Title page

Integrative multispecies omics reveals a hierarchy of cold-responsive network launched by circadian components in rosids

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Abstract

Elucidating regulators and molecular mechanisms underlying gene transcriptional and post-transcriptional co-regulatory network is key to understand plant cold responses. Previous studies were mainly conducted on single species and however, whether the regulators and mechanisms are conserved across species remains elusive. Here, we select three species that diverged at early evolution of rosids (93–115 million years ago) and integrate phylotranscriptome and regulome data to investigate cold-responsive regulators and their regulatory networks across rosids. First, we identify over ten thousand cold-responsive genes including differentially expressed genes (DEGs) and alternative splicing genes (DASGs) in each species. Among DEGs, a set of transcription factor (TF) families (AP2/ERF, MYB, WRKY, NAC, etc.) and RNA binding protein (RBP) families (Ribosomal, RRM, DEAD, Helicase_C, etc.) are conserved in cold responses in rosids. Compared to TFs, RBPs show a delayed cold-responsive pattern, likely suggesting a hierarchical regulation of DEGs and DASGs. Further, we identify 259 overlapping DE-DASG orthogroups between DEGs and DASGs and interestingly, pathway analysis on each dataset of DEGs, DASGs, and DE-DASGs coincidently shows an enrichment of circadian rhythm. Evidentially, many circadian components are cold-regulated differentially at both transcriptional and post-transcriptional level. Moreover, we reason 226 cold-responsive genes regulated by at least two of five circadian components (CCA1, LHY, RV4, RVE8, and RVE7) in rosids. Finally, we unveil a conserved hierarchical network in dynamic transcriptional and post-transcriptional regulation launched by circadian components in rosids. Together, our results provide insights into core regulators and mechanisms underlying cold-responsive network across rosids, despite a long evolutionary history.

Key words: transcription factor; RNA binding protein; circadian clock; hierarchical network; cold stress; rosids

Introduction

For adaptation to environmental stressors, angiosperms have evolved sophisticated and advanced mechanisms and become the largest land plant lineage with more than 350,000 known species widely distributed from tropical to polar terrestrial zones (Wu et al., 2020). Among environmental stressors, seasonal and daily variation of temperature is a fundamental component that affects plant growth, productivity, and geographical distribution (Creux et al., 2019; Kidokoro et al., 2022). Under low temperature, plants undergo physiological and molecular changes that increase the levels of specific proteins, metabolites, and phytohormones, which are generally regulated by transcription factors (TFs) and RNA binding proteins (RBPs) at transcriptional and post-transcriptional levels (Zhu, 2016; Ding et al., 2019; Kidokoro et al., 2022).

Thousands of differentially expressed genes (DEGs) were identified involved in cold stress (Calixto et al., 2018; Zheng et al., 2022) and many TFs have been suggested in regulating and managing their expression (Chinnusamy et al., 2007; Ding et al., 2019; Kidokoro et al., 2022). The best characterized TFs are C-repeat binding factor (*CBF*) genes that are rapidly and transiently induced by cold stress and can enhance freezing tolerance of plants through binding to *cis*-elements (CCGAC) in the promoters of cold-responsive (*COR*) genes (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998). RNA-seq analysis of *Arabidopsis cbf123* triple mutant showed that hundreds of *CORs* are regulated through CBF-dependent pathway (Jia et al., 2016; Zhao et al., 2016). Activation of CBF target *CORs*, such as *COR15A*, *COR47*, *KIN1*, and *RD29A*, leads to an increase in plant freezing tolerance (Jaglo-Ottosen et al., 1998; Novillo et al., 2007; Wang et al., 2009). In addition, cold responsiveness of plants is also regulated by circadian clock system, which is involved in plant stress response through controlling daily energy expenditure and enabling plants to grow and reproduce under seasonal and daily temperature variation (Greenham et al., 2015; Markham et al., 2021). In the clock system, some circadian clock-related TFs, such as CIRCADIAN

75 CLOCK ASSOCIATED 1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), and
76 REVEILLES (RVEs), have been proven involved in cold tolerance by directly
77 regulating the expression of downstream *CORs*, such as *CBFs*, *COR15A*/*15B*, *COR28,*
78 and *COR27* (Dong et al., 2011; Wang et al., 2017; Kidokoro et al., 2021).

79 On the other hand, post-transcriptional regulation is also a key processing in
80 reprogramming gene expression to tolerate and survive constantly temperature
81 changes (Ding et al., 2019; Kidokoro et al., 2022). As well as TFs in regulating gene
82 transcription, RBPs are essential regulators in regulating post-transcriptional RNA
83 processing, including capping, polyadenylation, alternative splicing (AS), RNA export,
84 translation, and turnover (Calixto et al., 2018; Bach-Pages et al., 2020). Under cold
85 stress, upregulation and/or downregulation of RBP genes may cause differentially
86 post-transcriptional RNA processing. During RNA processing, AS is an important
87 post-transcriptional mechanism by producing different transcripts and protein variants
88 from single gene for increasing transcriptome and proteome diversity (Wu et al., 2018).
89 The concentration, localization, and activity of RBPs determine AS events to produce
90 different transcripts (Fu et al., 2014; Lee et al., 2015). In plants, extensive AS events
91 have been implicated in various abiotic stresses, including cold, salt, heat and other
92 environmental stresses (Calixto et al., 2018; Liu et al., 2018; Zhao et al., 2021; Zheng et
93 al., 2022). For example, genome-wide AS profiling in *Arabidopsis* showed a massive
94 differentially alternative splicing genes (DASGs) after cold treatment and suggested
95 that plants may fine-tune AS events of gene transcripts for adapting to cold stress
96 (Calixto et al., 2018). Among DASGs, *CCA1*, a core component of circadian clock,
97 has two splice variants: CCA1α and CCA1β. CCA1β competitively inhibits the DNA
98 binding activity of CCA1α and LHY by forming nonfunctional heterodimers of
99 CCA1β-CCA1α and CCA1β-LHY (Seo et al., 2012). Under cold stress, CCA1β
100 production is repressed to release the activity of CCA1α and LHY, whereby CCA1α
101 and LHY promote the expression of downstream *CORs* for improving plant cold
102 tolerance (Seo et al., 2012).

103 In the past years, numerous cold-induced DEGs and DASGs were identified in
104 different species by high-throughput methods (Calixto et al., 2018; Liu et al., 2018;
105 Wang et al., 2021; Zhao et al., 2021; Zheng et al., 2022). Identifying core regulators
106 (e.g., TFs and RBPs) and elucidating molecular mechanisms underlying the outcome
107 of DEGs and DASGs are key to understand the reprogramming of gene regulatory
108 network in response to cold stress. The previous studies were generally performed on
109 single species, especially the reference model *Arabidopsis*, and however, whether the
110 regulators and mechanisms underlying transcriptional and post-transcriptional
111 changes are conserved in different lineages remains elusive. In this study, we focus on
112 rosids, a large group of angiosperm eudicots, and select three species: *Carya*
113 *illinoinensis* and *Populus trichocarpa* from fabids (rosid I) and *Arabidopsis thaliana*
114 from malvids (rosid II). Fabids and malvids are two main clades of rosids, comprising
115 of >40,000 and ~15,000 species, respectively (Chase et al., 2009). The three species
116 diverged during early evolution of rosids and evolved independently for over 100
117 million years (Kumar et al., 2017). The long evolutionary history caused great
118 differences in their growth habits and geographic distributions, and accordingly, we
119 ask whether the regulators and mechanisms underlying gene transcriptional and
120 post-transcriptional regulation are conserved (or not) across rosids in response to low
121 temperature.

122 To solve the question, we perform omics analysis of genomes, transcriptomes, and
123 regulomes and identify common core regulators in regulating cold responses across
124 the three species. First, by phylotranscriptome sequencing (RNA-seq), we investigate
125 global expression profiles of cold-induced DEGs and DASGs in each of the studied
126 species. Subsequently, we analyze cold responses of TFs and RBPs and identify those
127 TF and RBP families that are conserved in cold responses, which likely contribute to
128 DEGs and DASGs in rosids. Moreover, we explore overlapping genes (i.e.,
129 DE-DASGs) of DEGs and DASGs and perform homologous analysis to identify
130 conserved DE-DASG orthogroups in rosids. Finally, we select five circadian

131 components and integrate transcriptomes and regulome maps to identify their target
132 *CORs* for constructing a hierarchical cold-responsive regulatory network.

