Two Types of Cinnamoyl-CoA Reductase Function Divergently in Tissue Lignification, Phenylpropanoids Flux Control, and Inter-pathway Cross-talk with Glucosinolates as Revealed in *Brassica napus*

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28 **Running title:** Cinnamoyl-CoA reductases function divergently in *Brassica*

29 **Highlight:** Brassicaceae contains two types of Cinnamoyl-CoA reductase. As
30 revealed in *Brassica napus*, they are divergently involved in lignin monomer
31 biosynthesis, tissue lignification, phenylpropanoid flux control, and
32 inter-pathway crosstalk with glucosinolates.

33 **Abstract**

34 Cinnamoyl-CoA reductase (CCR) is the entry point of lignin pathway and a
35 crucial locus in dissection and manipulation of associated traits, but its
36 functional dissection in Brassicaceae plants is largely lagged behind though
37 *Arabidopsis thaliana* *CCR1* has been characterized to certain extent. Here, 16
38 *CCR* genes are identified from *Brassica napus* and its parental species *B. rapa*
39 and *B. oleracea*. Brassicaceae *CCR* genes are divided into *CCR1* subfamily
40 and *CCR2* subfamily with divergent organ-specificity, yellow-seed trait
41 participation and stresses responsiveness. *CCR1* is preferential in G- and
42 H-lignins biosynthesis and vascular development, while *CCR2* has a deviation
43 to S-lignin biosynthesis and interfascicular fiber development. *CCR1* has
44 stronger effects on lignification-related development, lodging resistance,
45 phenylpropanoid flux control and seed coat pigmentation, whereas *CCR2*
46 controls sinapates levels. *CCR1* upregulation could delay bolting and flowering
47 time, while *CCR2* upregulation weakens vascular system in leaf due to
48 suppressed G lignin accumulation. Besides, *CCR1* and *CCR2* are deeply but
49 almost oppositely linked with glucosinolates metabolism through inter-pathway
50 crosstalk. Strangely, upregulation of both *CCR1* and *CCR2* could not enhance
51 resistance to UV-B and *S. sclerotiorum* though *CCR2* is sharply induced by
52 them. These results provide systemic dissection on *Brassica* *CCRs* and
53 *CCR1-CCR2* divergence in Brassicaceae.

54 **Keywords:** *Brassica*, Cinnamoyl-CoA reductase (CCR), Functional
55 divergence, Lignin, Sinapates, Flavonoids, Glucosinolates, Lodging,
56 *Sclerotinia sclerotiorum*.

57

58 **Introduction**

59 Lignins constitute one important group of phenylpropanoids, are deposited in
60 plant secondary cell walls, and are the second most abundant biopolymers on
61 the planet (Huang *et al.*, 2010; Vanholme *et al.*, 2010). They perform many
62 functions, providing structural support, giving rigidity and strength to stems to
63 stand upright, and enabling xylems to withstand the negative pressure
64 generated during water transport (Escamilla-Treviño *et al.*, 2010; Labeeuw *et al.*, 2015). In addition, lignins have been suggested to be induced upon various
65 biotic and abiotic stresses, such as pathogen infection, insect feeding, drought,
66 heat and wounding (Caño-Delgado *et al.*, 2003; Weng and Chapple, 2010;
67 Chantreau *et al.*, 2014).

69 To date, engineering of lignins mainly pursues reduced lignin content or
70 altered lignin composition to meet the demands of agro-industrial processes,
71 such as chemical pulping, forage digestibility, and the bioethanol production
72 from lignocellulosic biomass (Baucher *et al.*, 2003; Li *et al.*, 2008; Weng *et al.*,
73 2010; Ko *et al.*, 2015; De Meester *et al.*, 2020). However, when altering the
74 expression of lignin pathway genes, the metabolic flux of its neighbor
75 pathways would be changed correspondingly (Hoffmann *et al.*, 2004; Li *et al.*,
76 2010; Thévenin *et al.*, 2011). Furthermore, dramatic modification of lignin
77 content or lignin composition may provoke deleterious effects on plant growth,
78 such as dwarfism and collapsed xylem vessels, with concomitant loss of
79 biomass and yield (Piquemal *et al.*, 1998; Ruel *et al.*, 2009; De Meester *et al.*,
80 2020).

81 Cinnamoyl-CoA reductase (CCR) is the entry point for the lignin-specific

branch of the phenylpropanoid pathway and catalyzes the monolignol biosynthesis (Lacombe *et al.*, 1997; Kawasaki *et al.*, 2006). *Arabidopsis thaliana* possesses 11 annotated CCR homologs (Costa and Dolan, 2003), but only *AtCCR1* and *AtCCR2* encode true CCR enzyme (Lauvergeat *et al.*, 2001). *AtCCR1* is preferentially expressed in tissues undergoing lignification, while *AtCCR2* is poorly expressed during development but is strongly and transiently induced by *Xanthomonas campestris*, suggesting that *AtCCR1* might be involved in constitutive lignification whereas *AtCCR2* might be involved in resistance (Lauvergeat *et al.*, 2001). However, to date there is no functional verification of this assumption through over-expression transgenic study in *A. thaliana*, and the function of *CCR2* in Brassicaceae is not dissected. In monocot grasses, *CCR1* expression can be detected in various organs with a relatively high transcription level in stem (Tu *et al.*, 2010; Giordano *et al.*, 2014), thus is thought to be involved in constitutive lignification. In poplar and switchgrass, *CCR2* is expressed at very low levels in most organs, but can be significantly induced by biotic and abiotic stresses (Escamilla-Treviño *et al.*, 2010). Manipulation of *CCR* (mainly through downregulation) typically results in a significant variation of lignin content and composition (Goujon *et al.*, 2003; Zhou *et al.*, 2010; Wagner *et al.*, 2013). Moreover, plants with dramatically downregulated *CCR* genes usually showed a stunted growth and delayed development (Tamasloukht *et al.*, 2011; Thévenin *et al.*, 2011; De Meester *et al.*, 2020), and the alternation of carbon flux between lignin and other metabolic pathways was also accompanied (van der Rest *et al.*, 2006; Dauwe *et al.*, 2007; Wagner *et al.*, 2013).

Lodging and diseases are fatal problems in field production of most crops. Reducing plant height has been proven to be a useful strategy for improving lodging resistance, but dwarfism will reduce canopy photosynthetic capacity and yield (Zhang *et al.*, 2001; Acreche and Slafer, 2011; Peng *et al.*, 2014). Increasing stem strength would be a very promising strategy for breeding crops with high lodging resistance (Ma, 2009). Moreover, lignin is a physical

112 barrier which can restrict pathogens to the infection site and confer disease
113 resistance in plants (Lee *et al.*, 2019). There are few reports at the molecular
114 level to address how the regulation of lignin biosynthesis affects crop lodging
115 and pathogen resistance via gene manipulation. In wheat, accumulation of
116 lignin is closely related to lodging resistance, and wheat culms with higher
117 lignin content could have a better lodging resistance (Peng *et al.*, 2014).
118 Regarding defense, the CCR-like gene *SnI6* is required for NH1-mediated
119 resistance to bacterial pathogen *Xanthomonas oryzae* pv. *Oryzae* in rice (Bart
120 *et al.*, 2010). However, there is few reports on overexpressing CCR in
121 enhancing lodging or disease resistance with molecular breeding values in
122 crops.

123 *Brassica*, a relative genus of model plant *A. thaliana*, is of great importance
124 since it contains various important oilseed, vegetable and ornamental crops.
125 Unfortunately, *Brassica* crops especially rapeseed (*B. napus*) frequently suffer
126 from some detrimental stresses such as lodging (Liu *et al.*, 2010; Peng *et al.*,
127 2014) and stem rot disease caused by *Sclerotinia sclerotiorum* with serious
128 yield penalty and quality deterioration (del Río *et al.*, 2007; Ding *et al.*, 2015).
129 In rapeseed, lodging could lead to 20%–46% yield loss and about four percent
130 points of oil content reduction, and limits the efficiency of mechanical harvest
131 (Pan *et al.*, 2012; Kendall *et al.*, 2017; Berry, 2018). There is little knowledge at
132 present concerning the functional genes involved in lignin biosynthesis in
133 rapeseed or even in *Brassica* species. Comprehensive characterization of the
134 lignin biosynthesis in rapeseed will enable us to manipulate the lignin content
135 or composition through genetic engineering, and to strengthen the capacity for
136 lodging and pathogen resistance.

137 In this study, the CCR1 and CCR2 subfamily members from *B. napus* and its
138 parental species *B. rapa* and *B. oleracea* were isolated, their expression
139 patterns in different organs and under various stresses were investigated, and
140 overexpression of *BnCCR1* and *BnCCR2* was performed for deciphering their
141 biological functions and biotechnological potentials. Our results demonstrated

142 that *BnCCR1* was mainly involved in the biosynthesis of H- and G-lignins,
143 while *BnCCR2* showed a preference in the biosynthesis of S-lignin and in plant
144 defense. A dramatic shift of carbon flux in phenylpropanoid pathway and a
145 strong crosstalk effect on glucosinolate pathway was demonstrated in both
146 *BnCCR1*- and *BnCCR2*-transgenic plants, especially for *BnCCR1*
147 manipulation. Besides, *BnCCR1* and *BnCCR2* showed distinctly different
148 association with flux regulation and development control, which accounted for
149 distinct phenotypic modification in vascular system, lodging resistance, seed
150 color, flowering time and glucosinolate profiles in corresponding
151 overexpressors. Surprisingly and unexpectedly, both *BnCCR1*- and
152 *BnCCR2*-overexpressors did not show increased resistance to *S. sclerotiorum*
153 and UV-B light, implying complicated association of CCR and lignin pathway
154 with disease resistance.

