINGCDGVFHTASPVTDDPEQMV CCR2
 HLRELEGAKERLSLHRADLLDYPSLKEAISGCDGVFHTASPVTDDPEQMV CCR1
 *****:***** ***** ***** ***** ***** ***** ***** *****
 EPAVNGTKNVIMAAAEAKVRRVVFTSSIGAVYMDPNRSPDVVVDESCWSD CCR2
 EPAVNGTKNVIMAAAEAKVRRVVFTSSIGAVYMDPNRSPDVVVDESCWSD CCR1
 ***** ***** ***** ***** ***** ***** ***** ***** *****
 LEFCNKTKNWYCYGKAVAEQAAWETAKEKGVDLVVVTPVLVLGPLLQSTV CCR2
 LEFCNKTKNWYCYGKAVAEQAAWETAKEKGVDLVVVAPVLVLGPLLQSTV CCR1
 *****:***** ***** ***** ***** ***** ***** ***** *****
 NASIVHILKYLTGSAKTYANSVQAYVHVRDVALAHILVFENPSASGRYLC CCR2
 NASTVHILKYLTGSAKTYANSVQAYVHVRDVALAHILVFENPSASGRYLC CCR1
 *** ***** ***** ***** ***** ***** ***** ***** ***** *****
 AESVLHRGEVVEILAKFFPEYPIPTKCSDENNPRKKPYKFSNQKLRDLGL CCR2
 AESVLHRGEVVEILAKLFPEYVPTKCSDESNPRKKPYKFSNQKLRDLGL CCR1
 *****:*****:***** ***** ***** ***** ***** ***** *****
 EFTPVKQCLYETVKSLQEKGHVAIPAHHQEDSVLRIQS CCR2
 EFTPVKQCLYETVKSLQEKHGLAIPA-QQQEDPVLRIQS CCR1
 *****:*****:***** ***** ***** ***** ***** ***** *****

LEGEND:

Alpha Helix, Beta Sheet, Random Coil

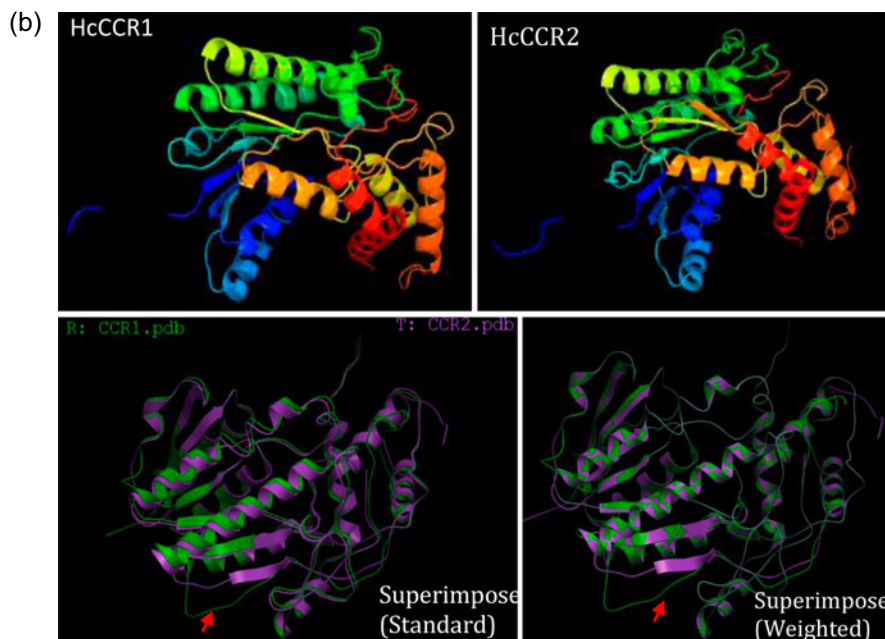


Figure 1. (a) Global alignment and predicted secondary structure of two kenaf homologs. NAD(P)-binding domain at the N-terminal and catalytic domain (NWYCYGK) are underlined with solid and dashed lines, respectively. Amino acids in red show predicted palmitoylation sites. (b) Predicted tertiary structure of HcCCR1 and HcCCR2 protein and their superimposition. Structural differences are indicated by the red arrow. Tertiary structure of HcCCR1 and HcCCR2 was predicted using the Phyre2server. Superimposition of the two structures was conducted using BioSuper.

