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REVIEW PAPER

The sucrose non-fermenting-1-related (SnRK) family of protein kinases: potential for manipulation to improve stress tolerance and increase yield

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Abstract

Sucrose non-fermenting-1 (SNF1)-related protein kinases (SnRKs) take their name from their fungal homologue, SNF1, a global regulator of carbon metabolism. The plant family has burgeoned to comprise 38 members which can be subdivided into three sub-families: SnRK1, SnRK2, and SnRK3. There is now good evidence that this has occurred to allow plants to link metabolic and stress signalling in a way that does not occur in other organisms. The role of SnRKs, focusing in particular on abscisic acid-induced signalling pathways, salinity tolerance, responses to nutritional stress and disease, and the regulation of carbon metabolism and, therefore, yield, is reviewed here. The key role that SnRKs play at the interface between metabolic and stress signalling make them potential candidates for manipulation to improve crop performance in extreme environments.

Key words: ABA, biotic stress, carbon metabolism, crop yield, plant nutrition, salt tolerance, signalling, stress.

Introduction

Due to the close and direct dependence upon the land and agriculture of much of the African population, it is likely that changes in weather patterns and climate will be keenly felt by many on the continent. The Intergovernmental Panel on Climate Change (IPCC), in their report of 2007 on Regional Climatic Projections (Christensen *et al.*, 2007), predicted that climate change will impact heavily on Africa over the coming century. It concludes that all of Africa is very likely to get warmer during this century, with the drier subtropical regions warming more than the moister tropics. In the west, east, south, and Saharan sub-regions, the projected median temperature increase in the 100 years between the 1980–1999 and 2080–2099 20-year averages, lies between 3 °C and 4 °C, roughly 1.5 times the global mean increase. Smaller increases, near 3 °C, are predicted in the equatorial and coastal areas, but larger increases, above 4 °C, are predicted for the Western Sahara. Annual rainfall is predicted to decrease in much of Mediterranean Africa, the Northern Sahara, and the winter rainfall region and western margins of Southern Africa, although increases in rainfall are predicted for East Africa. In addition, a general increase is predicted in the intensity of high rainfall events, in conjunction with a decrease in the number of rain days in regions of mean drying (Christensen *et al.*, 2007).

Decreasing rainfall and rising temperatures will increase both the requirement for irrigation and the rates of evapotranspiration, in turn exacerbating the risk of soil salination. Salts from precipitation and irrigation water remain in the soil and accumulate in the root zone when water evaporates from the soil or is taken up by the crop. If the salt is not leached from the soil by a sufficient flow of water, either from precipitation or by the application of excess irrigation water, the concentration increases until it reduces crop yields by impeding water uptake by the plant. Conversely, the more frequent and severe flooding events predicted for parts of East Africa could potentially lead to

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increased hypoxia of crop roots. These additional challenges will further compound those already faced by African agriculture, such as the often poor nutrient status of its older soils.

In the face of these challenges, there is clearly scope for research into developing crops with improved resistance to nutrient limitation, drought, heat, salt, and flooding, to be exploited by African agriculture. As a further consequence of predicted climatic changes, it is also to be expected that crop diseases and insect pests may be able to spread to regions where they have not posed a serious problem in the past and where traditional agricultural practices may not be best suited to protect against them. Development of crops with improved disease resistance could offer a degree of protection from yield losses under these circumstances. Development of crops better able to tolerate adverse conditions would be of particular value in places like Africa where resources to ameliorate the environment, such as fertilizers, irrigation water or pesticides, are often difficult or uneconomic to access given the farming practices frequently employed.

Many of the reductions in yield that occur in response to stress are evolutionary adaptations that enable the plant to conserve energy and resources in the expectation of better future conditions either for itself or its offspring, thereby increasing its long-term chances of survival. In the artificial conditions imposed by agriculture, depending on the particular crop product being produced, long-term survival strategies may be less important than the crop's value to the farmer. It is therefore likely that potential exists for manipulating plant responses to stress to improve yields significantly under adverse conditions.

Interactions between metabolic and stress signalling networks

Recent developments in plant cell signalling have highlighted intricate interconnections between metabolic regulation and stress signalling systems which could enable the development of crops better able to resist and adjust to environmental stresses, whilst maintaining yields. High salt concentrations, drought, and heat stress in plants have much in common. All lead to cellular osmotic stress and are associated with increases in the concentration of the plant hormone, abscisic acid (ABA), which acts as a trigger for a number of physiological responses that can help to ameliorate the effects of stress on the plant. It is becoming clear that metabolic regulation is yet another common factor linking these stresses. This is, perhaps, not surprizing, because plants modify the balance between soluble and insoluble compounds in the cell in order to cope with osmotic stress. Recently, considerable developments have been made in elucidating the signalling networks that have formed to enable metabolic and stress signalling pathways to interact and cross-talk (Hey *et al.*, 2010).