155 **Materials and methods**

156 **Plant Materials**

157 *B. napus*: black-seed varieties 5B and ZS10, yellow-seed variety 09L587. *B.*
158 *rapa*: black-seed variety 09L597 and yellow-seed variety 09L600. *B. oleracea*:
159 black seed variety 09L598 and yellow-seed variety 09L599. T1 transgenic lines
160 together with non-transgenic wild-type (WT) ZS10 plants were grown in
161 artificial growth room (25°C, 16-h photoperiod/20°C, 8-h dark period). Later
162 generations of transgenic lines and all other materials were planted in field
163 cages, with common plantation conditions. Samples were immediately frozen
164 in liquid nitrogen and stored at -80°C for gene expression analysis,
165 biochemical and histochemical assay, gas chromatography–mass
166 spectrometry (GC-MS) detection and UPLC-HESI-MS/MS analysis. Mature
167 seeds, stems and roots (about 65 DAP) were harvested for agronomic traits
168 investigation and biochemical analysis.

169 **Southern hybridization analysis**

170 Total genomic DNA was extracted from leaves of the 3 *Brassica* species using
171 a standard cetyltrimethylammonium bromide protocol (Porebski *et al.*, 1997). It

172 was digested with restriction enzymes *DraI*, *EcoRI*, *EcoRV*, *HindIII* and *XbaI*
173 (70 µg for each enzyme) respectively, and separated on a 0.8% (w/v) agarose
174 gel. Following electrophoresis, DNA was transferred to a positively charged
175 nylon membranes (Roche, Switzerland) using established protocols. The
176 *Brassica CCR1*- and *CCR2*-specific probes were amplified by PCR with primer
177 pairs FCCR1C1+RCCR1 and FBCCR2I+RBCCR2I respectively, and labelled
178 using digoxigenin (DIG) Probe Synthesis Kit (Roche, Switzerland), with an
179 annealing temperature of 61°C and extension time of 1 min. Sequences of all
180 primers are provided in [Supplementary Table S8](#). Hybridizations with the
181 probes were performed at 43°C overnight, with chemiluminescent detection
182 using DIG Luminescent Detection Kit (Roche, Switzerland).

183 **Detection of Transcription Levels**

184 Total RNA of each sample was extracted using EASYspin Kit (Biomed, China)
185 and RNAPrep pure plant kit (TIANGEN, China), and treated with DNase I to
186 eliminate contaminated gDNA. Equal quantities of RNA (1µg) were adopted for
187 the synthesis of total cDNA using the PrimeScriptTM RT reagent kit with gDNA
188 Eraser (TaKaRa Dalian, China). The transcript levels of *CCR1*, *CCR2* and
189 other target genes were detected using both quantitative real-time PCR
190 (qRT-PCR) and semi-quantitative RT-PCR (sRT-PCR) as described previously
191 ([Zhao et al., 2007b](#)). The *25SrRNA* primer pair was used as an internal control
192 in qRT-PCR. A 25-fold dilution series of original reverse-transcription products
193 was used for qRT-PCR using SsoAdvanedTM Universal SYBR Green Supermix
194 (BioRad, USA) on CFX96TM Real-Time System (BioRad, USA). Conditions for
195 qRT-PCR were as follows: 95°C for 2 min; 40 cycles of amplification with 95°C
196 for 30 s and 62°C for 30 s. A melting curve was obtained after amplification by
197 heating products from 60 to 95°C. Transcript levels were determined based on
198 changes in Cq values relative to the internal control. Results were analyzed
199 using the CFX ManagerTM 3.0 software (Bio-Rad, USA). Conditions for
200 sRT-PCR on Veriti Thermal Cycler (ABI, USA) were as follows: 94°C for 2 min;
201 31 cycles of amplification with 94°C for 0.5 min, 60-64°C for 0.5 min and 72°C
202 for 1 min; followed by 72°C for 10 min. The *26SrRNA* primer pair was used as
203 an external control in sRT-PCR. Sequences of all primers for qRT-PCR and
204 sRT-PCR are listed in [Supplementary Table S8](#).

205 **Rapeseed Genetic Transformation and Screening**

206 A modified *Agrobacterium*-mediated transformation protocol according to
 207 (Cardoza and Stewart, 2003) was used to transform “double low” (low erucic
 208 acid and low glucosinolates) rapeseed commercial cultivar Zhongshuang 10
 209 (ZS10) with overexpression vectors pCD-*BnCCR1-2ox* and pCD-*BnCCR2-4ox*,
 210 using hypocotyl segments as explants (Fu et al., 2017). The regenerated
 211 plants were identified first by leaf β -glucuronidase (GUS) staining and leaf
 212 Basta-resistance test (200 ppm), then by Taq-PCR detection of target genes
 213 (as those for engineering strains verification) and the selectable and
 214 screenable marker gene *BAR* (primer pair FBar+RBar, annealed at 58°C,
 215 extension for 30 s). The T1 transgenic plants were grown in artificial growth
 216 room and were selfed. Representative T2 and T3 transgenic lines and WT
 217 were grown in field cages, and positive plants of above identifications were
 218 subjected to traits investigation and further studies.

219 **Agronomic Traits Investigation**

220 The area and length of the 4th or the 5th leaves which was fully expanded
 221 were surveyed during vegetative stage, and the length and width of the
 222 petioles of these leaves were measured at the same time. The primary branch
 223 numbers, middle stem diameter and stem strength at reproductive stage, and
 224 the plant traits at harvest stage, were recorded and measured. The weight of
 225 1000 seeds and yield per plant were measured after the harvested seeds were
 226 dry. Stem strength determination: Freshly collected middle stem segments
 227 were placed horizontally, and the force exerted to break the stem was recorded
 228 with a universal force testing device (model DC-KZ300, Sichuan, China) to
 229 determine the stems rigidity, which was normalized with the stem's length and
 230 diameter.

231 **Quantification of Insoluble Condensed Tannins of Seed Coat**

232 The modified method for quantification of insoluble condensed tannins was

233 according to (Auger *et al.*, 2010) and (Naczki *et al.*, 2000). The oven-dried seed
234 coat was milled to fine powder using a microball mill, and extracted with
235 hexane for 12 h using a Soxhlet apparatus and then dried at room temperature.
236 10 mg sample of seed coat powder was extracted with the extraction solution
237 of three milliliters of butanol-HCl (95:5; v/v), 300 µL methanol and 100 µL of 2%
238 ferric ammonium sulfate (w/v) in 2 N HCl. The tubes were heated for 3 h at
239 95°C in a water bath, centrifuged after cooling, and extracted again with the
240 same extraction solution for 1 h. The absorbance of the pooled supernatant
241 was measured at 550 nm against a reagent-only blank using UV-VIS
242 spectrophotometer (UV-5100B, Shanghai, China). A calibration curve was
243 prepared using procyanidin with amounts ranging from 0 to 200 µg/mL.

244 **Histochemical Staining, Autofluorescence and Anatomical Studies**

245 Cross sections were obtained by using a frozen section machine (Leica
246 CM1850, Germany). Fresh petioles at vegetative stage, fresh stems and roots
247 of the plants at reproductive stage, fresh mature silique coat and dry mature
248 seeds coat were cut into slices of 60, 60, 60, 60 and 5 µm thick, respectively.
249 Phloroglucinol-HCl staining and Mäule staining of those plant organ sections
250 were performed as previously described (Chapple *et al.*, 1992; Chen *et al.*,
251 2002). Stained sections were observed on a stereoscopic microscope (Nikon
252 C-BD230, Japan; OLYMPUS SZX2-FOA, Japan) and a fluorescence
253 microscope (Nikon ECLIPSE E600W, Japan).

254 **Determination of Lignin Content and Composition**

255 Lignin content was quantified using a modified acetyl bromide soluble lignin
256 method (Fukushima and Hatfield, 2001; Chang *et al.*, 2008), and lignin
257 composition was determined with a modified thioacidolysis method according
258 to previous reports (Lapierre *et al.*, 1995; Yosef and Ben-Ghedalia, 1999;
259 Robinson and Mansfield, 2009).