HcCCR2 also showed the presence of a N-terminal palmitoylation site (CSNGTTVCVTGAGGF) (Figure 1a). The TMHMM prediction indicated that HcCCR1 and HcCCR2 do not contain any transmembrane domains and may therefore be localized in cytosol (Figure 2). To confirm whether the two *H. cannabinus* homologs were true CCR or CCR-like, a phylogenetic tree was

constructed using *Arabidopsis* CCR (AT1G15950, AT1G80820) and CCR-like protein sequences (AT1G76470, AT2G02400, AT2G33590, AT2G33600) along with the deduced protein sequence of switchgrass (*Panicum virgatum*) CCR (GQ450296,GQ450301) and CCR-like (GQ450304,GQ450305) (Figure 3) (Raes et al. 2003; Escamilla-Treviño et al. 2010). Our results showed

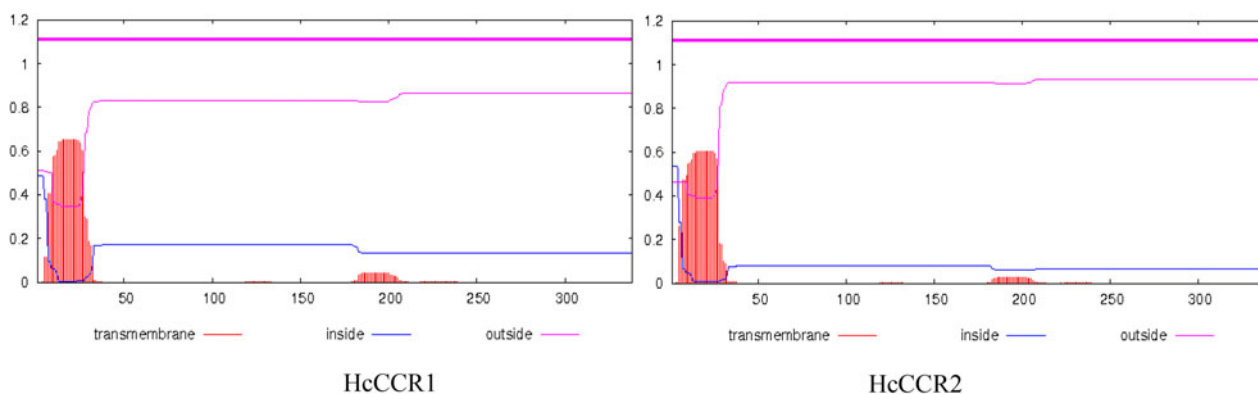


Figure 2. Transmembrane domain prediction of HcCCR1 and HcCCR2. HcCCR1 and HcCCR2 do not contain any transmembrane helices and are therefore assumed to be localized in cytosol. The vertical axis and horizontal axis represents posterior probability and amino acid number, respectively.

that CCR and CCR-like proteins were distinctly separated. Both *H. cannabinus* CCR were closely related to *Arabidopsis* CCR when compared to monocot (switch-grass) CCR. In the phylogenetic tree, CCR-like proteins fell in a distant group relative to true CCR.

