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Meta-analysis of transcriptomic studies of cytokinin-treated rice roots defines a core set of cytokinin response genes

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SUMMARY

Cytokinins regulate diverse aspects of plant growth and development, primarily through modulation of gene expression. The cytokinin-responsive transcriptome has been thoroughly described in dicots, especially Arabidopsis, but much less so in monocots. Here, we present a meta-analysis of five different transcriptomic analyses of rice (*Oryza sativa*) roots treated with cytokinin, including three previously unpublished experiments. We developed a treatment method in which hormone is added to the media of rice seedlings grown in sterile hydroponic culture under a continuous airflow, which resulted in minimal perturbation of the seedlings, thus greatly reducing changes in gene expression in the absence of exogenous hormone. We defined a core set of 205 upregulated and 86 downregulated genes that were differentially expressed in at least three of the transcriptomic datasets. This core set includes genes encoding the type-A response regulators (RRs) and cytokinin oxidases/dehydrogenases, which have been shown to be primary cytokinin response genes. GO analysis revealed that the upregulated genes were enriched for terms related to cytokinin/hormone signaling and metabolism, while the downregulated genes were significantly enriched for genes encoding transporters. Variations of type-B RR binding motifs were significantly enriched in the promoters of the upregulated genes, as were binding sites for other potential partner transcription factors. The promoters of the downregulated genes were generally enriched for distinct *cis*-acting motifs and did not include the type-B RR binding motif. This analysis provides insight into the molecular mechanisms underlying cytokinin action in a monocot and provides a useful foundation for future studies of this hormone in rice and other cereals.

Keywords: cytokinin, plant hormones, rice, transcriptomics, signaling.

INTRODUCTION

Cytokinins are N^6-substituted adenine derivatives that regulate diverse aspects of plant growth and development, including meristem function, leaf senescence, vascular development, gynoecium development, and biotic and abiotic interactions (Davies, 2004; Jameson and Song, 2016; Kieber and Schaller, 2014, 2018; Mok and Mok, 2001; Schaller *et al.*, 2015). Much has been learned in the past few decades regarding cytokinin metabolism, signaling, and function, primarily from studies of the dicot *Arabidopsis thaliana*, with additional substantial contributions from studies in rice (*Oryza sativa*) and maize (*Zea mays*). The biosynthesis of cytokinin starts with the addition of a prenyl group derived from dimethylallyl diphosphate to the N^6 position of ADP/ATP, catalyzed by isopentenyltransferases (IPTs) (Sakakibara, 2006), which are encoded by a multigene family in most plants (Kakimoto, 2001; Takei *et al.*, 2001). The iP ribotides made by IPT are subsequently converted to *trans*-zeatin (*t*Z)-type cytokinins by hydroxylation of the isoprenoid side chain by the cytochrome P450 enzymes CYP735A1 and CYP735A2 (Takei *et al.*, 2004). The active forms of cytokinins are the free bases, which are synthesized from cytokinin ribotides in a single enzymatic step catalyzed by the LONELY GUY (LOG) family of cytokinin nucleoside 5′ monophosphate phosphoribohydrolases, which were initially discovered in rice (Kurakawa *et al.*, 2007; Kuroha *et al.*, 2009). The level of active cytokinin can be decreased through conjugation to sugars (Bajguz and Piotrowska, 2009; Hoyerová and Hošek, 2020; Sakakibara, 2006) or by degradation by cytokinin oxidases/dehydrogenases (CKXs) (Schmülling *et al.*, 2003; Werner *et al.*, 2006).

The cytokinin signaling pathway is similar to bacterial two-component phosphorelays and is comprised of sensor

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histidine kinase (HK) receptors, histidine phosphotransfer proteins (AHPs), and response regulators (RRs) (Figure 1). The cytokinin signaling elements in rice and other plants are encoded by small gene families whose members generally have overlapping functions (Burr *et al.*, 2020; Du *et al.*, 2007; Pareek *et al.*, 2006; Pils and Heyl, 2009; Tsai *et al.*, 2012; Worthen *et al.*, 2019). Cytokinin HK receptors mostly localize to the ER membrane, with the cytokinin-binding CHASE domain oriented into the lumen (Caesar *et al.*, 2011; Lomin *et al.*, 2011; Romanov *et al.*, 2018; Wulfetange *et al.*, 2011), though recent evidence suggests that a portion of the receptors may also be located at the plasma membrane (Antoniadi *et al.*, 2020; Kubiasová *et al.*, 2020; Zürcher *et al.*, 2016). There are four CHASE domain-containing HKs in rice, and disruption of two of these (*HK5* and *HK6*) reduces the response to cytokinin and has substantial effects on many aspects of rice growth and development (Burr *et al.*, 2020). The AHPs shuttle between the cytoplasm and the nucleus and act as intermediates in the transfer of phosphate from the HKs to the downstream RRs (Hutchison *et al.*, 2006; Suzuki *et al.*, 1998; Tanaka *et al.*, 2004). The RRs, which fall into two major classes called types A and B, contain an N-terminal receiver domain that harbors an Asp residue that is the target of phosphotransfer. Type-B RRs mediate the transcriptional response to cytokinin and contain, in addition to the receiver domain, a DNA-binding MYB domain. Based on studies in Arabidopsis, phosphorylation of the type-B RRs increases binding to their genomic targets, thus mediating the initial transcriptional response to cytokinin (Xie *et al.*, 2018; Zubo *et al.*, 2017). Type-A RRs lack DNA binding output domains, are rapidly and specifically transcriptionally induced by cytokinin, and function as negative feedback regulators in cytokinin signaling (Brandstatter and Kieber, 1998; D'Agostino *et al.*, 2000; Jain *et al.*, 2006; To *et al.*, 2004). In addition to the type-A RRs, cytokinin signaling is also inhibited by the pseudo-phosphotransfer proteins (PHPs), in which the conserved His at the phospho-receiving site is replaced with an Asn or Gln residue (Mähönen *et al.*, 2006; Suzuki *et al.*, 2000; Vaughan-Hirsch *et al.*, 2020), and by *S*-nitrosylation of the AHPs (Feng *et al.*, 2013). There are three PHPs in rice with overlapping functions, some of which are distinct from the role of AHP6, the sole PHP in Arabidopsis (Vaughan-Hirsch *et al.*, 2020).

Here, we examined the transcriptional response of rice roots to exogenous cytokinin. We performed a meta-analysis of five distinct transcriptomic studies to define a core set of cytokinin-responsive genes in a monocot species. The core set consists of 205 upregulated and 86 downregulated genes and contains both previously described and novel cytokinin response genes. This core dataset defines molecular targets of cytokinin signaling in rice and should be useful in the analysis of cytokinin in rice and other monocots.

RESULTS

Treatment methods for phytohormone application can substantially affect the rice transcriptome in the absence of hormone

Transcriptional profiling of phytohormone-treated plants has provided valuable insights into the mechanisms by which these signaling molecules regulate plant growth and development. Various methods have been employed to treat plant tissues with exogenous hormones, including immersing seedlings or excised tissues in media with or without added hormone, transferring seedlings grown on sterile solid media to media containing hormone, and spraying intact or various plant tissues with the hormone of interest. All of these methods have potential drawbacks and likely impact gene expression. For example, removing seedlings or plant organs followed by immersion in hormone-containing media can lead to flooding and hypoxic stress. Furthermore, sterile growth of seedlings on solid media in an enclosed space (i.e., Petri plate), though

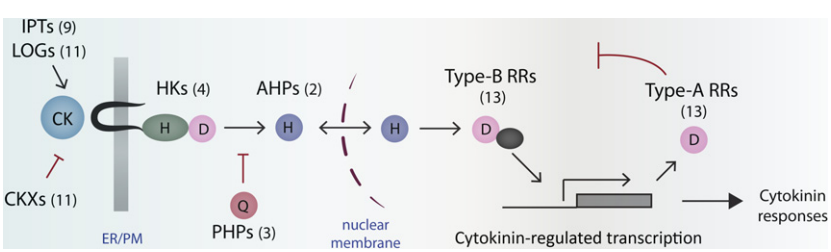

Figure 1. A diagram of the cytokinin signaling pathway in rice. Cytokinin binds to the sensor histidine kinase (HK) receptors, which are localized to the endoplasmic reticulum (ER) and the plasma membrane (PM). This induces autophosphorylation on a His residue, and the phosphate group is then relayed to the response regulators (RRs) through the AHP proteins. The Ds and As indicated in the various domains represent the sites of phosphorylation. Type-B RRs elicit the cytokinin responses by direct binding to target genes, whereas type-A RRs lack the DNA binding domain and act as negative feedback regulators. Additionally, cytokinin signaling is inhibited by the pseudo-phosphotransfer proteins (PHPs), in which the target His is replaced with a Gln in rice PHPs. The LOG/L and IPTs play a role in cytokinin biosynthesis, and the CKXs act to degrade cytokinin. The numbers in brackets indicate the numbers of genes encoded for each element in the rice genome.

