1 Cold tolerance in rice plants is partially controlled by root responses

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13

14 Abstract

15 Rice (*Oryza sativa* L.) ssp. *indica* is the most cultivated species in the South of Brazil.
16 However, these plants face low temperature stress from September to November, which
17 is the period of early sowing, affecting plant development during the initial stages of
18 growth, and reducing rice productivity. This study aimed to characterize the root
19 response to low temperature stress during the early vegetative stage of two rice
20 genotypes contrasting in their cold tolerance (CT, cold-tolerant; and CS, cold-sensitive).
21 Root dry weight and length, as well as number of root hairs, were higher in CT than CS
22 when exposed to cold treatment. Histochemical analyses indicated that roots of CS
23 genotype present higher levels of lipid peroxidation and H₂O₂ accumulation, along with
24 lower levels of plasma membrane integrity than CT under low temperature stress.
25 RNAseq analyses revealed that the contrasting genotypes present completely different
26 molecular responses to cold stress. The number of over-represented functional
27 categories was lower in CT than CS under cold condition, suggesting that CS genotype
28 is more impacted by low temperature stress than CT. Several genes might contribute to
29 rice cold tolerance, including the ones related with cell wall remodeling, cytoskeleton

and growth, signaling, antioxidant system, lipid metabolism, and stress response. On the other hand, high expression of the genes *SRC2* (defense), *root architecture associated 1* (growth), *ACC oxidase*, *ethylene-responsive transcription factor*, and *cytokinin-O-glucosyltransferase 2* (hormone-related) seems to be related with cold sensibility. Since these two genotypes have a similar genetic background (sister lines), the differentially expressed genes found here can be considered candidate genes for cold tolerance and could be used in future biotechnological approaches aiming to increase rice tolerance to low temperature.

Keywords

cell wall, cold tolerance, growth, oxidative stress, root responses, signaling

Abbreviations

CS, cold-sensitive; CT, cold-tolerant; DAB, diaminobenzidine; DEGs, differentially expressed genes; GA, gibberellin; QC, quiescent center.

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops, providing food for more than half of the world's population (Zhang et al. 2017). Rice production can be seriously affected by environmental stresses, including low temperature, since it is normally grown in tropical and temperate climate zones, and therefore is sensitive to chilling (Mukhopadhyay et al. 2004; Byun et al. 2017). Cold can be harmful from germination to reproductive stages, in most cases resulting in decreased crop yield (Ma et al. 2009; Cruz et al. 2013). Therefore, cold-tolerant plants are able to grow better than cold-sensitive ones under low temperature conditions (Cabello et al. 2014).

Given its tropical origin, cultivated rice germplasm genetic variability is very limited to identify cold stress tolerance genes and alleles (Byun et al. 2017). A few examples of alleles associated with low temperature tolerance are derived from *japonica* subspecies, as *COLD1^{jap}* and *bZIP73^{jap}* (Ma et al. 2015; Liu et al. 2018). Indeed,

transferring the *japonica* alleles to *indica* rice genotypes increased its cold tolerance. Even though *japonica* subspecies is more adapted to temperate climates (Cruz et al. 2013), few *indica* rice cultivars can tolerate stressful conditions and are able to grow under low temperature (Sanghera et al. 2011).

Our group previously identified two *indica* rice genotypes (sister lines with similar genetic background) with contrasting levels of cold tolerance during germination and early vegetative stage (Dametto et al. 2015; Adamski et al. 2016; Sperotto et al. 2018). Parallel physiological and transcriptomic analyses under cold treatment (13°C/ 7 days and 10°C/ 6 hours for germination and vegetative stage, respectively) indicated protective and more active processes in the cold-tolerant genotype when compared to the cold-sensitive one, including cell division and expansion processes, membrane fatty acid unsaturation, antioxidant capacity, cellulose deposition in the cell wall, photosynthetic performance under cold stress/ photosynthetic performance recovery after cold stress, and expression of numerous genes with diverse functions related to chilling stress response, including *Temperature-induced lipocalin-2* and transcription factors *SNAC1* and *OsERD15*.

Optimal root development and distribution play multiple roles in rice growth, including anchorage of the plant and acquisition of water and nutrient elements, which are heterogeneously distributed in soil (Gowda et al. 2011; Lynch 2013). Although the understanding about rice roots has been expanded in the last decades, and a wide natural variation of root system architecture has been reported (Uga et al. 2009), much remains to be investigated regarding root morphology and physiology, particularly in root genetics (Wu and Cheng, 2014), mostly because there is a close relation between above ground traits (including grain yield and quality) and underground roots (Yang, 2011; Ahamed et al. 2012; Neilson et al. 2013). Information on gene networks involved in root formation has been accumulated for *Arabidopsis thaliana*, but our knowledge of these aspects in rice is still limited (Rebouillat et al. 2009; Coudert et al. 2010). As roots can be considered the foundation of rice development, dissecting genetic and molecular mechanisms controlling rice root responses is critical for the development of new rice genotypes that are better adapted to adverse conditions (Rebouillat et al. 2009; Coudert et al. 2010; Wu and Cheng, 2014), including cold stress. Unfortunately, only a few studies of cold tolerance in rice have taken root responses into account (Hashimoto et al.

2009; Lee et al. 2009; Ahamed et al. 2012; Neilson et al. 2013; Xiao et al. 2014; Yang et al. 2015; Zhu et al. 2015).

In our previous works of rice genotypes with contrasting levels of cold-tolerance (cited above), we repeatedly noted that roots of the cold-tolerant and cold-sensitive genotypes seemed different under low temperature stress, being longer and thicker in cold-tolerant plants. For this reason, we decided to better understand the root responses of these contrasting rice genotypes to cold stress, and how that could be related to cold tolerance. In order to identify and characterize novel genes and processes involved with rice root cold tolerance, we performed parallel transcriptomic (10°C for 24 h) and physiological analyses. Our findings could be useful for future biotechnological and breeding efforts aiming to enhance cold tolerance in rice plants.