Early evidence of links between ABA, stress, and metabolic signalling pathways materialized when several mutant plants that were identified in screens for impairment in response to sugar, turned out also to be ABA signalling mutants (reviewed by Halford, 2006). This has since been further supported by evidence that the key metabolic regulator SnRK1 (sucrose non-fermenting-1-related protein kinase-1) is involved in stress signalling. SnRK1 regulates carbon metabolism, both through the modulation of enzyme activity by direct phosphorylation or redox activation of metabolic enzymes, and through the regulation of gene expression (reviewed by Halford and Hey, 2009). The enzymes that are known to be directly phosphorylated and inactivated by SnRK1 are 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase: sterol biosynthesis), sucrose phosphate synthase (SPS: sucrose synthesis), nitrate reductase (NR: nitrogen assimilation), trehalose-phosphate synthase (TPS: desiccation tolerance; signalling), and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (F2KP: signalling; photosynthate partitioning). Note that in the case of NR, TPS, and F2KP, inactivation also requires the binding of a 14-3-3 protein. Another key enzyme in carbon metabolism, ADP-glucose pyrophosphorylase, is regulated by SnRK1 through both modulation of its redox state and gene expression, while sucrose synthase, α-amylase, and sugar-repressed/dark-induced asparagine synthetase are all regulated by SnRK1 at the level of gene expression. Furthermore, a recent transcriptomics study has shown that SnRK1 is involved in responses to sugar, darkness, and a range of stresses that limit photosynthesis and respiration, including herbicide [(3,4-dichlorophenyl)-1,1-dimethylurea, (DCMU)] treatment and flooding/hypoxia (Baena-Gonzáles *et al.*, 2007).

SnRK1 is closely related to the metabolic regulators of mammals (5'-AMP-activated protein kinase, AMPK) and yeast (sucrose non-fermenting-1, SNF1), with which it shares about 47% amino acid sequence identity and similar substrate specificity. However, in plants, the SnRK family of protein kinases has proliferated, with the sub-families SnRK2 and SnRK3 having emerged during plant evolution since the divergence of plants, animals, and fungi. Unlike SnRK1, which in *Arabidopsis* has only two active representatives, these sub-families are large and diverse with 10 and 25 members, respectively, in *Arabidopsis* (Halford and Hey, 2009). SnRK2s and 3s have diverged further from AMPK and SNF1 than have SnRK1s; it is suggested that they emerged as a result of duplication and then evolved rapidly, taking on new roles to enable plants to link metabolic and stress signalling (Halford and Hey, 2009).

Compelling evidence suggests that members of the SnRK2 and SnRK3 sub-families are involved in signalling pathways that regulate plant responses to nutrient limitation, drought, cold, salt, and osmotic stresses. Expression of the entire SnRK2 sub-family of rice (10 members), for example, is induced by osmotic stress, and, of these, three are also induced by ABA (Kobayashi *et al.*, 2004). A SnRK2 in *Arabidopsis* has also been shown to control stress responsive gene expression and improve drought tolerance when over-expressed (Umezawa *et al.*, 2004), while PKABA1, a SnRK2 from wheat, mediates ABA-induced

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changes in gene expression in response to cold and other stresses (Gómez-Cadenas *et al.*, 1999). Very recently, a central role for SnRK2 in ABA signalling has been discovered with the identification of PYR/PYL/RCAR proteins as ABA receptors and elucidation of the ABA signalling pathway. PYR/PYL/RCAR proteins bind to ABA and inhibit the activity of phosphatase PP2Cs encoded by *ABSCISIC ACID-INSENSITIVE 1* and *2* (*ABI1* and *ABI2*) (Leung *et al.*, 1997). In the absence of ABA, these PP2Cs dephosphorylate and inactivate SnRK2, the phosphorylation of which is required to activate downstream transcription factors (ABF) (Ma *et al.*, 2009; Park *et al.*, 2009; Sheard and Zheng, 2009; Umezawa *et al.*, 2009). *Arabidopsis* mutants defective in three SnRK2s, SnRK2.2, 2.3, and 2.6, have been shown to be almost completely insensitive to ABA (Fujii and Zhu, 2009).

Several of the SnRK3-type protein kinases have also been implicated in stress responses. For example, one of the SnRK3s of *Arabidopsis* [SnRK3.11 or Salt Overly Sensitive 2 (SOS2)], is involved in conferring salt tolerance (Liu *et al.*, 2000), whilst another (SnRK3.1) functions as a global regulator of ABA responses in a calcium-responsive negative regulatory loop controlling ABA sensitivity (Guo *et al.*, 2002). SnRK3s are thought to be calcium-dependent because they interact with calcineurin B-like (CBL) calcium-binding proteins (Guo *et al.*, 2002) and for this reason are also known as CBL-interacting kinases (CIPKs) (reviewed by Luan, 2009). The involvement of Ca^{2+} signalling in mediating responses to abiotic stresses, and particularly those mediated by ABA, is well known.