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convenient and reproducible, results in reduced transpiration, which can skew the distribution of endogenous hormones, including cytokinin, which is transported shootward via the transpiration stream in the xylem. A recent study has shown that growing Arabidopsis plants on parafilm-wrapped Petri dishes caused hypoxia, resulted in higher carbon dioxide concentrations, and led to differential regulation of multiple stress- and defense-related genes (Xu *et al.*, 2019).

We sought to develop a method to grow and treat rice plants in sterile culture with transpiration intact and with minimal perturbation to the seedlings. As some cytokinin is synthesized in roots and transported to the shoots via the transpiration stream (Durán-Medina *et al.*, 2017; Kudo *et al.*, 2010; Osugi *et al.*, 2017), transpiration is likely important to maintain a normal endogenous level and distribution of cytokinin. Further, lowland rice, which includes the Kitaake variety used here, normally grows with its roots flooded, and thus growth in hydroponic media can more closely approximate a natural situation, as opposed to many plants, including Arabidopsis, in which growth submerged in hydroponic media likely induces flooding stress. To promote transpiration, sterile rice seedlings were grown in hydroponic media in a chamber through which a sterile airstream was maintained prior to and during treatment. This experimental design promoted transpiration of rice seedlings in sterile conditions and enabled facile treatment of roots with phytohormone.

To examine the effect of the treatment method on the transcriptome, we designed two experiments that differed in the manner of treating transpiring rice plants with cytokinin. In the first experiment (HM.1), we grew rice seedlings in sterile hydroponic culture, and on the day of treatment we replaced the media with fresh media identical to the original supplemented either with NaOH (vehicle control) or with cytokinin (6-benzylaminopurine [BA], C_f = 5 μM). The second experiment (HM.2) was conducted in a similar manner, but rather than changing the media, cytokinin or the control was added to the existing media. We harvested the entire rice root system from the seedlings at the start (0 h) of the experiment and after 2 h of treatment and performed RNA sequencing (RNA-seq) analysis. The transcriptome profiling revealed 255 differentially expressed genes (DEGs) ($|\log_2$(fold change [FC])$| \geq 0.7$; false discovery rate [FDR] < 0.05) in the HM.1 experiment (168 up- and 87 downregulated) after 2 h of cytokinin treatment as compared to the vehicle control (Figure 2). In the HM.2 experiment, we identified 473 DEGs (284 up- and 189 downregulated) after 2 h of treatment. We hypothesized that the lower number of DEGs in the HM.1 experiment is perhaps a result of noise that occurred due to medium replacement. We therefore examined gene expression changes over the 2-h time course and found a surprisingly large number (1170) of DEGs in the control treatment in

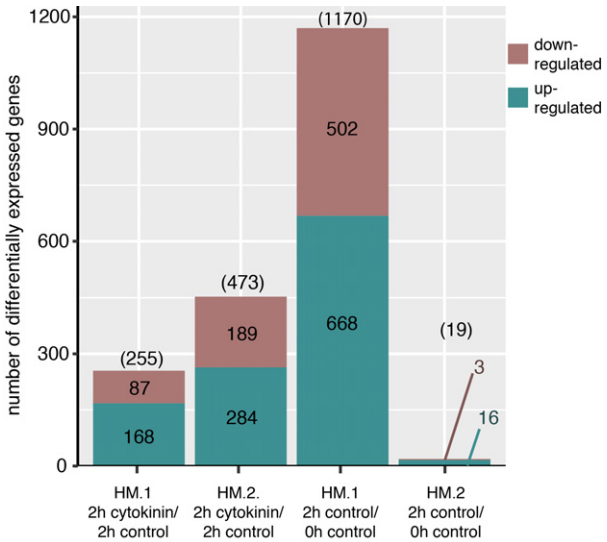

Figure 2. The number of up- and downregulated genes in the two hydroponic medium experiments, HM.1 and HM.2. The differentially expressed genes were identified between cytokinin- and control-treated roots and over the 2-h control treatment (2 h versus 0 h) ($|\log_2$FC$| \geq 0.7$, FDR < 0.05).

this experiment (Figure 2), even though the replaced medium was the same as the original medium used to grow the seedlings. Moreover, multidimensional scaling (MDS) analysis demonstrated a very small separation between the cytokinin and control treatments in HM.1, suggesting a greater impact of the media replacement on the transcriptome than the cytokinin treatment itself (Figure S2a). To test whether such a substantial number of DEGs are indeed a result of the treatment method, we examined transcriptome changes in the control treatment group in the HM.2 experiment. In this case, there were very few genes (16 up- and 3 downregulated) differentially expressed in the control conditions. Moreover, MDS analysis shows two distinct clusters, representing the control and cytokinin treatments (Figure S1b). Based on these results, we concluded that the minimal perturbation treatment used in the HM.2 experiment nearly eliminated transcriptional changes that occur due to the change of media and potentially increased the ability to detect hormone-related changes by reducing the noise in the system.

Comparative analysis of the cytokinin-induced transcriptional changes reveals a suite of core cytokinin response genes

To define a core set of cytokinin-regulated genes, we performed a comparative analysis of five differently conducted transcriptomic studies of cytokinin-treated rice roots, including four RNA-seq experiments (including the previously published study by (Raines *et al.*, 2016a) and one Affymetrix GeneChip® microarray experiment (Hirose

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et al., 2007)) (Table 1). The RNA-seq studies included the HM.1 and HM.2 experiments, an additional new study in which rice seedlings were grown in sterile conditions on solid agar medium (AM) and subsequently removed and treated with cytokinin in liquid media in Petri dishes while shaking. It is noteworthy that the 'Hirose' and 'Raines' experiments differed in several important aspects from the other three analyses. In the 'Hirose' study, the rice plants were grown in pH-adjusted water rather than nutrient media, and the treatment utilized *trans*-zeatin rather than the BA that was used in the other experiments. Moreover, in both the 'Raines' and 'Hirose' experiments, seedlings were grown in non-sterile hydroponic conditions, which likely substantially impacts gene expression and the response to hormones. To minimize differences resulting from data analyses, the raw data from the four RNA-seq experiments (including the previously published 'Raines' dataset) were analyzed (or re-analyzed) using the EdgeR package in R (Nikolayeva and Robinson, 2014; Robinson *et al.*, 2010) as described in the Experimental Procedures section.

