The interface between metabolic and stress signalling

Sandra J. Hey, Edward Byrne and Nigel G. Halford*

Plant Science Department, Rothamsted Research, Harpenden, Hertfordshire AL5 2JQ, UK
* For correspondence. E-mail nigel.halford@bbsrc.ac.uk

INTRODUCTION
The ability of plants to tolerate herbivory, pathogen attack and abiotic stresses such as drought, cold, heat and osmotic stresses is a key factor in determining crop yield and quality. Cold, heat and water limitation all cause osmotic stress, so they have much in common with each other and with direct osmotic stress caused by, for example, exposure to high salt concentrations. Not surprisingly, therefore, the hormone abscisic acid (ABA), which is particularly associated with cellular osmotic stress, is also a common factor in mediating cold, heat and water stress responses. It is becoming clear that another common factor is metabolic regulation, partly perhaps because plants modify the balance between soluble and insoluble compounds in the cell in order to cope with osmotic stress. It is not surprising, therefore, that metabolic and stress signalling pathways interact and cross-talk, and a full understanding of plant stress responses will not be achieved without elucidation of the signalling networks that they form. The present briefing reviews what is known about the signalling factors that lie at the interface between metabolic and stress signalling.

LINKS BETWEEN ABA, STRESS AND SUGAR SIGNALLING

The first evidence of links between ABA, stress and sugar signalling pathways came from screens carried out by several independent groups to identify Arabidopsis mutants that were impaired in their response to sugar (sugar response mutants). Several of the mutants identified in these screens turned out to be ABA-related (reviewed by Halford, 2006). The discovery of these mutants led to the proposal of several hypotheses concerning cross-talk between ABA and metabolic signalling pathways. One proposed that sugar signalling could be directly mediated by ABA, a second that ABA could modulate sugar signalling by priming tissues to respond to sugars, and a third that ABA and sugar signalling, although essentially separate, could converge and cross-talk through specific factors. The identification of such factors became a key target because they would be expected to be involved in the control of developmental events such as germination that are sensitive to both ABA and sugars.

Further evidence of cross-talk between stress and sugar signalling pathways came with evidence that the protein kinase SnRK1 (sucrose non-fermenting-1-related protein kinase-1), a key metabolic regulator, is involved in stress signalling. SnRK1 regulates carbon metabolism through the modulation of enzyme activity and gene expression (Halford, 2006). It is closely related to the metabolic regulators 5′-AMP-activated protein kinase (AMPK) of mammals and protein kinase sucrose nonfermenting-1 (SNF1) of yeast (Saccharomyces cerevisiae), sharing 47 % amino acid sequence identity and showing similar substrate specificity. SnRK1 channels...
carbon into the starch biosynthetic pathway in storage organs through modulation of sucrose synthase and ADP-glucose pyrophosphorylase gene expression (McKibbin et al., 2006) and, in the case of ADP-glucose pyrophosphorylase, redox activation (Tissien et al., 2003). Paradoxically, it is also required for the expression of α-Amy2 (α-amylase), a sugar-repressed gene that is involved in starch breakdown (Laurie et al., 2003). SnRK1 is involved in the re-allocation of carbon in response to herbivory (Schwachtje et al., 2006) and evidence from transcriptomic studies suggests that it is involved in a range of stress and darkness responses (Baena-González et al., 2007).

The metabolite or metabolites that are sensed by the SnRK1 system have been difficult to identify. However, glucose 6-phosphate and, more recently, trehalose 6-phosphate have been shown to inhibit SnRK1 (Toroser et al., 2000; Zhang et al., 2009), with trehalose 6-phosphate the more potent inhibitor of the two but requiring a hitherto unidentified intermediary factor to be present for inhibition to occur.

Evidence that ABA response element binding proteins are hubs linking metabolic and stress signalling pathways

One possible route by which SnRK1 could affect stress response pathways is through ABA response element binding proteins (AREBPs), a family of bZIP transcription factors. AREBPs contain highly conserved SnRK1 target sites, and peptides with amino acid sequences based on these sites are very good substrates for phosphorylation by SnRK1 (Zhang et al., 2008a).