Tissue specific expression of HcCCR1

Investigation of the expression pattern of *HcCCR1* in various tissues of 16-week-old kenaf plants revealed the highest and lowest level of expression in mature leaf and mature flower tissue, respectively, while intermediate levels were observed in root and stem tissue (Figure 4). We also investigated developmental stage specific expression patterns in leaf, stem, and flower tissues. No significant difference was observed in expression among young, immature and mature leaf tissues. The expression of *HcCCR1* was slightly higher in young flowers than immature and mature flowers, and its expression in stem tissues varied greatly according to the age. Initially (two weeks old), the expression of *HcCCR1* was lower, after which it was upregulated in 4-week-old stems. The expression was then downregulated gradually for up to 20 weeks. Overall, *HcCCR1* expression was ubiquitous,

similar to that of *HcCCR2* (Ghosh et al. 2014). However, a significant difference was observed between the expression of *HcCCR1* and *HcCCR2*. The higher expression of *HcCCR1* was observed in mature leaf while lower level of expression was observed in mature flowers. Conversely, the highest level of *HcCCR2* expression was observed in mature flowers, whereas the lowest was in stems and petioles (Ghosh et al. 2014). Different levels of expression between *HcCCR1* and *HcCCR2* were observed during stem development as well. Specifically, *HcCCR2* showed a steady level of expression in 2- and 4-week-old stem, after which it was downregulated. Conversely, *HcCCR1* showed lower expression in 2-week-old stems, was sharply upregulated at 4 weeks, and was then gradually downregulated.

Stress-mediated expression of HcCCR1

We analyzed the stress-mediated expression of *HcCCR1* (Figure 5). Wound and SA treated plants showed similar *HcCCR1* expression patterns. In both cases, *HcCCR1* was downregulated at 1 h, then upregulated at 6, 12, and 48 h. NaCl treatment also showed initial downregulation at 1 h, after which it was gradually upregulated to 24 h,

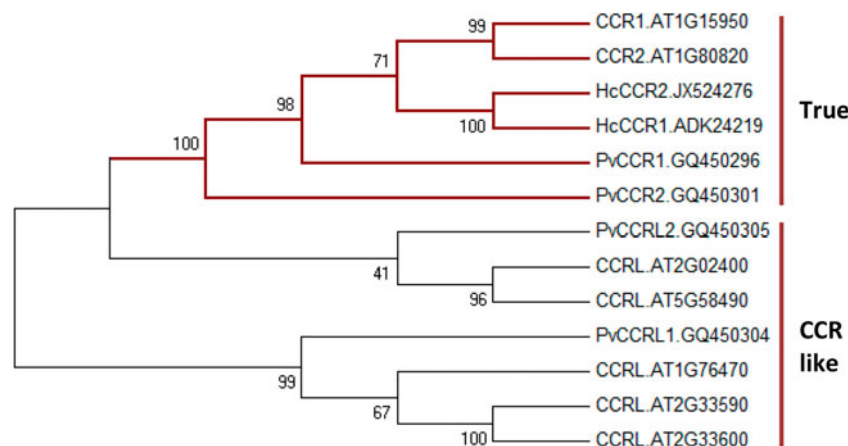


Figure 3. Phylogenetic analysis of the deduced amino acid sequences of kenaf CCR orthologs, including *Arabidopsis* and switch grass true and CCR-like protein sequences. The tree was constructed by the neighbor-joining method of ClustalW and MEGA5. The numbers at the nodes indicate bootstrap values from 1000 replications.