260 **Cell Wall Isolation**

Harvested stems were dried in a 70°C forced air oven for 72 h, and then were ground with a Wiley mill to pass a 40-mesh screen or with a microball mill to pass an 80-mesh screen using to meet the requirements of the subsequent extraction method. 60-70 mg samples were added into a 2 mL Sarstedt screw cap tube, adding with 1.5 mL of 80% aqueous ethanol to the dispensed ground material, and vortex thoroughly (10 min). This step was repeated for a total of three cycles, followed by centrifugation at 10,000 rpm for 10 min, and the supernatant was decanted. Add 1.5 mL of chloroform/methanol (2:1 v/v) solution to the residue, and shake the tube thoroughly to resuspend the pellet. Centrifuge at 10,000 rpm for 10 min, and decant the supernatant. Resuspend the pellet in 500 µL of acetone, and evaporate the solvent with a stream of air at 35°C until dry (If needed, dried samples can be stored at room-temperature until further processing). To initiate the removal of starch from the sample, resuspend the pellet in 1.5 mL of a 0.1 M sodium acetate buffer pH 5.0, cap the Sarstedt tubes, and heat for 30 min at 80°C in a heating block. Cool the suspension on ice, and add the following agents to the pellet: 35 µL of 0.01% sodium azide (NaN₃), 35 µL amylase (from *Bacillus* species, 50 µg/mL in H₂O, Sigma); 3.56 µL pullulanase (from *Bacillus acidopullulyticus*, 17.8 units, Sigma). Cap the tube, and vortex thoroughly. The suspension is incubated over night at 37°C in the shaker (Orienting the tubes horizontally aides improved mixing). After enzyme digestion, the suspension was heated at 100°C for 10 min in a heating block to terminate digestion. Centrifuge at 10,000 rpm for 10 min, and discard the supernatant containing solubilized starch. The remaining pellet was washed three times by adding 1.5 mL water, vortexing, centrifuging, and decanting of the washing water. For removing the water, 1.5 mL anhydrous ethanol was added to resuspend the pellet, followed by vortexing, centrifuging, decanting of the supernatant, and resuspending the pellet with 500 µL of acetone. Finally, evaporate the solvent with a stream of air at 35°C until dry. It may be necessary to break the material in the tube with a spatula for better drying. If needed, the dried samples can be stored at

291 room-temperature until further processing.

292 **Spectrophotometer Test of Lignin Content**

293 2 mg of prepared cell wall material was interacted with 200 µL of freshly
 294 made acetyl bromide solution (25% v/v acetyl bromide in glacial acetic acid) in
 295 a culture tube under 50°C for 3 h with shaking at 30 min intervals during first 2
 296 h and with vortex every 15 min during the last 1 h, and the reaction was
 297 stopped on ice for 5 min. Add 800 µL of 2 M sodium hydroxide and 140 µL of
 298 freshly prepared 0.5 M hydroxylamine hydrochloride, and vortex the tubes.
 299 Transfer the reaction solution to a 15 mL graduated test tube with stopper, and
 300 the tubes were rinsed with glacial acetic acid to complete the transfer. Fill up
 301 the tubes exactly to the 15.0 mL mark with glacial acetic acid, cap, and invert
 302 several times to mix. The absorbance of the solutions was read at 280 nm on a
 303 Varian Cary 50 spectrophotometer. A blank was included to correct for
 304 background absorbance by the reagents. Determine the percentage of acetyl
 305 bromide soluble lignin (%ABSL) using the coefficient (23.35) with the following
 306 formula: % ABSL Calc: $\frac{\text{abs}}{(\text{Coeff} \times 1 \text{ cm})} \times \frac{(15 \text{ ml} \times 100\%)}{\text{weight}(\text{mg})}$, 1 cm represents the
 307 pathlength, multiplication of %ABSL with 10 results in the ug/mg cell wall unit.

308 **GC-MS Test of Lignin Composition**

309 Transfer approximately 5 mg of cell wall material into a screw-capped glass
 310 tube for thioacidolysis. Add 1 mL freshly made thioacidolysis reagent
 311 consisting of 2.5% boron trifluoride diethyl etherate (BF₃) and 10% ethanethiol
 312 (EtSH) in dioxane solution, purge vial headspace with nitrogen gas (N₂), and
 313 cap immediately. After 4 h reaction at 100°C with gentle mixing every hour, the
 314 reaction was stopped by cooling on ice for 5 min. The pH of the reaction was
 315 adjusted to 3-4 by adding 300 µL of 0.4 M sodium bicarbonate. Add 2 mL water,
 316 200 µL tetracosane (0.5 mg/ml ethyl acetate) and 0.8 mL of ethyl acetate, and
 317 vortex. Transfer 300 µL of the ethyl acetate layer into a 2 mL Sarstedt tube
 318 (make sure no water is transferred), and evaporate the solvent with N₂. To

remove excess water, 200 μ L acetone was added and evaporated twice. The finally obtained oily residue was redissolved in 500 μ L of ethyl acetate, and 100 μ L of resuspended sample was added with 20 μ L of pyridine, and 100 μ L of N, O-bis(trimethylsilyl) acetamide for the TMS derivatization, and incubated for 2 h at 25°C. The GC-MS test was performed to identify and quantify the lignin monomer trimethylsilylated derivatives, as 1 μ L injection volume sample was separated with a Restek Rxi-5ms column (30 m X 0.25 mm X 0.25 μ m film thickness) under split mode, and the injector and detector temperatures were set to 250°C. Helium was the carrier gas. The following temperature gradient was used with a 30 min solvent delay and a 1.1 mL/min flow rate: Initial hold at 130°C for 3 min, a 3°C/min ramp to a 250°C which was hold for 5 min, and then equilibration to the initial temperature of 130°C. Quantitation of the main lignin-derived monomers was performed after an appropriate calibration relative to the Tetracosane internal standard, and the characteristic mass spectrum ions of 299 m/z, 269 m/z and 239 m/z were representative for S, G and H trimethylsilylated derivatives respectively.

Phenolic Profiling by UPLC-HESI-MS/MS

Extraction of Soluble Metabolites

Samples of stems, leaves, petioles and seeds (30 DAP) were ground under liquid nitrogen in a mortar and pestle, and freeze-dried by using a vacuum freeze drier (SCANVAC, Coolsafe 110 – 4, Denmark). 30 mg ground lyophilized stems, leaves and petiole material were extracted twice by sonication with 1.0 mL of 50% methanol plus 1.5% acetic acid for 1 h at 4°C, clarified at 15,000 $\times$ g for 10 min. The supernatants were combined and concentrated by using a vacuum concentration (SCANVAC, scan speed 32, Denmark), and redissolved in 0.5 mL of 50% methanol, filtered through a 0.22 μ m nylon syringe filter. 50 mg ground lyophilized seed material was extracted as previously described (Auger *et al.*, 2010) with a little modification. One ml of a methanol/acetone/water/TFA mixture (40:32:28:0.05, v/v/v/v) was added to

the seed samples, and sonicated for 1 h at 4°C. After centrifugation (15,000 ×g, 5 min), the pellet was extracted further with 1 mL methanol/acetone/water/TFA mixture overnight at 4°C under agitation (200 rpm), while the supernatant was stored at -80°C. Supernatants were pooled and clarified at 15,000 ×g for 10 min, then concentrated. To further remove the water, 200 µL methanol was added to the extracts two times during evaporation. The dried extracts were redissolved in 1 mL of 1% acetic acid in methanol and filtered through a 0.22 µm nylon syringe filter, then stored at -80°C before analysis.

UPLC-HESI-MS/MS Analysis

UPLC was performed on Dionex Ultimate-3000 UHPLC System (Thermo Fisher Scientific, Tacoma, Washington, USA), as 5 µL samples were separated on a Waters ACQUITY UPLC BEH C18 column (1.7 µm, 2.1 mm ×150 mm). The flow rate was 0.2 mL/min, and the oven temperature was 30°C. Eluent A was 0.1% formic acid in water, and eluent B was 0.1% formic acid in acetonitrile. The following gradient was applied for stems, leaves and petiole extracts elution: 5% B for 5 min, 5% B to 95% B for 20 min, 95% B for 5 min, followed by column wash and re-equilibration. For seed metabolites elution, gradient conditions were as follows: 5% to 9% B for 5 min, 9% B to 16% B for 10 min, 16% B to 50% B for 25 min, 50% B to 95% B for 15 min, 95% B for 5 min, followed by column wash and re-equilibration. Mass analyses were carried out with the mass spectrometer Thermo Scientific™ Q Exactive™ (Thermo Fisher Scientific, Tacoma, Washington, USA) equipped with a HESI source used in the negative ion mode. Source parameters were as following: spray voltage of 3.0 kV, sheath gas flow rate at 35 arbitrary units, auxiliary gas flow rate at 10, capillary temperature at 350°C, and aux gas heater temperature at 300°C. Nitrogen gas was used as sheath gas and auxiliary gas. Full MS/dd-MS2 were acquired from m/z 100 to m/z 1500. Thermo Xcalibur software version 3.0.63 (Thermo Fisher Scientific) was used for data collection and processing. Contents of the metabolites were expressed relative to the

377 calibration curves of available standards. Standard compounds, namely
378 *p*-coumaric acid, caffeic acid, ferulic acid, epicatechin, quercetin, kaempferol
379 and isorhamnetin (from PureChem-Standard Co., Ltd, Chengdu, China) as
380 well as sinigrin, sinapic acid, coniferyl aldehyde and abscisic acid (from
381 Sigma-Aldrich, USA) were analyzed under the same conditions described
382 above.

383 **Statistical Issues**

384 Except for non-necessary cases and specially indicated situation, all
385 experiments in this study were carried out with 3 replications, and all
386 experimental results were statistically analyzed. Statistical significance was
387 calculated by two-tailed Student's t test (*P < 0.05, **P < 0.01), and error bars
388 indicate SD. One-way ANOVA followed by Duncan's multiple comparisons test
389 was used to determine the differences. Values of P < 0.05 were considered to
390 be statistically significant.

391 **Accession Numbers**

392 Sequence data of the genes and proteins involved in this article can be found
393 in the NCBI (<http://www.ncbi.nlm.nih.gov/>) databases under the accession
394 numbers indicated in [Supplementary Table S1](#) and [Fig. 1](#).