AREBPs are unique to plants, so this activity for SnRK1 has evolved during plant evolution. Sub-families of protein kinases related to SnRK1 have also emerged during plant evolution: these are SnRK2 and SnRK3 (Halford and Hey, 2009), which are large and relatively diverse compared with SnRK1, with ten and 25 members, respectively, in Arabidopsis. There is now compelling evidence that members of the SnRK2 and SnRK3 sub-families of protein kinases are involved in ABA-mediated and other signalling pathways that regulate plant responses to drought, cold, salt and osmotic stresses. For example, an SnRK2-type protein kinase in Arabidopsis has been shown to control stress-responsive gene expression and improve drought tolerance when over-expressed (Umezawa et al., 2004), whilst expression of the entire SnRK2 sub-family of rice (ten members) is induced by osmotic stress and three of the sub-family members are also induced by ABA (Kobayashi et al., 2004). Similarly, PKABA1, an SnRK2 from wheat, mediates ABA-induced changes in gene expression in response to cold and other stresses (Gómez-Cadenas et al., 1999). Like SnRK1, SnRK2-type protein kinases have been shown to phosphorylate AREBPs (Kobayashi et al., 2005; Furihata et al., 2006).

SnRKs are closely related to calcium-dependent protein kinases (CDPKs); indeed, the SnRK3-type protein kinases are themselves likely to be calcium-dependent because they interact with calcineurin B-like (CBL) calcium-binding proteins (Guo et al., 2002). For this reason SnRK3s are also known as CBL-interacting protein kinases (CIPKs). The involvement of Ca²⁺ signalling in mediating responses to abiotic stress has been known and studied for many years, and several SnRK3-type protein kinases have been implicated in stress responses, although their substrates have not yet been identified. One of the 25 SnRK3-encoding genes of Arabidopsis [SnRK3-11, also known as CIPK24, and Salt Overly Sensitive 2 (SOS2)] is involved in conferring salt tolerance (Liu et al., 2000). Another SnRK3 (called SnRK3-1, CIPK15 or PKS3) functions as a global regulator of ABA responses, forming part of a calcium-responsive negative regulatory loop controlling ABA sensitivity (Guo et al., 2002). It is possible that SnRK3-type protein kinases phosphorylate AREBPs because a calcium-dependent activity from stressed Arabidopsis seedlings phosphorylates the same AREBP-based peptides that SnRK1 phosphorylates (Zhang et al., 2008a). This would make AREBPs potential convergence points for multiple signalling pathways (Fig. 1): hubs within an intricate signalling network (Halford and Hey, 2009).

SnRK3s have also been shown to interact with the type 2C protein phosphatases, abscisic acid insensitive-1 and -2 (ABI1 and ABI2), via a protein phosphatase interaction (PPI) domain in the regulatory region of the protein kinases and a protein kinase interaction (PKI) domain in the phosphatases (Guo et al., 2002; Ohta et al., 2003). Furthermore, part of the regulatory domain of SnRK3-11 is similar to that of the DNA repair and replication checkpoint protein kinase, CHK1, which also contains this conserved PPI domain (Ohta et al., 2003). CHK1 is required for cell cycle arrest in response to DNA damage and it has been reported that SnRK3-11 mutants show cell-cycle defects at the root meristem when subjected to salt stress (Liu et al., 2000). There is evidence to support a role for SnRK1 in cell-cycle control (Francis and Halford, 2006), and it is intriguing to consider the possibility that SnRK3s are also conduits for cross-talk between metabolic, stress and cell-cycle signalling.

SnRK2s and SnRK3s have diverged further from SNF1 and AMPK than has SnRK1. It is possible that they emerged as a result of duplication of a progenitor gene in ancestral eukaryotes before the divergence of plants, animals and fungi but were subsequently lost during animal and fungal evolution. However, we favour a hypothesis that they emerged only during plant evolution but then evolved rapidly as they took on new roles to enable plants to link metabolic and stress signalling.

CDPKs as conduits for cross-talk between metabolic and stress signalling

‘Classical’ CDPKs are also implicated in stress and ABA signalling (Hrabak et al., 2003). These protein kinases contain a calcium-binding, auto-regulatory domain, so do not require the interaction of a separate calcium-binding protein in the way that SnRK3 does. Binding of calcium ions to this domain increases kinase activity. Many CDPK genes themselves contain an ABA-response element in their promoter and, therefore not surprisingly, some have been shown to be induced at the transcriptional level by ABA.