102

103 **Material and methods**

104 *Plant materials and cold treatment*

Previously, we identified low temperature tolerant (IRGA 959-1-2-2F-4-1-4-A) and sensitive (IRGA 959-1-2-2F-4-1-4-D-1-CA-1) sister lines from the *indica* subspecies (Dametto et al. 2015; Adamski et al. 2016; Sperotto et al. 2018). Both genotypes were germinated for five days in distilled water and then transferred to holders positioned over plastic pots covered with aluminum foil and containing 300 mL of nutrient solution (as described by Ricachenevsky et al. 2011). The pH of the nutrient solution was adjusted to 5.4. Plants were kept at 28°C ± 1°C under photoperiod of 16 h / 8 h light/dark (150 µmol.m⁻².s⁻¹). Solutions were replaced every two days. At the three-leaf stage (approximately 30 days), plants were transferred to a growth chamber and maintained at 10°C (or control condition at 28°C) for up to 10 days (light intensity of 100 µmol.m⁻².s⁻¹).

116

117 *Root measurements*

Roots (n = 16) from plants submitted to control and cold treatments were measured and oven-dried at 65°C until constant weight, in order to obtain the length (cm) and dry weight (mg), respectively. Root diameter (mm) was measured 1 cm from

the apex, using ImageJ software (n = 15). Root hair density was measured in 1 cm sections (always derived from the same location, i.e. 1 cm from the apex) of roots from plants submitted to control and cold treatments (n = 16).

In situ histochemical localization of H₂O₂

In situ accumulation of H₂O₂ was detected by histochemical staining with diaminobenzidine (DAB), according to Shi et al. (2010), with minor modifications. Roots were excised and immersed in a 1 mg ml⁻¹ solution of DAB (pH 3.8) in 10 mM phosphate buffer (pH 7.8) at room temperature. Immersed roots were illuminated for 8 h until brown spots were visible, which were derived from the reaction of DAB with H₂O₂. Roots were kept in 70% ethanol for taking pictures with a digital camera coupled to a stereomicroscope.

Detection of cell death by loss of plasma membrane integrity

To determine changes in viability of cells by cold treatment, excised roots (n = 5 per genotype/ treatment) were immersed for 5 h in a 0.25% (w/v) aqueous solution of Evans Blue (Romero-Puertas et al. 2004). Roots were photo documented with a digital camera coupled to a stereomicroscope.

Evaluation of lipid peroxidation

Histochemical detection of lipid peroxidation was performed as described by Pompella et al. (1987). In brief, roots (n = 5 per genotype/ treatment) were stained with Schiff's reagent for 60 min, to detect aldehydes originating from lipid peroxidation. After the reaction with Schiff's reagent, roots were rinsed with a sulfite solution (0.5% [w/v] K₂S₂O₅ in 0.05 M HCl). The stained roots were kept in the sulfite solution to retain the staining color. The roots stained with Schiff's reagent were photo documented with a digital camera coupled to a stereomicroscope.

149 *Detection of cellulose deposition*

150 For analyzing the cellulose deposition in root cell walls of the CT and CS
151 genotypes, 30-day old plants (n = 10 per genotype/ treatment) were submitted to control
152 and cold conditions for 72 h. Root samples were washed and then fixed in a
153 glutaraldehyde 1% + formaldehyde 4% solution in 0.1 M phosphate buffer (pH 7.2), for
154 24 h, and subsequently went through a dehydration process through an alcoholic series,
155 starting at 10% up to 70% for 20 min, and maintained in 70% ethanol until cutting. Root
156 transversal sections were obtained 1 cm from the apex (3-4 sections per root) using
157 freehand technique. The cuts were hydrated and stained in Congo red 1% for 1 min,
158 which colors cellulose-rich cells (Zheng et al. 2018). After staining, the sections were
159 washed with MilliQ water until dye removal. The colored sections were observed using
160 a Zeis Scope A.1 optical microscope coupled to an Axio CAM 506 color digital camera.

161

162 *RNA extraction and comparative transcriptomic profiling by RNAseq*

163 Total RNA was extracted from rice roots using Concert Plant RNA Reagent
164 (Invitrogen[®]) and treated with DNase I (Invitrogen[®]). Approximately 20 µg of total
165 RNA were used in high-throughput cDNA sequencing by Illumina HiSeq 2000
166 technology (Fasteris SA, Plan-les-Ouates, Switzerland - <http://www.fasteris.com/>). We
167 constructed two individual single-end cDNA library for each rice genotype (cold
168 tolerant and sensitive), after 24 h of cold or control treatment, totalizing eight libraries
169 (four plants per library). The cDNA libraries were prepared according to Illumina's
170 protocols. Briefly, RNAseq was performed using the following successive steps: poly-A
171 purification; cDNA synthesis using a poly-T primer, shotgun method to generate inserts
172 of approximately 500 bp; 3p and 5p adapter ligations; pre-amplification; colony
173 generation; and Illumina single-end 50 bp sequencing.

174 Low quality reads (FASTq value <13) were removed, and 3p and 5p adapter
175 sequences were trimmed using Genome Analyzer Pipeline (Fasteris). The remaining
176 low-quality reads with 'n' were removed using Python script. After cleaning the data
177 (low quality reads, adapter sequences), the mRNAseq data from the eight libraries was
178 aligned to the rice genome using the software Spliced Transcripts Alignment to a
179 Reference (STAR) (Dobin et al. 2013). Only sequences with up to two mismatches to

180 the rice reference genome
 181 (http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs
 182 [/pseudomolecules/version_7.0/all.dir/all.seq](http://rice.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/all.dir/all.seq)) were used. The SAM files from STAR
 183 were then processed using Python scripts to assign the frequencies of each read and map
 184 them onto references. For data normalization, we used the scaling normalization method
 185 (Robinson et al. 2010). To assess whether the sequences were differentially expressed,
 186 we used the R package EdgeR (Robinson et al. 2010). We considered that the sequences
 187 were differentially expressed if they had an adjusted p-value < 0.05 and at least 2-fold
 188 change. The RNA-seq data obtained in this work was deposited on the Gene Expression
 189 Omnibus database (GEO - <https://www.ncbi.nlm.nih.gov/geo/> - Barrett et al. 2013) and
 190 can be accessed using the code

191

192 *Gene Ontology (GO) terms enrichment analysis*

193 The *loci* from differentially expressed genes with increased expression in plants
 194 from the cold-tolerant or cold-sensitive genotypes were used to search for enriched
 195 Gene Ontology (GO) terms comparing both datasets. Enrichment analysis was
 196 performed using Plant GeneSet Enrichment Analysis Toolkit (PlantGSEA -
 197 <http://structuralbiology.cau.edu.cn/PlantGSEA/> - Yi et al. 2013) built-in Fisher's Exact
 198 Test. The significance threshold of gene set enrichments was false discovery rate (FDR)
 199 < 0.05. From a total of 202 (control condition) and 582 (cold condition) differentially
 200 expressed genes, 200 and 569 could be assigned a GO term, respectively, and were thus
 201 considered in the GO enrichment analysis.

