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Regulation of plant NR activity by reversible phosphorylation, 14-3-3 proteins and proteolysis

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Abstract. This review highlights progress in dissecting how plant nitrate reductase (NR) activity is regulated by Ca^{2+}, protein kinases, protein kinase kinases, protein phosphatases, 14-3-3 proteins and protease(s). The signalling components that regulate NR have also been discovered to target other enzymes of metabolism, vesicle trafficking and cellular signalling. Extracellular sugars exert a major impact on the 14-3-3-binding status and stability of many target proteins, including NR in plants, whereas other stimuli affect the regulation of some targets and not others. We thus begin to see how selective or global switches in cellular behaviour are triggered by regulatory networks in response to different environmental stimuli. Surprisingly, the question of how changes in NR activity actually affect the rate of nitrate assimilation is turning out to be a tough problem.

Key words. Nitrate reductase; 14-3-3 proteins; phosphorylation; proteolysis; kinase.

Basics of a regulatory mechanism controlling NR activity

Plant eukaryotic NR is subject to rapid changes in activity in response to a variety of environmental factors. There have been long-standing reports of diurnal variations in the abilities of plant cells to reduce nitrate, too rapid to be accounted for by changes in gene expression. Experiments in the 1980s showed that the activity of NR in extracts did not always match the amount of NR protein measured by immunological methods [1]. But the first major advance in understanding the mechanisms underlying the posttranslational regulation of NR came when Werner Kaiser's group found that NR activity in leaf extracts dropped within minutes in response to inhibition of photosynthesis, that is in darkness or low CO_2, provided that assays were performed in the presence of Mg^{2+} or Ca^{2+} [2]. This inactivation of NR required protein phosphorylation [2, 3], and reactivation in vivo and in vitro was blocked by specific inhibitors of protein (serine/threonine) phosphatases [4]. Thus, the first regulatory models for posttranslational regulation of NR depicted an interconversion between high- and low-activity states of NR as a phosphorylation/dephosphorylation cycle.

It soon became clear, however, that this simple model was not sufficient to account for the control of NR. It was discovered that at least two protein components were required to convert purified NR from its high-activity to the low-activity state in vitro [5]. Furthermore, when attempts were made to purify the low-activity form of NR from leaves harvested in the dark, the enzyme became activated, even though dephosphorylation was prevented by the inclusion of protein phosphatase inhibitors in all the purification buffers. However, the low-activity form of NR could be reconstituted by mixing phosphorylated NR with other fractions from the purification that contained an NR-inhibitor protein (NIP) [6–8]. NIP was later purified and identified as one or more isoforms of 14-3-3 proteins [9]. Now each of the regulatory components of this system have been isolated, and it is possible to reconstitute the inactivation of NR in vitro. These experiments led to the conclusion that inactivation of NR occurs by phosphorylation (of serine-543 in

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the spinach enzyme [10]) by an NR kinase. Phosphorylation alone does nothing to the activity of NR; rather, phosphorylation creates a phosphopeptide motif that binds to 14-3-3 proteins, and it is the interaction with 14-3-3s that inhibits NR (fig. 1). In line with Kaiser's earlier findings [2], the inhibition of phosphorylated NR by 14-3-3 proteins requires millimolar concentrations of Me^{2+} ions, which means that the ratio of inactive NR to total NR can be estimated by comparing NR activity in the presence of excess Me^{2+} ions (= NR active) as a percentage of total maximum NR activity (NR maximum) which is assayed in buffer containing excess EDTA.

Further dissection of the components that regulate NR

Regulatory sites on NR

14-3-3 proteins are a highly conserved protein family that generally bind to phosphopeptide motifs in many target proteins [11, 12], and the 14-3-3s themselves are considered in more detail below. The regulatory phosphorylation site on NR is a conserved seryl residue (ser543 in spinach and ser534 in *Arabidopsis*) in the hinge 1 region between the MoCo and haem-Fe domains [10, 13, 14]. Phosphorylation of ser543 creates a canonical 14-3-3-binding site (R-X-X- pS/pT-X-P) that binds directly to 14-3-3s in vitro [9, 10, 15]. Consistent with the hinge 1 site being the relevant residue in vivo, an antibody which is reported to be specific for the phosphoser543 site recognises NR only from leaves harvested in the dark [16], and NR mutated at that site cannot be inhibited by 14-3-3s in planta or when examined in the yeast two-hybrid system [13, 17]. Whereas phosphorylation is essential for binding of 14-3-3s to the wild-type enzyme, Kanamura and colleagues have identified substitutions of amino acids other than the phosphorylation site that restored the ability of an S534D *Arabidopsis* NR mutant to bind to 14-3-3s [17]. The sequences generated by these second site mutations that allow binding to 14-3-3s independent of phosphorylation are presumably mimetics of the phosphorylated site in the wild-type protein.