SnRK1 and CDPKs have similar phosphorylation site requirements, with the minimal recognition motif for both generally given as a serine or threonine residue (SnRK1 much
prefers serine) with a hydrophobic residue at position –5 and +4 with respect to the serine or threonine and a basic residue at –3 or (less favourably) –4 (Sugden et al., 1999b; Huang and Huber, 2001). However, a proline residue at position –4 selectively inhibits phosphorylation by CDPK relative to SnRK1, possibly by incorporating a tight turn in the substrate (Huang and Huber, 2001). This proline residue may also be involved in the recruitment of 14-3-3 proteins to the phosphorylation site, a prerequisite for regulation of some enzymes by SnRK1. The similarity in substrate specificities of SnRK1 and CDPK suggests that cross-talk could occur in both directions. In other words, CDPKs, which are usually associated with stress responses, could initiate signals that affect metabolic regulation, just as the metabolic regulator, SnRK1, has links with stress signalling factors. However, we are not aware of experimental evidence to support this hypothesis at present.

Evidence that biotic challenges initiate signals through metabolic signalling pathways

SnRK1, like its animal and fungal counterparts, AMPK and SNF1, is regulated in part by phosphorylation of a threonine residue in its so-called T-loop (Sugden et al., 1999a). Upstream kinases responsible for this activation remained elusive for many years, but two have been identified recently (Hey et al., 2007; Shen and Hanley-Bowdoin, 2006; Shen et al., 2009). These have been called SnRK1-activating kinase-1 and -2 (SnAK1 and SnAK2) (Hey et al., 2007) or geminivirus rep-interacting kinases (GRIK1 and GRIK2) (Shen and Hanley-Bowdoin, 2006). The GRIK name derives from the fact that they are expressed in response to geminivirus infection and interact with geminivirus replication protein AL1 (Shen and Hanley-Bowdoin 2006), suggesting that metabolic signalling pathways could respond to pathogen attack. They are also expressed in young tissues, and it has been suggested that they initiate metabolic responses to meet the demands of rapidly growing tissues and geminivirus-infected cells that have been induced to re-enter the cell cycle (Shen et al., 2009).

THE INTERFACE BETWEEN STRESS AND NITROGEN/AMINO ACID SIGNALLING

General regulation of the level of free amino acids and protein synthesis in all eukaryotic cells is achieved through the action of a group of regulatory protein kinases that bring about the reversible phosphorylation of the α-subunit of the eukaryotic translation initiation factor eIF2 (eIF2α). Four different eIF2α kinases are known to be able to phosphorylate eIF2α and thus to regulate protein synthesis in response to free amino acid levels and environmental stresses in this manner (reviewed by Halford, 2006). These are: double-stranded RNA-dependent protein kinase (PKR), PKR-like endoplasmic reticulum kinase (PERK), haem-regulated inhibitor (HRI) and...
general control non-derepressible-2 (GCN2). All four of these related eIF2α kinases contain a highly conserved kinase catalytic domain linked to a unique regulatory domain, and it is the regulatory domain that confers the ability of each eIF2α kinase to respond to a different, specific stimulus.

The distribution of eIF2α kinases varies within different groups of organisms; mammals, for example, contain all four different eIF2α protein kinases, whereas fungi and plants only contain one, namely GCN2, although there remains a degree of mystery over the existence of a PKR-like activity in plants that is discussed later.

GCN2 was originally characterized in yeast, where it functions to sense and respond to amino acid starvation. In this organism, amino acid starvation not only causes a general reduction in protein synthesis but also initiates a change in expression of a large number of genes involved in, amongst other things, amino acid biosynthesis; this response is known as general amino acid control (Hinnebusch, 2005). During general amino acid control, GCN2 physically senses the reduction of cellular amino acids through the interaction of uncharged tRNA with its regulatory domain, and is activated to phosphorylate eIF2α. eIF2 can bind either GDP or GTP, but only when bound to GTP is it able to attach methionyl-tRNA to the ribosome and transfer it to the 40S ribosomal subunit. Following attachment of the [eIF2.GTP.Met-tRNA] complex to the 40S subunit, the GTP is hydrolysed to GDP and inorganic phosphate. Phosphorylation of eIF2α inhibits the recycling of bound GDP to GTP and the rate of protein synthesis decreases.

There is a second aspect of general control of amino acid metabolism in yeast: GCN2-mediated phosphorylation of eIF2α under conditions of amino acid deprivation increases expression of a large set of genes, including many encoding enzymes of amino acid biosynthesis, through the action of a transcriptional activator, GCN4 (Hinnebusch, 2005). While phosphorylation of eIF2α by GCN2 represses translation of most transcripts, translation of the GCN4 transcript is increased through a ribosome re-initiation mechanism. This apparent paradox enables the de-novo production of amino acids during periods of starvation, thus helping the yeast cell to maintain homeostasis and survive during adverse conditions.