202

203 *Gene expression analysis by quantitative RT-PCR*

204 To confirm the high-quality of deep sequencing results, RT-qPCR was used to
 205 check the gene expression of ten putative cold tolerance-related genes. Total RNA was
 206 extracted from rice roots of plants exposed to 10°C for 24 h using Concert Plant RNA
 207 Reagent (Invitrogen) and treated with DNase I (Invitrogen). First-strand cDNA
 208 synthesis was performed with reverse transcriptase (M-MLV, Invitrogen) using 1 µg of
 209 RNA. RT-qPCRs were carried out in a StepOne Real-Time Cyclers (Applied
 210 Biosystems). All primers (listed in Supplementary Table 1) were designed to amplify

100-150 bp of the 3'-UTR of the genes and to have similar T_m values (60 ± 2°C). Reaction settings were composed of an initial denaturation step of 5 min at 94°C, followed by 40 cycles of 10 s at 94°C, 15 s at 60°C (fluorescence data collection) and 15 s at 72°C; samples were held for 2 min at 40°C for annealing of the amplified products and then heated from 55 to 99°C with a ramp of 0.1°C /s to produce the denaturing curve of the amplified products. RT-qPCRs were carried out in 20 µl final volume composed of 10 µl of each reverse transcription sample diluted 100 times, 2 µl of 10X PCR buffer, 1.2 µl of 50 mM MgCl₂, 0.1 µl of 5 mM dNTPs, 0.4 µl of 10 µM primer pairs, 4.25 µl of water, 2.0 µl of SYBR green (1:10,000, Molecular Probe), and 0.05 µl of Platinum Taq DNA Polymerase (5 U/µl, Invitrogen, Carlsbad, CA, USA). Gene expression was evaluated using a modified 2^{-ΔCT} method (Schmittgen and Livak 2008), which takes into account the PCR efficiencies of each primer pair (Relative expression TESTED GENE / CONTROL GENE = (PCReff CG)^{Ct CG} / (PCReff TG)^{Ct TG}). Rice Ubiquitin (*OsUBQ5*) was used as the control gene. Experiments were performed with three biological and four technical replicates, collected in a second experiment (i.e. distinct from the one for RNAseq).

227

228 *Statistical analysis*

229 When appropriate, means were statistically compared by the Student's *t*-test (*p*-
230 value ≤ 0.05, 0.01 and 0.001) using SPSS Base 21.0 for Windows (SPSS Inc., USA).

231

232 **Results**

233 *Physiological characterization of cold-tolerant and cold-sensitive roots*

234 Thirty-day old plants were submitted to control (28°C) or cold (10°C) condition
235 for up to 10 days. As seen in Figure 1, root dry weight of both cold-tolerant (CT) and
236 cold-sensitive (CS) plants decreased in cold conditions. Also, root dry weight of CT
237 plants under cold condition was higher than CS ones during the entire evaluated period.
238 Under control condition we detected higher variation, even so the root dry weight was
239 higher in CT than CS plants in most of the evaluated periods. Root length and root hair
240 density presented higher values in CT plants than in CS ones submitted to cold stress,

while no difference was detected under control condition (Figure 1). Histochemical analyses showed that CS roots present high levels of lipid peroxidation (Figure 2a), loss of plasma membrane integrity (indicative of cell death - Figure 2b), and H₂O₂ accumulation (Figure 2c), suggesting that roots from CT plants are more efficiently protected of and less damaged by the oxidative stress caused by low temperature than roots from CS plants. Such differences in physiological responses, together with the fact that most cold-tolerance studies are performed only with rice leaves, encouraged us to better understand the molecular responses of these roots under low temperature stress using RNAseq.

250

251 *Overview of cold-tolerant and cold-sensitive root cDNA libraries sequencing*

Roots from plants of both genotypes (CT and CS) under cold (10°C) and control (28°C) conditions for 24h were used to identify differentially expressed mRNAs using the Illumina Platform. Since we were interested in understanding the molecular basis of tolerance, we focused on the differences between genotypes in the same experimental condition. Considering the two comparisons performed (Control: CT x CS; and Cold: CT x CS), we found 784 differentially expressed sequences, which are all listed in Supplementary Tables 2 and 3. Under control condition, 104 sequences were more expressed in the CT genotype, and 98 in the CS genotype. Under cold condition, 235 sequences were more expressed in the CT genotype, and 347 in the CS one (Figure 3a). As these genotypes are sister lines (Sperotto et al. 2018), and therefore share a similar genetic background, the low number of differentially expressed sequences between CT and CS was expected. Interestingly, 71 and 69 sequences were more expressed in the CT and CS genotypes, respectively, regardless the tested condition (Figure 3b). These sequences with common expression patterns on the CT and CS genotypes under both treatments are listed in Supplementary Table 4. Also, three sequences presented opposite expression pattern in the CT and CS genotypes: LOC_Os09g29490 (*peroxidase precursor*) and LOC_Os07g14740 (*harpin-induced protein 1 domain containing protein*) were more expressed in the CT genotype under control condition, and more expressed in the CS genotype under cold condition. On the other hand, LOC_Os02g17780 (*ent-kaurene synthase*) was more expressed in the CS genotype

under control condition, and more expressed in the CT genotype under cold condition (Supplementary Table 4, marked in gray color).

GO terms and metabolism categories

The GO categories with the highest percentage of annotated genes within each main GO division (biological process, cellular component and molecular function) are shown for both genotypes under control and cold conditions (Supplementary Figure 1). Comparing both genotypes under control condition, GO analysis indicates that the CT genotype presents more active protein metabolic processes (phosphorylation and modification), phosphorus metabolic processes, macromolecule modification, and transferase/kinase activity. On the other hand, other biological processes such as response to stress, defense response, cell death, and apoptotic process, are more active in the CS genotype, along with the molecular function of general binding (Supplementary Figure 1). Under cold treatment, more GO categories were found enriched in both genotypes. Several GO categories were more active in the CT genotype, including general metabolic/biosynthetic processes, cellular component (mostly related to cytoskeleton), protein binding, and synthesis of cellulose. On the other hand, the CS genotype presented more active electron transport and response to stimulus/stress, along with ion binding, kinase activity, and membrane-related processes (Supplementary Figure 1).