There is also compelling evidence for some involvement of the N-terminal tail in the regulation of NR. A tobacco mutant NR containing a 56-amino acid deletion in the acidic domain of the N-terminus was reported not to be regulated by light/dark transitions in vivo and was not subject to ATP-dependent inactivation in vitro in crude extracts [18]. However, surprisingly, the mutated NR purified from leaves harvested in the dark could be inhibited by 14-3-3 proteins [19]. Furthermore, spinach leaf NR from which the N-terminal 45 amino acids had been removed by an endogenous protease could still be phosphorylated, but seemed to be less sensitive than full-length NR to inactivation in vitro [10]. Whether the regulatory events at the N-terminus involve binding to another protein, phosphorylation and/or conformational changes that are triggered by phosphorylation and binding of 14-3-3s is not known [18, 20].

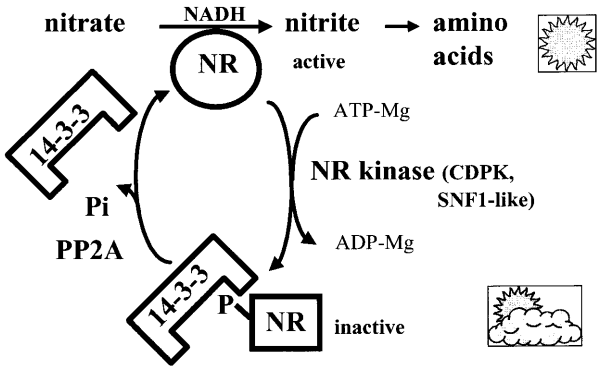

Figure 1. Regulation of NR in leaves in response to changes in photosynthesis. When leaves are actively photosynthesising, assimilation of nitrate is active because NR is in an active dephosphorylated form. However, if anything happens to block photosynthesis (depicted here as a cloud passing in front of the sun), NR becomes phosphorylated on a serine residue (serine 543 in the spinach enzyme). Phosphorylation alone does nothing to the activity of NR. However, in the plant, phosphorylation of serine 543 creates a phosphopeptide motif that binds directly to one or more 14-3-3 proteins. 14-3-3 binding inhibits NR activity. The inhibition of NR is reversed by dissociation of 14-3-3s, which may be caused by dephosphorylation of serine 543 by a type 2A protein phosphatase, or by other mechanisms. This scheme invokes some sort of signal reflecting photosynthetic activity being transmitted from the chloroplast to control the components that regulate NR in the cytosol.

NR kinases

After fractionation of spinach leaf extracts by anion-exchange chromatography, several peaks of NR kinase activity were identified, which each phosphorylated spinach NR on serine543 and inactivated the enzyme in the presence of 14-3-3s. One kinase was identified as a SNF1-related enzyme by the following properties: inactivation by dephosphorylation with protein phosphatases 2A or 2C; requirement for a hydrophobic residue in the +4 position of the peptide substrate AMARAASAAALARRR, its inactivation of *Arabidopsis* 3-hydroxy-3-methylglutary-coenzyme A (HMG-CoA) reductase-1 by phosphorylation of serine-577, a molecular mass of ~140 kDa, its Ca^{2+}-independent activity and recognition of its 58-kDa catalytic subunit by an antibody raised against the rye SNF1-related RKIN1 [21–23]. The term SNF1 comes from the sucrose nonfermenting *Saccharomyces cerevisiae* mu-

tant, which is unable to grow on carbon sources other than glucose because of a defect in this protein kinase. The mammalian homologue is the AMP-activated protein kinase, which phosphorylates and inactivates several biosynthetic enzymes in response to nutritional and environmental stresses that cause cells to be depleted of ATP [24]. It is intriguing to find that NR is inactivated by a plant version of a protein kinase required for 'nutrient sensing' in other eukaryotes.

