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Abstract:

Several factors affect the yield potential and geographical range of crops including the circadian clock, water availability, and seasonal temperature changes. In order to sustain and increase plant productivity on marginal land in the face of both biotic and abiotic stresses, we need to more efficiently generate stress-resistant crops through marker-assisted breeding, genetic modification, and new genome-editing technologies. To leverage these strategies for producing the next generation of crops, future transcriptomic data acquisition should be pursued with an appropriate temporal design and analyzed with a network-centric approach. The following review focuses on recent developments in abiotic stress transcriptional networks in economically important crops and will highlight the utility of correlation-based network analysis and applications.

Key words: Co-Expression Networks, Abiotic Stress, Circadian, Diurnal

Introduction

Current projections of crop productivity indicate that global demand will not be met by 2050 due to a growing and more affluent world population [1]. Climate change may make drought more prevalent worldwide [2] and may have significant effects on the range of both pests and pollinators [3]. The yield potential and geographical range of crops is modulated by abiotic stresses such as drought, low temperature and high temperature but also by the circadian clock [4,5]. The plant circadian clock coordinates plant growth and physiological responses to daily and seasonal environmental changes [4]. Clock-related genes have been found to underlie quantitative trait loci (QTL) for important adaptive traits such as flowering time in crops such as barley, rice and soybean [6–8]. In order to sustain and increase plant productivity on marginal land in the face of both biotic and abiotic stress, we need to generate stress-resistant crops more efficiently through genome-enabled computational breeding and genetic modifications including new genome-editing technologies. To economically leverage these strategies, future and existing transcriptomic data should be scrutinized with a network-based approach that incorporates circadian and diurnal data sets to develop integrative causal networks for generating predictive models as demonstrated for nitrate response in *Arabidopsis* [9].

In a plant system, the greatest body of knowledge on abiotic stress and circadian regulation is in the dicot model *Arabidopsis thaliana*. However, the majority of the world's diet is based on monocots, including the cereals maize, rice, and wheat, which diverged from dicots approximately ~127 to 191 million years ago [10]. There are fundamental differences between monocots and dicots which motivated the development of model monocot systems *Brachypodium distachyon* [11] and *Setaria viridis* [12]. Whole genome duplications (WGD), rearrangements and divergence complicates comparisons of crops to *Arabidopsis* or other model systems [13–15] but exploring duplicate gene function can be greatly improved through a network-based analysis where connections between genes are compared to infer pathway structure and membership. The following review will highlight the advantages of gene co-expression networks in temporal abiotic stress research.

Circadian Regulation: From *Arabidopsis* to Crops

The circadian clock is involved in a vast array of processes from stomatal conductance, photosynthesis, stem elongation, flowering time, leaf movement, pathogen and herbivory resistance to response to abiotic stress [16]. The importance of this master regulator in plants is evident by the breadth of circadian regulation of gene expression. As many as 90% of *Arabidopsis* genes have been shown to have rhythmic expression under at least one diurnal (under an entraining environmental cycle such as photo- or thermocycles) cycle or circadian (free run) condition [17–20]. Similar estimates of the proportion of the transcriptome under diurnal and circadian regulation have been made in other plant systems such as rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) [21], demonstrating the importance of considering temporal gene regulation in future expression studies. There is evidence of significant variation in the temporal regulation of key pathways between and within plant systems [22–26]. Variation in both circadian and diurnal patterning of transcriptomic pathways complicates single time-point comparisons between model systems and crops. A comparative analysis of microarray datasets for cold-treated *Arabidopsis* plants sampled at a single time-point revealed that circadian and diurnally regulated genes contributed the most to the differences between independent studies, thereby suggesting that a single time-point experiment cannot account for the effects of rhythmically-expressed genes [27]. For a

summary of the circadian and diurnal transcriptome data currently available in public databases please refer to Table 1.

