CHAPTER TEN

KIN10/11 are master regulators of the convergent stress transcriptome

[Au1] **Elena Baena-González[1*], Filip Rolland[1,2,3*], and Jen Sheen[1]**

Abstract Different types of stress result in both specific and convergent responses that modulate plant growth and development. The elucidation of common regulatory nodes may provide insight into the functional basis of stress tolerance and cross-protection. Large-scale generation and comparison of microarray data has recently revealed that part of the cross-talk among the various stress response pathways occurs at the gene expression level, with diverse types of stress triggering overlapping transcriptional responses. We have identified *Arabidopsis* KIN10 and KIN11 as central regulators of the convergent stress transcriptome. Sensing and signaling stress-associated energy deprivation, these protein kinases (PKs) trigger global gene expression reprogramming, enabling the adjustment of energy homeostasis necessary for coping with stress. The remarkably broad transcriptional program promotes catabolism and autophagy, and suppresses anabolism and ribosome biogenesis. Significantly, KIN10/11 also target a plethora of transcriptional and signaling regulators to orchestrate global responses beyond metabolic regulation. Analyses of gain- and loss-of-function mutants uncover the picture that KIN10/11 are central regulators for the integration of metabolic, environmental and hormonal cues during plant growth and development.

Keywords KIN10, KIN11, SnRK1, starvation, stress transcriptome, energy sensor, *Arabidopsis*

Introduction

Exposure to stress often triggers stress-specific adaptive responses as well as general responses that confer basic protection to adverse conditions. With the advent of large-scale microarray and metabolic studies it has become increasingly clear that this triggering is partly the result of an extensive cross-talk among stress response pathways that were

[1]Department of Genetics, Harvard Medical School, and Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114, USA
[2]Department of Molecular Microbiology, VIB, Leuven, Belgium
[3]Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, K.U. Leuven, Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Flanders, Belgium

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once considered linear (Yamaguchi-Shinozaki and Shinozaki 2006; Ma and Bohnert 2007). Although some of the stress-signalling cascades have been dissected in detail, the intersection points between different types of stress as well as the identity of the signalling intermediates and key regulators remain largely unknown. Interestingly, different stress conditions induce similar alterations in carbon and nitrogen metabolism and lead to overlapping patterns of gene expression, with many genes being induced or repressed by multiple stimuli. As a major target of diverse stress conditions is photosynthesis, decreased cellular energy levels are an obvious common consequence. Certain conditions, such as flooding, also impair mitochondrial respiration and result in similar energy deprivation. The ability to cope with both long-term and short-term stress and energy starvation is critical to prevent inhibition of growth and irreversible senescence and cell death.

In the present study, we developed sensitive and quantitative cell-based assays using a well-characterized stress–responsive marker gene and *Arabidopsis* leaves. This system facilitated the identification of the *Arabidopsis* SnRK1 (Snf1-related PKs) orthologs, KIN10 and KIN11, as central mediators of various stress responses that alter cellular energy homeostasis. Furthermore, it allowed the application of powerful global gene expression profiling to uncover a large array of metabolic and regulatory genes that are coordinately activated or repressed by KIN10/11 in leaf cells. Extensive microarray data and genetic analyses revealed that KIN10/11 play a central role in the regulation of anabolic and catabolic pathways linked to starvation and stress signalling, and, unexpectedly, to normal growth and development (Baena-González et al. 2007).

Materials and methods

Effector and reporter constructs. KIN10 and *KIN11* were fused to the HA tag and cloned between a 35S-derived promoter and *NOS* terminator. The 624 bp promoter/5′UTR sequence of *DIN6* was fused to the *LUC* reporter gene to generate *DIN6-LUC* as described (Lam et al. 1994; Baena-González et al. 2007).

Protoplast transient expression assay. Protoplasts ($1–4 \times 10^4$) were incubated for 6 h in 1 mL buffer in 6-well plates or submerged in 1 mL buffer in a 1.5 mL microfuge tube for hypoxia treatment as described (Baena-González et al. 2007). For dark treatment, plates were covered with aluminium foil. DCMU was added at 20 µM.