Involvement of SnRKs in salinity tolerance

Salinity has already become a serious risk to agricultural production, limiting plant growth and productivity worldwide. Salinity in the crop root zone is likely in drying areas of Africa because of the predicted reductions in rainfall and increased evapotranspiration caused by lower humidity (reviewed by Semenov and Halford, 2009). This will lead to reduced water uptake, with concomitant effects on growth and crop yield. It is also likely to activate SnRK stress signalling, potentially affecting carbon allocation. Sodium ions (Na^+) are cytotoxic to plants when they accumulate to high concentrations, causing an ionic stress resulting from a solute imbalance and an osmotic stress resulting from reduced water availability (Silva and Gerós, 2009). Plant responses involve changes in the expression of multiple genes, some of which are considered to be part of the general stress response, others of which are regulated specifically in response to high salinity (Chinnusamy *et al.*, 2004). High salinity produces dehydration, but dehydration can also be caused by drought and low temperatures, so some of the responses are common to these different abiotic stresses (Boudsocq and Lauriere, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). A critical component of the salt stress response is the maintenance of ion homeostasis.

The role of SnRK3-type protein kinases in salt-stress responses

To prevent Na^+ accumulation in the cytoplasm, plants can reduce its entry into cells, activate its efflux from cells or compartmentalize it in the vacuole (Bertorello and Zhu, 2009). In *Arabidopsis*, ion homeostasis is mediated by the Salt Overly Sensitive (SOS) signalling pathway, which consists of three main components: SOS1, which is a Na^+/H^+ antiporter that effluxes excess ions out of the cytosol; SOS2, which is a SnRK3-type protein kinase, and SOS3, which is a Ca^{2+} sensor protein with an N-terminal myristoylation site and four Ca^{2+}-binding EF hands (helix–loop–helix structures) (Sanchez-Barrena *et al.*, 2005; Mahajan *et al.*, 2008). *SOS1* gene expression is up-regulated in response to salt stress and its over-expression improves salt tolerance in transgenic plants (Shi *et al.*, 2003; Yang *et al.*, 2009). By contrast, *sos1* mutants are extremely sensitive to high salt concentrations and accumulate more salt than wild-type plants (Qiu *et al.*, 2004). *sos2* and *sos3* mutants show a similar phenotype, suggesting that the three genes function in the same pathway (Mahajan *et al.*, 2008).

SOS2 has an N-terminal catalytic domain and a C-terminal regulatory domain, both of which are important for its function in salt tolerance. *In vitro* and *in vivo* experiments have shown that SOS2 directly interacts with SOS3, and that a 21-amino acid motif in the C-terminal regulatory domain (FISL motif) is sufficient for the interaction to occur (Guo *et al.*, 2001). Moreover, the FISL motif serves as an autoinhibitory sequence, keeping SOS2 in an inactive state (Guo *et al.*, 2001). Increases in Ca^{2+} concentration in response to salt stress can be detected by SOS3, which, in turn, binds to and activates the kinase domain of SOS2 by releasing the autoinhibitory effect of the FISL domain. Myristoylation of SOS3 enables the complex to associate with the cell membrane and lack of such a modification leads to failure of SOS3 to confer salt tolerance (Ishitani *et al.*, 2000). The active SOS2/SOS3 complex phosphorylates and activates the SOS1 Na^+/H^+ antiporter. Other substrates of SOS2 may include vacuolar Na^+/H^+ antiporters and H^+-ATPases (Silva and Gerós, 2009).

All 25 members of the *Arabidopsis* SnRK3 family have a C-terminal FISL motif and expression analysis of seven of them showed four to be up-regulated by Na^+, two to be repressed, and one to be unaffected, This differential regulation of expression was organ-specific. Furthermore, all seven protein kinases that were studied interacted with SOS3, although the interactions were weak compared with the SOS2–SOS3 interaction (Guo *et al.*, 2001; Batistic and Kudla, 2009). SOS3 is a member of a small family of calcium sensors (CBL, calcineurin-B like proteins). Besides the SOS2 (CIPK24)/SOS3 (CBL4) partners, other CBL/SnRK3 partners have been shown to be involved in salt responses. SOS2 can also interact with CBL10, and the complex seems to regulate the transport of Na^+ to the vacuole (Kim *et al.*, 2007). It has also been shown that SOS2 phosphorylates CBL10 and that, in so doing, it

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stabilizes the complex at the membrane, so it has been proposed that SOS1 could also be a target of the SOS2/CBL10 complex (Lin *et al.*, 2009). Interestingly, SOS3 is preferentially expressed in roots, whereas CBL10 is mainly expressed in shoots, so SOS2/SOS3 partners might function in conferring root salt tolerance whilst SOS2/CBL10 could confer shoot salt tolerance (Quan *et al.*, 2007; Bertorello and Zhu, 2009; Lin *et al.*, 2009). CBL2 interacts with and activates a SOS2-like protein kinase (PKS5), and this complex negatively regulates the activity of a plasma membrane H^+-ATPase (Fugslang *et al.*, 2007). CBL1 and CBL9 interact with CIPK23 and this complex increases the activity of AKT1, a plasma membrane K^+ channel (Lin *et al.*, 2009).