To empirically establish the optimal $\log_2$FC cut-off for the DEG discovery, we examined the two most divergent RNA-seq experiments (HM.2 and 'Raines', as determined by hierarchical clustering) and the two most similar ones (HM.1 and HM.2) (Figure S2). Using the lowest applied $\log_2$FC cut-off ($|\log_2 FC| \geq 0.4$), the number of DEGs in the 'Raines' dataset was 6517, while there were only 473 DEGs in the HM.2 dataset, 391 of which were shared with 'Raines' (Figure S2a). We explored how more stringent cut-off values affected the shared versus unique DEGs in these two datasets, reasoning that the optimal cut-off would reduce the number of DEGs unique to each dataset, but would reduce the number of shared genes, which in general are more likely to be authentic, to a much lesser extent. Balancing the reduction in unique DEGs versus common DEGs between these datasets, we settled on a cut-off value of $|\log_2 FC| \geq 0.7$ (Figure S2a). In the more similar HM.1 and HM.2 datasets, the $|\log_2 FC| \geq 0.7$ cut-off value did not affect either shared or common DEGs as compared to lower cut-off values, and indeed these numbers were only nominally affected at even more stringent values (Figure S2b). We thus used a cut-off value of $|\log_2 FC| \geq 0.7$ for all four RNA-seq analyses and used the original published parameters for the 'Hirose' microarray dataset.

Using these parameters, we found that the 'Raines' dataset contained the highest number of DEGs (4075) and HM.1 had the smallest number of DEGs (255) (Table 2). We defined a core suite of genes involved in mediating cytokinin responses in roots as those identified as differentially expressed in a majority (three out of five) of the experiments. There was a relatively small number of DEGs shared by all experiments (eight up- and seven downregulated DEGs), with more genes shared by four out of five

Table 1 Summary of the experiments

	Plants/growth conditions				Treatment				
Name	Cultivar	Age	Medium	Day length	Cytokinin	Concentration, duration of treatment	Delivery method	Laboratory PI	Publication
Hirose	Nipponbare	14 days	Hydroponics, water	12 h	*trans*-Zeatin	5 μM; 2 h	Excised, immersed in water	Sakakibara, H.	Hirose *et al.* (2007)
Raines	Kitaake	10 days	Hydroponics, Kimura B	12 h	6-Benzylaminopurine	5 μM; 2 h	Excised, immersed in new media	Kieber, J.J.	Raines *et al.* (2016)
Agar medium (AM)	Nipponbare	10 days	Phytagel, Kimura B	14 h	6-Benzylaminopurine	5 μM; 2 h	Excised, immersed in new media	Kieber, J.J.	This work
Hydroponic medium (HM.1)	Kitaake	8 days	Hydroponics, Kimura B	14 h	6-Benzylaminopurine	5 μM; 2 h	Medium change	Kieber, J.J.	This work
Hydroponic medium (HM.2)	Kitaake	8 days	Hydroponics, Kimura B	14 h	6-Benzylaminopurine	5 μM; 2 h	Added to media	Kieber, J.J.	This work

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Table 2 Number of differentially regulated genes in discussed experiments ($|\log_2 FC| \geq 0.7$; FDR ≤ 0.05, except for 'Hirose')

Name	Upregulated	Downregulated	Total
Hirose (H)	611	737	1348
Raines (R)	2438	1637	4075
Agar medium (AM)	686	538	1224
Hydroponic medium 1 (HM.1)	168	87	255
Hydroponic medium 2 (HM.2)	284	189	473

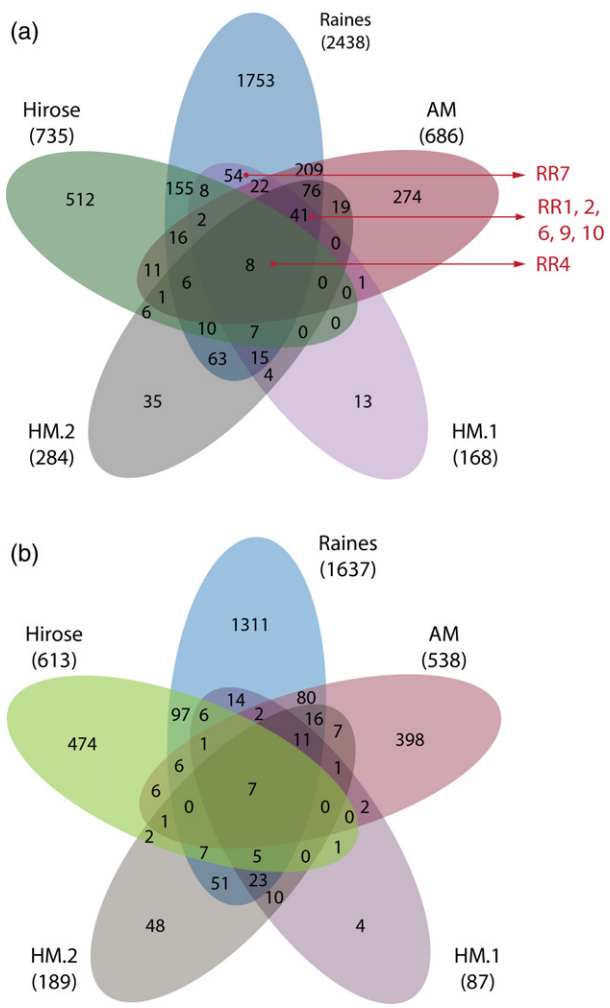

Figure 3. Identification of the core cytokinin response genes in rice roots. The Venn diagrams show overlaps between (a) the upregulated and (b) the downregulated genes from the five datasets discussed in this work ($|\log_2 FC| \geq 0.7$ [except for Hirose], FDR < 0.05).

experiments (57 up- and 26 downregulated DEGs) (Figure 3, Table S1). The core set of genes that were shared by at least three datasets consisted of 205 up- and 86 downregulated genes, representing a high-confidence core set of cytokinin-regulated genes in rice roots.

We queried this core set of cytokinin-regulated genes for overrepresented gene ontology (GO) terms using the g:GOst function on the g:Profiler webserver (Raudvere *et al.*, 2019). The analysis revealed a number of significant GO terms in the molecular function (MF), biological process (BP), cellular component (CC), and Kyoto Encyclopedia of Genes and Genomes (KEGG) categories (Figure 4a,b, Table S2). As expected, for the upregulated genes, among the top GO terms within the MF category was 'cytokinin dehydrogenase', which was previously found to be enriched among cytokinin-induced genes in Arabidopsis and suggests a feedback mechanism between cytokinin signaling and its metabolism (Bhargava *et al.*, 2013; Brenner *et al.*, 2005; Rashotte *et al.*, 2003). The next most overrepresented terms were 'heme' and 'tetrapyrrole binding', perhaps related to the activation of enzymes such as cytochrome P450, which participates in the synthesis of *trans*-zeatin and requires heme as a cofactor (Li *et al.*, 2008; Takei *et al.*, 2004). Alternatively, since 'oxidoreductase activity' emerged as another enriched GO term, the presence of heme and tetrapyrrole binding could indicate activation of stress signaling pathways and/or autophagy (Nagahatenna *et al.*, 2015; Shanmugabalaji *et al.*, 2020), the latter of which has recently been linked to cytokinin (Acheampong *et al.*, 2020). 'Adenine/purine phosphoribosyltransferase' (APT) activity was also overrepresented, which may reflect cytokinin metabolic processes (Zhang *et al.*, 2013). The BP category included terms such as 'cytokinin-activated signaling pathway', 'amine metabolic process', 'response to hormone', 'adenine salvage', and 'oxidation-reduction process'. The enriched terms in the KEGG category were 'zeatin biosynthesis' and 'plant hormone signal transduction', consistent with a response to the applied BA (Figure 4a).

The GO analysis of the downregulated genes revealed an enrichment of distinct terms, most notably several related to transmembrane transport (Figure 4b; Table S2). Previous studies have linked cytokinin to the acquisition of multiple nutrients, including iron (Fe), zinc (Zn), phosphorus (P), sulfur (S), potassium (K), and nitrogen (N) (Argueso *et al.*, 2009; Gao *et al.*, 2019; Kamada-Nobusada *et al.*, 2013; Kiba *et al.*, 2011; Pavlů *et al.*, 2018; Salama and Waering, 1979; Séguéla *et al.*, 2008). Together, these results confirm findings from previous studies regarding processes regulated by cytokinin in roots and indicate potential novel interactions.