It was possible to assign metabolism categories to 55 and 59 genes more expressed in the CT and CS genotype, respectively, after control treatment, and 134 and 223 genes more expressed in the CT and CS genotype, respectively, after cold exposure. It is interesting to note that cold treatment activates several metabolic pathways in both genotypes (Figure 4). Under control condition, protein metabolism- and signaling-related genes are more expressed on the CT genotype, while stress- and secondary metabolism-related genes are more expressed on the CS one (Figure 4a). Under cold condition, most of the metabolic pathways seem more active in the CS genotype (except cell wall- and carbohydrate metabolism-related - Figure 4b), suggesting that the CS genotype is deeply affected by low temperature stress, while the CT genotype presents active cell wall remodeling, probably to reinforce and thicken it. This hypothesis was strengthened by the higher deposition of cellulose in root cells of CT genotype under

304 cold condition when compared with CS (Figure 5a), which probably contributes to the
305 higher root diameter (Figure 5b).

306 All the 784 differentially expressed genes (DEGs) identified under control or
307 cold conditions were carefully analyzed and, based on the predicted molecular function
308 and available literature, were classified in functional categories (Supplementary Tables
309 2 and 3). In order to facilitate the overall understanding of the differential gene
310 expression in each functional category presented in Supplementary Tables 2 and 3, we
311 summarize these data in Table 1. Seven functional categories (antioxidant system,
312 carbohydrate metabolism/energy production, cell wall, lipid metabolism, others,
313 signaling, and stress response) were more represented in CT than in CS roots under
314 control conditions, while only five (defense, hormone-related, secondary metabolism,
315 transcription factor, and transport) were more represented in CS than in CT roots (Table
316 1). Under cold treatment, we detected four different patterns, based on the number of
317 differentially expressed sequences and expression level: 1) functional categories with
318 similar representation in both CT and CS genotypes (carbohydrate metabolism and
319 energy production, lipid metabolism, nucleotide metabolism, secondary metabolism,
320 stress response, and others); 2) functional categories more represented in CT than CS
321 genotype (cell wall, growth, and signaling); 3) functional categories uniquely
322 represented in only one of the genotypes (cytoskeleton in CT, and DNA structure
323 maintenance + photosynthesis in CS); and 4) the most common pattern, containing
324 functional categories more represented in CS than CT genotype (amino acid
325 metabolism, antioxidant system, calcium-related, defense, hormone-related, protein
326 degradation/modification, transcription factor, translation-related, transport, and
327 unknown). It is interesting to highlight the higher expression of cell wall-related genes
328 under control condition and the higher expression of cell wall/growth-related genes
329 under cold condition on the CT genotype (Table 1), which agree with the higher root
330 biomass, length and hair density of this genotype when compared with CS, especially
331 under cold condition (Figure 1). The high number of functional categories more
332 represented in CS than CT genotype (Table 1) agrees with the metabolic pathways data
333 presented in Figure 4b. Several genes belonging to these functional categories are
334 discussed later.

335

336 *Confirmation of RNAseq expression patterns using RT-qPCR*

337 To further confirm the quality of our libraries and to evaluate possible induction
338 or repression of expression by the cold treatment, 10 genes were randomly selected for
339 RT-qPCR analyses: six genes with higher expression in the CT plants under both tested
340 conditions, three genes with higher expression on the CS under cold, and one gene with
341 higher expression on the CS under both tested conditions (Supplementary Tables 1, 2
342 and 3). The differences in expression between the two genotypes after cold exposure,
343 found by the RNAseq method, were confirmed for all tested genes (Figure 6), endorsing
344 the good quality of the datasets.

345

346 **Discussion**

347 *Differential expression under control condition*

348 Root system architecture is a complex trait controlled by several genes
349 (Wachsman et al. 2015). Courtois et al. (2009) summarized 675 rice QTLs for 29 root
350 parameters detected in 12 mapping populations. Even though in our analyses the
351 absolute number of differentially expressed genes was similar under control condition
352 (104 more expressed in the CT genotype, and 98 more expressed in the CS - Figure 3
353 and Supplementary Table 2), the number of over-represented functional categories was
354 higher in CT than CS under control condition (Table 1). According to Janiak et al.
355 (2018), drought tolerance in barley may be attributed to stressed-like expression
356 patterns present before the occurrence of stress. Therefore, the over-representation of
357 functional categories in roots of CT than CS plants under control condition could be
358 representative of a constitutive tolerance mechanism, as previously suggested for cotton
359 (Zheng et al. 2012).

360 Under control condition, the CT genotype stood out with higher expression of
361 genes related to antioxidant system, cell wall, lipid metabolism, signaling, stress
362 response, and others (Table 1 and Supplementary Table 2). However, for most of these
363 genes, we cannot establish a direct link with the higher root dry mass of CT than CS
364 plants seen under control condition (Figure 1). Root growth is caused by cell division
365 and elongation. Mutant analyses revealed that genes related to cell wall growth, cell
366 expansion, and auxin signaling are involved in the division and elongation of root cells

(Kitomi et al. 2018). Even though the CS genotype presented higher expression of hormone-related genes, the CT genotype presented higher expression of *OsIAA2*, an auxin-responsive gene, along with a cell wall-related gene, *glycosyl hydrolase* (Supplementary Table 2). *OsCel9A*, a rice *glycosyl hydrolase* family gene, plays an essential role in regulating auxin-induced lateral root primordia formation (Yoshida et al. 2006). Auxin signaling is important in overall root morphogenesis/elongation (Kitomi et al. 2018). A mutation in a member of the Auxin (Aux)/Indole-3-acetic acid (IAA) gene family, *OsIAA23*, causes defects in postembryonic quiescent center (QC) maintenance due to the disintegration of the root cap and termination of root growth, suggesting the importance of auxin in rice QC maintenance (Ni et al. 2011). Also, plants overexpressing the auxin biosynthesis gene *OsYUCCA1* have higher auxin content, which leads to more crown roots (Yamamoto et al. 2007). Susuki et al. (2003) reported the first root-hairless mutant in rice, *rh2*, whose absence of root hairs is likely caused by a shortage of endogenous auxin. Also, WUSCHEL-related homeobox 3A (*OsWOX3A*) was reported to control root hair formation through the regulation of auxin transport genes (Yoo et al. 2013), further suggesting that auxin is required for root hair development. We also detected higher expression of *phospholipase D*, *phytosulfokine receptor*, and *wali7* in CT than CS plants. The phospholipase D family plays an important role in the regulation of cellular processes in plants, including root growth (Liu et al. 2010). Phytosulfokine- α (PSK- α) is a disulfate pentapeptide described as a growth factor. In Arabidopsis, PSK- α induced root growth (mainly by an increase in cell size) in a dose-dependent manner without affecting lateral root density (Kutschmar et al. 2009). Also, *pskr1-3 pskr2-1* mutant seedlings had shorter roots and hypocotyls than the wild type, whereas *35S:PSKR1* or *35S:PSKR2* seedlings were larger (Hartmann et al. 2013). Janiak et al. (2012) reported that a *wali7* domain-containing protein accumulates during the initial stage of root hair development in barley. It is important to highlight that this same gene (*wali7*) was also detected more expressed in the CT than CS under cold condition (Supplementary Table 3).