The SOS pathway for salt tolerance appears to be conserved in other plant species. In rice the *Sos1, Sos2*, *and Sos3* homologues have been identified and *OsSos2* and *OsSos3* also belong to small gene families, suggesting that some other SnRK3/CBL partners could also be involved in salt tolerance (Martinez-Atienza *et al.*, 2007). In maize, a SOS3 homologue, ZmCBL4, can complement a *sos3* mutant and reconstitute salt-tolerance responses (Wang *et al.*, 2007) whilst a *Sos2* homologue, ZmCIPK16 enhances salt tolerance when expressed in *Arabidopsis* (Zhao *et al.*, 2009).

Interconnection with other signalling networks can be inferred from the analysis of other interacting proteins. For example, interactions between SOS2 and members of the protein phosphatase 2C (PP2C) have been demonstrated using the yeast two-hybrid assay. Mutational analysis of the protein phosphatase interaction (PPI) domain, demonstrated that a region of 37 amino acids was sufficient and necessary for interaction with ABI2. The PPI domain was found to be conserved in all the SnRK3s that were analysed and differences in the interactions between the kinases and the *ABI1*- and *ABI2*-encoded PP2Cs were discovered (Ohta *et al.*, 2003). Presently, it is not known if the phosphatase is able to dephosphorylate the kinase or if the kinase can phosphorylate the phosphatase. However, what is known is that binding of the phosphatase at the PPI domain might prevent interaction with the CBL protein and vice versa (Sanchez-Barrena *et al.*, 2007). The functional relevance of this interaction clearly requires further study.

The role of SnRK2-type protein kinases in salt-stress responses

The SnRK2 family of *Arabidopsis* contains 10 members and, with the exception of SnRK2.9, all of them are activated by saline stress (Boudsocq *et al.*, 2004). Furthermore, over-expression of one member of the family, SnRK2.8, has been shown to increase drought tolerance and up-regulate stress-induced genes (Umezawa *et al.*, 2004). Activation of SnRK2 in response to hyperosmotic treatments depends on phosphorylation of a specific serine residue located in the activation loop, although some other sites may also be phosphorylated (Burza *et al.*, 2006; Boudsocq *et al.*, 2007). Other plant species also have members of the SnRK2 family that have been shown to be involved in salt signalling. In rice, for example, the entire SnRK2 family has been shown to be activated by hyperosmotic stress and this activation involves phosphorylation (Kobayashi *et al.*, 2004). Interestingly, not all members of the family are activated similarly in response to various salt concentrations. Activation of one member, SAPK1, for example, has been observed at NaCl concentrations higher than 300 mM, whereas another, SAPK2, becomes active at lower concentrations. Domain exchange experiments have revealed that the C-terminal domain is responsible for these responses (Kobayashi *et al.*, 2004). The C-terminal domain of SnRK2s is short and contains a characteristic acidic patch. Paradoxically, for understanding the evolution of the family, the acidic patch is highly aspartic acid-rich in some SnRK2s but glutamic acid-rich in others (Halford and Hardie, 1998).

Members of the soybean SnRK2 family, SPK1 and SPK2, have also been shown to be activated by NaCl when expressed in yeast, although concentrations higher than 0.5 M are required, and to phosphorylate a soybean phosphatidylinositol transfer protein (Ssh1p) in response to saline stress. Ssh1p might be involved in phosphoinositide metabolism, playing an essential role in hyperosmotic signalling (Monks *et al.*, 2001). In wheat, three SnRK2 family members have been shown to be induced by saline treatments. Expression of *PKABA1*, *W55a*, and *TaSnRK2.4* is stimulated by high salt treatment and expression of W55a and TaSnRK2.4 in *Arabidopsis* has been shown to enhance salt tolerance (Holappa and Walker-Simmons, 1995; Xu *et al.*, 2009; Mao *et al.*, 2010).

Interactions between ABA and stress signalling through SnRKs

It has been known for some time that an important set of genes induced by drought, salt, and cold stress, are also activated by ABA. As already described, several studies have shown that *Arabidopsis* and rice SnRK2s are activated in response to salinity stress. Some are also regulated by ABA, although not all are, demonstrating that osmotic and ABA signalling networks are distinct. Analysis of the C-terminal domain of SnRK2 showed that this region was responsible for ABA activation (Boudsocq *et al.*, 2004; Kobayashi *et al.*, 2004). Furthermore, *Arabidopsis* SnRK2.6, which is activated by osmotic stress and is involved in stomatal closure in response to ABA (Merlot *et al.*, 2002; Mustilli *et al.*, 2002; Yoshida *et al.*, 2002), has two regulatory domains at its C-terminus. When part of the Domain II was deleted, ABA-dependent activation was inhibited, whereas osmotic-dependent activation remained as normal. The PP2C encoded by *ABI1* might play an important role in the ABA-dependent activation of SnRK2.6, since the *abi1-1* mutation inhibits its ABA-dependent activation and the PP2C interacts directly with SnRK2.6 (Yoshida *et al.*, 2006). Just recently, HAB1, another type of PP2C in *Arabidopsis*, was shown to regulate

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the abscisic acid-dependent activation of SnRK2.6 (Vlad *et al.*, 2009). A simple model depicting the interaction of Ca^{2+}, ABA, and SnRKs in salinity responses is shown in Fig. 1.