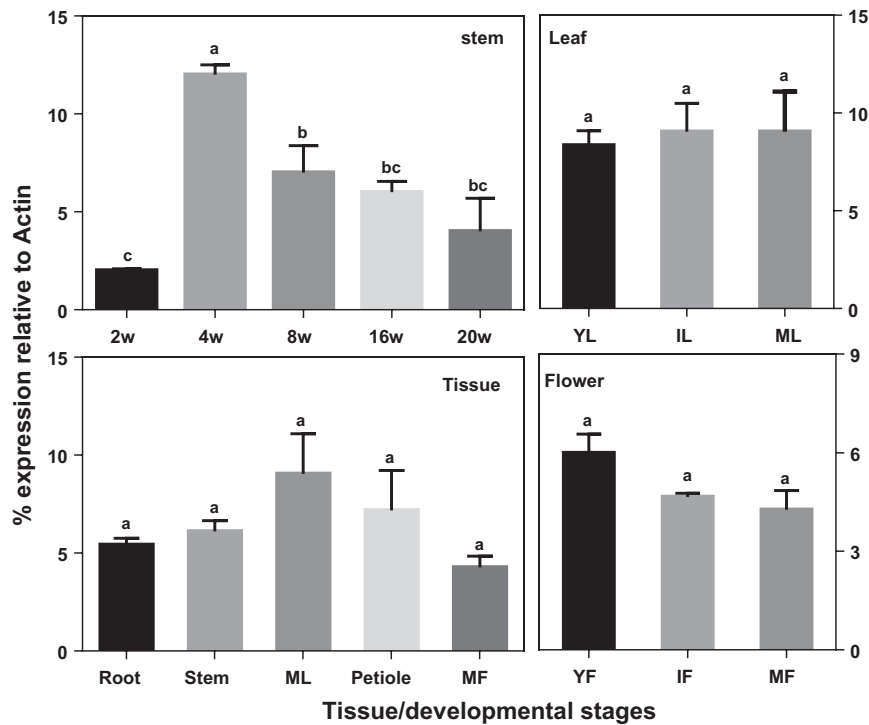


Figure 4. Transcript expression patterns of *HcCCR1* in various tissues and organs during developmental stages. Relative transcript levels were measured using qRT-PCR with *ACTIN* as the internal control. The transcript levels of the kenaf *CCR* ortholog were adjusted after deduction of the control transcript level. (a) During stem development (2, 4, 8, 16, and 20 weeks after sowing); (b) during leaf development (YL, young leaf; IL, immature leaf; ML, mature leaf); (c) during flower development (YF, young flower; IF, immature flower; MF, mature flower), and (d) expression pattern in various tissues and organs from 16-week-old kenaf plants. Bars show the means \pm standard error of three biological replications. Letters above the error bars indicate significant differences among each other with a $p < 0.05$ as determined by Duncan's multiple range test (DMRT).

and then downregulated at 48 h. Cold treatment downregulated the expression of *HcCCR1* at 1, 6, 12, and 48 h, although it was upregulated at 24 h. Conversely, in H_2O_2 treated samples, expression of *HcCCR1* was upregulated at all time points except 1 h. The ABA treated samples exhibited early downregulation (1, 6, and 12 h), followed by strong upregulation at 24 and 48 h. In MeJA treated samples, *HcCCR1* was highly upregulated at 24 h, while drought treated samples showed continuous upregulation at all time points. Downregulation of *HcCCR1* was observed at early time points, especially at 1 h, in response to all treatments except drought. The greatest downregulation of *HcCCR1* was observed at 1 h in cold treated sample. Based on the highest upregulation time, the treatments could be classified into two categories: early (1, 6, and 12 h) and late (24 and 48 h) response. Wound and SA falls in the first category, while cold, ABA, NaCl, and MeJA showed late response. H_2O_2 and drought treated samples showed upregulation in almost all time points. Among all treatments, maximum upregulation of *HcCCR1* was occurred by MeJA at 24 h. *HcCCR1* expression also showed differences at many points relative to *HcCCR2* (Ghosh et al. 2014). Previous experiments showed that, expression of *HcCCR2* was unaffected after 1 h of stress treatments, whereas *HcCCR1* was downregulated by many treatments. In wound, SA, NaCl, and H_2O_2 treated samples; *HcCCR1* was downregulated at 1 h, whereas

HcCCR2 expression was remained unaffected. In cold treated samples, *HcCCR2* was highly downregulated in all time points. However, *HcCCR1* was strongly upregulated at 24 h. Differences also existed in NaCl treated samples at 48 h, with *HcCCR2* and *HcCCR1* being highly up and downregulated, respectively. In wound treated samples, *HcCCR2* showed high upregulation at 24 h, while *HcCCR1* remained unchanged. In H_2O_2 treated samples, significant upregulation of *HcCCR2* was only observed at 12 and 24 h, whereas *HcCCR1* was highly upregulated at all time points except at 1 h. Both of these homologs showed similar expression patterns in drought and MeJA treatments. Both homologs were also highly upregulated at 24 h and 10 days after MeJA and drought treatment, respectively. Conversely, ABA treated samples showed similar expression patterns at early time points (1, 6, and 12 h), while *HcCCR1* was highly upregulated at 24 and 48 h.