133 ## Results

134 **Global gene expression profiles in rosids under cold stress**

135 Physiological and molecular changes in plants at low temperature are usually caused
136 by up- or downregulated expression of genes (Kidokoro et al., 2022). To capture the
137 landscape of DEGs at low temperature, we performed RNA-seq experiments in three
138 rosid species before and after cold treatments (**see Methods and materials**). Based on
139 RNA-seq analysis, we identified a huge number of cold-induced DEGs: 7,579, 8,878,
140 and 8,480 respectively in *A. thaliana*, *C. illinoinensis*, and *P. trichocarpa* (**Table S1**).
141 Notably, more upregulated than downregulated DEGs are observed in all three plants
142 (**Fig 1A**). Expression profiles show that these upregulated DEGs are mainly clustered
143 in three expression patterns following three timepoints of 2 hours (h), 24 h, 168 h
144 after cold treatments (**Fig 1B**). Inspecting the expression patterns, we identified that
145 the number of upregulated DEGs in *A. thaliana* is significantly more at 2 h but less at
146 168 h than the counterparts in other two plants after cold treatments. Likewise, there
147 are significantly more downregulated DEGs at 2 h and less downregulated DEGs at
148 168 h in *A. thaliana* than that in *C. illinoinensis* and *P. trichocarpa* (**Fig 1B**). These
149 findings show that over seven thousand genes are differentially cold-regulated in
150 rosids with three common expression patterns but likely in different cold-responsive
151 speeds: an early extensive response in *A. thaliana* but a delayed extensive response in
152 *C. illinoinensis* and *P. trichocarpa*.

153 **Common biological processes of cold responses in rosids**

154 Despite of different cold-responsive speeds between the three plants, Gene Ontology
155 (GO) term enrichment analysis of the DEGs identifies a very similar enrichment of
156 biological processes (**Fig 1C, 1D**). The upregulated DEGs are mainly enriched in
157 abiotic stress processes, e.g., response to hypoxia, water, and cold, and circadian

158 rhythm (**Fig 1C**). Additionally, regulations of transcription and RNA processing are
159 also amongst the most enriched terms, which imply that the regulators (e.g., TFs and
160 RBPs) at transcriptional and post-transcriptional level are likely to be more activated
161 at low temperature (**Fig 1C**). The downregulated DEGs are mainly enriched in
162 catalytic activity, photosynthesis, developmental growth, small molecular and/or lipid
163 biosynthetic/metabolic process, etc (**Fig 1D**). It is reasonable that for improving cold
164 tolerance, plants reduce expression of the genes involved in activities of plant
165 development and growth (Kidokoro et al., 2022).

166 **Conserved cold-responsive TF families in rosids**

167 Differential changes of gene expression at transcriptional level are likely caused by
168 fluctuations of TFs (Calixto et al., 2018; Kidokoro et al., 2022). To detect the roles of
169 TFs under cold stress, we first identified a total of 1,717, 1,899, and 2,466 TFs in *A.*
170 *thaliana*, *C. illinoinensis*, and *P. trichocarpa* genomes, respectively (**Table S2, see**
171 **Methods and materials**). Transcriptome analysis shows that over five hundred TFs
172 are differentially cold-regulated, covering a high proportion of the genome TFs, that is,
173 32%, 35%, and 22% respectively in *A. thaliana*, *C. illinoinensis*, and *P. trichocarpa*
174 (**Fig 2A, Table S2**). Notably, there are more upregulated than downregulated TFs and
175 their expression patterns in cold-responsive speeds agree well with the
176 aforementioned global expression patterns of DEGs (**Fig 1B, Fig 2B**). This finding
177 might suggest more the upregulated TFs, more the regulatory links, and more the
178 differentially regulated genes under cold stress.

179 Moreover, we classified all TFs into gene families based on their protein domain
180 architectures (**Table S2, see Methods and materials**). Interestingly, the up- and
181 downregulated TFs are respectively clustered in common gene families in three plants
182 (**Fig 2C, 2D)**. For examples, the main families of upregulated TFs are AP2/ERF,
183 MYB, WRKY, and NAC, which are the best-known TF families involved in abiotic
184 stresses (Khan et al., 2018; Guo et al., 2022). The counterparts of downregulated TFs
185 are MYB, bHLH, Homeobox, and AP2/ERF. Notably, AP2/ERF and MYB are two

186 most prominent families with many more upregulated members but also a
187 considerable number of downregulated members in all three plants under cold stress.
188 It is explainable that members from the same TF families can often function in a
189 negative feedback mechanism in abiotic responses (Dubos et al., 2010; Xie et al.,
190 2019). According to the maximum parsimony principle, we suggest that the conserved
191 cold-responsiveness of those TF families in three plants is largely descended from
192 their common ancestor.

193 **Global alternative splicing profiles in rosids under cold stress**

194 In addition to transcriptional regulation, we also identified an enrichment of
195 post-transcriptional regulation, such as RNA processing, in response to cold stress
196 (**Fig 1C**). Based on transcriptome analysis, we detected 2,572, 7,428, and 7,817
197 cold-induced DASGs, which occupy 10%, 24%, and 18% of coding genes in *A.*
198 *thaliana*, *C. illinoinensis*, and *P. trichocarpa*, respectively (**Fig 3A**, **Table S3**). Among
199 DASGs, many have multiple AS sites, producing 3,192, 11,972, and 13,373
200 differentially AS events (DASEs), respectively in *A. thaliana*, *C. illinoinensis*, and *P.*
201 *trichocarpa* (**Fig 3A**, **Table S3**). This is mainly caused by AS occurrences at the same
202 or different exon/intron boundaries across a gene, which leads to more diversities of
203 RNA transcripts. In consistence with previous studies (Calixto et al., 2018; Zhao et al.,
204 2021), intron retention (IR) is the most common AS mode in three plants (**Fig 3B**).
205 Despite a relatively small role of skipping exon (SE) in *A. thaliana*, SE provides a
206 crucial role in other two plants (*C. illinoinensis* and *P. trichocarpa*). Further, GO
207 enrichment analysis shows that these DASGs from three different plants are enriched
208 in very similar biological processes, such as metabolic processes, gene expression,
209 RNA splicing, circadian rhythm, etc (**Fig 3C**). Given a key role of RBPs in RNA
210 processing (Calixto et al., 2018; Bach-Pages et al., 2020), thousands of cold-induced
211 DASEs might at least partially result from fluctuations of RBP genes. Next, we
212 determine expression changes of RBP genes under cold stress.

213 **A delayed cold responsivity of RBP genes**

214 In *A. thaliana*, we collected a total of 788 high-confident RBPs that were
215 experimentally confirmed by RNA-binding (Marondedze et al., 2016; Bach-Pages et
216 al., 2020). Using the RBPs for homologs searching, we identified 1,110 and 1,379
217 RBPs in *C. illinoinensis* and *P. trichocarpa*, respectively (**Fig 4A**, **Table S4**).
218 Transcriptome analysis shows that 36%, 31%, and 35% RBP genes are cold-regulated
219 differentially in *A. thaliana*, *C. illinoinensis*, and *P. trichocarpa*, respectively (**Table**
220 **S4**). Like TFs, we identified more upregulated RBPs than downregulated ones,
221 especially in *A. thaliana* (**Fig 4A**). Interestingly, in contrast to TF genes, RBP genes
222 show a delayed responsive pattern after cold treatment in all three plants (**Fig 2B, 4B**).
223 For example, only a relatively small number of RBP genes are observed to be
224 differentially regulated at early stage (2 h) of cold stress. This finding shows that cold
225 responsivity of RBP genes lags after that of TF genes, which might suggest a
226 hierarchy of temporal dynamics of transcriptional and post-transcriptional network in
227 response to cold stress.

228 **Conserved cold-responsive RBP families in rosids**

229 Based on RNA binding domains, we classified RBP genes into different gene families
230 (**Table S4**). Among the top 15 cold-responsive RBP families, Ribosomal and RRM
231 are two most prominent families with more genes being upregulated than
232 downregulated under cold stress in all three plants (**Fig 4C-D**). DEAD and
233 Helicase_C families are mainly enriched in the upregulated RBPs (**Fig 4C**), while
234 Chloroa_b-bind and MIP families are more clustered in the downregulated RBPs (**Fig**
235 **4D**). Like TF families, according to the maximum parsimony principle, we suggest
236 that the conserved cold responsivity of the RBP families in three plants is largely
237 descended from their common ancestor.

238 **Cold association of transcriptional and post-transcriptional activities in rosids**

239 As described above, thousands of genes were identified as cold-induced DEGs and/or

240 DASGs in three plants (**Fig 1, Fig 3**). Further, we identified that upregulated DEGs
241 show a significant overlapping association with DASGs, that is, 558, 1340, and 1419
242 up-DE-DASGs respectively in *A. thaliana*, *C. illinoinensis*, and *P. trichocarpa* (**Fig**
243 **5A**, **Table S5**). Similarly, downregulated DEGs also show a significant association
244 with DASGs in the three species. The findings suggest that many cold-responsive
245 genes are differentially regulated not only by TFs at transcriptional level but also by
246 RBPs at post-transcriptional level, and these genes might play an important role in
247 rosids under cold stress. In the follows, we determine whether these genes across the
248 three studied plants are homologous and function in a conserved way.