Two other major NR kinase activities in spinach leaf extracts are calcium-dependent protein kinases (CDPKs), and one has been purified and identified by amino acid sequencing as an isoform with closest sequence similarity to the *Arabidopsis* CDPK6 (also known as CPK3) [25]. The CDPKs have been found only in plants and protozoa, not mammalian cells. These enzymes have a calmodulin domain on the same polypeptide as the protein kinase domain. Binding of calcium triggers activation of CDPKs by dislodging an autoinhibitory junction sequence from the active site.

Plants contain several members of the SNF1-related kinase family and many CDPK isoforms, and it will be important to determine whether all of these or only particular forms are NR kinases in vivo. Another critical question is which kinase is responsible for phosphorylating NR under which condition. It is not known how the activities of the SNF1-related kinases or CDPKS respond to changes in photosynthesis or other signals that regulate NR. Perhaps the only clue is that cytosolic Ca^{2+} concentrations have been reported to increase in leaf cells in the dark [26], a time when phosphorylation and inactivation also occur.

14-3-3 proteins

The 14-3-3 proteins were first identified as abundant brain proteins, and thus named because of their mobility on ion-exchange chromatography and two-dimensional gel electrophoresis. We now know that 14-3-3s are an unusually highly conserved protein family with central regulatory roles in plant, fungal and mammalian cells [27]. 14-3-3s bind to phosphopeptide motifs in diverse target proteins [11, 12, 28], and through these interactions, 14-3-3s have been reported to modulate the activities of enzymes [28, 29], regulate subcellular location of targets [30–32] and act as 'adaptor proteins' that mediate interactions between components of signal transduction pathways [33].

In plants, 14-3-3s have been discovered primarily as regulators of NR and other enzymes of cytosolic metabolism, and the plasma membrane proton-adenosyl triphosphatase (ATPase) [28, 29, 34], whereas in mammalian cells 14-3-3s are best known as regulators of many components of oncogenic signalling pathways and cell cycle checkpoints [27, 33].

Activation of NR: NR phosphatase(s) and other mechanisms

The activation of NR in vitro, or in leaves in response to illumination, is blocked by microcystin or okadaic acid, suggesting that activation is catalysed by one of the PPP family of protein phosphatases, most likely a type 2A enzyme [4, 35]. Using microcystin-Sepharose chromatography, plant extracts have been found to contain at least two members of this family, namely the canonical trimeric PP2A, and PP5 [35]. Which of these or related enzymes actually dephosphorylates NR in vivo is unknown.

Having 14-3-3s bound directly to the ser-543 phosphorylation site poses the question of how the phosphatase accesses its substrate. 14-3-3-bound NR can be activated in vitro by competition with a 14-3-3-binding phosphopeptide, so presumably the NR/14-3-3 interaction must be sufficiently dynamic to allow the phosphopeptide to exchange with NR for 14-3-3 binding. However, the 14-3-3s have been found to provide a barrier to dephosphorylation, because phosphorylated NR with bound 14-3-3s is dephosphorylated more slowly than free phosphorylated NR in vitro [14]. It has been speculated that 14-3-3 inhibition of dephosphorylation may be important physiologically: if 14-3-3s ensure that NR in leaves is only activated slowly by dephosphorylation, fully active nitrate reduction may be delayed to coincide with complete induction of photosynthesis, when the 'surplus' C skeletons needed for incorporation of ammonia into amino acids would become available [14].

There are signs that dephosphorylation may not be the only mechanism for activating NR. We [S.E.M.M. and C.M., unpublished] have found that the 14-3-3 proteins themselves are posttranslationally modified in response to hormones and nutrients, and that these modifications change the binding properties of the 14-3-3s in in vitro assays. In addition, small molecules including phosphate ions, EDTA and AMP [36, 37] activate NR in vitro. EDTA acts by chelating the Me^{2+} ions that are essential for 14-3-3 binding to NR [2, 38], and Athwal and colleagues [38] have identified a specific binding site for AMP on 14-3-3s whose occupation is proposed to disrupt the inactivated phosphorylated NR/14-3-3 complex.

Pharmacological agents and conditions that activate NR in leaves, leaf discs and/or roots include 5-aminoimidazole-4-carboxamide riboside (AICAR), which gives rise to an AMP mimetic in cells [39]; the cytosolic protein synthesis inhibitor, cycloheximide [40, 41]; mannose; organic acids and anoxia [42]. Although there are some clues and speculations in the cited papers, it is not clear how the actions of these compounds are mediated [23].

Why is NR activity posttranslationally regulated?