Global profiling of circadian-regulated genes by microarray analysis in *Arabidopsis* has revealed extensive circadian control of abiotic stress-related genes as well as genes involved in all hormone pathways [20,28]. Microarray studies have estimated that 50% of heat-responsive genes and 40% of cold-responsive genes are circadian regulated with an average difference in phasing of peak expression of 12 hours [20]. A temporal study of drought response in *Arabidopsis* found that the time of day had more effect on the transcriptome than the response to drought, making a time-course study necessary to capture the drought response [29]. A diurnal and circadian microarray and metabolite profiling study of cold-treated *Arabidopsis* plants revealed extensive diurnal regulation of cold-responding genes as well as diurnal regulation of roughly 47% of identified metabolites [30]. A large portion of stress transcripts show time-of-day dependence in two ways that are not mutually exclusive: Type A) rhythmic transcriptomic changes that occur under stress and non-stress conditions which can be characteristic of rhythmic basal expression and rhythmic response to progressive stress; Type B) variation in the amplitude of the transcriptional response that is gated by the time of day that the stress is applied which can be characteristic of response to acute application of stress.

The rhythmic expression of genes associated with cold, heat, osmotic, ROS, and salt stresses under non-stress conditions [17,20] and a maximum transcriptomic response to progressive drought late in the day in *Arabidopsis* and *Populus* [29] would both be examples of Type A time dependence. In *Arabidopsis*, the level of accumulation of low-temperature induced transcription factor (TF) *C-Repeat Binding Factor (CBF)/Dehydration Response Element Binding (DREB)* Factor was much higher when a cold treatment was applied 4 hours after dawn than if it was applied 16 hours after dawn [31]. This gated expression of *CBF* would be an example of Type B time dependence [31]. Type B gated transcript expression has been observed in *Arabidopsis*, grape [32], peach [33] and potato [34] under many abiotic stress signaling pathways including cold stress [31,35–37], heat stress [32], ROS stress [38], salt stress [34,39] and UV-B stress [40,41]. Common to all the aforementioned stress studies is the suggestion that sampling the transcriptome during an off-peak period will systematically underestimate the magnitude of the response within a species. The importance of minding the temporal regulation of abiotic stress response is further exacerbated when trying to compare data from two species taken at discordant times.

Abiotic Stress, Time, and Expanding Gene Families

Identification of the crop primary stress response (PSR) TFs that are functional orthologs to *Arabidopsis* PSR TFs [42] is complicated by the differential expansion of these gene families in higher plants. For example, there are 6 *CBF/DREB* TFs in *Arabidopsis* [43] but possibly 14 in rice [44], 19 in the model grass *Brachypodium distachyon* [45], 20 in barley [44], 21 in cotton [46], and 25 in wheat [47]. Identifying orthologous PSR TFs does not guarantee similar stress induction patterns, especially if there are overarching differences in the circadian regulation or simply differences in the timing of the expression data. Hierarchical clustering of microarray analysis under 2, 8, 24 and 48 h of cold, in chilling-sensitive and chilling-tolerant rice genotypes, revealed differences in transcriptomic response between genotypes and also in the response of *CBF1* duplicate genes, suggesting possible sub-functionalization of *CBF1* [48].

Even in cases where sub-functionalization of orthologous PSR TFs is not suggested (e.g. stress-induced expression of orthologous PSR TFs are similar), conservation of all target gene regulation (regulon) is not implicit [49,50]. The bulk of abiotic stress expression data has been examined using paired (stress vs. non-stress) differential gene expression analysis with limited time resolution [51–54]. Accordingly, the temporal dynamics of many PSR TF regulons remain unknown since identification of PSR TF targets often examines limited time resolution or relies on overexpression, which obfuscates temporal information. There is a compelling need for generating and examining temporal abiotic stress datasets in non-Arabidopsis models and crop plants to both better identify functionally orthologous PSR TFs and predict novel PSR TFs. Because pairwise comparison of datasets cannot be easily expanded to include a temporal component [55], both existing single time-point data and new time series data will likely require the application of network-based approaches for analysis.

Transcriptional Networks for Abiotic Stress and Circadian Regulation

The rapid progress in Next Generation Sequencing (NGS) has revealed the evolutionary role of polyploidy in shaping crop genomes. Addressing the challenges of understanding the function and variation in temporal regulation of abiotic stress pathways that influence yield mandates the application of network-based approaches that are amenable to inclusion of different technologies (RNA-seq vs. microarrays) into the analysis.