Discussion

Our analysis suggests that both *H. cannabinus* CCR homologs are closely related at the sequence level (Supplementary table). However, tertiary structure prediction revealed slight differences in their structural features. Bioinformatics prediction showed the absence of transmembrane domain in the *HcCCR1* and *HcCCR2* protein. A previous study showed that the *HcCCR* protein

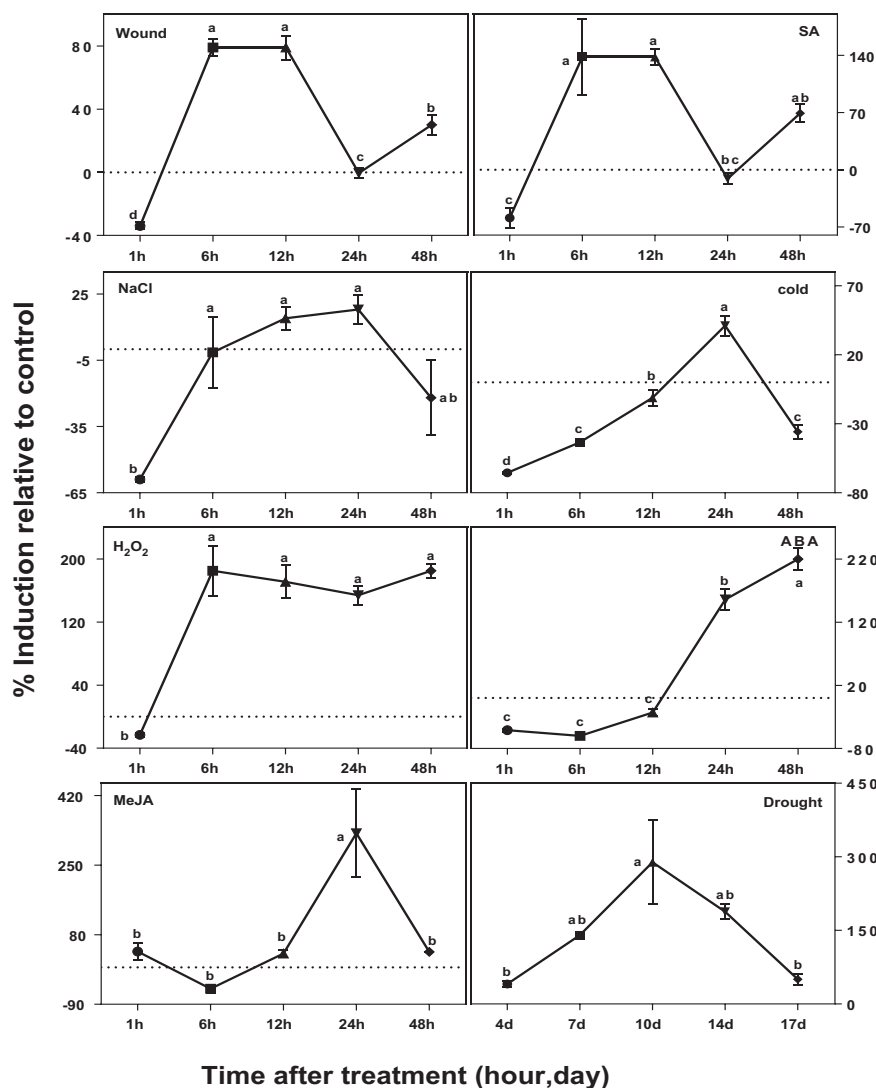


Figure 5. Transcript expression patterns of *HcCCR1* in response to various abiotic stresses. Three-week-old stem tissues were subjected to different stresses including wounding, salicylic acid (SA), NaCl, cold, H₂O₂, methyl jasmonate (MeJA), abscisic acid (ABA), and drought. Bars show means \pm the standard error of three biological replications. Different letters above the error bars indicate significant differences among groups at $p < 0.05$ as determined by Duncan's multiple range test (DMRT).