On the other hand, under control condition the CS genotype showed higher expression of genes related to defense, hormone-related, secondary metabolism, transcription factor, and transport (Table 1 and Supplementary Table 2). As previously discussed, there is no direct relation between the expression of most of these genes with lower root growth of CS than CT plants seen under control condition (Figure 1). One of

the hormone-related genes detected with higher expression in the CS than in the CT genotype, *2OG-Fe oxygenase*, participates in ethylene biosynthesis (Dalal et al. 2018). Also, the CS genotype presented higher expression level of the genes *brassinosteroid LRR receptor kinase* and *ent-kaurene synthase* than CT. The first one is known to be involved in normal brassinosteroid perception and plant development, including root growth via hormonal crosstalk with ethylene (Clouse, 2011; Lv et al. 2018). The second one encodes the enzyme catalyzing the second step of the gibberellin (GA) biosynthesis pathway (Margis-Pinheiro et al. 2005), also interacting with ethylene to regulate root growth and defense (Saithong et al. 2015; Tezuka et al. 2015). Under control condition, we detected higher expression of genes related to terpene biosynthesis (*cycloartenol synthase*, *phytoene synthase* and *terpene synthase*) in roots of CS than CT (Supplementary Table 2). It was shown that ethylene treatment can up-regulate *cycloartenol synthase* in the perennial medicinal herb *Dioscorea zingiberensis* (Diarra et al. 2013). Also, ethylene regulates carotenoid biosynthesis (which includes phytoene synthase protein) in rice (Yin et al. 2015), and sesquiterpene biosynthesis in citrus, through the activation of a *terpene synthase* gene (*CsTPS1* - Shen et al. 2016). Therefore, several ethylene-related genes were found more expressed on the roots of CS than CT plants. It is important to highlight that high levels of ethylene can inhibit root growth (Yin et al. 2015; Qin and Huang, 2018). In Arabidopsis, exogenous application of ethylene inhibits the primary root elongation (Ruzicka et al. 2007). Thus, we hypothesize that roots of CS plants present lower growth under control condition (Figure 1) due to higher ethylene levels than CT.

422

423 *Differentially expressed genes under cold condition*

Under low temperature, the absolute number of differentially expressed genes was lower in CT than CS (235 more expressed on the CT genotype, and 347 more expressed on the CS - Figure 3 and Supplementary Table 3). Also, the number of over-represented functional categories was lower in CT than CS under cold condition (Table 1), suggesting that the CS genotype is more prone to sense and more impacted by low temperature stress than the CT. Under cold treatment, the CT genotype showed higher expression of genes related to only four categories: cell wall, cytoskeleton, growth, and signaling (Table 1 and Supplementary Table 3).

432 The cell wall senses and broadcasts stress signals to the interior of the cell, by
 433 triggering a cascade of reactions leading to plant tolerance/resistance. Therefore, the
 434 study of wall-related genes is particularly relevant to understand the metabolic
 435 remodeling triggered by plants in response to exogenous stresses (Guerriero et al.
 436 2014). Several genes involved in the formation and remodeling of plant cell wall,
 437 including *glycosyl hydrolase*, *cellulose synthase*, *glycosyl transferase*, *wall-associated*
 438 *kinase (WAK)*, and *glycine-rich cell wall structural protein*, were detected with higher
 439 expression in the CT than CS under cold condition (Supplementary Table 3). Glycosyl
 440 hydrolases have been implicated in physiologically important processes in plants, such
 441 as response to stresses, activation of phytohormones, lignification, and cell wall
 442 remodeling (Opassiri et al. 2006). Cellulose, hemicelluloses, and pectins are the main
 443 structural polysaccharides of the plant cell wall. Enzymes as glycosyl transferases and
 444 cellulose synthases are involved in the synthesis of these polysaccharides and other
 445 glycans (De Caroli et al. 2014) and have been previously related with cold tolerance in
 446 young rice plants (Dametto et al. 2015; Sperotto et al. 2018; Kong et al. 2019). To our
 447 knowledge, this is the first time that these cell wall-related genes are detected with
 448 higher expression in rice roots of CT plants when compared to CS ones. Biochemical
 449 studies demonstrated that WAK proteins (which play important roles in cell expansion)
 450 are covalently bound to pectin in the cell wall and serve as physical links between the
 451 extracellular matrix and the cytoplasm and as a signaling component between the cell
 452 wall and the cytoplasm (Zhang et al. 2005; de Oliveira et al. 2014), interacting with
 453 glycine-rich proteins (Park et al. 2001; Giarola et al. 2016).