249 **Identification of 259 conserved orthogroups in rosids**

250 Biased retention and loss of gene duplicates in plants after independent
251 whole-genome and/or small-scale duplications lead to complex and intertwined
252 evolution of co-orthologs and in-paralogs across different species (Koonin, 2005; Guo
253 et al., 2022). To determine common cold-responsive genes between species, we
254 classified those genes that are differentially regulated at both transcriptional and
255 post-transcriptional level into orthogroups: sets of homologous genes descended from
256 the recent common ancestor of the plants (Emms et al., 2019) (**see Methods and**
257 **materials**). For example, 558, 1,340, and 1,419 up-DE-DASGs are classified into 305,
258 626, and 625 orthogroups, respectively in *A. thaliana*, *C. illinoinensis*, and *P.*
259 *trichocarpa* (**Table S6**). Among these orthogroups, Venn diagram analysis shows a
260 significant overlapping of 150 orthogroups among three plants (**Fig 5B, Table S6**).
261 From the 150 orthogroups, we extracted 186 *A. thaliana* genes and performed GO
262 term enrichment analysis (**Fig 5C**). As expected, the genes are mainly enriched in
263 abiotic stresses, such as response to abiotic stresses (salt and cold stress) and circadian
264 rhythm (**Fig 5C**). The same process was also conducted for down-DE-DASGs, and
265 we identified 109 overlapping orthogroups with many genes enriched in diverse
266 enzymatic actives (**Fig 5D, 5E, Table S7**). The findings together show a total of 259
267 orthogroups (150 + 109) that are regulated at both transcriptional and

post-transcriptional levels in a conserved manner in rosids under low temperature, likely suggesting a conserved role of these orthogroups under cold stress.

Seven striking TF orthogroups involved in circadian rhythms

Among the 259 orthogroups, there are 27 upregulated TF orthogroups (**Table S8**) and it is noteworthy that seven are known circadian clock components including *CCA1*/*LHY*, *RVE4*/*8*, *RVE7*, *PRR9*, *BBX19*, *COL9*, and *JMJD5* genes (**Table 1**). In agreement, previous studies have reported that *CCA1, LHY* and *PRR9* can be regulated at transcriptional and post-transcriptional levels under cold stress in *Arabidopsis* (James et al., 2012; Seo et al., 2012). Here, they are also cold-regulated in *C. illinoinensis*, and *P. trichocarpa* and besides, we found other more circadian clock or related genes that are differentially cold-regulated at both transcriptional and post-transcriptional levels in rosids (**Table 1**). This explains well aforementioned repeated observations that the biological process of circadian rhythm is enriched (**Fig 1C**, **Fig 3C**, **Fig 5C**). The seven orthogroups are upregulated under cold stress in all three plants, and in contrast, AS types of genes in each orthogroup show a great diversity, which suggest a conservation of gene expression but a rapid evolution of AS events after rosid radiation (**Table 1**). Moreover, MYB is the most prominent family including three orthogroups (*CCA1/LHY*, *RVE7*, and *RVE4/8*). The finding suggests that circadian clock components might play important roles in regulating cold responses in a conserved manner in rosids.

Cold-responsive genes regulated by circadian clock components

First, we collected experimentally genome-wide regulome maps of *A. thaliana CCA1*, *LHY*, *RVE4*, *RVE7*, and *RVE8* from chromatin immunoprecipitation (ChIP-seq) (Kamioka et al., 2016) and DNA affinity purification followed by deep sequencing (DAP-seq) (O'Malley et al., 2016). Subsequently, we developed a stringent pipeline to identify cold-responsive genes regulated by the five TFs (see **Materials and methods**). In brief, we performed ChIP-seq and DAP-seq analysis to identify *A.*

295 *thaliana* genes bound by either of the five TFs and investigated their orthogroup
296 genes in *C. illinoinensis* and *P. trichocarpa* whose promoters (1-Kb) also have at least
297 one *cis*-regulatory binding site of the TFs. Finally, and of importance, we integrated
298 phylotranscriptomic data to identify those orthologous genes that have DNA binding
299 sites of the five TFs and are differentially cold-regulated as well as the five TFs in all
300 three species. Thus, we designated those orthologous genes as Conserved COR genes
301 (CoCORs) targeted by clock components *CCA1*, *LHY*, *RVE4*, *RVE7*, or *RVE8* in
302 rosids (**Fig 6A, Table S9**).

303 By inspecting Fig 6A for closer analysis, the five circadian components regulate
304 different numbers of CoCORs but with a common pattern of dominant upregulation in
305 three rosids. For instance, as compared to only 17 downregulated CoCORs, there are
306 101 CoCORs identified to be upregulated by CCA1 (**Fig 6A, Table S9**). GO analysis
307 further shows that these CoCORs are enriched in environmental stresses, such as
308 response to light, cold, and circadian rhythm (**Fig 6B**). Moreover, Venn diagram
309 shows a significance of overlapping CoCORs regulated by the five TFs (**Fig 6C**), and
310 among the overlapping CoCORs, 16 are regulated by all five TFs and 210 are
311 regulated by at least two of the five TFs. The intertwined DNA binding of CoCORs
312 by the five circadian TFs suggests a complex regulatory network launched by
313 circadian clock components.

314 **A conserved hierarchy of cold-responsive network in rosids**

315 To construct the network, we employed the CoCORs involved in abiotic stresses that
316 are directly regulated by the five circadian components. Taking account of
317 cold-responsive speeds of the genes under cold treatments at 2, 24, and 168 h and also
318 their regulatory relationships, we constructed a hierarchical regulatory network with
319 three layers launched by the five circadian clock components and elaborated this
320 network as follows.

321 The first layer is composed of 30 TFs including many well-known cold-regulated

322 genes, such as *CBFs*, *PRR5*, *ZAT6*, *ZAT10*, *COL9*, *BT2* etc. In this layer, the five
323 clock components play a dominant role to upregulate the expression of 21 TFs and in
324 turn work together with these upregulated TFs to further regulate the expression of
325 downstream genes (e.g., RBPs and structural genes in the second and third layers). In
326 addition to upregulated TFs launched by circadian clock components, there are four
327 downregulated TFs (*TCP7*, *EBS*, *IBH1*, and *PIF1*). This demonstrates a potential
328 negative regulatory loop between differentially regulated TFs for controlling the
329 balance between plant growth and cold tolerance. Evidentially,
330 phytochrome-interacting factor 1 (PIF1) negatively regulates plant freezing tolerance
331 likely through repressing *CBFs* expression (Jiang et al., 2020).

332 The second layer is composed of 14 RBPs. Among the 14 RBPs, 12 are upregulated
333 by the first layer TFs and function in a feedback mechanism by potentially regulating
334 AS of TFs in the first layer and also function in regulating AS of the downstream
335 layer genes. For example, by analyzing individual nucleotide resolution crosslinking
336 and immunoprecipitation (iCLIP)-seq experimental data (Meyer et al., 2017), we
337 found that GRP7 can directly bind to transcripts of *BT2* and *HB6* in the first layer and
338 *COR27*, *KCS6*, *BAP1*, *COR413-PM1*, *ERD15*, *LTL1*, *PSBTN*, and *MEE14* in the third
339 layer, and many of these genes undergo differentially AS events under cold stress.

340 The third layer is composed of 140 *COR*s mainly encoding for structural proteins.
341 These genes are differentially regulated in expression and/or alternative splicing by
342 the first and/or second layer regulators. Among the genes, overexpression or knockout
343 of some genes (e.g., *COR47*, *TIL*, *SLD1*, *LOS2*, and *ADC1/2*) can affect plant freezing
344 tolerance (Lee et al., 2002; Cao et al., 2005; Charron et al., 2008; Cuevas et al., 2008;
345 Chen et al., 2012). Altogether, our findings suggest that circadian clock components
346 are actively regulated under cold stress to launch a hierarchy of gene regulatory
347 network for rosid adaptation to cold stress.