The molecular cloning of an Arabidopsis homologue of GCN2 (AtGCN2) was reported by Zhang et al. (2003). Recent experiments demonstrated that AtGCN2 does indeed function as an eIF2α kinase, phosphorylating Arabidopsis eIF2α at serine-52 in response to, for example, treatment with herbicides such as glyphosate, chlorsulfuron or IRL 1803 (Zhang et al., 2008b). This supports the hypothesis that general amino acid control is conserved, at least in part, in plants. However, no obvious candidate for a GCN4 homologue has been identified in plants, despite the extensive genome data now available (Halford, 2006). Furthermore, although genes encoding enzymes of amino acid biosynthesis in plants do respond to treatments that perturb amino acid metabolism, they appear to respond in similar fashion whether GCN2 is present or not (Zhang et al., 2008b).

It is possible that GCN2-dependent phosphorylation of eIF2α is involved in the regulation of expression of amino acid biosynthesis genes in plants but that other regulatory systems are able to compensate for GCN2 when GCN2 is not present. However, given that no plant GCN4 homologue has been identified, on balance the evidence to date suggests that the regulation of expression of genes of amino acid biosynthesis and other systems through a GCN4-like transcription factor does not occur in plants. Nevertheless, regulation of the translation of GCN4 mRNA in response to general amino acid control in yeast requires the presence of short open reading frames in the GCN4 transcript, upstream of the GCN4 coding sequence, and many plant genes contain similar upstream open reading frames (uORFs). These genes include a number that encode transcription factors (Kawaguchi and Bailey-Serres, 2005) and it is possible that, although these transcription factors do not have the same function as GCN4 in yeast, they are regulated in a similar fashion. Furthermore, promoter elements similar to that recognized by GCN4 are present in some plant genes, notably the prolamin storage protein genes of cereals in which they act as positive elements when nitrogen is in plentiful supply and negative elements when nitrogen is limiting.

**GCN2-mediated phosphorylation of eIF2α as a general stress response**

Studies in fungal and mammalian systems suggest that GCN2 may be activated and protein synthesis inhibited in response to stresses in addition to that of amino acid deprivation, including purine deprivation, exposure to UV-B, oxidative and osmotic stress, and glucose deprivation (Yang et al., 2000; Hinnebusch, 2005; Mascarenhas et al., 2008). In a recent study, the effect of some of these and other stresses on GCN2-dependent eIF2α phosphorylation was studied in Arabidopsis (Lageix et al., 2008): UV-B, cold-shock, wounding, methyl jasmonate, 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid, and the purine analogue 8-azaadenine all caused GCN2-dependent phosphorylation of eIF2α, while treatment with salt, hydrogen peroxide or heat-shock did not. Methyl jasmonate and salicylic acid are signals of tissue injury and are involved in the activation of defence mechanisms in response to insect herbivores, so GCN2, like SnRK1, could be involved in the response to herbivory.

**Links between carbon metabolic signalling and nitrogen/amino acid signalling**

In plants, expression of many nitrogen-regulated genes is strongly influenced by sugar, suggesting an extensive interaction between sugar and nitrogen signalling pathways (Price et al., 2004). Such cross-talk seems logical because amino acids are, of course, based on carbon skeletons. SnRK1 may be known principally for its role in regulating carbon metabolism, but there are also routes through which it could affect nitrogen and amino acid metabolism (Fig. 2). Most obviously, nitrate reductase, the key enzyme for assimilation of inorganic nitrogen, is a substrate for SnRK1 in vitro (reviewed by Halford, 2006) and a recent study has provided strong evidence that it is regulated in part by SnRK1 in vivo (Polge et al., 2008). A CDPK has also been shown to phosphorylate nitrate reductase at the same target site (serine-543 in the
spinach enzyme) (Douglas et al., 1998), and there is evidence that expression of a nitrate reductase gene, NIA1, of Arabidopsis is affected by GCN2 (its expression is reduced in a mutant lacking GCN2) (Zhang et al., 2008b), making nitrate reductase another convergence point for multiple signalling pathways (Fig. 2).

Another route through which SnRK1 could affect nitrogen metabolism is through the regulation of asparagine synthetase gene expression: normal expression of one of the two SnRK1 genes of Arabidopsis has been shown to be required for sugar- and dark-responsive expression of an asparagine synthetase gene (Baena-González et al., 2007). Asparagine is required for protein synthesis, like any other amino acid, but free asparagine accumulates in response to a variety of abiotic and biotic stresses and to deficiencies in minerals, particularly sulphur, possibly acting as a nitrogen store when protein synthesis is impaired (Lea et al., 2007).