395 **Other Methods**

396 Details of the stress treatments, bioinformatic methods for this study,
397 construction of overexpression vectors, measurement of leaf spad readings,
398 and near-infrared reflectance spectroscopy (NIRS) measurements are
399 provided in supplementary methods.

400 **Results**

401 **Isolation and characterization of *CCR* genes from *Brassica* species**

402 *CCR* genes were isolated from *B. napus* and its parental species *B. rapa* and *B.*
403 *oleracea* using RACE strategy. Full-length cDNAs and corresponding gDNA

404 sequences of three, four and six *CCR1*-subfamily gene sequences were
 405 isolated from *B. rapa*, *B. oleracea* and *B. napus*, while three, three and four
 406 *CCR2*-subfamily gene sequences were isolated from *B. rapa*, *B. oleracea*,
 407 respectively. Besides, one *CCR2* pseudogene from each parental species and
 408 two *CCR2* pseudogenes from *B. napus* were also isolated ([Supplementary](#)
 409 [Table S1](#)). To determine the copy number of the *BnCCR1*, *BoCCR1*, *BrCCR1*,
 410 *BnCCR2*, *BoCCR2* and *BrCCR2* genes, Southern hybridization analysis was
 411 performed ([Supplementary Fig. S1](#)), and the numbers of the clear bands were
 412 well correlated with the cloned gene sequence numbers. [Supplementary Table](#)
 413 [S1](#) shows all identity parameters of *CCR1*-subfamily and *CCR2*-subfamily
 414 genes/pseudogenes from *B. napus* and its parental species *B. oleracea* and *B.*
 415 *rapa* in terms of our cloning work and three genome datasets (NCBI GenBank,
 416 Genoscope and BRAD). The basic features of the cDNA and gDNA sequences
 417 are displayed in [Supplementary Table S2](#), and the deduced protein features
 418 are depicted in [Supplementary Table S3](#).

419 Multiple alignments showed that the conserved NADP binding domain and
 420 the CCR-featured traditional motif (KNWYCYGK, which was believed as the
 421 catalytic site) and novel motif H₂₀₂K₂₀₅R₂₅₃ (CCR-SBM or CCR substrate
 422 binding motif) are conserved in all *Brassica* CCRs ([Lacombe et al., 1997](#); [Chao](#)
 423 [et al., 2019](#)). Therefore, it is speculated that these *Brassica* CCRs have
 424 catalytic activities. A phylogenetic tree was constructed to reveal the
 425 relationships of *Brassica* CCR1 and CCR2 proteins with CCRs from all
 426 whole-genome-sequenced species of other Brassicales species and other
 427 malvids orders ([Supplementary Fig. S2](#)). Firstly, it is clear that an early
 428 intrafamily duplication event generated the CCR1 and CCR2 groups within
 429 Brassicaceae, i.e. AtCCR1 and AtCCR2 have respective CCR1 and CCR2
 430 orthologs only within Brassicaceae family. Secondly, in Brassicaceae the
 431 CCR1 group is much more conserved than the CCR2 group, which could be
 432 reflected by the great difference in branch lengths. Thirdly, outside the

Brassicaceae family, other Brassicales families (e.g. Cleomaceae and Caricaceae) and other malvids orders (e.g. Malvales, Myrtales and Sapindales) have similar trends in CCR evolution (gene duplication and unequal divergence of paralogs) as revealed in Brassicaceae. However, paralog numbers (numbers of duplication events) vary distinctly among relative families and orders, and the single-copy CCR from Brassicales species *Carica papaya* is nearer to non-Brassicales CCRs than to other Brassicales CCRs.

Divergent involvement in organ-specificity and seed coat color among *Brassica* CCR family members

In all the three *Brassica* species the highest *CCR1* subfamily expression was detected in silique pericarp, while the highest *CCR2* subfamily expression varied among species (bud in *B. napus*, silique pericarp in *B. rapa*, and root in *B. oleracea*), implying faster divergence of organ-specificity in *CCR2* than in *CCR1* among species. Within each species, divergence of organ-specificity and expression intensity among CCR paralogs were distinct, and generally stronger divergence of organ-specificity among *CCR2* paralogs could be observed than among *CCR1* paralogs. For *CCR1* subfamily, *BnCCR1-2*, *BrCCR1-2* and *BoCCR1-1* were the dominant paralog within respective species. For *CCR2* subfamily, *BnCCR2-4*, *BrCCR2-1A* and *BoCCR2-2* were the dominant paralog within respective species ([Supplementary Figs S3-S6](#)). In all the three species, *CCR1* subfamily overall expression was distinctly lower in developing seeds especially in late-stage seeds of yellow-seed stocks than in black-seed stocks, whereas *CCR2* subfamily showed an opposite trend ([Supplementary Figs S3-S6](#)).

Differential responsiveness to various stresses between *BnCCR1* and *BnCCR2* subfamilies

For *BnCCR1*, its overall expression showed limited upregulations by NaCl, drought & high temperature and UV-B treatment, while kept almost constant when treated with other stresses ([Supplementary Fig. S7](#)). Under the same stresses, both the overall and the member-specific expressions of *BnCCR2* subfamily were dramatically upregulated. Its overall expression in leaves was

upregulated by 400.3 folds at 48 h after *S. sclerotiorum* inoculation, by 49.59 folds at 80 min after UV-B treatment, by 10.75 folds at 48 h after *P. rapae* inoculation, and with slow and slight increase after high temperature & drought treatment (Supplementary Figs S7 and S8). All *BnCCR2* subfamily members could be triggered by these stresses, and *BnCCR2-4* was dominant among paralogs (Supplementary Fig. S8). Although both *BnCCR1* and *BnCCR2* subfamilies can be distinctly regulated by multiple stresses, *BnCCR1* only mildly responds to abiotic stresses, while *BnCCR2* responds sharply and intensively to both biotic and abiotic stresses, thus *BnCCR2* is speculated to play more important roles than *BnCCR1* in coping with various stresses in *B. napus*.

BnCCR* over-expression dramatically influenced plant morphology, but did not increase resistance to UV-B and *S. sclerotiorum

To further uncover the function of *Brassica CCR* genes, overexpression transgenic plants were generated. *BnCCR1-2* and *BnCCR2-4* were selected for transgenic study as they are dominant members within respective subfamilies. The transgenic lines were coded as *BnCCR1-2ox* or *ox1* lines for *BnCCR1-2* overexpression, and *BnCCR2-4ox* or *ox2* lines for *BnCCR2-4* overexpression, in the following analysis. The transgenic lines were screened along with non-transgenic controls (WT) by GUS staining, Basta resistance and PCR detection (Supplementary Fig. S9). Six *BnCCR1-2ox* and seven *BnCCR2-4ox* triple-positive lines were obtained, with different over-expression levels revealed by qRT-PCR detection (Supplementary Fig. S10).

All *BnCCRox* plants showed a stronger morphological development than WT throughout the whole life (Fig. 2). Both *BnCCR1-2ox* and *BnCCR2-4ox* had larger and longer leaves, higher leaf chlorophyll content, larger stem diameter, wider silique, higher breaking-resistant stem strength, better lodging resistance and more siliques per plant, with distinctly stronger effects in *BnCCR1-2ox* lines than in *BnCCR2-4ox* lines (Fig. 2; Supplementary Figs S11 and S12). Phytohormone detection showed that abscisic acid (ABA) increased significantly in the leaves of *BnCCRox* lines compared with WT (Supplementary Fig. S13). The leaves of *BnCCR1-2ox* had more obvious

wrinkles and less leaf margin serrates than WT (Fig. 2c; Supplementary Fig. S12E). *BnCCR2-4ox* plants had a looser morphology with larger leaf angles (Fig. 2B and J), and their leaves were more easily to show bending and rollover phenomenon than WT when under strong sunlight and high temperature conditions (Supplementary Fig. S12D). On the other hand, upper stems of *BnCCR1-2ox* plants at late bolting stage were more easily to appear bending phenomenon, but this phenomenon would disappear after flowering stage (Fig. 2F-I; Supplementary Fig. S12A). Moreover, *BnCCR1-2ox* lines flowered 7-10 days later than WT on average, and this phenomenon varied among years and environments. However, *BnCCR2-4ox* plants had no significant difference in flowering time when compared with WT. Interesting phenomenon was observed on petiole-vein system, which was distinctly larger in transgenic plants than WT, but phloroglucinol-HCl staining showed that petiole-vein lignification was strengthened only in *BnCCR1-2ox* plants while was weakened in *BnCCR2-4ox* plants (Fig. 2D; Supplementary Fig. S12F, H and J). Seed yield per plant of *BnCCR1-2ox* had little difference compared with WT, but *BnCCR2-4ox* had slightly decreased seed yield (Supplementary Fig. S11L). Besides, both *BnCCR1-2ox* and *BnCCR2-4ox* plants exhibited no better resistance to *S. sclerotiorum* inoculation and UV-B treatment when compared with WT, even some transgenic lines had a little reduction in disease resistance in leaf identification (Supplementary Fig. S14A, B, E and H).