348 ## Discussion

349 When exposed to low temperature, plants undergo a series of molecular changes to
350 protect themselves from freezing damage (Ding et al., 2019; Kidokoro et al., 2022). By
351 high-throughput methods, previous studies provided insights into the reprogramming
352 of gene regulation in response to cold stress (Calixto et al., 2018; Liu et al., 2018;
353 Wang et al., 2021; Zhao et al., 2021; Zheng et al., 2022). However, the studies were
354 generally performed on single species and focused on the best-known CBF-dependent
355 pathway genes. Here, we used three species (*A. thaliana*, *C. illinoinensis*, and *P.*
356 *trichocarpa*) form different rosid lineages which diverged during the early evolution
357 of rosids (~100 million years ago) and performed RNA-seq experiments under cold
358 stress to investigate conserved regulators and mechanisms underlying rosids in
359 response to cold stress. Over seven thousand DEGs were identified in each species
360 and show common responses of biological processes, such as cold stress process and
361 circadian rhythm (**Fig 1C**). Interestingly, the species show different cold-responsive
362 speeds: an early in *A. thaliana* but a delayed extensive cold-response in *C.*
363 *illinoinensis* and *P. trichocarpa* (**Fig 1B, 2B**). The difference in cold-responsive
364 speeds maybe due to the different growth characteristics between herbs (e.g., *A.*
365 *thaliana*) and trees (e.g., *C. illinoinensis* and *P. trichocarpa*). Compared to trees, herbs
366 are more sensitive to temperature change (Cornelius et al., 2013), which might cause
367 an early extensive cold-response.

368 In addition to transcriptional regulation, post-transcriptional regulation is also a key
369 processing in reprogramming gene expression under cold stress (Calixto et al., 2018;
370 Zhao et al., 2021). Like DEGs, thousands of DASGs were identified in rosids under
371 cold stress. Interestingly, the number of DASGs in *A. thaliana* is significantly much
372 less than the counterparts in *C. illinoinensis* and *P. trichocarpa* (**Fig 3A**). This
373 difference may be due to shorter introns in *Arabidopsis* than in *C. illinoinensis* and *P.*
374 *trichocarpa* (**Fig S1**), and alternative splicing tends to occur around the exons
375 interrupted by long introns (Fox-Walsh et al., 2005; Roy et al., 2008). In consistence

376 with DEGs, DASGs show an enrichment of circadian rhythm (**Fig 3C**). Unexpectedly,
377 enrichments of biological processes related to cold or abiotic stress are not observed
378 for DASGs. This is probably because a limited knowledge of cold-induced DASGs
379 and their genetic roles as compared to an extensive study of cold-induced DEGs.
380 Regarding this, we provide a complete list of cold-induced DASGs in three rosid
381 species (**Table S3**) and hope this list will be a potential reference for future research
382 of splicing regulation at low temperature.
383 Moreover, association analysis of transcriptional and post-transcriptional activities
384 revealed 259 conserved DE-DASG orthogroups in rosids. From DE-DASG
385 orthogroup genes, circadian rhythm is again significantly enriched (**Fig 5C**).
386 Circadian clock system can allocate stress-responsive processes to specific times of
387 the day, which provides a control of over daily energy expenditure and enables plants
388 to grow and reproduce under temperature changes (Greenham et al., 2015; Markham
389 et al., 2021). Here, we found that some conserved circadian clock components are
390 reprogrammed at both transcriptional and post-transcriptional levels under cold stress
391 in rosids (Fig 5C). Among them, MYB is the most prominent TF family including
392 three orthogroups with five *Arabidopsis* genes (*CCA1/LHY*, *RVE7*, and *RVE4/8*),
393 which likely contribute to the flexibility and controllability of the cold response
394 network. Thus, by integrative omics of phylotranscriptomes and regulome maps
395 (RNA-seq and ChIP/DAP-seq data), we identified their direct targets and constructed
396 a conserved hierarchy of cold-responsive network launched by the five MYB TFs.
397 The existence of many well-known cold-response genes, such as *CBFs*, *RBG7/8*,
398 *ZAT6/10*, and *COR27*, in the network demonstrate a high confidence of the network
399 but also imply an importance of circadian clock for plants in response to cold stress.
400 Altogether, our research shed light on conserved hierarchical cold-responsive network
401 in rosids at transcriptional and post-transcriptional levels via circadian clock
402 components, which provide insights into conserved core regulators and ancestral
403 mechanisms underlying cold-responsive gene regulatory network across rosids.

404

405 ## Materials and Methods

406 ### Identification and characterization of TFs and RBPs

407 Proteome sequences and gene annotations of three rosid species (*A. thaliana*, *C.*
408 *illinoinensis*, and *P. trichocarpa*) were directly downloaded from the public databases
409 (Goodstein et al., 2012; Aken et al., 2016). Their divergence times were extracted and
410 assessed from the website TimeTree (Kumar et al., 2017). TFs and RBPs in the species
411 were obtained through the following strategies. For TFs, we downloaded *A. thaliana*
412 and *P. trichocarpa* TFs from plantTFDB 4.0 (Jin et al., 2017) and identified TFs in *C.*
413 *illinoinensis* according to the method as described (Jin et al., 2017)(**Table S2**). For
414 RBPs, we obtained *A. thaliana* RBPs, which were experimentally confirmed
415 RNA-binding (Marondedze et al., 2016; Bach-Pages et al., 2020). By using *A. thaliana*
416 RBPs as queries, we employed BLASTP 2.5.0 (Altschul et al., 1990) to retrieve RBP
417 homologs in *P. trichocarpa* and *C. illinoinensis* (E-value < 1e–10, identity > 60%)
418 (**Table S4**).

419 To identify protein families of TFs and RBPs, we downloaded all Hidden Markov
420 Model (HMM) profiles (>19,000) of protein domains from Pfam database (Finn et al.,
421 2016) and searched them against the proteome sequences of the three plants by
422 HMMER 3.1b2 with an E-value < 1e–5 (Eddy, 1998). Accordingly, we obtained gene
423 families for each TF and RBP (**Table S4**).

424 ### Genome-wide analysis of cold-induced DEGs and DASEs

425 To explore molecular changes of rosids under low temperature, we performed
426 RNA-seq experiments in three above plants with cold treatment (4°C) at 0, 2, 24, and
427 168 hours (h). The experiment and analysis were described as a previous report (Nie
428 et al., 2022). Gene expression in normal and cold treatment conditions was
429 represented by Trimmed Means of M value (TMM) calculated from StringTie v2.0.3
430 (Pertea et al., 2015). Subsequently, cold-induced DEGs were defined based on three

431 criteria: at least one sample with TMM greater than 5, more than two-fold change
432 expression before and after cold treatment, and a statistical difference with *P* value <
433 0.01. Additionally, five type AS events, including intron retention (IR), alternative 3'
434 splice site (A3SS), alternative 5' splice site (A5SS), skipping exon (SE), and mutually
435 exclusive exons (MXE), were identified by rMATS v4.0.2 (Shen et al., 2014) coupled
436 with CASH v2.2.1 (Wu et al., 2018). A false discovery rate (FDR) ≤ 0.05 was set as
437 the criterion for identifying cold-induced DASEs between normal and cold
438 treatments.

439 **GO term enrichments**

440 The GO term annotations of *A. thaliana* genes were directly acquired from The
441 *Arabidopsis* Information Resource (TAIR) database (Rhee et al., 2003). According to
442 method described by the previous reports (Salojärvi et al., 2017; Guo et al., 2022), the
443 genes in *P. trichocarpa*, and *C. illinoinensis* were annotated with the highest
444 alignment score matches against *A. thaliana* proteins from BLASTP searches (E-value
445 < 1e–5) (Altschul et al., 1990). The GO term enrichment analysis was performed
446 using the OmicShare tools (www.omicshare.com/tools).

447 **Identification of DE-DASG orthogroups**

448 To explore whether DE-DASGs across the rosids are homologous, we classified the
449 up- and down-DE-DASGs of three rosids into different orthogroups using Orthofinder
450 v2.3.8 (Emms et al., 2019) with the parameters (-M dendroblast; -S diamond; -A mafft;
451 -I 1.5). Subsequently, we obtained a total of 1,532 DE-DASG orthogroups and among
452 which, 259 were identified to be present in all three species. In 259 DE-DASG
453 orthogroups, 150 and 109 are respectively upregulated and downregulated under cold
454 stress (**Table S6, S7**).

455 **Identification of CoCORs targeted by circadian components**

456 To determine CoCORs regulated by circadian components in rosids, we developed the
457 following pipeline. First, we obtained *A. thaliana* ChIP-seq (CCA1) and DAP-seq

458 (LHY, RVE7, RVE4, and RVE8) datasets (Kamioka et al., 2016; O'Malley et al., 2016)
459 and performed ChIP/DAP-seq analysis as described (36070250). The genes with at
460 least one binding peak of the five TFs in the 1-Kb upstream of translation initiation
461 site (TIS) were considered as direct target genes of the corresponding TFs in *A.*
462 *thaliana*. Second, we collected *cis*-regulatory binding motifs of CCA1, *LHY*, *RVE7*,
463 *RVE4*, and *RVE8* (Kamioka et al., 2016; O'Malley et al., 2016) and searched them
464 against the 1-Kb promoter sequences of *P. trichocarpa* and *C. illinoinensis* genes
465 using FIMO (E-value <1e–3) (Grant et al., 2011). The genes with at least one
466 *cis*-regulatory binding site for the five TFs were identified as potentially targets of the
467 TFs in *P. trichocarpa* and *C. illinoinensis*. Subsequently, all coding genes in the three
468 species were classified into orthogroups according to their homology. The orthogroup
469 genes that have binding sites of the five TFs and are differentially regulated after cold
470 stress in all three rosids are defined as CoCORs. Finally, a total of 101/17, 167/67,
471 193/93, 110/34, and 38/10 CoCORs (**Fig 6A**, **Table S9**) were identified as to be
472 up/down-regulated by CCA1, LHY, RVE7, RVE4 and RVE8, respectively.