Global gene expression analyses. For global gene expression analyses, protoplast transfection experiments were scaled up 50-fold. RNA was extracted from cells transfected with control or *KIN10*-expressing plasmid DNA and used for hybridization of *Arabidopsis* ATH1 GeneChips. Original data (GEO accession number GSE8257) was filtered following three independent strategies based on: (i) Affymetrix GCOS presence (P) or absence (A) calls and a twofold cutoff, (ii) rma or gcrma normalization (http://www.bioconductor.org/) and RankProd analysis as described (Baena-González et al. 2007), using a *p*-value cut-off (0.012) based on the validated *MYB75* marker gene expression. Only genes overlapping in the three independently generated lists were considered ("KIN10-regulated genes"). An additional filtering step was used to provide physiological significance to the data: using a set of KIN10 marker genes (At3g47340, At1g12780, At4g15530, At2g33830, At3g15450, At5g18630, At5g20250, At5g22920, At4g35770, At1g12240, At1g70290) matching transcriptional profiles were identified through Genevestigator (https://www.genevestigator.ethz.ch/) and only genes with consistent expression patterns in all datasets were considered (induced in all positively correlated profiles and repressed in all negatively correlated profiles, "KIN10 and starvation-regulated genes") (see Baena-González et al. 2007 for further details).

Filtered genes were classified according to the MapMan functional categories (Thimm et al. 2004) with some modifications.

Results

Dark-inducible DIN genes are activated by multiple stresses and repressed by sugar

We surveyed public microarray databases using Genevestigator and found that *DIN* genes, originally identified as dark-inducible genes (Lam et al. 1994) are induced by multiple other stresses, as diverse as heat, wounding and high salt concentrations. Accordingly, *DIN* genes are strongly induced in *Arabidopsis* protoplasts when subjected to darkness, hypoxia, or herbicide (3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU) treatment (Fig. 1a). Importantly, this induction can be blocked, in a hexokinase 1 (HXK1)-independent manner [Au4] (Baena-González et al. 2007), by the addition of sugar, as well as by K-252a, a serine/threonine PK [Au5] inhibitor (Fig. 1a; Fujiki et al. 2000), suggesting that the signal may be metabolic and that it may be mediated through a PK.

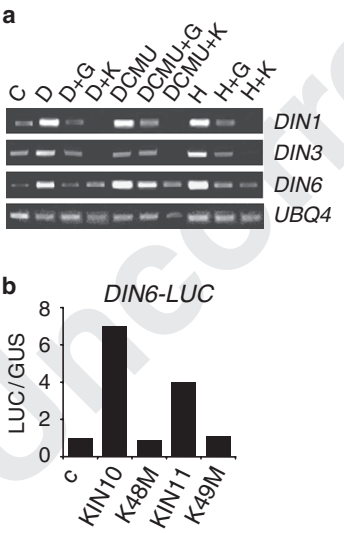

Fig. 1 *DIN* genes are regulated by stress and KIN10/11. (**a**) Various types of stress induce *DIN* gene expression in *Arabidopsis* protoplasts. This induction is repressed by glucose (25 mM) and the PK inhibitor K252a (2 µM). (**b**) *DIN6-LUC* is activated by KIN10 and KIN11. The induction is impaired in the kinase dead mutants (K48M and K49M). D, dark; H, hypoxia; C, control DNA

DIN6 is specifically induced by the SnRK1 PKs KIN10 and KIN11

To test the possibility of a convergent metabolic signal in the stress response we developed a cell model system using a sensitive reporter by fusing the putative *DIN6* (encoding the glutamine-dependent asparagine synthetase, *ASN1*) promoter to the luciferase (*LUC*) gene. Regulation of *DIN6-LUC* expression in transfected protoplasts was similar to that of the endogenous gene (Baena-González et al. 2007). Since energy levels in other organisms are sensed by a highly conserved group of serine/threonine PKs, comprising yeast Snf1 and animal/human AMP-activated PK (AMPK), we hypothesized that the orthologous plant PKs, SnRK1s, might be implicated in the *DIN6* activation triggered by various stresses. *DIN6-LUC* was indeed activated by the two SnRK1 members, KIN10 and KIN11, and the kinase activity was required for this activation (Fig. 1b). Related PKs from the SnRK2 and SnRK3 families did not have an effect on *DIN6-LUC* expression (Baena-González et al. 2007).

Global gene expression regulation by KIN10

To determine the extent of KIN10 transcriptional regulation and identify its downstream target genes, we performed global gene expression profiling using the 22 K ATH1 GeneChips, taking advantage of the protoplast system transiently expressing KIN10. To increase the robustness of our data, data was imported and processed following three independent sets of criteria and only genes overlapping in the resulting three lists were considered. A final filtering step involved the cross-comparison with published microarray datasets identified as exhibiting matching expression profiles (see Materials and Methods). A striking positive correlation (Pearson coefficients 0.85–0.87) of KIN10-regulated gene expression was found with profiles obtained under extended night-induced carbon deprivation (Thimm et al. 2004), short-term sucrose starvation (Contento et al. 2004), and starvation- (Buchanan-Wollaston et al.