Genes encoding proteins involved in cytokinin signaling and metabolism are overrepresented in the rice core list

We investigated the expression of the core set of genes across the five experiments using hierarchical clustering (Figure 5; Figure S3, Table S1). As expected for robustly regulated genes, the core DEGs are predominantly regulated in the same direction and rarely show opposing

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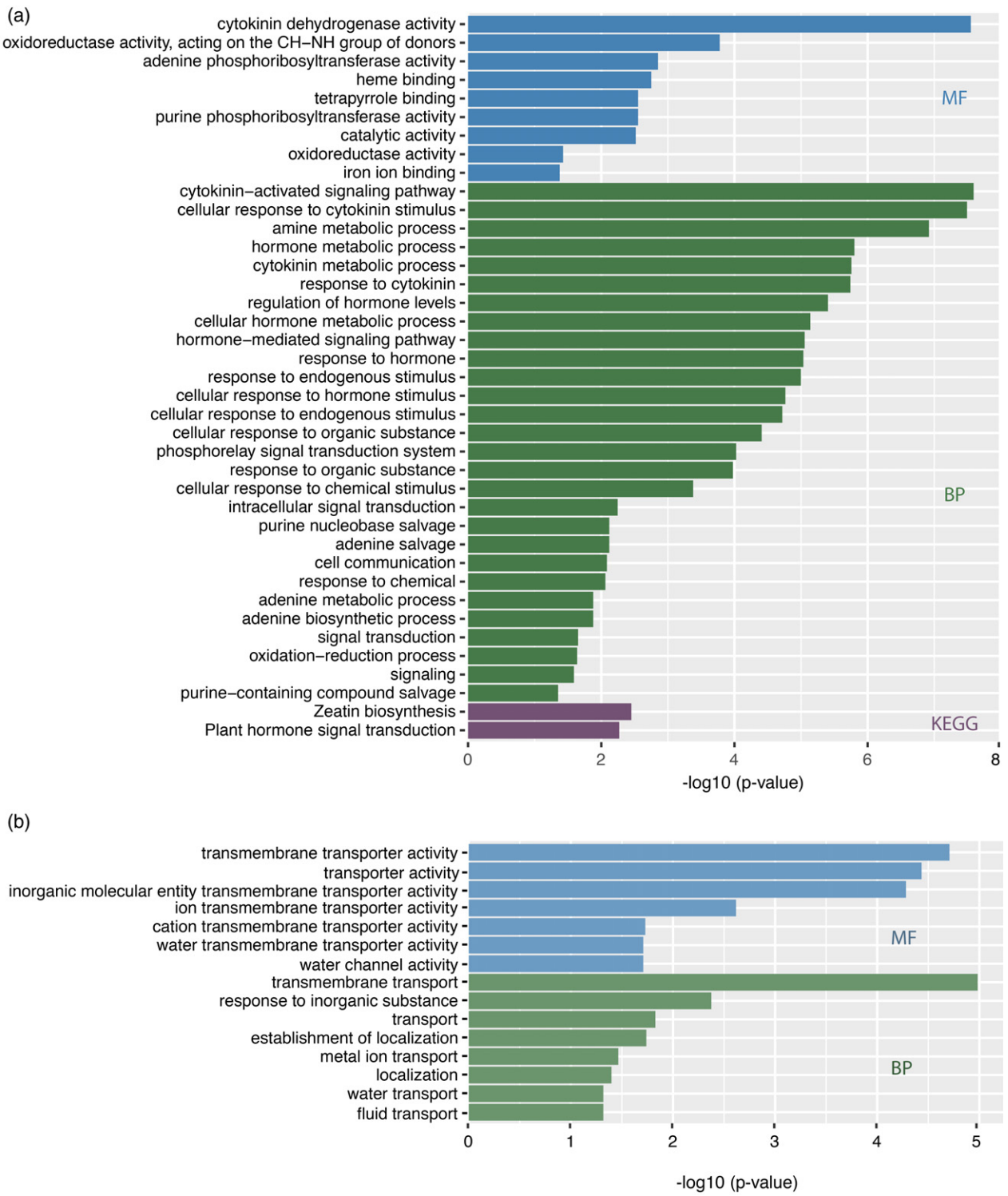

Figure 4. Gene ontology analysis. Overrepresented gene ontology terms in (a) the upregulated and (b) the downregulated genes from the core rice cytokinin set as determined by g:Profiler (version e102_eg49_p15_7a9b4d6). The g:SCS multiple testing correction method was used, applying a significance threshold of 0.05 (for the detailed g:Profiler results, see Table S2).

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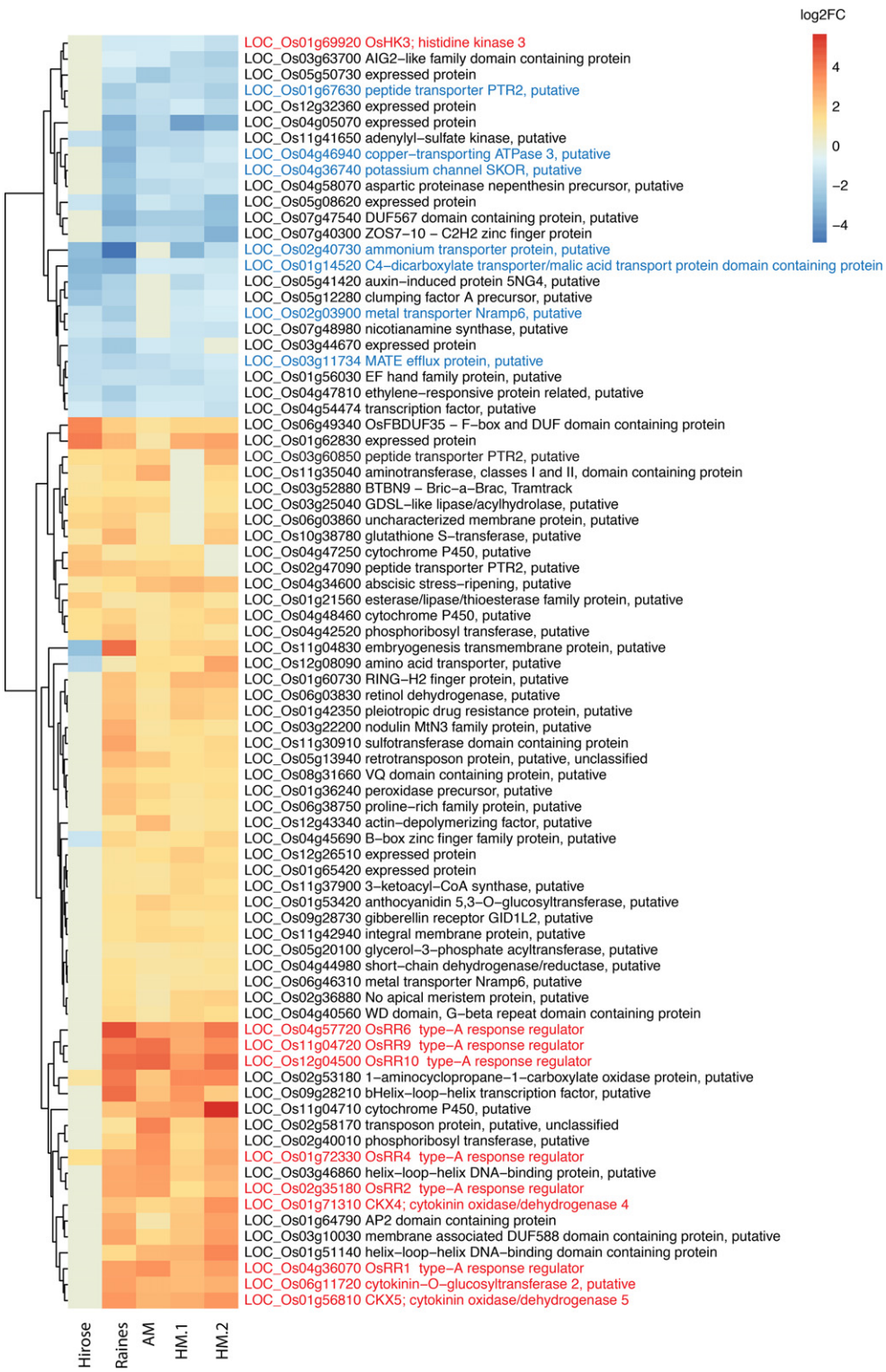

Figure 5. Heatmap of the differentially expressed genes shared by at least four datasets, which represents a subset of 86 genes from the 'core list' of 291 genes. Hierarchical clustering was done in R using the pheatmap package. The color scale represents the $\log_2$FC value. *CKX2* (LOC_Os01g10110) was excluded from the clustering analysis due to the high expression levels (Table S1). For the heatmap of all the 291 DEGs, see Figure S3. Genes related to cytokinin signaling/metabolism are highlighted in red. Genes encoding transporters are depicted in blue.