473 **Statistical analysis**

474 The significance of enrichment analysis between gene groups was calculated by
475 Fisher Exact test through R programming.

476 ## Acknowledgments

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479 National Natural Science Foundation of China (grant number 31871233).

480 ## Contributions

481 WW, ZJ, and JX designed the research; LG and XZ performed the study; SW did the
482 RNA-seq experiments of the plants and YX performed the RNA-seq analysis; WW
483 and ZJ wrote and edited the manuscript.

484 ## Data availability statement

485 The RNA-seq datasets of the three plants including *A. thaliana*, *C. illinoinensis*, and *P.*

486 *trichocarpa* under cold stress have been deposited to NCBI BioProject (Accession:
487 PRJNA767196) and CNGBdb (Accession: CNP0002243).

488 Conflict of interests

489 There are no conflicts of interest.

490 Supplementary information

491 Fig S1. Alternative splicing seems to occur in the genes with longer introns.

492 Table S1. RNA-seq values of DEGs in rosids at four timepoints (0, 2, 24, and 168 h)
493 of cold treatments (4°C).

494 Table S2. TF family genes and expression in rosids under cold stress.

495 Table S3 A list of cold-induced DASGs and their AS types in rosids.

496 Table S4. RBP family genes and expression in rosids under cold stress.

497 Table S5. The detailed information of DE-DASGs.

498 Table S6. The orthogroups of up-DE-DASGs in rosids.

499 Table S7. The orthogroups of down-DE-DASGs in rosids.

500 Table S8. Details of 27 up-DE-DASG TF orthogroups in rosids.

501 Table S9. The predicted CoCORs targeted by clock components of CCA1, LHY,
502 RVE4, RVE8, and RVE7 in rosids.

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645
646

647 ## Figure legends

648 **Fig 1. Identification and enrichment analysis of cold-induced DEGs.**

649 (**A**) Overview of DEGs under cold stress. The number in parentheses below each
650 species indicates the number of all coding genes in the genome. The evolutionary
651 relationship and divergence times of the species were obtained from the website
652 TimeTree (Kumar et al., 2017). Bar heights indicate gene numbers of upregulated
653 DEGs (in red) and downregulated DEGs (in green). (**B**) Expression profiles of DEGs
654 before and after cold treatments. The expression values of DEGs at four different
655 timepoints (0, 2, 24, and 168 h) of cold treatments were normalized by row scale.
656 (**C-D**) GO term enrichments of upregulated DEGs and downregulated DEGs. The top
657 20 significant GO terms were shown in a conserved manner with common
658 cold-responsive biological processes in three species.

659

660 **Fig 2. Cold-responsive TFs in conserved TF families.**

661 (**A**) Upregulated and downregulated TFs under cold stress. Bar percentage indicates
662 the proportion of upregulated, downregulated, and no significant expression changed
663 TFs to all TFs. The number in parentheses after the species indicates the number of
664 TFs in the genome. (**B**) Expression profiles of up- and downregulated TFs under cold
665 stress. The expression values of the TFs at four different timepoints (0, 2, 24, and 168
666 h) of cold treatments were normalized by row scale. (**C-D**) The numbers of up- and
667 downregulated TFs in TF families. The top 15 up- and downregulated TF families
668 were selected for visualization.

669

670 **Fig 3. Identification and enrichment analysis of cold-induced DASGs.**

671 (**A**) Overview of DASGs and DASEs under cold stress. The number in circle indicates
672 the number of cold-induced DASGs or DASEs in the corresponding species. (**B**)
673 Proportions of five AS types of cold-induced DASEs. IR: intron retention; SE:
674 skipping exon; A3SS: alternative 3' splicing site; A5SS: alternative 5' splicing site;
675 MXE: mutually exclusive exons. (**C**) GO term enrichments of DASGs. The top 20
676 significant GO terms were shown in a conserved manner with common
677 cold-responsive biological processes in three species.

678

679

680 **Fig 4. Cold-responsive RBPs in conserved RBP families.**
681 (**A**) Upregulated and downregulated RBPs under cold stress. Bar percentage indicates
682 the proportion of upregulated, downregulated, and no significant expression changed
683 RBPs to all RBPs. The number in parentheses after the species indicates the number
684 of RBPs in the genome. (**B)** Expression profiles of up- and downregulated RBPs
685 under cold stress. The expression values of the RBPs at four different timepoints (0, 2,
686 24, and 168 h) of cold treatments were normalized by row scale. (**C-D**) The numbers
687 of up- and downregulated RBPs in RBP families. The top 15 up- and downregulated
688 RBP families were selected for visualization.
689
690 **Fig 5. Association of cold-induced DEGs and DASGs and identification of**
691 **conserved DE-DASG orthogroups in rosids.**
692 (**A**) Overlapping and unique genes between DEGs and DASGs in three species. The
693 overlapping significance was calculated by Fisher's Exact Test. (**B-E**) Identification
694 and enrichment analysis of conserved DE-DASG orthogroups in three species. Venn
695 diagram shows overlapping and unique orthogroups of up- (**B**) and down-DE-DASGs
696 (**D**) in three species. Of the 150 up- and 109 down-DE-DASG orthogroups, we
697 extracted 186 (**C**) and 157 (**E**) *A. thaliana* genes representatively for GO term analysis.
698 The top 15 significant enriched terms were selected to be shown.
699
700 **Fig 6. A hierarchical cold-responsive regulatory network launched by circadian**
701 **clock components in rosids.**
702 (A) Numbers of up- and downregulated CoCORs targeted by CCA1, LHY, RVE4,
703 RVE7, and RVE8. These CoCORs were predicted by a stringent pipeline (see
704 Materials and methods). (**B**) GO enrichment analysis of CoCORs. The top 10
705 significant GO terms were selected to be shown. (**C**) Overlapping and unique
706 CoCORs regulated by the five circadian-related TFs. (**D**) A hierarchical network
707 launched by the five circadian-related TFs. The network is arranged in three layers
708 (TFs, RBPs, and Structural proteins). To improve the robustness of the network, we
709 also integrated the regulatory relations between CBFs, ZAT10, HB6, HB-7, NAC053,
710 WRKY7, MYB73, and TCP7 on CoCORs from ChIP/DAP-seq data (O'Malley et al.,
711 2016; Song et al., 2021) and identified GRP7 target CoCORs from iCLIP-seq data
712 (Meyer et al., 2017). Solid arrow lines indicate that the regulatory relations between
713 TFs (or RBPs) and CoCORs were from ChIP/DAP-seq (or iCLIP-seq) experiments.

714 # Tables

715 **Table 1. Cold-responsive circadian-related TFs in rosids.**

Family(orthogroup)	Cold regulation *Ath*/*Cil*/*Ptr*	*Ath* (AS-type)	*Cil* (AS-type)	*Ptr* (AS-type)
MYB (OG0000072)	UP/UP/UP	*CCA1* (A5SS) *LHY* (A3SS,A5SS,RI,SE)	*CIL1205S0016* (A3SS,A5SS,MXE,RI,SE)	*Potri.014G106800* (A3SS,A5SS,MXE,IR,SE)
MYB (OG0000110)	UP/UP/UP	*RVE4 (A3SS,SE)* *RVE8 (A3SS,IR)*	*CIL0982S0042* (A5SS)	*CIL0982S0042* (A5SS)
MYB (OG0000043)	UP/UP/UP	*RVE7* (SE)	*CIL1030S0151* (IR) CIL1245S0064 (IR)	*Potri.004G074300 (IR)* *Potri.012G038300 (IR)*
PRR (OG0000018)	UP/UP/UP	*PRR9* (A5SS)	*CIL0897S0115* (IR) *CIL1083S0010* (IR) *CIL1194S0023* (IR)	*Potri.002G179800 (A3SS,IR,SE)* *Potri.014G106000 (A3SS,IR,SE)*
BBX (OG0000101)	UP/UP/UP	*COL9* (IR)	*CIL1155S0003* (IR)	*Potri.002G214500 (IR)* *Potri.014G170600 (IR)*
BBX (OG0000088)	UP/UP/UP	*BBX19* (IR,SE)	*CIL0978S0047* (IR)	*Potri.001G061800* (A3SS)
Cupin (OG0000219)	UP/UP/UP	JMJD5 (A3SS,IR)	*CIL1465S0014* (IR)	*Potri.001G016200* (IR)