[Au6] 2005) and dark-induced senescence (Lin and Wu 2004). Importantly, KIN10 target genes also exhibited strong negative correlations (Pearson coefficients −0.87 to −0.92) with gene expression profiles obtained from glucose-(Price et al. 2004) or sucrose-treated seedlings (Palenchar et al. 2004) and differentially CO_2-fixing plants (Bläsing et al. 2005). The stringent and multi-step filtering process selected a reliable list of 278 genes co-activated by KIN10 and sugar starvation conditions, but co-repressed in sugar-treated seedlings or during maximal CO_2 fixation. A second list of 322 genes was also identified based on their co-repression by KIN10 and sugar starvation conditions, but co-activation in sugar-treated seedlings or during maximal CO_2 fixation.

In general, a clear trend of gene expression reprogramming from anabolic to catabolic processes was observed in response to transient expression of KIN10 in mesophyll protoplasts. Previous functional studies of mammalian AMPK and plant SnRK1 have mainly focused on enzyme activity regulation and expression of selected marker genes (Halford et al. 2003; Kahn et al. 2005). The present work provides for the first time a detailed overview of the extensive transcript changes induced by these conserved kinases in a multicellular organism.

KIN10-upregulated (but sugar-repressed) genes represent a variety of mainly catabolic pathways, consistent with a global metabolic switch induced by SnRK1 to provide an alternative source of metabolite and energy supplies through amino acid catabolism, protein degradation, starch, sucrose, cell wall and polysaccharide hydrolysis, and lipid mobilization and beta-oxidation (Fig. 2a; Table 1). Conversely, genes involved in the biosynthesis of these and other compounds (e.g. nucleotides) were repressed by KIN10 and starvation conditions. The most prominent and evolutionarily conserved set of repressed genes included 87 genes involved in protein synthesis (Fig. 2a).

The central and previously unrecognized regulatory role of SnRK1 was further illustrated by the large number of transcription regulation genes that are activated or repressed by KIN10 and starvation (Fig. 2a). In addition, a significant number

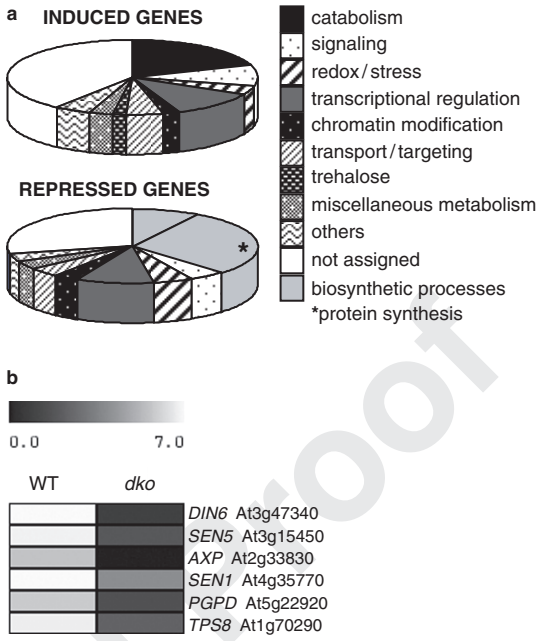

Fig. 2 Global gene expression regulation by KIN10. (**a**) The transcriptional program induced by KIN10 markedly overlaps with that induced by various starvation conditions and is antagonized by increased sugar availability (see text for details on the compared conditions). The induced and repressed genes comprise a wide range of functional categories. (**b**) Silencing of *KIN10* and *KIN11* ("double knockout", *dko*) abrogates the induction of a set of KIN10 marker genes in response to darkness

of hormone metabolism and hormone responsive genes were affected, as well as many genes encoding other signal transduction components, including PKs, protein phosphatases, and calcium modulators. Changes in metabolism genes were also accompanied by increased expression of genes encoding carbohydrate, amino acid, peptide, ion transporters and aquaporins, presumably to facilitate mobilization and recycling of these molecules.