Yellow-seed traits generated in *BnCCRox* lines

As displayed in Fig. 3A, the seed color of both *BnCCR1-2ox* and *BnCCR2-4ox* turned lighter compared with WT, and *BnCCR1-2ox* showed a stronger effect than *BnCCR2-4ox*. Microscopic investigation of the frozen sections of seed coat (Fig. 3B) and R value obtained through NIRS assay (Fig. 3E) further proved this effect. However, the thickness of the seed coat had no significant difference between *BnCCRox* and WT, which was different from traditionally bred yellow-seeded cultivars which usually had thinner seed coat than black-seeded cultivars (Qu *et al.*, 2013; Zhang *et al.*, 2013). As expected, the *BnCCR1-2ox* seed coat had an apparent reduction of condensed tannin compared with WT, e.g. ox1-5, ox1-8, ox1-12 and ox1-14 had a decrease of 69%, 29%, 40% and 57% respectively (Fig. 3C and G). But unexpectedly, a

significant increase was found for *BnCCR2-4ox* seed coat when compared with WT, e.g. ox2-4, ox2-11, ox2-16 and ox2-25 showed an increase of 88%, 300%, 158% and 83% respectively (Fig. 3C and G), implying looser condensation of the tannin structure caused by unknown mechanisms. Furthermore, all *BnCCRox* lines had a significant reduction of the thousand-seed weight, with *BnCCR1-2ox* lines reduced by 10%-20%, and *BnCCR2-4ox* lines reduced by 15%-30% (Fig. 3D). Surprisingly, the NIRS detection results showed that the total glucosinolates content of *BnCCR1-2ox* lines had a considerable increase compared with WT and a decline in *BnCCR2-4ox* lines (Fig. 3F).

***BnCCR* over-expression greatly changed lignification phenotypes**

The frozen cross sections of stem displayed that both *BnCCR1-2ox* and *BnCCR2-4ox* stems had an changed shape with more concave and convex and a wider xylem parts with deeper histochemical staining (Fig. 4A-C), and some extreme *BnCCR1-2ox* lines appeared an ectopic lignin deposition as shown in Fig. 4C. Observed with higher amplification folds of the sections, *BnCCR1-2ox* showed a better developed xylem part, had more number and more concentrated vessels with deeper brown color (indicating more G-lignin unit) in Mäule staining in which G- and S-type lignin units could be stained brown and red respectively (Fig. 4F and G, early flowering stage), with brighter red phloroglucinol-HCl staining (Fig. 4H, mature stage) and brighter blue fluorescence (Fig. 4I and J, early flowering stage) as compared with WT. These indicated that *BnCCR1-2ox* stems contained higher level of lignin content compared with WT. *BnCCR2-4ox* stem sections also displayed a better developed xylem part and interfascicular fiber part. Although its vessel number and size were not obviously different from WT, there was a significant deeper red color (indicating S-lignin unit) in both Mäule staining (Fig. 4F and G) and phloroglucinol-HCl staining (Fig. 4H), especially to the interfascicular fiber cells wall, and brighter blue florescence under UV light (Fig. 4I and J) compared with WT. This result indicated that *BnCCR2-4ox* stems had higher proportion of

559 S-lignin besides with higher total lignin content. Similar enhancement trends of
560 the lignification pattern were also found in detection results of siliques (Fig.
561 4K-N).

562 This study also reveals that rapeseed has two types of roots: type I with
563 higher proportion of interfascicular fibers, while type II with higher proportion of
564 vessels (Fig. 4D and E). In *BnCCR1-2ox* and *BnCCR2-4ox* lines, both type I
565 and type II roots displayed a better developed xylem tissues with bigger size
566 and more concentrated vessels, and had a deeper staining by
567 phloroglucinol-HCl method compared with WT (Fig. 4D and E). Besides, type II
568 roots of *BnCCR1-2ox* showed a significantly better vessel development (Fig.
569 4E) than WT and *BnCCR2-4ox*. Through comparison of the above-mentioned
570 histochemical staining assay or fluorescence excitation assay of stems and
571 roots, *BnCCR1-2ox* had better developed vascular bundles, while
572 *BnCCR2-4ox* had better developed interfascicular fibers than WT (Fig. 4D, G,
573 H and J).

574 For leaf traits, the petiole of *BnCCR1-2ox* tended to be more circular with
575 more and better developed vascular bundles (Fig. 4O and Q). But for
576 *BnCCR2-4ox* lines, it was smaller than WT and developed asymmetrically with
577 fewer and less developed vascular bundles (Fig. 4O and Q), maybe this was
578 one reason that caused *BnCCR2-4ox* leaves much easier to bend and rollover
579 than WT when under strong sunlight and high temperature conditions.
580 Moreover, *BnCCR1-2ox* lines had a deeper brown color in Mäule staining and
581 had a brighter blue fluorescence under UV light than WT (Fig. 4P and Q),
582 which indicated that *BnCCR1-2ox* petiole xylem contained higher lignin
583 content as well as higher G-lignin proportion. The *BnCCR2-4ox* petiole
584 sections displayed no better Mäule staining, and the blue fluorescence was
585 significant weaker than WT (Fig. 4Q), which indicated that *BnCCR2-4ox*
586 petiole xylem contained less lignin content which was consistent with
587 phloroglucinol-HCl staining of leaves (Supplementary Fig. S12J).

588 **Notable changes in content and structure of lignins in *BnCCR*** 589 **over-expression lines**

590 Biochemical analysis showed that the acetyl-bromide soluble lignin content of
591 both *BnCCR1-2ox* and *BnCCR2-4ox* was significantly increased compared to
592 WT (Fig. 4R-T). The lignin content of stems of *BnCCR1-2ox* lines ox1-5, ox1-8,
593 ox1-12 and ox1-14 increased by 24%, 34%, 23% and 15%, respectively (Fig.
594 4R). For *BnCCR2-4ox* lines ox2-4, ox2-11, ox2-16 and ox2-25, their stem
595 lignin content increased by 40%, 25%, 31% and 51%, respectively (Fig. 4R). In
596 roots, the lignin content of ox1-5, ox1-8, ox1-12, ox1-14, ox2-4, ox2-11, ox2-16
597 and ox2-25 increased by 0.4%, 18%, 15%, 5%, 18%, 25%, 12% and 23%
598 compared with WT, respectively (Fig. 4S). Similar trends also happened in
599 seed coat of *BnCCR1-2ox* lines (Fig. 4T). Similar to tannin detection result, the
600 seed coat lignin content of the *BnCCR2-4ox* lines increased by 92%-130% (Fig.
601 4T), implying possible decreased lignin polymerization.

602 S/G ratio, which was typically used to characterize the lignin structure,
603 changed significantly in both stems and roots of *BnCCRox* lines. In the stems
604 of both *BnCCR2-4ox* and WT, their lignin had higher content of S-unit than
605 G-unit, and the S/G ratio was increased by 20.2% and 22.5% in ox2-4 and
606 ox2-16 compared with WT (Fig. 4W). It was mainly attributed to their greater
607 proportion of S-lignin content (Fig. 4U and W; Supplementary Fig. S15), which
608 was in consistent with the Mäule staining results. However, in the case of the
609 stems of *BnCCR1-2ox* lines, the G-unit, other than S-unit, served as the main
610 lignin units, so it was contrary to *BnCCR2-4ox* and WT. Due to increased
611 G-unit proportion, the S/G ratio in the stems of ox1-5 and ox1-12 decreased by
612 47.3% and 24.0%, respectively, compared to WT (Fig. 4U and W;
613 Supplementary Fig. S15). In roots, the alteration tendency of S/G ratio of ox1-5
614 and ox2-16 was similar to that in stems, decreased to 0.22 and increased to
615 0.78, respectively as compared with WT (0.49) (Fig. 4X).

616 Another striking change was H-lignin proportion, which generally had trace
617 amount in dicotyledonous stems. The H-lignin content of ox1-5 and ox1-12
618 was about four and three folds of the WT respectively, while was increased by
619 only about 50% in ox2-4 and ox2-16 (Fig. 4U; Supplementary Fig. S15). In the

620 root of ox1-5, the H-lignin content also had an obvious increase, reaching
621 about two folds of WT amount (7.43% of WT VS 15.59% of ox1-5) (Fig. 4V;
622 Supplementary Fig. S15). In conclusion, the lignin structure of *B. rapus* was
623 undoubtedly modified after the manipulation of *BnCCR* genes. However, the
624 NIRS results indicated that cellulose and hemicellulose contents of both
625 *BnCCR1-2ox* and *BnCCR2-4ox* lines had no significant difference when
626 compared with WT (Supplementary Fig. S16).

627 **Metabolic remodeling of phenylpropanoid and glucosinolate pathways** 628 **by over-expressing *BnCCR1* and *BnCCR2***

629 The drastic alteration of seed color, seed coat condensed tannin content, lignin
630 content, lignin structure and plant phenotypes all indicated flux change within
631 and outside the lignin biosynthetic network in *BnCCR* overexpressors.