716 Note: The short names (*Ath*, *Cil*, and *Ptr*) indicate the species (*A. thaliana*, *C.*
717 *illinoinensis*, and *P. trichocarpa*)

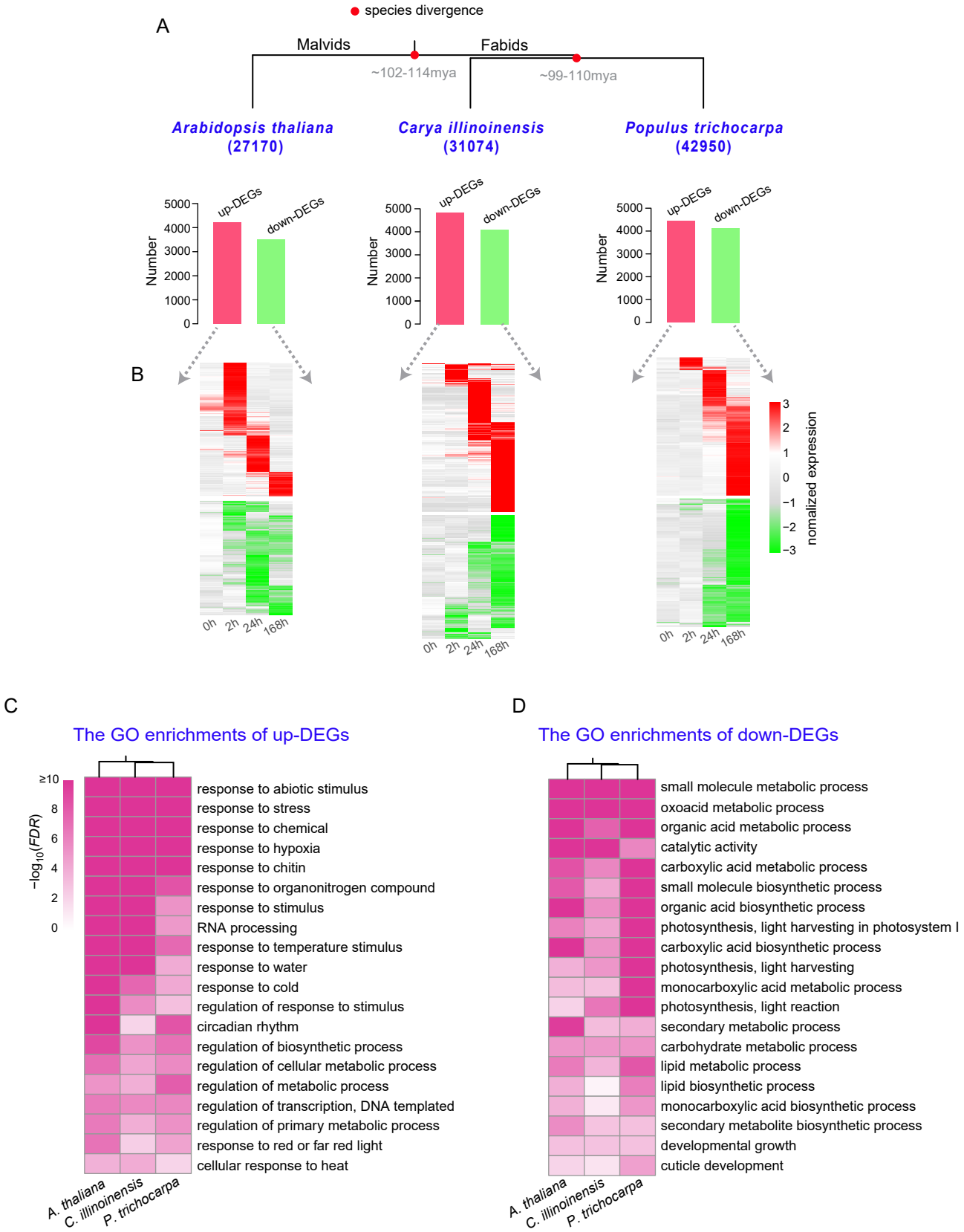

Fig 1. Identification and enrichment analysis of cold-induced DEGs.
(A) Overview of DEGs under cold stress. The number in parentheses below each species indicates the number of all coding genes in the genome. The evolutionary relationship and divergence times of the species were obtained from the website TimeTree (Kumar et al., 2017). Bar heights indicate gene numbers of upregulated DEGs (in red) and downregulated DEGs (in green). (B) Expression profiles of DEGs before and after cold treatments. The expression values of DEGs at four different timepoints (0, 2, 24, and 168 h) of cold treatments were normalized by row scale. (C-D) GO term enrichments of upregulated DEGs and downregulated DEGs. The top 20 significant GO terms were shown in a conserved manner with common cold-responsive biological processes in three species.

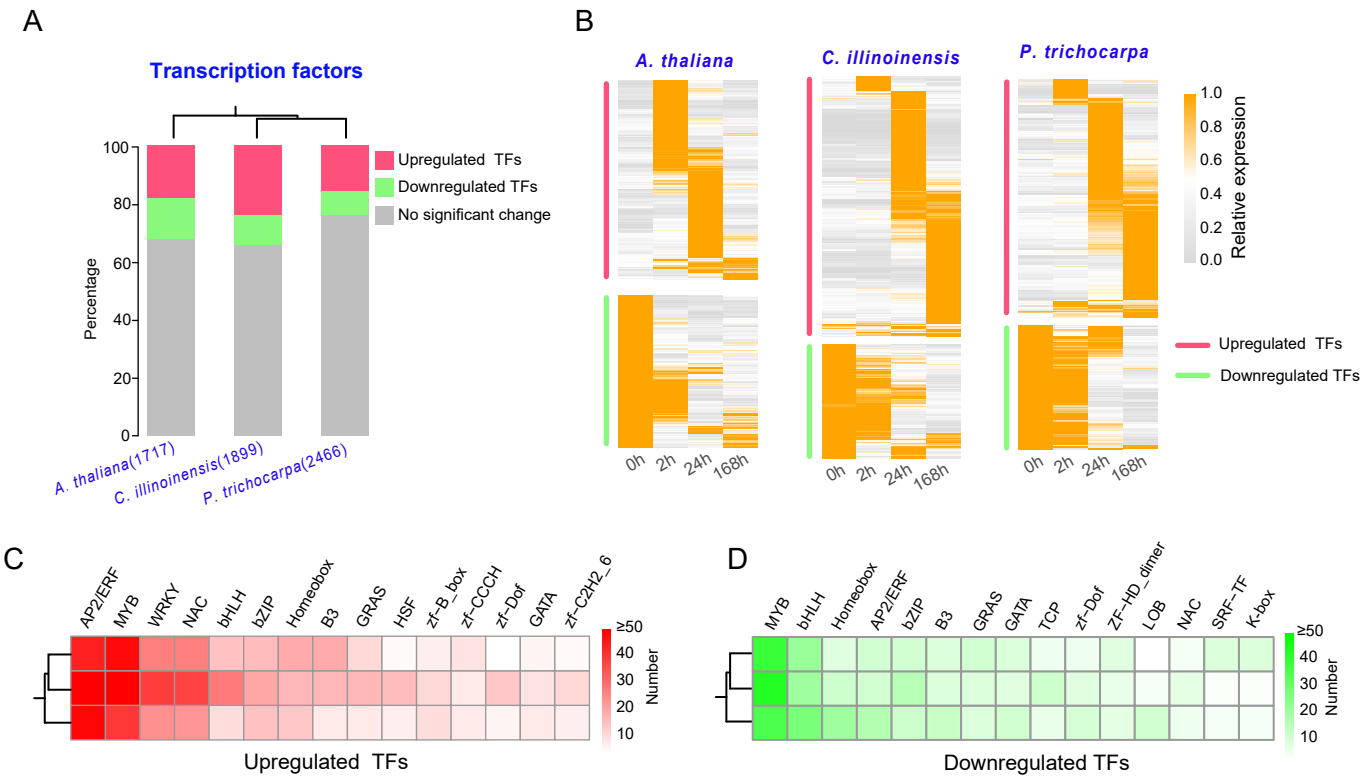

Fig 2. Cold-responsive TFs in conserved TF families.

(A) Upregulated and downregulated TFs under cold stress. Bar percentage indicates the proportion of upregulated, downregulated, and no significant expression changed TFs to all TFs. The number in parentheses after the species indicates the number of TFs in the genome. (B) Expression profiles of up- and downregulated TFs under cold stress. The expression values of the TFs at four different timepoints (0, 2, 24, and 168 h) of cold treatments were normalized by row scale. (C-D) The numbers of up- and downregulated TFs in TF families. The top 15 up- and downregulated TF families were selected for visualization.