Being the major source of energy, photosynthesis was also affected by KIN10 expression, probably in an attempt to increase ATP production. KIN10 induced the expression of several photosynthesis-related genes, encoding both components of the electron transport chain and the carbon assimilation pathways (Table 1). These genes were filtered out during the cross-comparison with the

[Au7] **Table 1** Selection of KIN10- and starvation-regulated genes. Complete lists are provided in Baena-González et al. (2007). Values correspond to KIN10-mediated induction. Stars denote regulation by KIN10 but not jointly by all described stresses. Functional categories are based on the classification in MapMan with some modifications

AGI number		Signal
Photosynthesis	Functional category	log2ratio
At2g13360*	PS.aminotransferases	2.9
At3g62410*	PS.calvin cyle.CP12-2	2.4
At1g12900*	PS.calvin cyle.GAP	2.1
At1g80380*	PS.calvin cyle.PRK	2.2
At3g16250*	PS.lightreaction.ferredoxin	1.8
At1g76100*	PS.lightreaction.plastocyanin	2.4
At1g03130*	PS.lightreaction.PS I	1.8
At1g14150*	PS.lightreaction. PS II	2.3
At3g50820*	PS.lightreaction. PS II	1.8
At1g51400*	PS.lightreaction. PS II	1.5
Amino acid metabolism		
At3g47340	aa synthesis asparagine DIN6	5.3
At5g18170*	aa degradation.glutamate GDH	2.4
At1g03090	aa degradation.leucine	4.6
At4g34030	aa degradation.leucine	3.0
At3g45300	aa degradation.leucine	3.4
At4g33150	aa degradation.lysine	2.7
At1g64660	aa degradation.methionine	6.2
At3g30775	aa degradation.proline	4.5
At3g06850	aa degradation.shared	3.9
At3g13450	aa degradation.shared	4.0
At1g55510	aa degradation.shared	3.2
At5g54080	aa degradation.tyrosine	2.3
At2g14170	aa degradation.valine	2.0
Carbon metabolism		
At4g37870*	gluconeogenesis.PEPCK	1.8
At4g15530	gluconeogenesis.PPDK	2.2
At1g43670*	gluconeogenesis.FBPase	2.6
At1g07110	glycolysis.Fruc2,6BisPase	1.6
At3g49160	glycolysis.PK	2.0
At5g18670*	starch.degradation BMY3/BAM9	3.6
At1g70290	trehalose.AtTPS8	3.0
At1g23870	trehalose.AtTPS9	3.3
At1g60140	trehalose.AtTPS10	1.8
At2g18700	trehalose.AtTPS11	2.2

public datasets, due to their reduced expression in the experiments involving dark treatment (e.g. Thimm et al. 2004; Lin and Wu 2004). Another gene, *BMY3/BAM9* β-amylase, is also induced by KIN10 but not by some of the starvation treatments (Table 1). Altered *BMY3/BAM9* expression may be related to the more than sevenfold higher starch levels measured in *KIN10/11* silenced plants than in the WT (Baena-González et al. 2007). Under starvation conditions, another potentially important source of energy is the cell wall, and starvation indeed triggers hydrolysis of some of its main components (Lee et al. 2007). Extensive transcriptional changes in genes related to cell wall modification, synthesis and degradation (Fig. 2a) might reflect cell wall utilization as an alternative energy source as well as a restriction of cell growth and elongation. Interestingly, the trehalose metabolism genes *TPS8-11* were also upregulated by KIN10 and starvation conditions (Table 1), possibly altering the level of trehalose-6-P (T6P), a regulator of plant carbohydrate metabolism, growth and development (Ramon and Rolland 2007). [Au8]

While Snf1 mediates the switch from fermentation to respiration in yeast and AMPK stimulates mitochondrial biogenesis in muscle, KIN10 expression appears to downregulate respiration in mesophyll protoplasts with repression of several mitochondrial electron transport and TCA cycle genes. This contrasting regulation of conserved genes in different systems may be explained by the unique role of leaf cells as the main energy suppliers from photosynthesis in plants. Leaf cells may lack external carbon sources to promote respiration and the TCA cycle and need to stimulate a global metabolic switch for an alternative energy supply under starvation conditions. However, similar to the situation in yeast and liver cells (DeRisi et al. 1999; Kahn et al. 2005), starvation might stimulate [Au9,10] gluconeogenesis in leaf cells through transcriptional upregulation of the rate-limiting anaplerotic enzymes fructose 1,6-bisphosphatase (FBPase) and phosphoenol pyruvate (PEP) carboxykinase (PEPCK). Conversely, expression of glycolytic enzymes such as glyceraldehyde 3-P dehydrogenase (GAPDH), PEP carboxylase (PEPCase) and phosphofructokinase (PFK) was downregulated by KIN10 expression in protoplasts (Table 1). AMPK activity, however, represses gluconeogenic gene expression in liver cells and stimulates glucose uptake and glycolytic flux in muscle cells (Kahn et al. 2005). Direct comparison between different experimental systems and organs is complicated

by the fact that the specific responses likely depend on the severity of the starvation condition (requiring mere 'balancing' of metabolism, e.g. for blood glucose homeostasis, or a more dramatic switch to 'survival' mode), or the availability of alternative carbon sources and the specific functions of the cell type or tissue under study. The coordinated induction of *PEPCK*, pyruvate phosphate dikinase (*PPDK*) and pyruvate kinase *(PK)*, together with glutamate dehydrogenase (*GDH*) and asparagine synthetase (*DIN6/ASN1*) may be related to a novel cycle proposed to generate asparagine for more energy-economical nitrogen remobilization under darkness, stress and starvation conditions (Table 1) (Lam et al. 1996; Lin and Wu 2004).