632 Conforming to prediction, most phenolic compounds synthesized at the
633 downstream of CCR were apparently increased in the stems of ox1-5 and
634 ox2-16 lines, including sinapoylhexose, sinapic acid, sinapoyl malate, ferulic
635 acid, feruloyl malate, *p*-coumaraldehyde and 1,2-disinapoylglucoside, with an
636 increase of about 1-10 folds, and ox2-16 displayed stronger effects than ox1-5
637 (Fig. 5A; Supplementary Table S4). However, flavonoids in ox1-5 stems were
638 reduced to 3%-71% of WT, including km-3-O-sophoroside-7-O-glucoside, rutin,
639 is-3-sophoroside-7-glucoside, km-3-O-sinapoylsophoroside-7-O-glucoside,
640 qn-3-O-sophoroside, qn-3-O-glucoside, km-3-O-glucoside and
641 is-3-O-glucoside; and ox2-16 showed the same trend as ox1-5 but with a less
642 extent (Supplementary Table S4). Leaf extracts of ox1-5 and ox2-16 had
643 similar variation tendency as in stems, but some compounds were
644 undetectable in leaves or had an opposite change (Supplementary Table S4).
645 Metabolites profiling was also performed on 30 DAP seeds. As expected, the
646 CCR-downstream compounds sinapic acid and disinapoylgentiobiose were
647 significantly increased in ox1-5 and ox2-16 (Supplementary Table S5). The
648 contents of the most compounds of epicatechin, procyanidin, epicatechin
649 polymers and other important flavonoids were significantly reduced (by even

more than 90% for some flavonoids) in ox1-5 compared with wild type, and ox2-16 showed the same trend as ox1-5 but with a less extent (Fig. 5B; Supplementary Table S5).

In metabolic profiling, glucosinolates were unexpectedly found to be drastically changed by *BnCCR* overexpression. A variety of aliphatic glucosinolates were distinctly differentially deposited between *BnCCRox* lines and WT. For example, 2(R)-2-hydroxy-3-butenyl glucosinolate, 1-S-[(3S)-3-Hydroxy-N-(sulfooxy)-5-hexenimidoyl]-1-thio-beta-D-glucopyranoside, 3-butenylglucosinolate, isobutyl glucosinolate, 4-pentenyl glucosinolate, 5-methylsulfinylpentyl glucosinolate and 5-methylthiopentyl glucosinolate were upregulated to hundreds of folds or even more than 1000 folds in ox1-5 stems as compared with WT, while in ox2-16 stems they were just slightly upregulated or even downregulated (Fig. 5C; Supplementary Table S4). In addition, 4-methylthiobutyl glucosinolate and 6-methylthiohexyl glucosinolate were obviously accumulated in ox1-5 stems (not in ox2-16 and WT stems). According to the quantitative results, glucosinolates had larger amounts of accumulation in leaves than in stems (Supplementary Table S4), and most of them in leaves were several folds higher in ox1-5 than in WT. However, most of them were downregulated by hundreds of folds in the leaves of ox2-16 in comparison with WT. 2(R)-2-Hydroxy-3-butenyl glucosinolate, 1-S-[(3S)-3-Hydroxy-N-(sulfooxy)-5-hexenimidoyl]-1-thio-beta-D-glucopyranoside and 5-methylthiopentyl glucosinolate were even not detectable in the leaves of ox2-16 (Supplementary Table S4). Glucosinolates variation in seed coat was similar to that in the leaf (Supplementary Table S5). Modification of secondary metabolites in petioles was ultimately similar to that in stems (Supplementary Table S6).

Differential redirection of gene expression in lignin, flavonoid and glucosinolate pathways in *BnCCR1* and *BnCCR2* over-expressors

In *BnCCR1-2ox* lines, *BnCCR1* subfamily itself was significantly upregulated,

679 and *BnCCR2* subfamily showed no significant upregulation. In *BnCCR2-4ox*
680 lines, the *BnCCR2* subfamily itself was undoubtedly greatly upregulated, while
681 *BnCCR1* subfamily was also significantly upregulated (1-3 folds) in the stems,
682 petioles and 30DAP seeds but significantly downregulated by 70% in the
683 leaves (Supplementary Fig. S17). These results indicated that over-expression
684 of *BnCCR1* subfamily had little impact on the expression of *BnCCR2* subfamily,
685 but over-expression of *BnCCR2* subfamily could significantly upregulate or
686 downregulate the expression of *BnCCR1* subfamily depending on different
687 organs.

688 In the stems of both *BnCCR1-2ox* and *BnCCR2-4ox* lines, the common
689 phenylpropanoid pathway loci *C4H* and *4CL* and the lignin-pathway early-step
690 loci *C3H*, *HCT* and *CCoAOMT* were mildly upregulated (Fig. 6A), and
691 *CCR*-downstream loci *CAD* and *F5H* were slightly upregulated. *COMT* was
692 specific, apparently upregulated in the stems of *BnCCR1-2ox*, but
693 downregulated to less than 10% in the stems of *BnCCR2-4ox* as compared
694 with WT.

695 On the other hand, the expression of the flavonoid biosynthesis pathway
696 was significantly downregulated in *BnCCRox* lines. Regulatory genes *TT1*,
697 *TT2*, *TT16*, *TTG1* and *TTG2*, and structural genes *CHS*, *CHI*, *F3H*, *F3'H*, *FLS*,
698 *ANR*, *TT19*, *GSTF11* and *TT12* were all suppressed to certain extent in 30DAP
699 seeds of both *BnCCR1-2ox* and *BnCCR2-4ox* (Fig. 6B), with less extent in
700 *BnCCR2-4ox* than in *BnCCR1-2ox*. *CHS*, *CHI*, *F3H*, *ANR* and *TT19* were
701 downregulated to less than 20% in the 30DAP seeds of ox1-5 when compared
702 with WT (Fig. 6B). The structural genes *AHA10* and *TT10* and the regulatory
703 genes *TT8* and *MYB111* were significantly downregulated in 30DAP seeds of
704 *BnCCR2-4ox*, but were unexpectedly significantly upregulated in the 30DAP
705 seeds of *BnCCR1-2ox* lines (Fig. 6B). The overall downregulation of the whole
706 flavonoid pathway could account for the reduction of the flavonoids in
707 *BnCCRox* lines.

Most of the genes associated with the aliphatic glucosinolate biosynthesis, such as *MYB28*, *MYB29*, *CYP79F1*, *CYP83A1*, *AOP2* and *GSL*, were significantly upregulated in *BnCCR1ox* lines (Fig. 6C). Especially, the expression of *MYB29*, *CYP79F1* and *GSL* could hardly be detected in WT and *BnCCR2ox* lines, but was considerably expressed in *BnCCR1ox* lines. *AOP2* was a specific locus, which showed considerable downregulation in *BnCCR2ox*, a trend opposite to that in *BnCCR1ox* (Fig. 6C). The expression of most genes involved in indole glucosinolate biosynthesis, such as *MYB122*, *CYP79B2*, *ST5a* and *IGMT1*, was dramatically decreased in both *BnCCR1ox* and *BnCCR2ox* lines, except that *MYB34* was significantly upregulated in both *BnCCR1ox* and *BnCCR2ox* lines (Fig. 6C). *MYB51* was significantly upregulated in *BnCCR1ox* lines but extremely downregulated in *BnCCR2ox* lines, implying that *MYB51* may also be involved in aliphatic glucosinolate biosynthesis in *B. napus*.

Discussion

Both *BnCCR1* and *BnCCR2* are crucial for lignification, associated with different monolignols and cellular types

Plants with downregulated CCR activities often displayed reduction in lignin content and alteration in lignin structure depending on different species (Goujon *et al.*, 2003; Zhou *et al.*, 2010; Prashant *et al.*, 2011). Lignin content had a great decrease in *Arabidopsis CCR1* mutant *irx4* (Jones *et al.*, 2001) and CCR-suppressed transgenic *Arabidopsis* (Goujon *et al.*, 2003), tobacco (Piquemal *et al.*, 1998), poplar (Leplé *et al.*, 2007), *Medicago truncatula* (Zhou *et al.*, 2010) and perennial ryegrass (Tu *et al.*, 2010).

In this study, anatomical observation and histochemical assays both indicated significant improvement of lignification in stems and roots in both *BnCCR1* and *BnCCR2* over-expressors, and *BnCCR1* over-expressor showed stronger effect on development of vessels and vascular bundles in xylem,

736 while *BnCCR2* over-expressor showed stronger effect on interfascicular fibers.

737 Furthermore, the S/G ratio was significantly decreased in *BnCCR1-2ox*,
738 mainly due to the stronger increase of G-lignin, the S/G ratio in *BnCCR2-4ox*
739 was significantly increased, mainly attributed to stronger increase of S-lignin.
740 The alteration of S/G ratio caused by CCR manipulation always had different
741 trends in different species. A lower S/G ratio, mainly caused by relatively
742 stronger effect of CCR downregulation on S-units, was observed on
743 *Arabidopsis irx4* mutants (Patten *et al.*, 2005), CCR-downregulated poplar
744 (Leplé *et al.*, 2007) and *ccr1*-knockout *M. truncatula* mutants (Zhou *et al.*,
745 2010). A higher S/G ratio, mainly caused by relatively stronger effect of CCR
746 downregulation on G-units, was found in CCR-downregulated tobacco
747 (Chabannes *et al.*, 2001), maize (Tamasloukht *et al.*, 2011), dallisgrass
748 (Giordano *et al.*, 2014) and *CCR2*-knockout *M. truncatula* mutants (Zhou *et al.*,
749 2010). No obvious change of S/G ratio was recorded in poplar (Leplé *et al.*,
750 2007) and perennial ryegrass with CCR manipulation (Tu *et al.*, 2010).