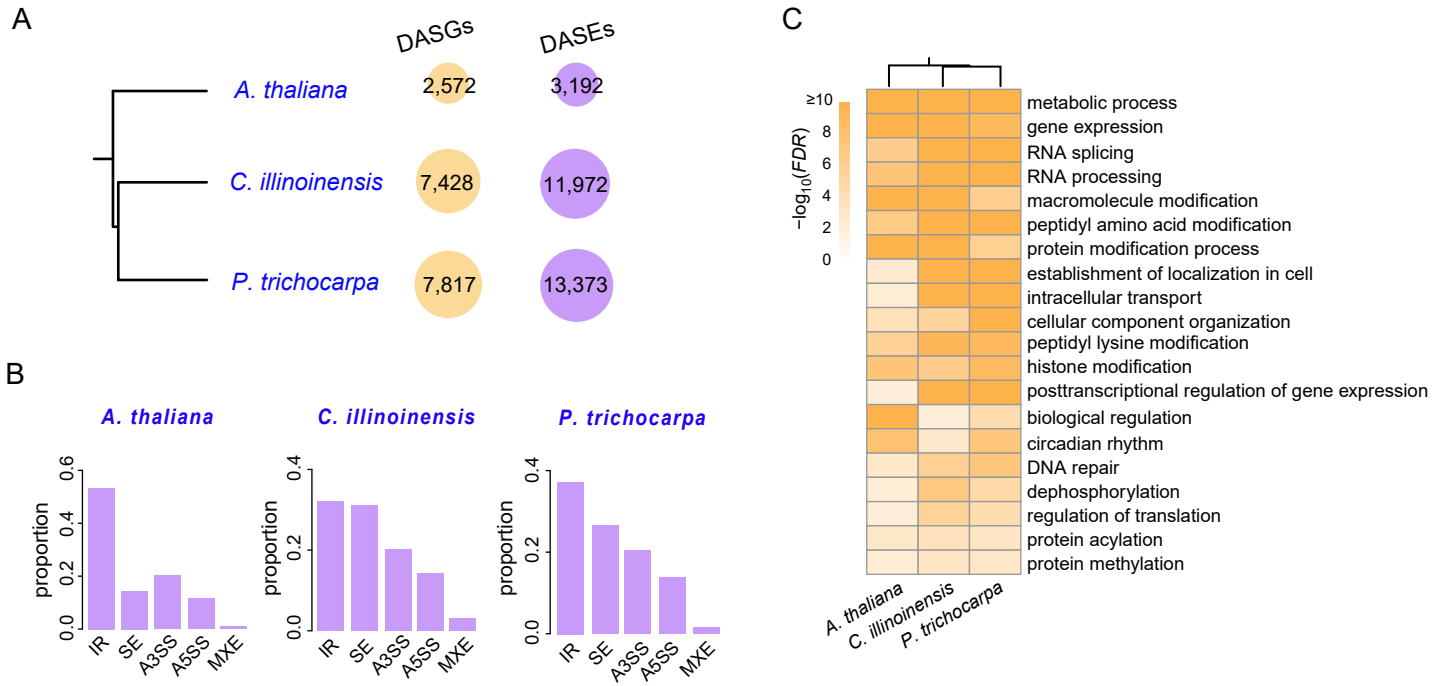

Fig 3. Identification and enrichment analysis of cold-induced DASGs.
(A) Overview of DASGs and DASEs under cold stress. The number in circle indicates the number of cold-induced DASGs or DASEs in the corresponding species. (B) Proportions of five AS types of cold-induced DASEs. IR: intron retention; SE: skipping exon; A3SS: alternative 3' splicing site; A5SS: alternative 5' splicing site; MXE: mutually exclusive exons. (C) GO term enrichments of DASGs. The top 20 significant GO terms were shown in a conserved manner with common cold-responsive biological processes in three species.

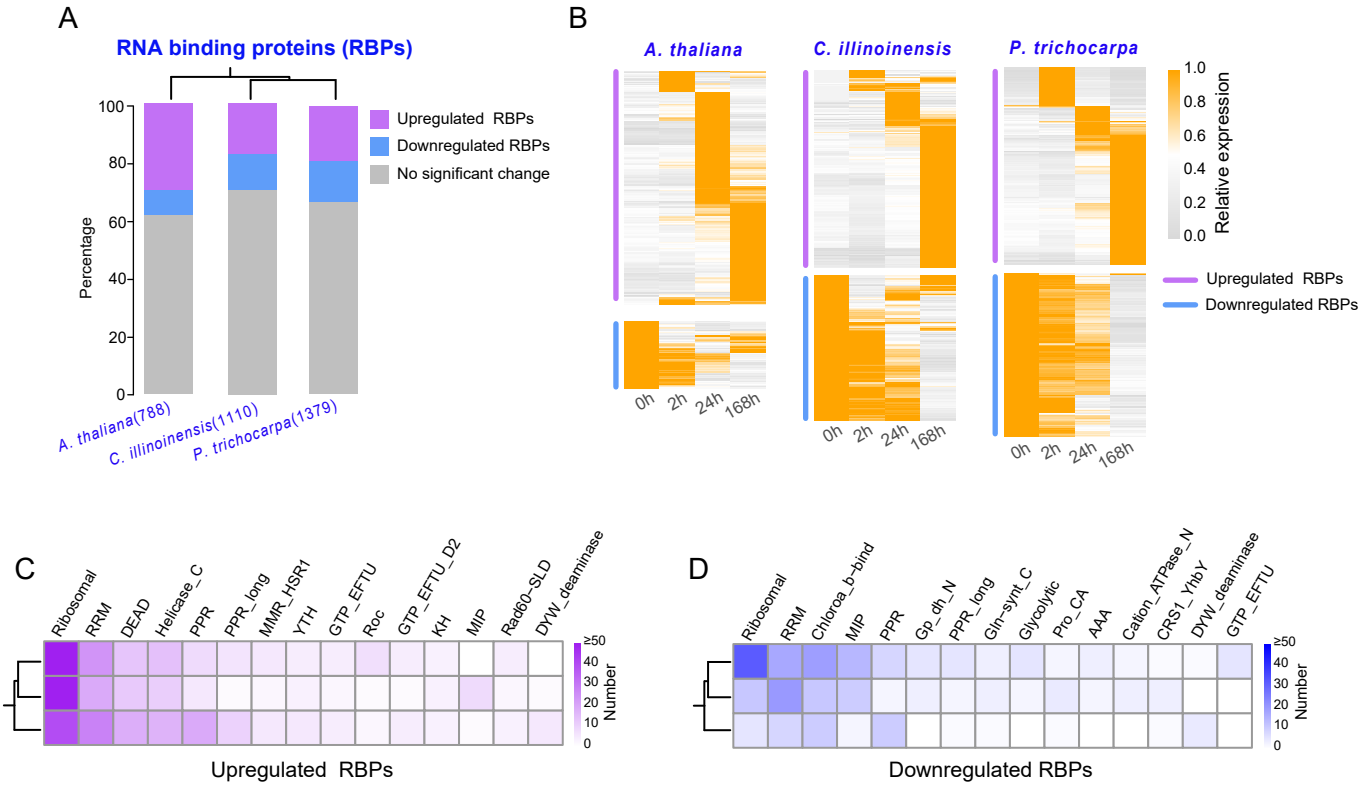

Fig 4. Cold-responsive RBPs in conserved RBP families.
(A) Upregulated and downregulated RBPs under cold stress. Bar percentage indicates the proportion of upregulated, downregulated, and no significant expression changed RBPs to all RBPs. The number in parentheses after the species indicates the number of RBPs in the genome. (B) Expression profiles of up- and downregulated RBPs under cold stress. The expression values of the RBPs at four different timepoints (0, 2, 24, and 168 h) of cold treatments were normalized by row scale. (C-D) The numbers of up- and downregulated RBPs in RBP families. The top 15 up- and downregulated RBP families were selected for visualization.