454 The maintenance of cellulose synthesis under stress is necessary for proper stress
 455 response. Interestingly, a new protein family named Cellulose Synthase Companion
 456 (CC) proteins, was shown to be crucial to the stability of the Cellulose Synthase
 457 Complex during salt stress in Arabidopsis. CC proteins promotes the reassembly of
 458 microtubules, which are important to drive cellulose deposition at the cell wall (Kesten
 459 et al. 2019). Interestingly, some genes related with the functional categories
 460 cytoskeleton remodeling and growth were detected with higher expression in CT than
 461 CS under cold condition, including *microtubule-associated protein 70 (MAP70)*, *kinesin*
 462 *motor domain containing protein*, *growth regulating factor protein*, *auxin-independent*
 463 *growth promoter protein*, and *RopGEF7* (Supplementary Table 3). Microtubules are a
 464 component of the plant cytoskeleton composed of heterodimers of $\alpha\beta$ -tubulin. They

perform several cellular roles, perhaps most notably coordinating the deposition of cellulose microfibrils in the cell wall (Gardiner, 2013). Kinesins, the ATP-driven microtubule (MT)-based motor proteins, have been reported to be involved in many basic processes, including cell division and organ development (Fang et al. 2018). Recently, Xu et al. (2018) showed that a rice class-XIV kinesin (coded by one of the kinesin genes detected in our study - Os04g53760) actively transports microtubules along each other in a unidirectional manner and enters the nucleus in response to cold, stimulating cell elongation. Growth regulating factors are plant-specific transcription factors important for developmental processes, including root development and coordination of growth processes under adverse environmental conditions (Omidbakhshfard et al. 2015). In Arabidopsis, RopGEF7 (a GTPase activator) is required for root meristem maintenance, which is essential for the maintenance of root growth (Chen et al. 2011). AtRopGEF7 is induced transcriptionally by auxin, while its function is required for the maintenance of normal auxin levels in seedling roots, suggesting that RopGEF7 may integrate auxin-derived positional information (Chen et al. 2011). Even though, we were not able to detect differential expression of auxin-related genes in the CT genotype, what could explain the higher expression of *auxin-independent growth promoter protein* in CT than CS (Supplementary Table 3).

Signaling-related genes were found more expressed in the CT than CS under cold condition, including *receptor-like protein kinase* and *Rapid Alkalinization Factor (RALFL21)* (Supplementary Table 3). Receptor-like kinases are a prominent class of surface receptors that regulate many aspects of the plant life cycle, including cell expansion (Yang et al. 2015). FERONIA (FER), a receptor-like kinase, is involved in brassinosteroids and auxin signaling pathways, which have positive roles in cell growth and development. Such activation occurs through the interaction with a Rapid Alkalinization Factor (RALF1) peptide, triggering a series of downstream events related with plant growth and stress response (Li et al. 2018).

Other genes with higher expression in CT than CS under cold condition are not related with over-represented categories but will be discussed here based on their likely relation with low temperature response/tolerance, according to the literature: *glutathione peroxidase* and *metallothionein* (antioxidant system), *fatty acid desaturase* and *phosphatidylinositol transfer protein* (lipid metabolism), *Tetratricopeptide Repeat-Containing Protein (TTL1)* (stress response). Glutathione peroxidases are thiol

498 peroxidases that catalyze the reduction of H₂O₂ to water, being induced in response to
 499 cold stress. Passaia et al. (2013) showed that silencing the cold-inducible *OsGPX3* gene
 500 impairs normal plant development and leads to a stress-induced morphogenic response
 501 via H₂O₂ accumulation, which leads to smaller roots. Such response is similar to what
 502 we found in the CS genotype under low temperature stress (reduced root growth and
 503 H₂O₂ accumulation – Figures 1 and 2). Recently, Hsu and Hsu (2019) tested eight rice
 504 cultivars under cold treatment and found that the cultivars with higher growth rate had
 505 lower levels of H₂O₂ in the roots when compared with the low growth rate cultivars.
 506 Metallothioneins, cysteine-rich proteins ubiquitous in eukaryotic organisms, are known
 507 to be involved in developmental processes and stress response, including low
 508 temperature in *Cicer microphyllum*, a wild relative of cultivated chickpea (Singh et al.
 509 2011). Polyunsaturated fatty acids (PUFAs) play an important role in cold tolerance,
 510 since membrane fluidity is important to sustain the functional activity of membrane
 511 proteins and the membranes themselves (Beney et al. 2001). Recently, Wang et al.
 512 (2019) over-expressed an ω-3 fatty acid desaturase from *Glycine max* (*GmFAD3A*) in
 513 rice, obtaining a significant improvement in cold tolerance and survival ratio. Also, rice
 514 mutants deficient in ω-3 fatty acid desaturase (*OsFAD8*) fail to acclimate to low
 515 temperature stress (Tovuu et al. 2016). It is well known that root hair growth requires
 516 extensive cell wall modification. *OsSEC14-NODULIN DOMAIN-CONTAINING*
 517 *PROTEIN 1* (*OsSNDP1*) gene, encoding a phosphatidylinositol transfer protein (PITP),
 518 promotes root hair elongation via phospholipid signaling and metabolism, suggesting
 519 that the mediation of these processes by PITP is required for root hair elongation in rice
 520 (Huang et al. 2013). Mutations in the Arabidopsis *Tetratricopeptide-Repeat*
 521 *Thioredoxin-like 1* (*TTL1* - At1g53300) gene cause reduced tolerance to NaCl and
 522 osmotic stress that is characterized by reduced root elongation (Rosado et al. 2006).
 523 Therefore, we hypothesize that the *TTL1* gene found in our work could be the rice
 524 functional ortholog of Arabidopsis *TTL1*, functioning in cold stress response and
 525 contributing to the cold tolerance and maintenance of root growth under cold condition
 526 presented by the CT genotype (Figure 1).

527 On the other hand, under cold condition the CS genotype showed higher
 528 expression of genes related to several categories: amino acid metabolism, antioxidant
 529 system, calcium-related, defense, DNA structure maintenance, hormone-related, protein
 530 degradation/modification, transcription factor, translation, transport, and unknown

(Table 1). For this genotype, only the genes that based on the literature are involved with cold sensitivity will be discussed. Among the defense-related genes, we found *SRC2* gene (Supplementary Table 3). In Arabidopsis, low temperature treatment induces expression of *AtSRC2* gene in roots, which binds to *AtRbohF* to enhance the Ca^{2+} -dependent ROS production (Kawarazaki et al. 2013). Therefore, we hypothesize that the cold-inducible protein *SRC2* found in CS genotype can function in cold response by controlling the Ca^{2+} -dependent ROS production, which agrees with the ROS accumulation seen in this genotype under cold condition (Figure 2). The CS genotype presented higher expression of only one gene related with growth, *OsRAAI* (*ROOT ARCHITECTURE ASSOCIATED1* – Supplementary Table 3), a GTP-binding protein. Overexpression of *OsRAAI* promotes abnormal cell division (blocked transition from metaphase to anaphase during mitosis) and inhibits the growth of primary roots in rice (Xu et al. 2010), what could explain, at least partially, the reduced root growth presented by this genotype under cold condition (Figure 1). As seen under control condition, the CS genotype presented higher expression of ethylene-related genes than CT, including *1-aminocyclopropane-1-carboxylate oxidase* and *ethylene-responsive transcription factor* (Supplementary Table 3). As previously discussed, high levels of ethylene can inhibit root growth (Qin and Huang, 2018), and therefore we believe the root growth inhibition seen on CS genotype under cold condition (Figure 1) can be related with an increased ethylene level. A cytokinin-related gene (*cytokinin-O-glucosyltransferase 2*) was also found more expressed in CS than CT. It is already known that decreased cytokinin levels in roots results in increased root growth and higher drought tolerance in barley (Ramireddy et al. 2018). Therefore, higher cytokinin levels in CS than CT under cold condition probably contributed to the reduced root growth.