751 Additionally, the H-lignin percentage in stems of *BnCCR1-2ox* was 2-3 folds
752 higher than WT, and also more than one-fold higher in roots. Which could be
753 caused by the enhanced accumulation of *p*-coumaraldehyde and expression
754 of *F5H* and *COMT* in *BnCCR1-2* over-expressor. Reasonably,
755 *p*-coumaroyl-CoA and caffeoyl-CoA might serve as the primary substrates for
756 *BnCCR1* (as suggested in Fig. 7). In *CCR1*-downregulated perennial ryegrass
757 (Tu *et al.*, 2010) and Mu-insertion maize mutant *Zmccr1* (Tamasloukht *et al.*,
758 2011), the H-subunit level was reduced by about 50% and 31% respectively,
759 suggesting that the optimal substrate of CCR1 of perennial ryegrass and
760 maize was *p*-coumaroyl-CoA. On the other hand, *BnCCR2* might prefer
761 feruloyl-CoA as its major substrate, because of higher accumulation of S-units
762 and its downstream derivatives sinapate esters, and higher increase in
763 transcript level of *F5H* in *BnCCR2ox* as compared with *BnCCR1ox* (Fig. 7).

764 **Upregulation of *BnCCR* genes enhanced lodging resistance, modified**
765 **morphology, but did not improve disease and UV-B resistance**

766 The expression perturbation of the genes located at the lignin biosynthetic
767 pathway was often accompanied with defects in plant growth and development
768 depending on which gene was targeted. For the severely silenced *CCR* plants,
769 phenotypic abnormalities with irregular vessels usually arise, including plant
770 size reduction, delayed flowering, delayed senescence, retarded seed
771 development, biomass yield reduction and compromised pathogen defense
772 (Leplé *et al.*, 2007; Zhou *et al.*, 2010; Vanholme *et al.*, 2012; Van Acker *et al.*,
773 2014; Xue *et al.*, 2015; De Meester *et al.*, 2018; De Meester *et al.*, 2020).

774 Firstly, *BnCCR* over-expression improved lodging resistance compared with
775 WT, especially for *BnCCR1-2ox* (Fig. 2; Supplementary Fig. S12). When *CCR1*
776 was reintroduced into *A. thaliana ccr1* mutants under the control of the
777 ProSNBE promoter, specific expression of *CCR1* in the protoxylem and
778 metaxylem vessel cells showed a full recovery in vascular integrity (De
779 Meester *et al.*, 2018). The breaking-resistance of both *BnCCR1-2ox* and
780 *BnCCR2-4ox* plants was greatly enhanced in comparison with WT, with larger
781 effect in *BnCCR1-2ox* lines than in *BnCCR2-4ox* lines. These results imply an
782 improved lodging resistance of *BnCCRox* lines due to better development and
783 growth in both morphology and lignification of roots and stems, which provides
784 significant potential in molecular breeding of rapeseed with enhanced lodging
785 resistance through over-expressing *BnCCR* genes.

786 Secondly, *BnCCR* over-expression also modified leaf morphology. Our
787 Mäule staining results indicated that rapeseed petiole xylem was exclusively
788 composed of vascular bundles without interfascicular fibers, which could
789 account for better development of leaf veins in *BnCCR1-2ox* plants, since
790 vascular bundles contained higher proportion of G-lignin and *BnCCR1-2* was
791 mainly involved in the biosynthesis of G- and H-lignins. In *Arabidopsis*, *CCR1*
792 also mediated cell proliferation exit for leaf development, and *ccr1-4* mutant
793 had a significantly reduced leaf and plant size compared with WT (Xue *et al.*,
794 2015). However, *BnCCR2-4ox* plants showed less developed leaf vascular
795 bundles (Fig. 4O; Supplementary Fig. S12J), which could possibly be resulted
796 from two reasons: weakened G-unit synthesis (since *BnCCR2-4*
797 overexpression mainly forced S-unit synthesis), and downregulated *BnCCR1*
798 expression in leaves of *BnCCR2-4ox* plants (Supplementary Fig. S17B).

Enhanced levels of ABA might also play a role in the alteration of leaf phenotypes of *BnCCRox* plants (Supplementary Fig. S13), as ABA plays important roles in plant growth and development (Cutler *et al.*, 2010).

Thirdly, resistance of *BnCCRox* plants to *S. sclerotiorum* and UV-B was not improved, which was in conflict with traditional notion and our original expectation. There are many factors involved in plant disease resistance, such as the epidermal cuticle-wax layers, trichomes, lignified cell walls, phenolics, phytoalexins and pathogenesis-related proteins (Zhao *et al.*, 2007a). In the present study, although the lignin content was increased through *BnCCR* over-expression, the other important factors correlated to the plant resistance were not enhanced or even suppressed by flux competition between lignin pathway and flavonoid and other pathways, which could be reflected by the changes of flavonoid and glucosinolate metabolic profiles. Besides, decrease of epidermal wax layer was observed on leaves of *BnCCR1-2ox* plants compared with *BnCCR2-4ox* and WT (Supplementary Fig. S18).

***BnCCR1* and *BnCCR2* differentially affect plant development progress**

During the progress of the growth and development of *BnCCRox* plants, the upper stem of *BnCCR1-2ox* plants at bolting stage was more easily to bend, but *BnCCR2-4ox* plants had no such case (Fig. 2F; Supplementary Fig. S12A). As reported, S-lignin percentage in stems would gradually increase during plant growth process (Tu *et al.*, 2010; Giordano *et al.*, 2014). Mäule staining of stem cross sections in this study revealed similar trend (Supplementary Fig. S19), suggesting that S-lignin might play an important role for maturation and mechanical strength of stems. (Kaur *et al.*, 2012) found that the stem of *CAD*-downregulated *Nicotiana* plants (*ir-CAD*) presented a rubbery phenomenon, and the S/G ratio was significantly reduced. Here the S/G ratio was also significantly decreased in *BnCCR1-2ox* plants (1.29 in WT, 0.68 in *ox1-5*, and 0.98 in *ox1-12*). Moreover, our results showed that in WT the S/G ratio in stem (1.29) was significantly higher than in root (0.49), which might be one factor contributing to the more flexible texture of roots compared with

829 stems, although total lignin content was higher in root than in stem (Fig. 4R
830 and S). Lower percentage of S-units might contribute to a lower level of
831 stiffness of the stem, together with the lower lignin content in the upper stem,
832 which might be one reason of the bending phenomenon of *BnCCR1-2ox* at
833 bolting stage. However, when *BnCCR1-2ox* plants entered flowering stage, the
834 bending phenomenon disappeared, which might be due to increase in total
835 lignin content and S-lignin percentage in stems (Supplementary Fig. S19).
836 Relationship between S/G ratio and the texture of the plant stems was
837 summarized in the Supplementary Table S7. These results suggest that higher
838 S/G ratio might contributes to stronger stiffness in plant stems, which is
839 manifested during reproductive growth.

840 In this study, *BnCCR1-2ox* plants flowered distinctly later than WT and
841 *BnCCR2-4ox* plants, implying that the function of Brassicaceae *CCR1* is also
842 associated with plant development progress. There was an evolutionarily
843 conserved mechanism between cell wall biosynthesis and production of
844 flowers (Vermerris *et al.*, 2002). The delayed accumulation of S-lignin and
845 lowered stem stiffness at bolting stage were reflections of delayed
846 development progress and flowering in *BnCCR1-2ox* plants.

847 ***BnCCR1* and *BnCCR2* exert different flux control on flavonoid pathway** 848 **and lignin-derivative pathways in *B. napus***

849 In metabolic engineering, products, precursor steps as well as associated
850 neighbor pathways would be affected via metabolic flux redirection. In lignin
851 engineering, for example, significantly higher amounts of vanillin, ferulic acid,
852 *p*-coumaric acid, coniferaldehyde and syringaldehyde were released from the
853 cell wall samples of *MtCAD1* mutants than the wild-type (Zhao *et al.*, 2013). In
854 the *C3'H* (Abdulrazzak *et al.*, 2006) or *HCT* (Besseau *et al.*, 2007) defect
855 *Arabidopsis*, *C3H1*-downregulated maize (Fornalé *et al.*, 2015), *CCR*-silenced
856 tomato (van der Rest *et al.*, 2006) and perennial ryegrass (Tu *et al.*, 2010), the
857 accumulation of flavonoids was significantly increased.

858 Besides enhancement of lignin monomers, our results also showed that both

859 *BnCCR1-2* and *BnCCR2-4* over-expressors had significantly increased levels
860 of various CCR-downstream pathway products when compared with WT,
861 especially for sinapate esters (Fig. 5; Supplementary Tables S4-S6), which
862 could be caused by the enhanced expression levels of corresponding genes
863 *CCR*, *F5H*, *COMT* and *CAD*. Moreover, *BnCCR2-4ox* plants had a stronger
864 effect on the accumulation of sinapate esters in stems than *BnCCR1-2ox*
865 plants, implying that the metabolic route related to *BnCCR2* catabolism (mainly
866 S-units synthesis) might be closer to sinapate ester pathway than *BnCCR1*
867 (Fig. 5A; Fig. 7).

868 More strikingly, many compounds synthesized in flavonoid pathway were
869 significantly reduced in *BnCCRox* plants compared with WT. The dramatic
870 reduction in flavonoids was theoretically caused by the reduced availability of
871 *p*-coumaroyl-CoA precursor for CHS as *BnCCR* overexpression attracted more
872 of this common precursor into lignin pathway (Fig. 7). This kind of flux shift
873 could be further evidenced on molecular level (Fig. 6). Seed color degree,
874 metabolic profile and gene expression profile all indicated that suppression
875 impact on flavonoid pathway was greater in *BnCCR1-2ox* plants than in
876 *BnCCR2-4ox* plants (Figs 3 and 6; Supplementary Tables S4-S6). Which could
877 result from the fact that the metabolic routes of *BnCCR1* catabolism (mainly H-
878 and G-units synthesis) were closer to flavonoid pathway than that of *BnCCR2*
879 (mainly S-unit synthesis) as suggested in Fig. 7.