Another route whereby the SnRKs could affect stress signalling is through ABA response element binding proteins (AREBPs), a family of bZIP transcription factors that are unique to plants and which regulate the expression of ABA responsive genes. 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.*, 2008). Like SnRK1, SnRK2-type protein kinases have also been shown to phosphorylate AREBPs (Kobayashi *et al.*, 2005; Furihata *et al.*, 2006). It is also possible that SnRK3s phosphorylate AREBPs because a calcium-dependent activity from stressed *Arabidopsis* seedlings has been shown to phosphorylate the same AREBP-based peptides, which would make AREBPs potential hubs in a signalling network where multiple pathways converge (Halford and Hey, 2009).

Nutritional stress

Drying and increasing salinity are not the only challenges faced by African farmers. African soils are very ancient and degraded and nutrient poor because of weathering and failure to replace nutrients that are removed in cropping (Keith Goulding, Rothamsted, personal communication). Artificial fertilizers are expensive and often difficult to obtain in remote areas, leaving many farmers to rely on traditional inputs from livestock, which can be limited and patchy in their availability. Nitrogen (N), phosphorus (P), and sulphur (S) are essential macronutrients required by plants for growth and productivity (Gojon *et al.*, 2009). Although their availability in soils is generally low, their amount can fluctuate greatly due to factors such as soil type, soil pH, temperature, and precipitation. As sessile organisms, plants have developed adaptations on morphological, biochemical, and molecular levels that allow them to cope with nutrient limitation. These adaptive responses include improvements in nutrient uptake and the modulation of metabolic processes to optimize the use of assimilated nutrients (Poirier and Bucher, 2002). Increasing evidence has shown that members of the different SnRK sub-families play central roles in deciphering stress signals and their participation in NPS signalling has been documented.

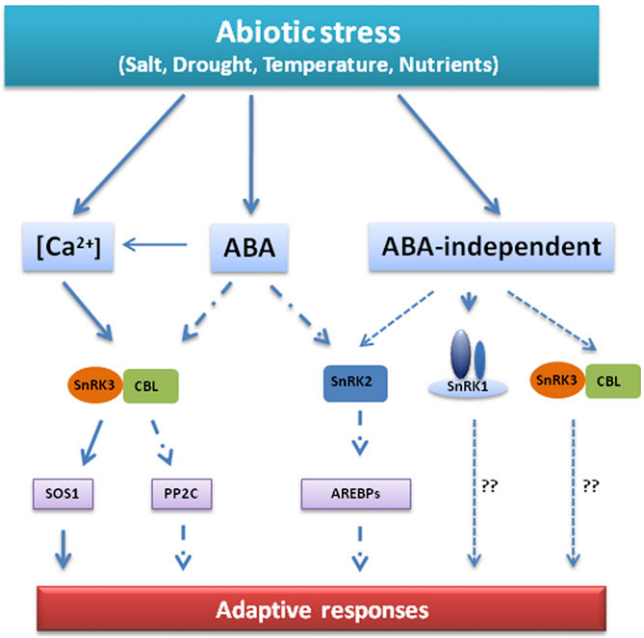

Fig. 1. Schematic diagram showing calcium- and ABA-dependent and -independent signalling pathways involving SnRK1, SnRK2, and SnRK3. High accumulation of Na^+ in the cytoplasm triggers a cytosolic Ca^{2+} signal, which is sensed by an SnRK3/calcineurin B-like (CBL) calcium-binding protein complex (SOS2/SOS3). ABA regulates the activity of SnRK2, which, in turn, activates AREBPs inducing gene expression. ABA might also regulate the activity of SnRK3 through the binding of PP2C. Other abiotic stresses such as nutrient deprivation induce or repress the expression/activity of SnRKs. However, additional components of the signal transduction pathways involved need to be identified.

Much of the work on N sensing has focused on the induction of N metabolism after the addition of nitrate (Schachtman and Shin, 2007). Nitrate reductase (NR) is the first enzyme involved in nitrate assimilation; it catalyses the transfer of two electrons from NAD(P)H to nitrate, which is further reduced to nitrite and ammonium (Kaiser and Huber, 2001). Regulation of NR occurs on at least two levels: NR genes can be induced by addition of N to starved plants (Wang *et al.*, 2000, 2003), and the activity of the NR enzyme can be rapidly and reversibly modulated by phosphorylation (Lillo, 2008). Phosphorylation occurs on a light/dark cycle, with the active, dephosphorylated form of NR being mainly present in the day, whereas the inactive, phosphorylated enzyme is present at night (Kaiser and Huber, 1994). NR is phosphorylated and inactivated by Ca^{2+}-dependent and -independent protein kinases, including SnRK1 (Douglas *et al.*, 1997; Sugden *et al.*, 1999*b*). Inactivation by SnRK1 occurs in a two-step mechanism, in which phosphorylation takes place first, followed by the binding of 14-3-3 proteins to the regulatory phosphorylation site (Ser 543) (Moorhead *et al.*, 1999).