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dynamics across the five datasets. The exceptions generally come from the 'Hirose' microarray experiment, which may reflect differences in the experimental design (Table 1). We also observed two distinctive subclades that contained highly and moderately upregulated DEGs. The two upregulated clusters included most of the type-A *RRs* (*RR1*, *2*, *4*, *6*, *9*, and *10*), *CKX4* and *CKX5* (*CKX2* was excluded from the heatmap due to its extremely high log_2FC values), and *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID OXIDASE 3* (*ACO3*). The type-A gene *RR7* is differentially expressed only in the 'Raines' and HM.1 datasets and, therefore, does not meet the criteria of the core list (Figure 3a). *RR3* is not differentially expressed in any of the datasets and *RR5*, *8*, *11*, *12*, and *13* are generally not expressed at detectable levels or are present at very low levels, consistent with a previous analysis of the expression of these genes in response to cytokinin in rice roots using NanoString technology (Tsai *et al.*, 2012). In addition, the highly upregulated clusters contain a *CYTOCHROME P450* and three genes encoding unknown bHLH transcription factors (LOC_09g28210, LOC_Os03g46860, and LOC_Os0151140). Overall, the two most highly upregulated sets of genes were the *RRs* and *CKXs* (Figure 5, Figures S4 and S5). Some of the cytokinin response genes exhibit a high degree of variance, which likely reflects their relatively low expression in the absence of cytokinin (Figure 6, Figures S4 and S5). Surprisingly, the core list contains no WRKY genes despite these transcription factors being highly overrepresented in the 'Raines' dataset (Raines *et al.*, 2016a). This may reflect the non-sterile conditions in which the seedlings were grown for this experiment, as multiple *WRKY* genes have been linked to biotic interactions (Eulgem and Somssich, 2007; Ryu *et al.*, 2006). In agreement with the GO analysis, among the most highly downregulated genes, we found a number of transporters (Figure 5; highlighted in blue). In conclusion, the genes in the core list display a fairly similar expression pattern across the experiments and contain many previously described, well-characterized cytokinin response genes, as well as novel loci.

Ortholog and motif enrichment analysis

Bhargava *et al.* (2013) constructed the Arabidopsis 'Golden list' by performing a meta-analysis of 13 microarray experiments of cytokinin-treated Arabidopsis seedlings and incorporating genes that were >1.5-fold differentially regulated in 40% of the experiments. Some of the genes from the 'Golden list' were further validated through an RNA-seq experiment, targeted quantitative real-time PCR, and/or a NanoStrings nCounter system. The Arabidopsis 'Golden list' consists of 226 DEGs, many of which are related to the cytokinin response and metabolism, secondary metabolite synthesis, and regulation of the redox state of the cell (Bhargava *et al.*, 2013), similar to what we find with the rice core set. We sought to determine whether any of the core rice genes are shared with the Arabidopsis 'Golden list' (Table 3). Overall, we found a fairly modest overlap that, perhaps unsurprisingly, included the type-A *RRs*, *CKXs*, a *CYP450*, *ADENINE PHOSPHORIBOSYLTRANSFERASE 3* (*APT3*), and *CYTOKININ RESPONSE FACTOR 5* (*CRF5*). Additionally, we found several genes encoding transcription factors whose orthologs appear in the Arabidopsis list, including the multifunctional transcription factor TGACG SEQUENCE-SPECIFIC BINDING

Table 3 Orthologs of the core rice genes shared with the Arabidopsis 'Golden list' (Bhargava *et al.*, 2013)

Rice locus ID	Ortholog locus ID	Ortholog name	Description
LOC_Os01g09260	N/D	*CKX*	Cytokinin dehydrogenase
LOC_Os01g10110			
LOC_Os01g56810			
LOC_Os02g56310	AT3G04530	*PPCK2*	Phosphoenolpyruvate carboxylase kinase 2
LOC_Os03g04190	AT2G46660	*CYP78A6*	Cytochrome P450 78A6
LOC_Os03g42710	AT1G49450	*AT1G49450*	F13F211 protein
LOC_Os04g31290	AT2G28160	*FIT*	Transcription factor FER-LIKE IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR
LOC_Os04g44670	AT2G46310	*CRF5*	Ethylene-responsive transcription factor CRF5
LOC_Os04g45690	AT4G39070	*BBX20*	B-box zinc finger protein 20
LOC_Os04g49550	AT1G76410	*ATL8*	RING/U-box superfamily protein
LOC_Os04g54474	AT5G65210	*TGA1*	TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 1
LOC_Os04g57720	N/D	*ARR*	Type-A ARR
LOC_Os11g04720			
LOC_Os12g04500			
LOC_Os05g44210	AT1G78580	*TPS1*	TPS1
LOC_Os06g03830	AT1G64590	*AT1G64590*	NAD(P)-binding Rossmann-fold superfamily protein
LOC_Os07g30150	AT4G22570	*APT3*	Adenine phosphoribosyltransferase 3
LOC_Os08g36040	AT1G49470	*AT1G49470*	Transmembrane epididymal protein (DUF716)
LOC_Os12g06060	AT5G04770	*CAT6*	Cationic amino acid transporter 6

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PROTEIN 1 (TGA1). TGA1 plays a central role in the response to nitrate levels, perhaps reflecting a link between cytokinin and nitrogen uptake/response, as well as defense, abiotic stress responses, and development (Alvarez *et al.*, 2014; Canales *et al.*, 2017; Li *et al.*, 2019; Sun *et al.*, 2018; Wang *et al.*, 2019). Genes from an independent meta-analysis of Arabidopsis cytokinin transcriptomic studies (Brenner and Schmülling, 2015) show a similarly small number of shared orthologs (11), which included all of the abovementioned genes.

To identify transcription factor families involved in mediating the transcriptional response to cytokinin in rice, we performed a *de novo* motif enrichment analysis of the promoters of the core genes. We examined the 1-kb regions upstream of the transcription start sites of up- and downregulated genes using the findMotifs function in HOMER (Heinz *et al.*, 2010) and focused on the motifs with $P < 1 \times 10^{-4}$ (Figure 7). Consistent with the known role of the type-B RRs in mediating the transcriptional response to cytokinin, we found several motifs that contained the canonical [A/G]TAG core sequence of type-B-binding site (Godoy *et al.*, 2011; Hosoda *et al.*, 2002; Imamura *et al.*, 2003; Taniguchi *et al.*, 2007; Xie *et al.*, 2018; Zubo *et al.*, 2017) upstream of the upregulated DEGs (Figure 7a), similar to what was found in the Arabidopsis 'Golden list' (Bhargava *et al.*, 2013). This element was found in nearly 20% of all the upregulated genes, consistent with a primary role in mediating cytokinin induction of gene expression (Mason *et al.*, 2005; Worthen *et al.*, 2019). Interestingly, this motif was not found enriched in the downregulated genes, which was also true of the Arabidopsis 'Golden list' (Bhargava *et al.*, 2013). Other enriched motifs among the upregulated genes include binding sites for the CRFs, which have been linked to mediating a subset of the cytokinin-responsive transcriptome in Arabidopsis (Raines *et al.*, 2016b) and WRKYs, which is consistent with their overrepresentation in the 'Raines' dataset, despite their not being present in the core gene list. A binding motif for the SQUAMOSA PROMOTER BINDING-LIKE (SPL) family of transcription factors (Birkenbihl *et al.*, 2005; Yamasaki *et al.*, 2009) was found enriched in both the up- and downregulated genes. SPLs are involved in a multitude of developmental processes, including floral transition and lateral root development (Chen *et al.*, 2010; Liang *et al.*, 2008; Xu *et al.*, 2016). We also identified the Teosinte branched/Cycloidea/PCF (TCP)