Gene co-expression networks (GCN) provide a means to examine the correlations in gene expression generated from complex microarray and RNAseq datasets across treatments, developmental stages and time courses. GCNs provide pathway structure for extracting modular responses that are often missed by differential expression or ANOVA approaches. A GCN is a set of relationships between genes where a node is defined as a gene (more accurately defined as a gene expression profile) connected to other genes by edges based on pairwise similarities. Genes clustered together in a module provide insight into potential regulatory functions (Figure 1). The similarities between gene expression profiles can be determined by various correlative, mutual information-based or context likelihood-of-relatedness metrics [56–59]. A weighted gene co-expression network approach (WGCNA) assigns weights to the edges based on the strength of the correlation between genes whereas an unweighted network assigns a connection if the correlation exceeds a chosen threshold [60,61]. Another approach for assigning expression data to a network structure is to apply a linear model to determine regulation based on an existing network. The edges in these networks can also be weighted by correlation strength but they are assigned based on *a priori* network information; an example of this is Network Component Analysis (NCA)[62,63].

WGCNA [60] was applied to existing microarray data generated from leaf, root and young panicle tissue of drought-treated rice plants at three growth stages [64]. GCNs were generated for genes responding to drought in at least 3 tissue samples. Network modules, or gene clusters, were used to identify highly coordinated genes across the tissue samples that were defined as hub genes and showed enrichment for the ABRE cis-element. The conservation of these gene patterns across tissue samples suggests that a conserved drought responsive pathway is maintained across tissue types [64]. WGCNA analysis of 10 Arabidopsis ecotypes following cold, heat, high-light, salt and flagellin treatment either separately or in combination revealed several stress regulatory modules. Many of the highly connected TFs in these modules were PSR TFs as well as circadian clock TFs [65], suggesting that these TFs are important mediators of the regulatory response characterized by those modules. Network analysis of gene-

expression changes following heat, salinity and drought stress over 24 h in *Brachypodium distachyon* identified 22 modules. All but one of these modules responded to drought stress with varying responses from the other stress treatments for each module. These modules were further interrogated for GO ontology and promoter motif enrichment to uncover sequences that may contribute to a pathway-specific stress response [66].

An essential goal of network analysis is to assign gene significance measures using the available expression data to the trait or stress response phenotype. Hub genes that are highly connected in the network are not always the genes of significant biological meaning for the trait of interest [67]. It has been suggested that intramodular hubs, genes that are highly connected within a module, are more likely to be biologically significant if that module is associated with the trait [61,67]. WGCNA applied to human and chimpanzee gene expression in brain tissue identified conserved intramodular hubs enriched for neuroanatomical structures, providing significant insight into primate brain organization [68]. Although the use of conserved intramodular hubs for trait discovery is untested in plants, for the purpose of this review, we refer to hub genes as highly connected genes within a module rather than between modules.

GCNs can be helpful in assigning candidates to identified QTL regions over standard clustering methods. Applying hierarchical clustering to drought-treated upland and paddy rice identified 213 candidate genes that colocalized with 17 QTL intervals [69]. These clustering methods have been widely used in expression analysis to generate lists of similarly responding genes. Genes with a pattern of interest are then interrogated against GO ontology categories to look for enrichment of biological processes. However, there is no regulatory information assigned to the list of genes, leaving it up to the investigator to assign priority to gene candidates. Modeling gene expression changes in combination with *cis*-regulatory motif analysis can provide regulatory predictions [70,71]. GCNs generated using 815 Affymetrix rice arrays resulted in the identification of 15 gene modules with enrichment for stress-related biological processes as well as *cis*-regulatory elements including ABRE [48]. Several hub genes identified overlapped with known drought tolerant QTLs, resulting in a short list of candidate genes for further study [48]. This highlights the advantage of applying a network approach to identify candidate genes for known QTLs and emphasizes the value in assigning genes to modules that are likely pathway specific as supported by GO ontology enrichment.

Although GCNs provide valuable insight into pathway regulation, they cannot differentiate between transcriptional co-regulation and post-transcriptional modification of steady state levels which has been shown to be especially relevant for circadian clock-mediated stress response [72,73]. The most attractive applicability of GCNs is their utility for building predictive models that integrate protein-protein interactions, TF-*cis*-element interactions, microRNA-mRNA interactions, alternative splicing dynamics and other ‘-omic’ datasets for inferring causality. For example, available biochemical, genetic and pharmacological information on ABA and stomatal closure was used to generate a dynamic boolean network model [74]. This dynamic signal transduction network can be used to predict the involvement of various components of the network on stomatal closure [74]. An extensive transcriptomic study of rice paddy fields over two years with temporal sampling at 2 h and weekly resolution was combined with meteorological data to generate a predictive transcriptional model. The model successfully predicted transcriptional changes for the following growing season when the environmental data were provided.