SnRK1 is a heterotrimeric complex comprising a catalytic α subunit with β and γ regulatory subunits. Uniquely, plants also have a protein that appears to be a fusion of the two regulatory subunits, called the βγ subunit (this is encoded by a single gene; it does not arise from a post-translational fusion) and there is further complexity in that there are three different forms of the β subunit. At the transcriptional level, all of these subunits are modulated by nutrient availability, as well as other stresses (Buitink *et al.*, 2004; Polge *et al.*, 2008). Evaluation of publicly-available microarray expression data of the genes that encode the SnRK1 complex subunits using Genevestigator shows that low N conditions have a strong influence on the expression of the α, β1, and γ subunits (Fig. 2). Variations in the amount of some of the β subunits have also been detected

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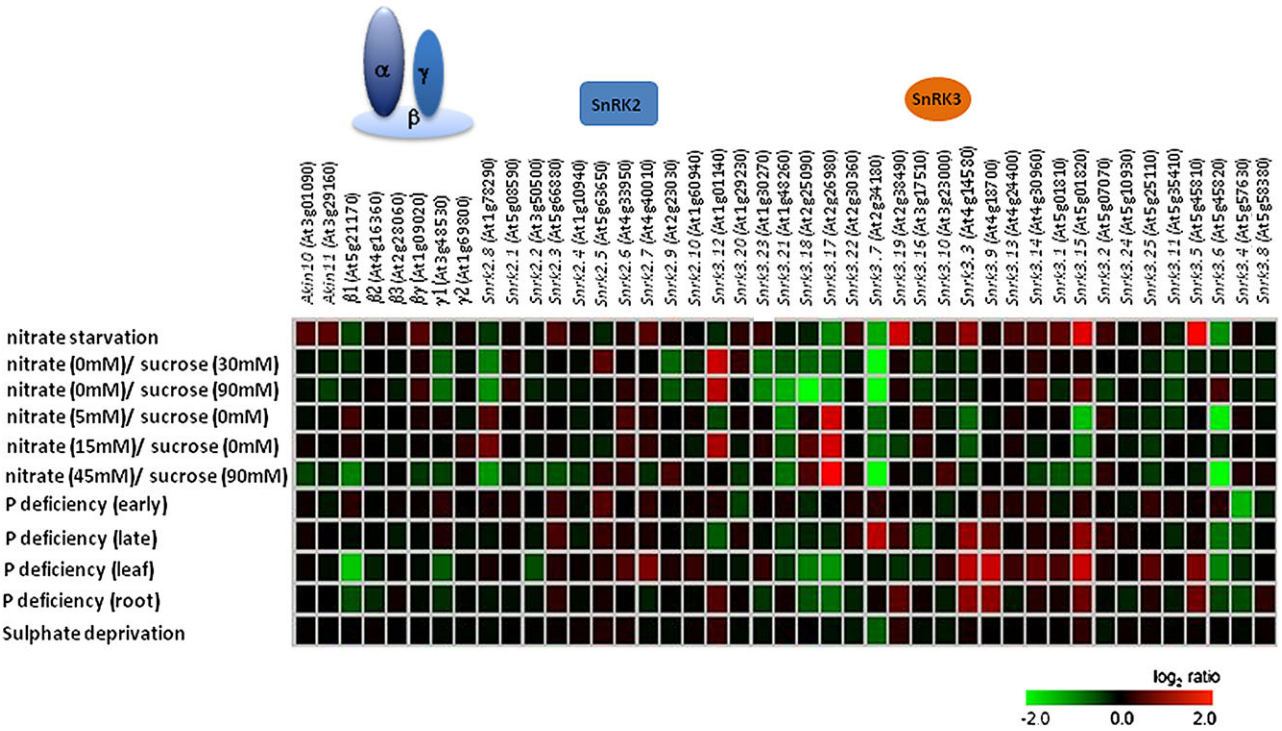

Fig. 2. Meta-analysis of microarray data for expression of genes encoding SnRK1, SnRK2, and SnRK3 in *Arabidopsis* in response to nitrate starvation, different combinations of nitrate and sucrose, phosphorus (P) deficiency, and sulphate deprivation. Results are shown for genes encoding all three subunits (α, β, and γ/βγ) of the SnRK1 complex (the α subunit is encoded by *AKIN10* and *AKIN11*), all ten members of the SnRK2 family, and all 25 members of the SnRK3 family. The SnRK2s have an N-terminal catalytic domain and a C-terminal domain containing an acidic 'patch' that is important for activation, while the SnRK3s have an N-terminal catalytic domain and a C-terminal domain containing a FISL motif that is important for binding the calcineurin B-like (CBL) calcium-binding protein partner. These structures are represented above the expression data. The data were obtained using the microarray database Meta-Analyzer provided by Genevestigator (Zimmermann *et al.*, 2004).

during the light/dark transition, the β1 and β3 genes being up-regulated in the dark (Polge *et al.*, 2008). Experiments using the two hybrid system to characterize the interaction between different β subunits and NR revealed that both β1 and β2 subunits were associated with the enzyme (Polge *et al.*, 2008; Li *et al.*, 2009). These results support the idea that the β subunits could interact specifically with different targets, as has been proposed in yeast (Vincent *et al.*, 2001).