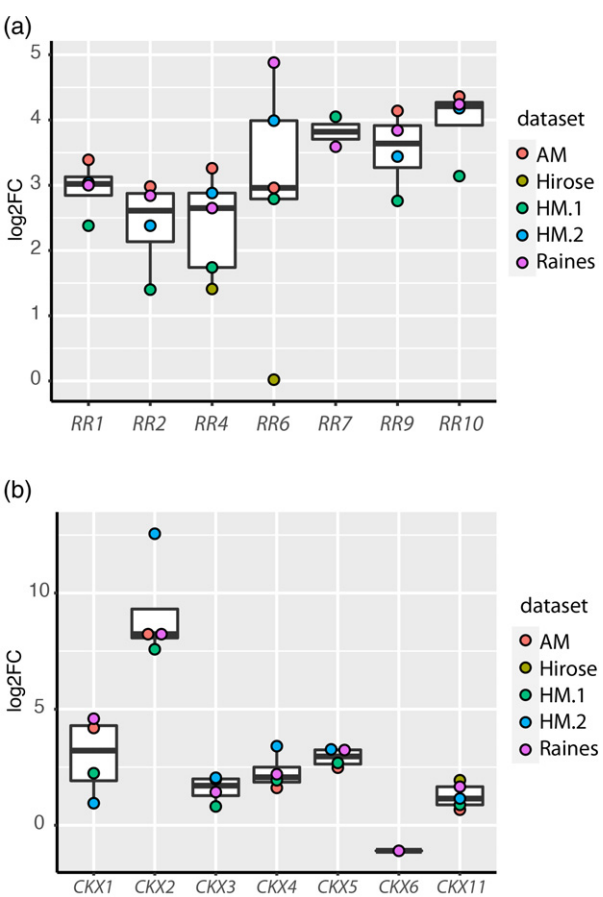

Figure 6. Expression levels of the upregulated genes present in the 'core list' related to cytokinin signaling and cytokinin metabolism. (a) Log_2FC values of the rice type-A *RRs*. (b) Log_2FC values of the rice cytokinin oxidase/dehydrogenase (*CKX*) genes. Each colored datapoint represents a log_2FC value from an individual experiment.

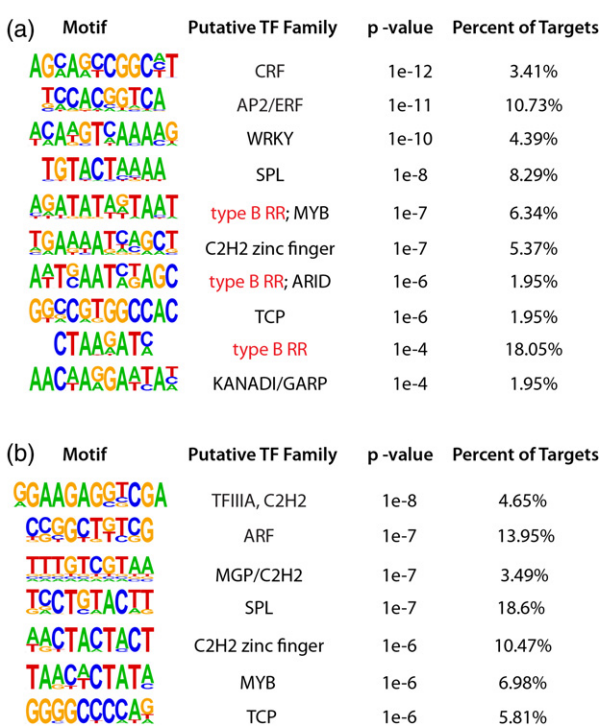

Figure 7. Motif enrichment analysis. Motif enrichment within 1-kb regions upstream of the transcriptional start sites of the upregulated (a) and downregulated (b) transcripts from the 'core list'. The list was generated using the findMotifs function in HOMER (Heinz *et al.*, 2010) and the elements with $P < 1 \times 10^{-4}$ were considered to be enriched.

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binding motifs in both the up- and downregulated genes, consistent with studies linking TCPs to cytokinin (Das Gupta *et al.*, 2014; Efroni *et al.*, 2013; Lucero *et al.*, 2015; Steiner *et al.*, 2012, 2016). These results are in agreement with a recent study of cytokinin-induced open chromatin that identified a number of similar enriched elements, including the type-B's, TCPs, and C2H2 zinc finger elements (Potter *et al.*, 2017).

Among the downregulated genes, there are two unique overrepresented motifs. The first corresponds to the binding motif for MAGPIE (MGP), a C2H2 zinc finger transcription factor that regulates asymmetric cell division in Arabidopsis root meristems (Welch *et al.*, 2007a). The expression of *MGP* is downregulated by cytokinin in Arabidopsis calli (Furuta *et al.*, 2011). The second unique overrepresented element in the downregulated genes corresponds to the binding motif for AUXIN RESPONSE FACTORs (ARFs), which is present in nearly 14% of the sequences. There is a substantial interaction between auxin and cytokinin in many developmental programs, and cytokinin and auxin regulate the expression of subsets of genes involved in each other's signaling (Moubayidin *et al.*, 2009; Schaller *et al.*, 2015). For example, auxin regulates the expression of a subset of type-A *RRs* in Arabidopsis (Hwang and Sheen, 2001) and cytokinin regulates the expression of several *Aux-IAA* genes (Ioio *et al.*, 2008). In conclusion, we confirmed that the promoters of core genes contain a number of known motifs involved in cytokinin responses in other species and contexts. Despite some overlap, there are interesting differences in the motifs of the upregulated and downregulated genes that may reflect the involvement of distinct partner transcription factors involved in mediating cytokinin induction versus repression by the type-B RRs.

DISCUSSION

While there have been multiple studies examining gene expression in response to cytokinin in dicots, including two meta-analyses of such data (Bhargava *et al.*, 2013; Brenner and Schmülling, 2015), there are relatively few such analyses in monocots. In any transcriptome study, given the large number of genes being analyzed, there will be false positives and false negatives among the defined DEGs. Various criteria are applied to reduce the number of false positives while only minimally eliminating authentic positives, including minimal FDR values, minimal $\log_2$FC cut-off values, and minimal expression values. Noise in the system resulting from the treatment method likely exacerbates this problem as it can contribute to increased biological variation in the expression of many genes. Here, we attempted to address this issue in three ways. First, we examined several transcriptomic experiments, reasoning that genes whose expression is truly influenced by cytokinin would be identified in multiple studies. Second, we used an empirical method to identify optimal FC cut-off values for the RNA-seq experiments. Finally, we developed a method that minimized the effects of treatment to reduce the noise in the system. Using these approaches allowed us to define a core set of genes that we conclude with fairly high confidence are regulated by cytokinin in rice roots. Nevertheless, it is likely that this list still contains a small number of false positives and that some cytokinin DEGs have been missed. This latter category will include genes whose expression is only modestly affected by exogenous cytokinin or which are expressed at low levels, genes that are only induced in particular conditions, or genes that are regulated in only a subset of cells within the root or only in other tissues not included in this analysis. Nonetheless, we suggest that this core list will be a valuable resource in the analysis of cytokinin function in rice and other cereal crops.