Concluding remarks

The genetic improvement of root system has been regarded as an important approach to enhance crop production (Kitomi et al. 2018). Here we present a list of differential expressed genes that might be involved with cold tolerance and sensibility in rice plants. The model in Figure 7 summarizes the molecular and physiological responses of CT and CS genotypes under control and cold conditions. According to our data, high

expression of genes related with cell wall remodeling (*glycosyl hydrolase, cellulose synthase, glycosyl transferase, wall-associated kinase, glycine-rich cell wall structural protein*), cytoskeleton and growth (*microtubule-associated protein 70, kinesin motor domain containing protein, growth regulating factor protein, auxin-independent growth promoter protein, RopGEF7*), signaling (*receptor-like protein kinase, Rapid Alkalinization Factor 21*)), antioxidant system (*glutathione peroxidase, metallothionein*), lipid metabolism (*fatty acid desaturase and phosphatidylinositol transfer protein*), and stress response (*Tetratricopeptide Repeat-Containing Protein*) might contribute to rice cold tolerance. On the other hand, high expression of the genes *SRC2* (defense), *root architecture associated 1* (growth), *ACC oxidase*, *ethylene-responsive transcription factor*, and *cytokinin-O-glucosyltransferase 2* (hormone-related) seems to be related with cold sensibility. Understanding more about the processes taking place in roots of plants subjected to cold stress can therefore not only provide a clearer picture of this complex phenomenon, but also disclose important information that can be used to develop engineering strategies aimed at improving plant tolerance to low temperature stress.

579

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838

839 **Figure legends**

840 **Figure 1.** Root dry weight, length, and hair density of thirty-day old cold-tolerant and
841 cold-sensitive plants under control (28°C) or cold (10°C) condition. Represented values
842 are the averages of sixteen samples $\pm$ SE. Mean values with one, two or three asterisks
843 are different by Student's *t* test (*p*-value $\leq$ 0.05, 0.01 or 0.001, respectively).

844 **Figure 2.** Root histochemical analysis. Lipid peroxidation (a), loss of plasma membrane
845 integrity (b), and H₂O₂ accumulation (c) by Schiff, Evans Blue, and DAB reagents,

846 respectively, in thirty-day old roots of cold-tolerant (CT) and cold-sensitive (CS) plants
847 after ten (a) or three days (b and c) under cold (10°C) condition. The positive staining
848 (detected in higher levels in cold-sensitive roots) in the photomicrographs shows as
849 bright images (pink-color for Schiff, blue-color for Evans Blue, and brown-color for
850 DAB). Bars in figures indicate 1.0 cm.

851 **Figure 3.** Differentially expressed sequences and similarity between libraries. Number
852 of differentially expressed sequences in different tested conditions (a). Blue bars:
853 enriched in cold-tolerant (CT) plants; Red bars: enriched in cold-sensitive (CS) plants.
854 Venn diagram showing the number of different expressed sequences (specific to each
855 group or common to both analyzed groups) in different conditions (b). Numbers in blue
856 color: enriched in CT plants; numbers in red color: enriched in CS plants; number in
857 black color: number of genes with opposite expression pattern on the two genotypes.
858 Tol = tolerant; Sen = sensitive.

859 **Figure 4.** Overview of the MapMan visualization of differences in transcript levels
860 between root transcriptomes from cold-tolerant and cold-sensitive rice genotypes under
861 control (a) and cold (b) conditions. Genes associated with metabolic pathways were
862 analyzed by the MapMan software (<http://mapman.gabipd.org/web/guest/mapman>).

863 **Figure 5.** Cellulose staining (a) and root diameter (b) of thirty-day old cold-tolerant
864 (CT) and cold-sensitive (CS) plants under control (28°C) and/or cold (10°C) condition
865 for 72 h. Represented values are the averages of sixteen samples $\pm$ SE. Mean values
866 with two asterisks are different by Student's *t* test (p -value ≤ 0.01).

867 **Figure 6.** Relative expression levels (RT-qPCR, relative to *OsUBQ5* expression) of
868 selected genes identified by RNAseq in roots of rice plants (CT: cold-tolerant; CS: cold-
869 sensitive) submitted to control or cold conditions: (a) LOC_Os04g02490 (*Expressed*
870 *protein*), (b) LOC_Os04g01330 (*Expressed protein*), (c) LOC_Os07g26100 (*Expressed*
871 *protein*), (d) LOC_Os11g09850 (*Expressed protein*), (e) LOC_Os07g12540 (*Expressed*
872 *protein*), (f) LOC_Os07g20164 (*Expressed protein*), (g) LOC_Os04g02110 (*Disease*
873 *resistance protein RGA3*), (h) LOC_Os07g15440 (*Alanyl-tRNA synthetase family*
874 *protein*), (i) LOC_Os09g25934 (*Expressed protein*), and (j) LOC_Os09g26554
875 (*Expressed protein*). Values are the averages of three samples $\pm$ SE. Mean values with
876 one or two asterisks are different by Student's *t* test ($p \leq 0.05$ and 0.01, respectively).
877 ND = not detected.

878 **Figure 7.** Gene expression profile and physiological responses of cold tolerant (CT) and
879 cold sensitive (CS) genotypes under control (a) and cold (b) conditions.