Examination of the contributions of terms within the model revealed plant age, circadian clock and environmental factors as the major determinants of transcriptome dynamics [75].

Conclusions and Future Applications

To leverage the vast wealth of existing data from model systems and to better plan future temporal stress datasets, network-based approaches should be utilized. Network cross-species comparative studies can be used to uncover conserved regulatory pathways and enable better functional characterization of orthologs [76]. An example of this is the Comparative analysis of Plant co-Expression networks (ComPIEx) tool that was developed following the analysis of diverged and conserved ortholog regulation across co-expression networks in Arabidopsis, poplar and rice [77]. In addition to improving cross-species comparisons, integrative regulatory networks that incorporate additional ‘omics’ datasets, such as high-throughput phenomics, ENCODE, proteomics and metabolomics, will provide the necessary framework for developing predictive models of seasonal crop fitness in the field. If future abiotic stress data is particularly mindful of the importance of time-to-stress and tissue specificity, thoughtful integration of temporal metadata could further improve user-friendly co-expression network databases such as the Bio-Analytic Resource for Plant Biology (BAR; <http://bar.utoronto.ca/>), RiceFRIEND [78], VirtualPlant [79] or future co-expression network tools.

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Table 1.

Publically available circadian and diurnal datasets. Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and Short Read Archive (SRA; <http://www.ncbi.nlm.nih.gov/sra>) databases were searched for clearly annotated circadian and diurnal datasets with associated publications (please see Supplemental References). No published RNA-seq circadian or diurnal datasets were found through this search. Datasets under photocycling (LDHH), thermocycling (LLHC) or photocycling and thermocycling (LDHC) conditions are referred to as diurnal because they were sampled under entrainment conditions. Datasets that have been entrained but are sampled under freerun conditions (LDHH-F, LLHC-F, LDHC-F) are considered circadian datasets. The list of available Arabidopsis circadian and diurnal datasets is not exhaustive, rather they are the microarray datasets that can be easily queried by the DIURNAL webtool [80].

Figure 1:

A network approach to gene expression studies for discovering gene function and cross-species comparisons. An important goal of gene expression studies is to uncover the underlying transcriptional networks, genes and pathways that are mediating a physiological process. These biological pathways have inherent dynamic network properties that are best revealed with a complementary network-based approach. The extent of diurnal and circadian control of abiotic stress response suggests that temporal resolution is necessary to capture the appropriate transcriptome response. These expression profiles can then be used to assemble co-expression networks using a variety of techniques. The advantage of using a network approach over standard pairwise comparisons is the regulatory information that is conveyed by the network; genes with greater module connectivity (A) are candidates for important regulatory hubs in the pathway. These networks can also be used to compare and identify orthologs (B) across species by analyzing their network membership to uncover conserved as well as possible diverged regulatory functions. Stress-responding modules (D) may also provide insight into the regulatory roles of genes of unknown function (C) by examining their associations within the network.

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Plant Species	LDHC	LDHC-F	LDHH	LDHH-F	LLHC	LLHC-F	Publication(s)
<i>Arabidopsis thaliana</i>	Yes	Yes	Yes	Yes	Yes	Yes	[17,18,81–83]
<i>Brachypodium distachyon</i>	Yes	Yes	Yes	Yes	Yes	-	[11]
<i>Glycine max</i>	-	-	-	Yes	-	-	[84]
<i>Hordeum vulgare</i>	Yes	-	-	-	-	-	[85]
<i>Nicotiana attenuata</i>	Yes	-	-	-	-	-	[86]
<i>Oryza sativa</i>	Yes	Yes	Yes	Yes	Yes	Yes	[21,78,87–89]
<i>Ostreococcus tauri</i>	-	-	Yes	-	-	-	[90]
<i>Populus trichocarpa</i>	Yes	Yes	Yes	Yes	Yes	Yes	[21,91]
<i>Saccharum officinarum</i>	-	-	-	Yes	-	-	[92]
<i>Vitis vinifera</i>	Yes	-	-	-	-	-	[93]
<i>Zea mays</i>	Yes	-	-	Yes	-	-	[94,95]