To construct the list, we first established the optimal $\log_2$FC cut-off by comparing the two most similar (HM.1 and HM.2) and dissimilar ('Raines' and HM.2) RNA-seq experiments and selected an empirically determined reasonable cut-off ($|\log_2 FC| \geq 0.7$). We concluded that this cut-off likely eliminated the majority false positives without affecting the abundance of true positive genes. We considered only genes that were differentially expressed in the same manner in at least three out of five experiments to be included in the core gene list. We suggest that this relatively stringent criterion should increase the robustness and, therefore, the usefulness of the core gene list. When comparing the core gene dynamics within each dataset, we found that the 'Hirose' experiment is quite distinctive from the rest of the datasets, which may reflect the method of treatment (*trans*-zeatin versus BA) or analysis (microarray versus RNA-seq) or the developmental stage of the plants (14 versus 10 days). Both the 'Hirose' and 'Raines' data were generated from plants grown in non-sterile conditions, which may contribute to expression differences observed. Furthermore, biotic interactions may potentiate the regulation of some genes by cytokinin, potentially accounting for the large number of DEGs in response to cytokinin.

As expected, the GO analysis of the 205 upregulated genes from the core list revealed overrepresentation of the terms 'cytokinin signaling' and 'cytokinin metabolism'. Nearly all of the type-A *RRs* that were reproducibly detected at significant levels in the root (*RR1*, *RR2*, *RR4*, *RR6*, *RR9*, and *RR10*) were highly upregulated in response to cytokinin, consistent with previous studies in various species, including rice (e.g., Camacho *et al.*, 2008; Cortizo *et al.*, 2010; D'Agostino *et al.*, 2000; He *et al.*, 2020; Ishida *et al.*, 2008; Lohar *et al.*, 2004; Papon *et al.*, 2003; Tsai *et al.*, 2012). The one exception is *RR3*, which is expressed in roots but not significantly induced after 2 h of cytokinin treatment here or in prior studies (Tsai *et al.*, 2012).

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Consistent with Tsai *et al.* (2012), we found that *RR8*, *11*, *12*, and *13* were not expressed at all in any of the root RNA-seq datasets. Other highly induced genes from this category were six *CKX* genes (*CKX1*, *CKX2*, *CKX3*, *CKX4*, *CKX5*, and *CKX11*), which likely reflect a negative feedback between cytokinin signaling and synthesis (Reid *et al.*, 2016; Sakakibara, 2006; Schmülling *et al.*, 2003; Werner *et al.*, 2006). The upregulation of the *ACO* gene in all datasets likely reflects crosstalk between cytokinin and ethylene biosynthesis (Hansen *et al.*, 2009; Lee and Yoon, 2018; Vogel *et al.*, 1998).

Hierarchical clustering of the core DEGs shows that the type-A *RRs*, *CKXs*, *CYP450*, and *ACOs* belong to the same highly induced clade. Interestingly, this group also includes three genes encoding bHLH transcription factors (LOC_Os09g28210, LOC_Os03g46860, and LOC_Os01g51140). LOC_Os09g28210 was identified in a quantitative trait locus study for root length and thickness in upland rice and has been shown to be induced under abiotic stress conditions, including drought and salt stress and ABA (Li *et al.*, 2015). LOC_Os03g46860 is transcriptionally induced by rice blast (*Magnaporthae oryzae*) infection and is suppressed by NH_4^+ (Kumar *et al.*, 2021; Wang *et al.*, 2017). Thus, these bHLH transcription factors may play a role in the cytokinin - stress response crosstalk.

The GO analysis of the 86 downregulated genes showed a distinct pattern of enriched GO terms, including significant overrepresentation in the 'transporters' category, which included transporters from multiple classes, such as amino acid, peptide, metal, and ammonium transporters. Cytokinin has been linked to the assimilation and metabolism of many micro- and macronutrients such as Pi, N, S, and Fe (Argueso *et al.*, 2009; Pavlů *et al.*, 2018). In Arabidopsis, cytokinin reduces iron responses, possibly through its negative effect on plant growth rates (Séguéla *et al.*, 2008). Interestingly, our core list of downregulated transcripts contains a gene encoding a putative transcription factor, LOC_Os04g31290, whose ortholog, the Arabidopsis *Fe DEFICIENCY-INDUCED TRANSCRIPTION FACTOR1* (*FIT1*), plays a central role in mediating responses to Fe deficiency (Colangelo and Guerinot, 2004; Schwarz and Bauer, 2020). A recent study demonstrated that rice plants with lower endogenous cytokinin levels have higher accumulation of Zn (Gao *et al.*, 2019). The availability of Zn transporters is regulated by cytokinin signaling and, conversely, Zn availability feeds back to cytokinin metabolism (Gao *et al.*, 2019). Lower cytokinin levels induce the expression of Pi transporters and, conversely, addition of exogenous cytokinin negatively affects the Pi starvation responses in Arabidopsis and rice (Franco-Zorrilla *et al.*, 2002; Martin *et al.*, 2000). Cytokinin also downregulates the expression of root sulfate transporter genes *SULTR1;1* and *SULTR1;2* (Maruyama-Nakashita *et al.*, 2004), and tobacco (*Nicotiana tabacum*) plants overexpressing *CKX1* retained high chlorophyll content under S deficiency (Werner *et al.*, 2010). Similarly, reduction in cytokinin levels leads to higher tolerance to K deficit (Nam *et al.*, 2012).

Low nitrogen has been shown to reduce cytokinin biosynthesis in rice shoots, and its optimal availability enhances cytokinin biosynthesis by promoting the expression of *IPT* genes (Kiba *et al.*, 2011; Sakakibara *et al.*, 2006). In Arabidopsis, cytokinin affects the systemic N demand signaling and the root–shoot–root communication regulating compensatory architectural changes (Poitout *et al.*, 2018; Ruffel and Gojon, 2017). Furthermore, in rice and other plants, organic N is primarily transported in the form of amino acids (Tegeder and Masclaux-Daubresse, 2018), and the regulation of amino acid/peptide transporters by cytokinin may reflect the link between cytokinin and nitrogen levels.

Our analysis of the motif enrichment in the *cis*-regulatory elements of the core genes consistently revealed the presence of the canonical type-B binding sites, consistent with their role in mediating the transcriptional induction of genes in response to cytokinin (Mason *et al.*, 2005; Nguyen *et al.*, 2016; Worthen *et al.*, 2019). Interestingly, the type-B motifs are present only in the upregulated genes in agreement with what was observed in the Arabidopsis 'Golden list' (Bhargava *et al.*, 2013). This result is at odds with the finding that Arabidopsis RR10 binds to the promoters of both up- and downregulated genes (Zubo *et al.*, 2017). Only 25% of the downregulated Arabidopsis 'Golden list' genes were direct targets of ARR10 as determined by ChIP-seq (Zubo *et al.*, 2017); the enrichment of the type-B binding motif may not be found when such a small fraction of the genes is directly targeted. The most overrepresented motif in terms of *P*-value is the AP2/ERF motif, which includes the binding site for the CRFs. CRFs are involved in the response to cytokinin and regulate different aspects of plant development (Raines *et al.*, 2016b; Rashotte *et al.*, 2006). Interestingly, the promoters of the core genes are also enriched for WRKY binding sites, which is consistent with a link between cytokinin and abiotic/biotic stress responses, despite the absence of *WRKY*s from the core DEGs. It is plausible that the regulation of WRKYs by cytokinin is potentiated by biotic interactions, which would explain why they are highly enriched in the 'Raines' cytokinin DEGs, but not in datasets derived from plants grown in sterile conditions. We also found that both the up- and downregulated DEGs contain the SPL and TCP binding motifs. A member of the SPL family, UNBRANCHED3 (UB3), has recently been shown to play a role in regulating endogenous cytokinin levels and cytokinin signaling (Du *et al.*, 2017). UB3 negatively regulates tillering and panicle branching, and directly binds to the promoters of *LOG1*, *RR1* and RR*6* (type-A RRs), and *CKX2*. The presence of the

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MGP binding motif suggests a link between rice root stem cell function and cytokinin. In Arabidopsis, MGP is involved in regulating asymmetric cell division in root apical meristems (Welch *et al.*, 2007b).