In other eukaryotic systems, GCN2 itself is a convergence point for carbon and nitrogen metabolic signalling pathways. In yeast, glucose limitation leads to an increase in GCN4 levels through the action of GCN2. This process is completely independent of the amino acid deprivation response (Yang et al. 2000) and may involve TOR (target of rapamycin), a protein kinase that acts as a central regulator of cell growth in yeast in response to nutrient and growth factors (Schmelzle and Hall, 2000). In mammals, the SnRK1 homologue AMPK negatively regulates TOR (Avruch et al., 2006) and although more research is required in both yeast and mammalian systems to fill in gaps in the signalling pathways, this may be the route through which carbon metabolic signalling interacts with nitrogen/amino acid signalling in these systems.

A TOR homologue (AtTOR) has been identified in Arabidopsis and implicated in a number of plant processes including embryo development, meristem growth, osmotic stress responses and mRNA translation (Menand et al., 2002). Silencing AtTOR by RNA interference (RNAi) leads to an inhibition of translation initiation, with severe effects on shoot and root growth, while over-expressing it has the opposite effect (Deprost et al., 2007). TOR RNAi and over-expressing lines respond to herbicide-induced amino acid depletion in similar fashion, suggesting that TOR is not involved in the response to free amino acid depletion in Arabidopsis (Lageix et al., 2008). However, this does not mean that TOR and GCN2 do not interact in the response to other stimuli.

**Phosphorylation of eIF2α in response to viral infection**

AtGCN2 is the only eIF2α kinase-encoding gene in the Arabidopsis and rice genomes and it is present as a single copy (Halford, 2006). Screening of expressed sequence tag
P58IPK is switched off, PKR dimerizes and becomes active, allowing protein synthesis to proceed normally. Under virus attack, PKR is suppressed, thereby allowing the eIF2α-dependent protein kinase (PKR), which has been reported in plants. In humans and other mammals, PKR is activated in response to viral infection, and a PKR-like activity (pPKR) was reported to be induced by viral infection of plants (Crump et al., 1988). Further, a plant homologue of another protein kinase in the animal viral response pathway that involves PKR, PS8IPK, has been identified (Bilgin et al., 2003). In animal cell cultures, PS8IPK is active under normal conditions, suppressing PKR activity and thereby allowing protein synthesis to proceed normally. Under virus attack, PS8IPK is switched off, PKR dimerizes and becomes active, eIF2α is phosphorylated and protein synthesis is shut down, leading eventually to programmed cell death (Lee et al., 1994).

One possible explanation of this was that the PKR and GCN2 activities had been consolidated in plants in a single protein kinase. However, in a recent study, phosphorylation of eIF2α could not be demonstrated in Arabidopsis plants infected with either Turnip crinkle virus or Turnip yellow mosaic virus, regardless of the presence or absence of AtGCN2 (Zhang et al., 2008b). The protein kinase responsible for the PKR-like activity reported in the earlier studies has therefore still to be identified, and the role of plant PS8IPK requires further investigation.

CONCLUDING REMARKS

The hypothesis that signalling pathways often comprise multiple phosphorylation/dephosphorylation steps in order to allow amplification of a signal as it is passed down a protein kinase cascade has been replaced by one in which multiple steps in pathways have evolved to enable linking between pathways to form networks (reviewed by Halford and Hey, 2009). In these networks, key protein kinases, phosphatases and target transcription factors represent hubs on/from which multiple pathways converge and emerge. In this review we have described conduits through which carbon and nitrogen metabolic signalling networks can cross-talk with each other and with stress signalling networks. This is particularly pertinent in plants because plants are sedentary organisms that have to cope with stresses where they stand and have evolved sophisticated cellular response systems in order to do so. These responses often involve changes in metabolism.

Global climate change is predicted to change the nature and increase the severity of stresses that crops are exposed to, and lead to new pressures from weeds, pests and diseases. In the next few decades in high latitudes, for example, crops that have to tolerate cold stress in the early stages of their development may be faced with heat, water and osmotic stresses over the summer months that are much more severe than anything that they experience at present (Semenov and Halford, 2009). Elucidating and understanding metabolic and stress signalling networks and how they interact is therefore likely to become even more important as global climate change progresses.

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