Remarkably, KIN10 activity induced expression of several *APG8/ATG8* (*AUTOPHAGY*) orthologs, whose induction also coincided with vacuolar autophagy triggered by sucrose starvation in *Arabidopsis* suspension culture cells (Contento et al. 2004). The APG8/12 conjugation pathways are conserved in plants and may have important roles in autophagic recycling during situations that require substantial nutrient mobilization (Thompson and Vierstra 2005). Other SnRK1 target genes included a large number of putative histone and histone deacetylase genes (Fig. 2a). KIN10 target genes include also highly regulated transcription factors (TFs), as well as chromatin proteins, photoreceptors, and signalling components that can amplify the regulatory impact through transcriptional and signalling cascades. Finally, global inhibition of protein synthesis, a conserved starvation response, is reflected by massive repression of RNA metabolism and ribosomal protein gene expression (Fig. 2a).

To establish a definitive molecular and quantitative link between KIN10/11 action and the ability to mount transcription activation in response to stress and energy deprivation, we examined the response of WT and *KIN10/11* silenced plant leaves to a 10 h dark treatment. As shown in Fig. 2b, transcriptional activation by darkness of a set of KIN10 marker genes was abolished in *KIN10/11* silenced plants. Impairment of marker gene induction was also observed in response to hypoxia and DCMU treatments (Baena-González et al. 2007).

Conclusion

The wealth of the whole-genome microarray data available for *Arabidopsis* offers new tools to examine the stress-specific and convergent transcriptomes triggered by multiple stress conditions. Applying integrative approaches, our studies have identified KIN10/11 as key regulators of the convergent stress responses, and established a molecular link between the cellular energy status and the response and tolerance to stress. Identification of common regulatory components may provide further understanding on the mechanisms of crosstalk between different types of stress and on the molecular basis of stress cross-protection. We have begun dissecting the components of the KIN10/11 signalling cascade through the identification of key TFs and common *cis*-elements using cellular and genomic tools (Fig. 3) (Baena-González et al. 2007). By integrating the new tools with the characterisation of gain- and loss-of-function mutants, we have provided compelling evidence not only for the involvement of KIN10/11 in plant protection and survival under stress, darkness and sugar deprivation conditions, but also for unexpected pivotal roles of KIN10/11 in growth and development under normal conditions (Baena-González et al. 2007).

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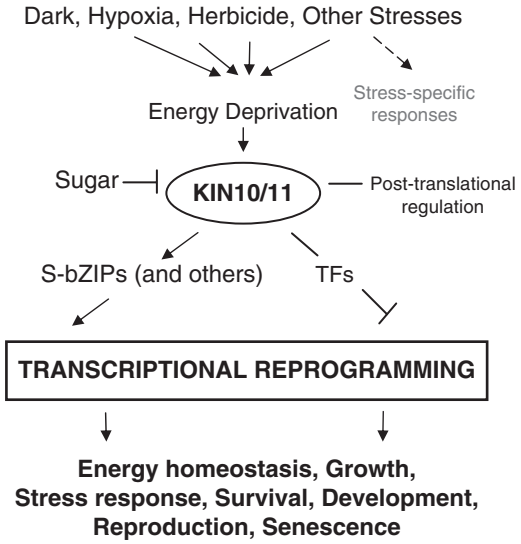

Fig. 3 KIN10/11 are central integrators of sugar, metabolic, stress, and developmental signals. Multiple types of stress ultimately converge as an energy-deficiency signal in the cell, triggering the activation of KIN10/11. Conversely, sugars have a repressive effect. Activated KIN10/11 initiates an energy-saving program at several levels, including massive transcriptional reprogramming that targets a wide range of cellular processes. This is partly mediated by the S-class of bZIP TFs. In addition to contributing to the maintenance of cellular energy homeostasis and tolerance to (nutrient) stress, KIN10/11 have profound effects at the whole organism level influencing growth, viability, reproduction and senescence and are thus proposed to be central in the integration of metabolic, stress and developmental signals

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