In contrast to the Arabidopsis 'Golden list' (Bhargava *et al.*, 2013), our rice core gene set consists only of transcriptomes derived from roots of comparable ages, and thus genes specifically regulated in other tissues will not be identified. Nevertheless, we found a number of orthologous genes shared between the two lists – namely the type-A *RRs*, *CKXs*, *CYP450*, and *CRFs*. Interestingly, both sets contain the *TGA1* transcription factor, which regulates a plethora of biological pathways, including stress and developmental pathways (Gatz, 2013; Jakoby *et al.*, 2002). In contrast to the Arabidopsis 'Golden list', we found that the rice core list is enriched for a relatively small number of consistent GO terms. The list of cytokinin DEGs described here should prove useful in the analysis of cytokinin function in monocots. Further, some of the genes identified here may provide direct clues to the molecular mechanisms underpinning the response to cytokinin in rice. Linking these datasets to other 'omics' datasets derived from the response to cytokinin perturbation in rice should further illuminate how this important signaling molecule modulates plant growth and development in monocots.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions for agar medium experiments

Rice seeds (cultivar Nipponbare) were surface-sterilized for 30 min in 25 ml of 2.5% sodium hypochlorite, washed with 50 ml sterile water five times, and then germinated on moist Whatman filter paper in the dark, overnight at 37°C. Eight germinated seeds were transferred into sterile cups with lids, each containing 250 ml Kimura B nutrient solution (Ma *et al.*, 2001) solidified with 1% gellan gum (PhytoTech Labs, Lenexa, KS, USA), and grown at 14 h light (28°C)/10 h dark (23°C). On day 10, eight plants were removed from the solid medium and treated with 5 μM BA dissolved in 40 ml Kimura B solution for 2 h while gently shaking at room temperature. NaOH (50 μM) was used as a vehicle control and added to the Kimura B solution. Three true biological replicates of the entire root system (from different cups and consisting of eight roots per replicate) were harvested and flash frozen in liquid nitrogen.

Plant material and growth conditions for hydroponic experiments 1 and 2 (HM.1 and HM.2)

Rice seeds (cultivar Kitaake) were sterilized and germinated as described in the previous subsection for the agar medium experiments. To maintain sterility, the following steps were performed in a laminar flow hood. On day 2, 60 germinated seeds were transferred to racks (see below) and placed in polycarbonate containers (13.5 × 9 × 7 cm) containing 500 ml sterile liquid Kimura B nutrient solution. The racks consisted of two 'tip wafers' (USA Scientific, Ocala, FL, USA; 1111–1810; 11 × 8 cm) with a 1-mm fiberglass mesh (Saint-Gobain ADFORS, La Défense, France) pressed in between. A single germinated seed was placed into a well of a 'tip wafer'; the buoyancy of the rack resulted in the germinated seeds floating in the media; however, they were not fully submerged. Four polycarbonate containers with a sterile stir bar were placed into a (64 × 35 × 21 cm) plexiglass chamber (the transpiration chamber). The transpiration chamber had been sterilized by washing with 20% bleach followed by 70% ethanol and the inlet and outlet ports of the chamber were covered with sterile aluminum foil. The chamber was closed, placed into a growth cabinet (14 h light [28°C]/10 h dark [23°C]) and placed on a digital stirrer (Thermolyne Cellgro 45700, Marshall Scientific, Hampton, NH, USA). On day 7, plants were transferred (under a laminar flow hood to maintain sterility) to new containers with fresh media and returned to the transpiration chamber, and a constant stream of sterile air filtered through a 0.2-μm venting filter (Whatman, Sigma Aldrich, St. Louis, MO, USA WHA2108) was initiated using the ports at a pressure of 75 kPa. On day 8, the medium was changed with fresh medium containing either 5 μM BA (dissolved in NaOH) or 50 μM NaOH (control) (HM.1). Alternatively, 25 μl of 0.1 M BA dissolved in 1 M NaOH (BA) or 25 μl of 1 M NaOH was added to the existing media (HM.2). Three true biological replicates (each replicate originated from a different experiment) consisting of five entire root systems per treatment were harvested (i) at the start of the experiment and (ii) after 2 h of treatment and flash frozen in liquid nitrogen.

Library preparation, sequencing (AM, HM.1, and HM.2), and data processing

Total RNA was extracted using the QIAGEN RNeasy Mini Kit as described by the manufacturer and samples were DNase-treated using the TURBO DNA-free kit (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were prepared using the TruSeq Stranded mRNA preparation Kit (AM; Illumina, San Diego, CA, USA) or the KAPA Biosystems Stranded RNA-seq kit (HM.1 and HM.2), as described by the manufacturer (Roche Sequencing and Life Science Kapa Biosystems, Wilmington, MA, USA). Samples were sequenced on one lane of a HiSeq2000 (AM) or HiSeq4000 (HM.1 and HM.2) platform with 50-bp single-end (AM) or paired-end (HM.1 and HM.2) reads. Sequences were trimmed using the BBDuk tool (BBMap v.37.50 package; https://sourceforge.net/projects/bbmap), aligned to the *Oryza sativa* genome (MSU6) using TopHat (Trapnell 2009) and Bowtie v.1.2.0 (Langmead 2009). Reads were quantified using featureCounts from subread v.1.5.2 (Liao 2014). RNA-seq analysis for each dataset was conducted with the EdgeR package (Nikolayeva and Robinson, 2014; Robinson *et al.*, 2010) in R with a cut-off value of $|\log_2 FC| \geq 0.7$ and an FDR below 5%.

Gene ontology, hierarchical clustering, and motif analyses

The functional enrichment analysis was performed using g:Profiler (version e102_eg49_p15_7a9b4d6) with the g:SCS multiple testing correction method applying a significance threshold of 0.05 (Raudvere *et al.*, 2019). Data sources were the MF, BP, CC, and KEGG categories. Hierarchical clustering was done in R using the pheatmap v.1.0.12 (Pretty Heatmaps) package. Motif enrichment analysis was performed within 1-kb regions upstream of the transcriptional start sites using findMotifs HOMER v.4.9.2 and the following length parameters: -len 6, 8, 10, 12.

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AUTHOR CONTRIBUTIONS

JKP and JJK conceived the project. JJK supervised the work. CAB and KCP performed the experiments. JKP, KCP, and CAB conducted the analyses. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The raw and processed RNA-seq data described in this study have been deposited into the NCBI Short Read Archive (SRA) database under PRJNA722497. The codes used in this study can be found on Github (https://github.com/KieberLab/Core_gene_list_rice).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. The upregulated genes from the core list.

Table S2. The downregulated genes from the core list.

Table S3. Detailed gene ontology results of the upregulated genes from the core list ($P < 0.05$); analysis was performed using the g:Profiler (Raudvere *et al.*, 2019).

Table S4. Detailed gene ontology results of the downregulated genes from the core list ($P < 0.05$); analysis was performed using the g:Profiler (Raudvere *et al.*, 2019).

Figure S1. The multidimensional scaling (MDS) plot showing clustering of the replicates across each time point in (a) the Hydroponic medium 1 (HM.1) experiment and (b) the Hydroponic medium 2 (HM.2) experiment based on the expression values.

Figure S2. Empirical determination of the $\log_2$FC cut-off applied to the RNA-seq analysis.

Figure S3. Heatmap of differentially expressed genes shared by at least three datasets.

Figure S4. Changes in the expression of type-A response regulator (RR) genes in the presence of exogenous cytokinin across all the RNA-seq experiments.

Figure S5. Changes in the expression of cytokinin oxidase/dehydrogenase (CKX) genes in the presence of exogenous cytokinin across all the RNA-seq experiments discussed.

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