is localized to the cytoplasm and does not contain any localization signal sequences (Kawasaki et al. 2006). Kawasaki et al. (2006) also reported that OsRac1 (Rac/Rop Family of small GTPases) protein interacts with OsCCR1 in the vicinity of plasma membrane and that OsRac1 is localized to plasma membrane (Kawasaki et al. 2006). It is very unlikely that interaction with a membrane bound OsRac1 protein would occur without localization. Therefore, they also predicted possible movement of OsCCR1 to the plasma membrane under certain circumstances, such as defense response and interaction with OsRac1, or activation of OsRac1 may affect the localization of OsCCR1 (Kawasaki et al. 2006). Accordingly, the presence of a transmembrane signal sequence is important in this event. Although *HcCCR1* and *HcCCR2* are cytosolic, upon activation of a signaling event they can undergo positional changes and interact with their partner in close vicinity to plasma membrane. Further in depth investigations are required to understand this event in detail. Moreover, co-translational modification of

protein is required to carry out some specific functions such as protein trafficking, aggregation, and affinity for membrane attachment (Adamson et al. 1992; Löffke et al. 2013). These co-translational modifications may be either palmitoylation or myristoylation (Linder & Deschenes 2007; Wright et al. 2010; Moriya et al. 2013). Sequence analysis revealed the presence of N-terminal palmitoylation sites in *HcCCR1* (SSNGMTVCVTGAGGF) and *HcCCR2* (CSNGTTVCVTGAGGF) protein. Protein palmitoylation occurs at the Cys amino acid in the N-terminal region of proteins (Linder & Deschenes 2007; Charollais & Van Der Goot 2009). In *HcCCR1* and *HcCCR2*, the Cys amino acid is present at the 14th position in the N-terminal region. N-terminal protein myristoylation is prerequisite for protein palmitoylation (Martín & Busconi 2001), and protein myristoylation occurs at the Gly amino acid in the N-terminal region. In *HcCCR1* and *HcCCR2*, Gly amino acid is present at the 10th position in the N-terminal region. The presence of a myristoylation and palmitoylation site reflects the fact that both *HcCCR*

homologs possess signal sequences and can be targeted to the plasma membrane under certain circumstances. The subcellular interaction of OsRac1 and OsCCR1 in the plasma membrane vicinity is closely correlated with the results of the present study (Kawasaki et al. 2006).

The results of our experiment suggest that, although both *H. cannabinus* CCR homologs are closely related at the sequence level and only slightly different at the structural level, they have significant differences in expression. The presence of slightly different sequences and structural features may lead to differential function, which might be due to the different mode of action and biochemical properties. The motif (TVCVTGAGGF IASWMVKLLLEKGYIVKGTV) has a sheet-helix-sheet structure (Figure 1a), common to the $\beta\alpha\beta$ -dinucleotide binding fold of NAD (H) and NADP(H)-dependent reductases and dehydrogenases (Larsen 2004; Hu et al. 2011). HcCCR1 and HcCCR2 have identical NADPH-binding motifs and catalytic domains, but showed differential expression patterns. The *TaCCR1* and *TaCCR2* also share nearly identical motifs for NADPH-binding and reaction active sites, but showed significant differences in substrate specificity and tissue specific expression (Ma 2007). These findings indicate that HcCCR1 and HcCCR2 are two different genes. Previous experiments also showed the diverse role of this gene family within same plant. Multiple homologs of CCR genes can be present in the same plant, even though only a few are involved in developmental lignification, while the rest are backups (Barakat et al. 2011). This diverse gene family can be classified as CCR and CCR-like genes, which differ in function and biochemical properties (Raes et al. 2003; Barakat et al. 2011). For example, *Arabidopsis* has only two real CCR genes (Zhou et al. 2010): *AtCCR1*, which is involved in developmental lignification, and *AtCCR2*, which is associated with stress and elicitor response (Raes et al. 2003). The *ccr1* knockout mutants displayed altered developmental program, dwarf phenotype and reduced lignin contents in *Arabidopsis* (Mir Derikvand et al. 2008). *AtCCR1* and *AtCCR2* showed differential expression in *Arabidopsis* upon infection with *Xanthomonas campestris*, although *AtCCR2* showed higher response than *AtCCR1* (Lauvergeat et al. 2001). The CCR enzymes can use various cinnamoyl-CoA esters as substrates (like *p*-coumaroyl-CoA, caffeoyl-CoA, feruloyl-CoA, 5-hydroxyferuloyl-CoA, and sinapoyl-CoA), each of which can be converted to corresponding cinnamaldehydes. Nevertheless, substrate specificity is highly variable between species, and even within the same species (Barakat et al. 2011). For example, *Medicago* CCR1 shows preference for feruloyl-CoA, whereas CCR2 prefers caffeoyl and 4-coumaroyl CoAs (Zhou et al. 2010). Variable substrate preferences were observed in *Arabidopsis*, wheat and tomato plants as well (Lauvergeat et al. 2001; van der Rest et al. 2006; Barakat et al. 2011). The differences in substrate specificity can lead to different modes of action during cellular events. Similar to biochemical properties, the expression of CCR homologs is highly variable within tissues. For example, wheat *ZmCCR1* was ubiquitous, whereas *ZmCCR2* was only