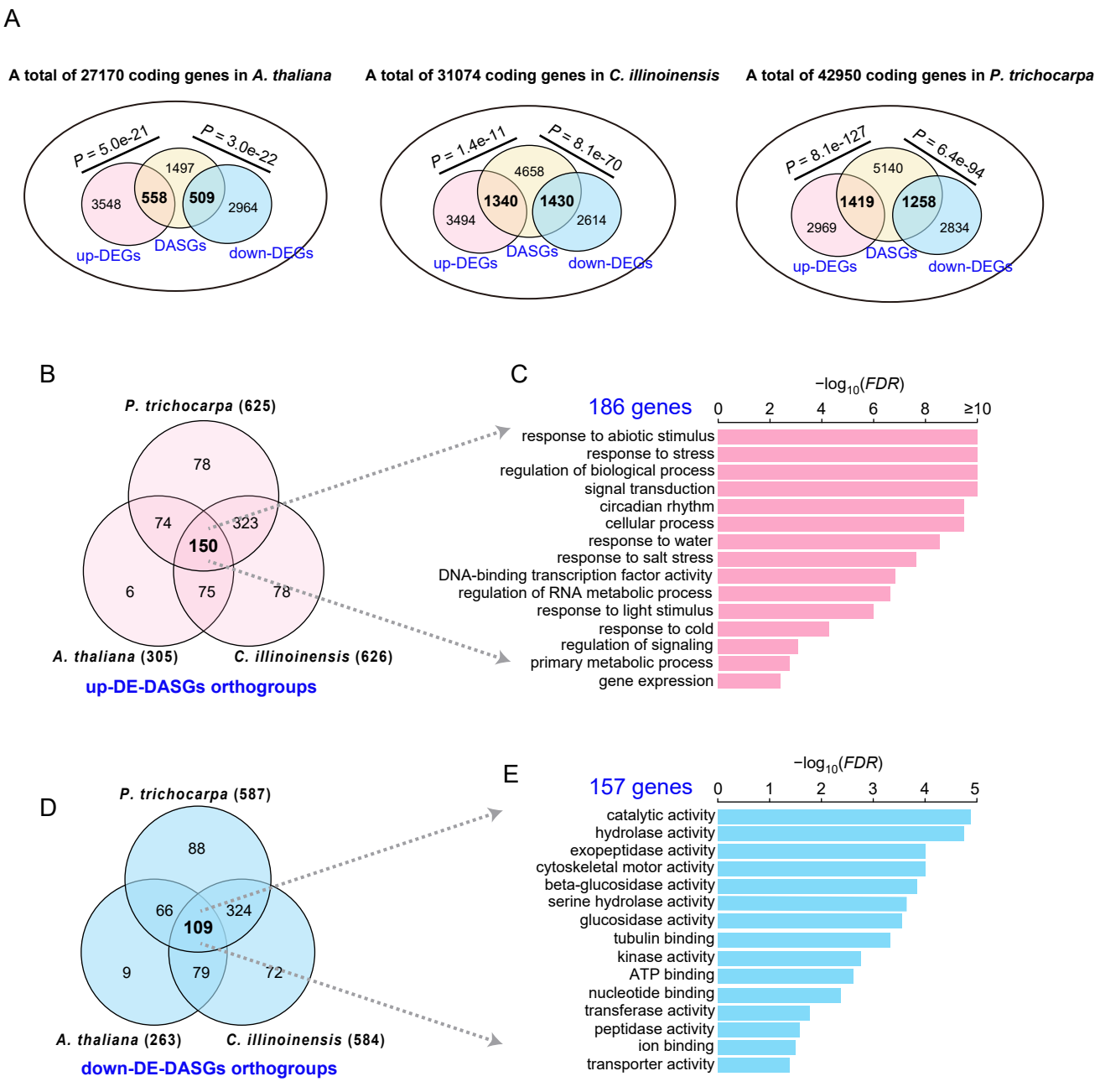

Fig 5. Association of cold-induced DEGs and DASGs and identification of conserved DE-DASG orthogroups in rosids.

(A) Overlapping and unique genes between DEGs and DASGs in three species. The overlapping significance was calculated by Fisher's Exact Test. (B-E) Identification and enrichment analysis of conserved DE-DASG orthogroups in three species. Venn diagram shows overlapping and unique orthogroups of up- (B) and down-DE-DASGs (D) in three species. Of the 150 up- and 109 down-DE-DASG orthogroups, we extracted 186 (C) and 157 (E) *A. thaliana* genes representatively for GO term analysis. The top 15 significant enriched terms were selected to be shown.

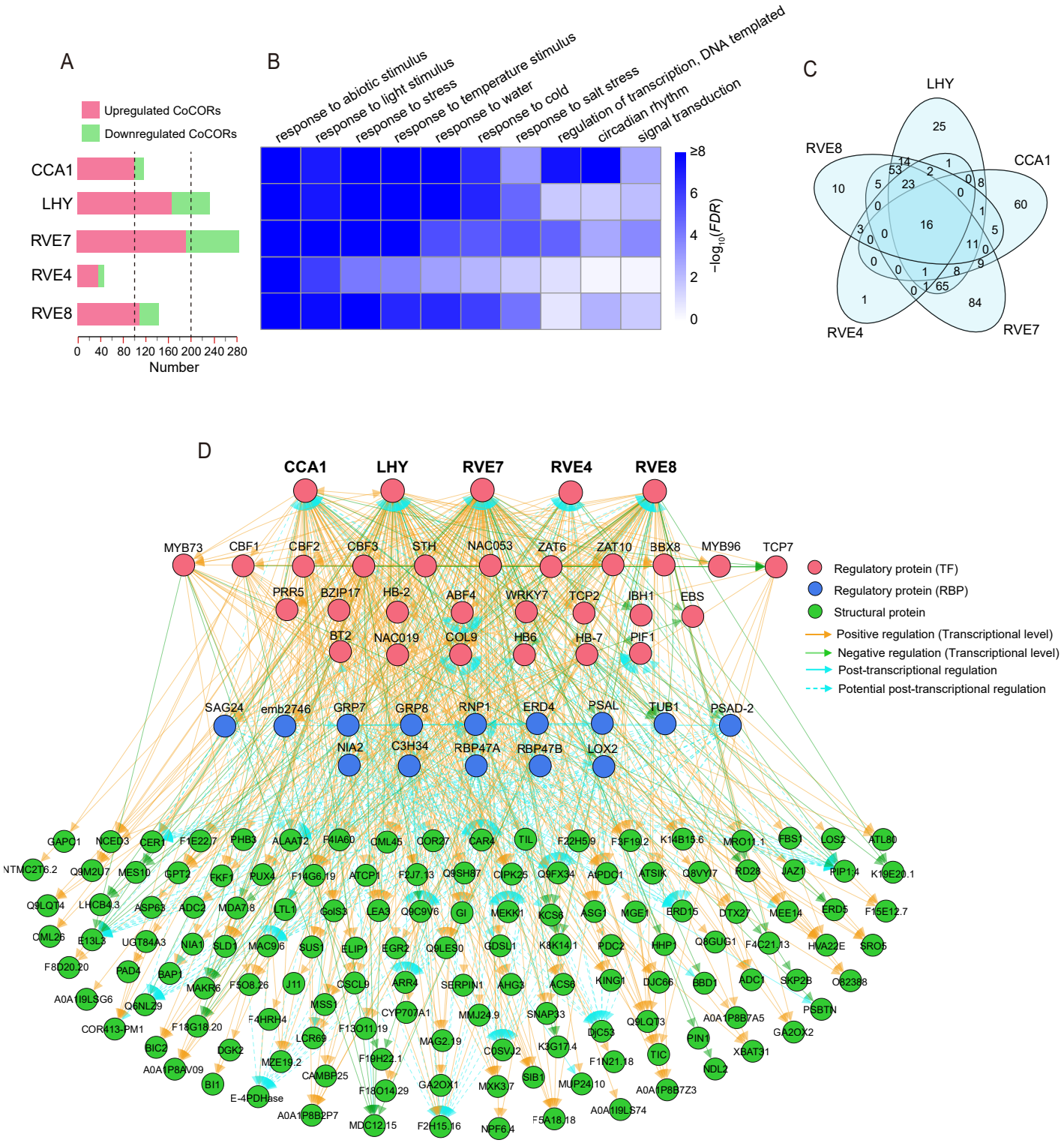

Fig 6. A hierarchical cold-responsive regulatory network launched by circadian clock components in rosids. (A) Numbers of up- and downregulated CoCORs targeted by CCA1, LHY, RVE4, RVE7, and RVE8. These CoCORs were predicted by a stringent pipeline (see Materials and methods). (B) GO enrichment analysis of CoCORs. The top 10 significant GO terms were selected to be shown. (C) Overlapping and unique CoCORs regulated by the five circadian-related TFs. (D) A hierarchical network launched by the five circadian-related TFs. The network is arranged in three layers (TFs, RBPs, and Structural proteins). To improve the robustness of the network, we also integrated the regulatory relations between CBFs, ZAT10, HB6, HB-7, NAC053, WRKY7, MYB73, and TCP7 on CoCORs from ChIP/DAP-seq data (O'Malley et al., 2016; Song et al., 2021) and identified GRP7 target CoCORs from iCLIP-seq data (Meyer et al., 2017). Solid arrow lines indicate that the regulatory relations between TFs (or RBPs) and CoCORs were from ChIP/DAP-seq (or iCLIP-seq) experiments.

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