Analysis of the expression of the SnRK3 sub-family in *Arabidopsis* shows that most SnRK3s are affected by N starvation and different combinations of N and sucrose, but that the expression of some increases while the expression of others decrease under the same treatment (Fig. 2). These results are consistent with other studies that have demonstrated the participation of SnRK3s in N and other nutrient signalling processes. WPK4, for example, a SnRK3 from wheat, is induced by low nutrient availability, in particular N, P, and S (Sano and Youssefian, 1994). WPK4 interacts with TaWIN1, which is a 14-3-3 protein that is able to bind WPK4-phosphorylated NR (Ikeda *et al.*, 2000). A model has been proposed in which WPK4 autophosphorylates on its catalytic domain in response to nutrient limitation, allowing the binding of the 14-3-3 protein. WPK4 then phosphorylates NR and transfers the 14-3-3 protein to inactivate the enzyme (Ikeda *et al.*, 2000). Just recently, another member of the SnRK3 sub-family, CIPK6, has been identified as being involved in N signalling: CIPK6 is partly responsible for the regulation of expression of some of the genes involved in N assimilation and transport in *Arabidopsis* (Hu *et al.*, 2009).

SnRK2-type protein kinases are also regulated at the transcript level by low N (Fig. 2) and over-expression of one, *SnRK2.8*, has been shown to lead to increased biomass accumulation in plants under nutrient-deprived conditions, particularly N, P, and potassium (K). Phosphoproteomic analysis of the *Arabidopsis snrk2.8* mutant showed one of its *in vivo* targets to be a 14-3-3 protein. The expression of *SnRK2.8* is regulated diurnally, with its activity enhanced during the day, leading to phosphorylation of the 14-3-3 protein and preventing the 14-3-3 protein's interaction with NR (Shin *et al.*, 2007).

S is another of the macronutrients that plants require for growth and development. The preferred form of S that is assimilated by plants is the sulphate ion, SO_4^{2-}. Most organisms have a limited capacity to store S and thus require a continuous supply of S-containing nutrients for survival. Once sulphate is taken up, it is assimilated to cysteine and then converted into methionine, glutathione (GSH) or other S-containing organic compounds (Schachtman and Shin, 2007). During the early stages of S deprivation, plants

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activate mechanisms which increase S acquisition from the soil, such as arylsulphatase and sulphate transporters (Gonzalez-Ballester *et al.*, 2008). However, if they still cannot get enough sulphate, leading to a decrease in the plant S content, this will lead to changes that reduce metabolism and growth rate (Nikiforova *et al.*, 2003) and affect composition (Muttucumaru *et al.*, 2006; Elmore *et al.*, 2007; Curtis *et al.*, 2009).

The first report on the involvement of SnRKs in S deprivation responses came from *Chlamydomonas* studies. *Chlamydomonas* cells have similar S limitation responses to those exhibited by vascular plants (Davies *et al.*, 1999) and SnRK2.2 (formerly named Sac3) regulates some of the responses to S starvation. *snrk2.2* mutants exhibit both positive and negative effects on regulation because they are unable to repress arylsulphatase activity fully when grown in nutrient-replete medium and are also unable to activate sulphate uptake fully upon S starvation (Davies *et al.*, 1999). In addition, the concentration of chloroplast RNA (cpRNA) in S-starved cells is associated with SnRK2.2 activity: mutant cells show higher levels of cpRNA than wild-type after several hours of S starvation. It has been proposed that a chloroplast sigma factor that controls chloroplast transcription might be deactivated by SnRK2.2 (Irihimovitch and Stern, 2006).

Another SnRK2 family member in *Chlamydomonas*, SnRK2.1, is also involved in regulating S-responsive gene expression, playing a crucial role in the control of S deprivation responses. *snrk2.1* mutants show little or no increase in the levels of known S deprivation-responsive transcripts when starved for S, and after transfer to medium lacking S they bleach rapidly. Furthermore, *snrk2.1* is epistatic to *snrk2.2*, reflecting its key position in the control of S-deprivation responses (Gonzalez-Ballester *et al.*, 2008).