detected in roots (Pichon et al. 1998). Moreover, *LeCCR1* was highly expressed in a wide range of organs in tomato plants, whereas *LeCCR2* was only expressed in roots and stems (van der Rest et al. 2006). Differential expression between CCR homologs was also reported in poplar, aspen, and switchgrass (Li et al. 2005; Escamilla-Treviño et al. 2010; Barakat et al. 2011). The differences in expression patterns between two kenaf homologs indicate their spatiotemporal nature. The CCR homologs also showed variable expression in response to various stresses. Strong upregulation of *HcCCR1* at early time points might be the direct effect of wound, SA, and H₂O₂ stresses, whereas changes in late time points in response to other stresses occurred due to the downstream process of an intricate cellular signaling network. When compared to *HcCCR1*, *HcCCR2* had a slower response for all stresses as it was primarily upregulated at later time points (Ghosh et al. 2014). Stress-mediated differential expression among CCR homologs was previously reported. For example, water deficit treatment induced the expression of both homologs in maize, though the response of *ZmCCR2* was much higher than that of *ZmCCR1* (Fan et al. 2006). In Norway spruce, expression of *PaCCR2* was more widespread than that of *PaCCR1*, and its expression was significantly upregulated after treatment with *Heterobasidion annosum* (Koutaniemi et al. 2007).

Phylogenetic diversity and functional differences are common among many lignin biosynthesis genes. In *Arabidopsis*, there are multiple homologs for PAL, 4CL, C3H, CCoAOMT, F5H, and CAD genes, and their expression pattern, biochemical properties, roles in plant development and stress responses also varied greatly. Different substrate specificity is probably assigned for different spatiotemporal expression of varying isoenzymes in plants (Raes et al. 2003), and it has been postulated that these variable expression patterns among homologs are caused by the presence of different *cis*-regulatory sites in various combinations in their promoters (Raes et al. 2003). In addition to well conserved AC-elements, which confer vascular expression of phenylpropanoid genes, other *cis*-regulatory elements are also present in the promoter (Raes et al. 2003). These include A-box, H-box, G-box, and AT-rich sequences, which impart diversity to homologs (Raes et al. 2003). In summary, HcCCR1 is a very close homolog of HcCCR2, but they have significantly different expression patterns. Further investigation is required to determine their substrate specificity and promoter elements, which will help to elucidate their modes of action.

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RG carried out the experiments and drafted the manuscript, BC carried out the experiments, JK analyzed the data, MJ revised the manuscript, TKM analyzed the results and drafted the manuscript, and HB conceived the idea and revised the manuscript. All authors read and approved the final manuscript.

Disclosure statement

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Supplemental data

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