880 *BnCCR1* and *BnCCR2* play different roles in crosstalk between 881 **phenylpropanoid pathway and glucosinolate pathway**

882 There were a few reports on crosstalk effect of glucosinolate pathway on
883 phenylpropanoid pathway (Hemm *et al.*, 2003; Kim *et al.*, 2015; Kim *et al.*,
884 2020). In *Arabidopsis*, the accumulation of phenylpropanoids were significantly
885 suppressed in *ref5-1* mutant, and *REF5* was proved to encode CYP83B1
886 which was involved in biosynthesis of indole glucosinolates (Kim *et al.*, 2015).
887 Defect of phenylpropanoids deposition was also detected in *Arabidopsis ref2*
888 mutant, as *REF2* encoded CYP83A1 which played a role in aliphatic
889 glucosinolate pathway (Hemm *et al.*, 2003). In low-lignin *c4h*, *4cl1*, *ccoamt1*
890 and *ccr1* mutants of *Arabidopsis*, transcripts of some glucosinolate

891 biosynthesis genes were more abundant (Vanholme *et al.*, 2012). However, to
892 date there is no systemic study on crosstalk effect of phenylpropanoid pathway
893 on glucosinolate pathway.

894 In present study, the aliphatic glucosinolates were significantly increased in
895 *BnCCR1-2ox* (Supplementary Table S4), which could result from upregulated
896 expression of *MYB28*, *MYB29*, *CYP79F1*, *CYP83A1*, *AOP2* and *GSL* in the
897 leaves of transgenic plants (Fig. 6). Among them, the expression of *MYB29*,
898 *CYP79F1* and *GSL* could almost only be detected in *BnCCR1-2ox*, not in WT.
899 Reasonably, the undetectable *MYB29* expression and extremely lower
900 expression of *MYB51*, *MYB122*, *AOP2*, *CYP79B2*, *ST5a* and *IGMT1* in
901 *BnCCR2-4ox* plants could be responsible for the decrease of glucosinolate
902 content. Because the WT material ZS10 of this study was a “double-low” (low
903 erucic acid, low glucosinolates) commercial cultivar, the low proportion of
904 indole and aromatic glucosinolates in WT and the limited change of indole and
905 aromatic glucosinolates in *BnCCR*-overexpressing plants might be caused by
906 breeding impairment of respective biosynthesis pathways of these two types of
907 glucosinolates. If a “double-high” stock was used as WT for *BnCCR*
908 transformation, the effect on indole and aromatic glucosinolates deposition
909 might not be so mild. Taken together, both *BnCCR1-2* and *BnCCR2-4* distinctly
910 affect glucosinolate biosynthesis, but have divergent or almost opposite
911 effects.

912 Crosstalk effect of glucosinolate pathway on phenylpropanoid pathway could
913 be linked through PAL degradation that mediated by Med5-KFBs-dependent
914 manner (Kim *et al.*, 2020). However, what mechanism is involved in change of
915 glucosinolate biosynthesis by manipulating phenylpropanoid genes, such as
916 *CCR*, needs to be clarified. Our qRT-PCR results indicate that at least
917 transcription regulation is involved, but whether mediator-mediated protein
918 degradation in glucosinolate pathway is also involved deserves future study.

919 **Supplementary data**

920 **Fig. S1.** Southern blot detection of *CCR* subfamily genes in *B. napus*, *B. rapa*
921 and *B. oleracea*.

922 **Fig. S2.** Multi-alignment indicates that *Brassica* CCRs contain complete
923 structural features as those in model plants *A. thaliana* and poplar.

924 **Fig. S3.** Overall expression patterns of *CCR1*-subfamily and *CCR2*-subfamily
925 show distinct organ-specificity and difference between black and yellow seeds
926 in *B. napus*, *B. rapa* and *B. oleracea*.

927 **Fig. S4.** Expression patterns of *CCR1*-genes and *CCR2*-genes show distinct
928 organ-specificity and difference between black and yellow seeds in *B. napus*.

929 **Fig. S5.** Expression patterns of *CCR1*-genes and *CCR2*-genes show distinct
930 organ-specificity and difference between black and yellow seeds in *B. rapa*.

931 **Fig. S6.** Expression patterns of *CCR1*-genes and *CCR2*-genes show distinct
932 organ-specificity and difference between black and yellow seeds in *B.*
933 *oleracea*.

934 **Fig. S7.** Overall expression of *BnCCR1*-subfamily and *BnCCR2*-subfamily
935 distinctly respond to various stresses in *B. napus* seedlings.

936 **Fig. S8.** *BnCCR1*-genes and *BnCCR2*-genes distinctly respond to various
937 stresses in *B. napus* seedlings.

938 **Fig. S9.** Basta-resistance, GUS-staining and PCR determination of transgenic
939 lines.

940 **Fig. S10.** qRT-PCR shows distinct upregulation of the target genes in
941 respective T2 lines of *BnCCR1-2ox* and *BnCCR2-4ox*.

942 **Fig. S11.** Multiple agronomic traits are significantly modified in *BnCCR1-2ox*
943 and *BnCCR2-4ox* lines.

944 **Fig. S12.** *BnCCR1-2ox* and *BnCCR2-4ox* lines show different growth
945 behaviors and leaf vein strengths.

946 **Fig. S13.** ABA content is upregulated in leaves of *BnCCR1-2ox* and
947 *BnCCR2-4ox* lines.

948 **Fig. S14.** Plants of both *BnCCR1-2ox* and *BnCCR2-4ox* do not show
949 enhanced resistance to *S. sclerotiorum* and UV-B.

950 **Fig. S15.** GC-MS chromatograms show different changes of monolignol
951 proportions in *BnCCR1-2ox* and *BnCCR2-4ox* lines.

952 **Fig. S16.** Contents of cellulose and hemicellulose in the stems of *BnCCR1-2ox*
953 and *BnCCR2-4ox* lines are not changed based on NIRS detection.

954 **Fig. S17.** *BnCCR1* expression is obviously altered in *BnCCR2-4ox* lines,
955 whereas *BnCCR2* expression has little change in *BnCCR1-2ox* lines.

956 **Fig. S18.** Leaf surfaces of *BnCCR1-2ox* and *BnCCR2-4ox* plants are less flat
957 with decreased wax deposition than WT.

958 **Fig. S19.** Mäule staining of stem sections indicates a trend of increase of
959 S-type lignins during developmental process in *B. napus*.

960 **Table S1.** Identity parameters of *CCR1*-subfamily and *CCR2*-subfamily genes
961 from *B. napus* and its parental species *B. oleracea* and *B. rapa*.

962 **Table S2.** cDNA and gDNA basic parameters of *Brassica CCR1* and *CCR2*
963 subfamily genes cloned in this study.

964 **Table S3.** Basic parameters and important features of *Brassica CCR1* and
965 *CCR2* proteins.

966 **Table S4.** Contents of major soluble secondary metabolites differentially
967 accumulated in stems and leaves of ox1-5 and ox2-16 compared with WT as
968 revealed by UPLC-HESI-MS/MS.

969 **Table S5.** Contents of major soluble secondary metabolites differentially
970 accumulated in 30DAP seeds of ox1-5 and ox2-16 compared with WT as
971 revealed by UPLC-HESI-MS/MS.

972 **Table S6.** Contents of major soluble secondary metabolites differentially
973 accumulated in petioles of ox1-5 and ox2-16 compared with WT as revealed
974 by UPLC-HESI-MS/MS.

975 **Table S7.** S/G ratio of stem lignin, stem stiffness, and plant phenotype of
976 different angiosperm species.

977 **Table S8.** Primers used in this study.

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988 **Conflict of interest**

989 The authors declare no conflict of interest.

990 **Author contributions**

991 Y.C., J.L. and N.Y. designed the study; N.Y., B.L., X.L. and Y.X. performed the
992 experiments; Y.C. did molecular and bioinformatic analysis, N.Y., J.L., M.C.,
993 K.L., L.W. and Y.L. analyzed or evaluated the results; J.L. and R.W. provided
994 some *Brassica* materials. N.Y. and Y.C. performed final analysis and wrote the
995 article.

996 **Data availability statement**

997 The data supporting the findings of this study are available from the

998 corresponding author (Jiana Li and Yourong Chai) upon request.

999

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Figure legends

Fig. 1. Phylogenetic analysis of CCR refseq_proteins from representative whole-genome-sequenced Malvids species including *B. napus*, *B. rapa* and *B. oleracea*.

Latin name, refseq_protein name and accession number are provided for each sequence. Names of cloned genes in this study are given after the accession numbers. Vertical bars on the right indicate corresponding order or order-family names.

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Bending phenomenon of the upper stem of BnCCR1-2ox plants at late bolting stage (F) will disappear after flowering (I).

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(K-N) Silique wall cross-sections at mature stage. Whole sections (K); Local sections ([I-N]). (K-L) Phloroglucinol-HCl staining; (M, N) Autofluorescence viewed under UV light. (K), bar = 500µm; (L) and (M), bar = 100µm; (N), bar = 50µm.

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