880

881 **Supplementary Figure 1.** Gene Ontology (GO) analysis of differentially expressed
882 sequences from root transcriptomes of cold-tolerant (Tol) and cold-sensitive (Sen)
883 genotypes under cold and control conditions through PlantGSEA
884 (<http://structuralbiology.cau.edu.cn/PlantGSEA/analysis.php>). Terms enriched in either
885 tolerant (black bars) or sensitive (white bars) datasets are shown as percentage of
886 annotated genes in the dataset. All GO terms shown are differentially enriched in either
887 group using Fisher's Exact Test ($p \leq 0.05$).

888 **Supplementary Table 1.** Gene-specific PCR primers used for RT-qPCR.

889 **Supplementary Table 2.** Differentially expressed sequences revealed by RNAseq in
890 roots of cold-tolerant (Tol) and cold-sensitive (Sen) rice genotypes exposed to control
891 (28°C) treatment for 24 h. Differential expression of the sequences marked with an
892 asterisk were confirmed by RT-qPCR.

893 **Supplementary Table 3.** Differentially expressed sequences revealed by RNAseq in
894 roots of cold-tolerant (Tol) and cold-sensitive (Sen) rice genotypes exposed to cold
895 (10°C) treatment for 24 h. Differential expression of the sequences marked with an
896 asterisk were confirmed by RT-qPCR.

897 **Supplementary Table 4.** Common genes present in the comparisons of cDNA libraries
898 from roots of rice plants. Sequences more expressed in the cold-tolerant (CT) or cold-
899 sensitive (CS) genotypes regardless the tested condition (control or cold treatment).
900 Sequences marked in gray color presented opposite expression pattern in the CT and CS
901 genotypes.

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Table 1. Schematic representation of the functional categories differently represented in roots of cold-tolerant (CT) or cold-sensitive (CS) rice plants under control and cold conditions. Different shades of gray color represent the four different patterns of response. Number of arrows (one or two) relates to the representation level, which is based on the number of differentially expressed sequences and gene expression level.

Functional categories	Control condition		Cold condition	
	CT	CS	CT	CS
Amino acid metabolism	-	-	↑	↑↑
Antioxidant system	↑↑	↑	↑	↑↑
Calcium-related	-	-	↑	↑↑
Carbohydrate metabolism/energy production	↑	-	↑	↑
Cell wall	↑↑*	↑	↑↑	↑
Cytoskeleton	-	-	↑	-
Defense	↑	↑↑	↑	↑↑
DNA structure maintenance	-	-	-	↑
Growth	-	-	↑↑	↑
Hormone-related	↑	↑↑	↑	↑↑
Lipid metabolism	↑	-	↑	↑
Nucleotide metabolism	-	-	↑	↑
Others	↑↑	↑	↑	↑
Photosynthesis	-	-	-	↑
Protein degradation/modification	↑	↑	↑	↑↑
Secondary metabolism	↑	↑↑	↑	↑
Signaling	↑↑	↑	↑↑	↑
Stress response	↑	-	↑	↑
Transcription factor	↑	↑↑	↑	↑↑
Translation-related	-	-	↑	↑↑
Transport	-	↑	↑	↑↑
Unknown	↑	↑	↑	↑↑

(-): functional category not detected as differentially represented.

(*): we considered more represented in CT than CS due to the high expression level.

● Cold-tolerant

■ Cold-sensitive

Control (28°C)

Cold (10°C)

Control (28°C)

Cold (10°C)

Control (28°C)

Cold (10°C)

(a) Lipid peroxidation

(b) Integrity of plasma membrane

(c) H_2O_2 accumulation

(a)

Differentially expressed genes

(b)

(a) Control condition

(b) Cold condition

↑ root dry weight

CT

- ↑ *OsIAA2* (auxin-responsive gene)
- ↑ *glycosyl hydrolase* (cell wall-related gene)
- ↑ *phospholipase D* (regulation of root growth)
- ↑ *phytosulfokine receptor* (receptor of a growth factor)
- ↑ *wali7* (root hair development)

↓ root dry weight

CS

- ↑ *2OG-Fe oxygenase* (ethylene biosynthesis)
 - ↑ *brassinosteroid LRR receptor kinase* (brassinosteroid perception)
 - ↑ *ent-kaurene synthase* (gibberellin biosynthesis)
 - ↑ *cycloartenol synthase*
 - ↑ *phytoene synthase*
 - ↑ *terpene synthase*
- } ethylene-related genes
- } terpene biosynthesis

(a) Control condition

- ↑ root dry weight
- ↑ root length
- ↑ root hair density
- ↑ root diameter
- ↑ cellulose deposition
- ↓ lipid peroxidation
- ↓ H_2O_2 accumulation
- ↑ integrity of plasma membrane

CT

- ↑ *glycosyl hydrolase*
 - ↑ *cellulose synthase*
 - ↑ *glycosyl transferase*
 - ↑ *wall-associated kinase (WAK)*
 - ↑ *glycine-rich cell wall structural protein*
 - ↑ *microtubule-associated protein 70 (MAP70)*
 - ↑ *kinesin motor domain containing protein*
 - ↑ *growth regulating factor protein*
 - ↑ *auxin-independent growth promoter protein*
 - ↑ *RopGEF7*
 - ↑ *receptor-like protein kinase*
 - ↑ *Rapid Alkalinization Factor (RALFL21)*
 - ↑ *glutathione peroxidase* (antioxidant system)
 - ↑ *metallothionein* (antioxidant system)
 - ↑ *fatty acid desaturase* (membrane fluidity - cold tolerance)
 - ↑ *phosphatidylinositol transfer protein* (lipid metabolism - root hair elongation?)
 - ↑ *Tetratricopeptide Repeat-Containing Protein (TTL1)* (stress response)
- } formation and remodeling of plant cell wall
- } cytoskeleton remodeling
- } growth
- } signaling

- ↓ root dry weight
- ↓ root length
- ↓ root hair density
- ↓ root diameter
- ↓ cellulose deposition
- ↑ lipid peroxidation
- ↑ H_2O_2 accumulation
- ↓ integrity of plasma membrane

CS

- ↑ *SRC2* (defense-related gene)
 - ↑ *OsRAA1 (ROOT ARCHITECTURE ASSOCIATED1)* (inhibition of root growth)
 - ↑ *1-aminocyclopropane-1-carboxylate oxidase*
 - ↑ *ethylene-responsive transcription factor*
 - ↑ *cytokinin-O-glucosyltransferase 2* (cytokinin-related gene - inhibition of root growth?)
- } ethylene-related genes

(b) Cold condition