In *Arabidopsis* plants, there is little evidence that members of the SnRK1, SnRK2 or SnRK3 sub-families are regulated at the transcriptional level by S deprivation (Fig. 2). In an attempt to determine whether SnRK2s play an important role in S deprivation responses in the way that they do in *Chlamydomonas*, mutations were induced in *snrk2.3*, the gene with most similarity with the *Chlamydomonas SnRK2.2* gene. The *Arabidopsis* mutant did not show the usual induction of *sultr2.2* sulphate transporter genes under S deprivation, but no other S-starvation responses were affected (Kimura *et al.*, 2006). These results may indicate that SnRK2s have a less important role in S signalling in higher plants than in *Chlamydomonas*, or that other members of the *Arabidopsis* SnRK2 family were able to fulfil the function of SnRK2.3 in its absence.

P, like N and S, is a vital macronutrient for living organisms. It forms part of important macromolecules such as nucleic acids, phospholipids, and adenosine triphosphate (ATP), and is directly involved in the regulation of diverse metabolic pathways through the participation of phosphorylated intermediates (Poirier and Bucher, 2002). Adaptations to P starvation include changes to optimize uptake from the soil (morphological modification of roots, induction of phosphatases, RNAses, phosphate transporters) and changes to promote the efficient use of internal phosphate (Yuan and Liu, 2008). Limited supplies of phosphate reduce the internal adenylate pools (ATP, ADP, and AMP), which could affect the energy status (Poirier and Bucher, 2002). The animal homologue of the SnRKs, AMP-activated protein kinase, as its name suggests, is activated allosterically by AMP. While this is not the case for SnRKs or the fungal homologue, SNF1, 5'AMP does modulate the phosphorylation state of SnRK1 (Sugden *et al.*, 1999*a*). It is therefore not surprizing that SnRKs could be involved in P-starvation responses.

Analyses of the expression of *SnRK* genes under phosphate starvation show that most of the genes encoding SnRK1, SnRK2, and SnRK3 are up- or down-regulated and variations in gene expression are dependent upon starvation time and the affected organ (Fig. 2). In the case of genes *AKIN10* and *AKIN11* (*SnRK1.1* and *SnRK1.2*), which encode the catalytic subunits of the SnRK1 complex in *Arabidopsis*, there is no regulation at the transcript level. However, the AKIN11/SnRK1.2 protein is specifically degraded under phosphate starvation, indicating that SnRK1 complexes under this nutritional condition contain AKIN10/SnRK1.1. *Akin10* mutants growing under P-starvation show reduced starch mobilization at night and important differences in the expression of genes involved in carbohydrate metabolism and general stress responses (Fragoso *et al.*, 2009).

Role of SnRKs in biotic stress responses

There is increasing evidence that metabolic signalling pathways in plants may also be activated by biotic stresses such as viral infection. 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.* 1999*a*). Two of the kinases responsible for this activation have recently been identified (Shen and Hanley-Bowdoin, 2006; Hey *et al.*, 2007; Shen *et al.*, 2009). These are known as 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). These kinases are expressed in response to geminivirus infection and interact with geminivirus replication protein AL1 (Shen and Hanley-Bowdoin, 2006), suggesting a metabolic signalling response to pathogen attack. As our understanding of the details of the pathways involved increases, manipulation of the interactions between metabolic and pathogen signalling might permit advanced priming of crops either to protect them from pathogen infection or from yield losses in the event of pathogen infection.

Potential for manipulation of carbon metabolism to increase yield

In addition to its potential for developing crops with greater tolerance to stress, SnRK1 regulation of starch accumulation

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in storage organs also gives us a potential handle on manipulating carbohydrate metabolism to increase the energy/nutritional value of crops and crop products. In this way, traditional crops, for which agricultural practices are already well established, could be adapted to provide better for the needs of the communities that rely on them. Sucrose synthesis and accumulation is co-ordinated with photosynthesis, the ultimate determinant of crop yield, and photosynthesis is inhibited if too much sucrose accumulates in the leaves (Stitt *et al.*, 1988; Smith and Stitt, 2007). Hypothetically, if the flow of carbon into starch and other storage products could be enhanced, sink strength would increase, reducing the build-up of sucrose. Feedback inhibition of photosynthesis would decrease, increasing carbon fixation and maximizing yield.

Biotechnologies could alternatively be used to improve productivity of particular products. For example, sucrose phosphate synthase (SPS) is an obvious target for manipulation to increase sucrose production in sugar cane. SPS is subject to inactivation by SnRK1, so simple over-expression of a wild-type SPS may not result in an increase in SPS activity in line with increased transcript and protein levels. This could be circumvented by uncoupling SPS from SnRK1 regulation by mutating the gene to remove the SnRK1 phosphorylation site. This technique has been used successfully to increase sterol production by uncoupling HMG-CoA reductase from regulation by SnRK1 (Hey *et al.*, 2006).

Maximizing yield is vital to provide food and fuel security to the rapidly increasing world population while reducing the contribution of agriculture to greenhouse gas emissions and climate change. Clearly, manipulation of the metabolic and stress signalling pathways mediated by the SnRK protein kinase families in plants has the potential to contribute to improving crop production and development, both in Africa and around the world.

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