As the mechanistic details emerge, we still do not know exactly why plants have evolved such intricate systems for regulating NR activity. One idea is that the flexible and rapid regulation of NR serves to minimize the major costs and hazards that are associated with plants' remarkable ability to synthesise amino acids from nitrogenous minerals and sugars.

Reduction of nitrate to amino acids is a heavy drain on the reductants NADH and NADPH. Intuitively then, one might expect this pathway to be highly regulated so that the rate of nitrate reduction exceeds neither the supplies of reductant nor the carbohydrates that are required to combine with reduced nitrogen, in the form of ammonia, to generate amino acids. Indeed, this expectation seems to hold generally true in reality.

A major potential hazard associated with assimilation of nitrate into amino acids is that the nitrite generated by in the cytosol by NR is highly toxic, and mutagenic because it can diazotise amino groups. In leaves that are actively photosynthesising, the risk of nitrite poisoning is minimal because photosynthetic electron transport maintains both the ΔpH across the chloroplast envelope that is required to translocate nitrite into the chloroplast, and the reduced ferredoxin used by nitrite reductase (NiR) to convert nitrite into ammonia. However, if photosynthesis and hence nitrite reduction are blocked, nitrite poisoning could become a dangerous prospect—prevented by downregulation of NR activity, which would ensure immediate cessation of nitrite production. Thus, the need to coordinate nitrate assimilation with supplies of NADH and carbon skeletons [43], and to prevent nitrite poisoning, certainly seems to provide a rationale for why regulation of NR in the cytosol should be so highly regulated in response to signals generated by changes in photosynthesis in the chloroplast.

Recently, however, it has been suggested that strict regulation of NR activity may go beyond what is needed to keep nitrate assimilation in check. The new proposal is that tight regulation of NR is linked to a novel 'signalling' role for NR as a producer of nitric oxide (NO) [44]. In mammals, NO is an important endogenous regulator of diverse biological functions, from muscle relaxation, neurotransmission and memory to cell-mediated immune responses. Animal cells synthesise NO from arginine, catalysed by nitric oxide synthases (NOSs), and different NOS isoforms have different modes of responsiveness to hormones and messengers such as interferon-γ. The biological effects of NO are mediated through its nitrosylation of, or redox reactions with, metal-, thiol- and tyrosine-containing protein targets, which include enzymes, signalling proteins such as guanylate cyclase and several transcription factors. There has been accumulating evidence that NO may be involved in plant signal transduction systems. However, the source of NO is still controversial. Neither a gene nor a protein homologous to mammalian NOS has been isolated from plants to date. Yamasaki and Sakihama [44] have reported that plant NR can produce NO, implicating NR as a NO signal emitter in plant cells. In this scenario, regulation of NO production by NR would be essential to its signalling role, but exactly how this process is envisaged to be controlled in a manner compatible with the assimilatory role of NR is not clear.

How do changes in NR activity affect nitrate assimilation in vivo?

Having just presented several cases for considering rapid posttranslational inhibition of NR to be vital to leaf survival, recent findings have ironically turned the question around, forcing us to ask why plants have as much NR as they do in the first place. Mark Stitt's group have discovered that plants with 40% wild-type NR protein have an apparently normal phenotype, albeit with altered diurnal regulation of NR protein and activity [45]. For example, darkening led to rapid inactivation of NR in the wild type and the mutants. However, in the low NR mutants only, this inactivation was reversed after 1–3 h of darkness. Thus, it appears that wild-type plants have excess capacity for nitrate assimilation, and that the regulatory mechanisms can adapt to a lower NR content to allow assimilation to continue for a longer period each day.

Another complication is that Kaiser's group have reported that whereas NRact measured in extracts in the presence of Mg^{2+} seemed to reflect the rates of nitrate reduction in high ambient CO_2 in vivo, a tight correlation does not always exist. For example, full activation of NR in the dark, which was promoted by anoxia or by AICAR feeding, did not result in increased rates of nitrate reduction in vivo [46]. These findings suggest either that the NRact measured in vitro is not an accurate reflection of the true operating NR activity, or that NR is not rate limiting for nitrate assimilation under some conditions. Surprisingly, then, the question of how changes in NR activity actually affect the rate of nitrate assimilation (or NO production) is turning out to be a tough problem.

Further plant proteins that bind to 14-3-3s in competition with synthetic 14-3-3-binding phosphopeptides

These difficulties in fully understanding 'why' NR is highly regulated prompted our growing realisation that

we should explore how control of NR is linked to control of other cellular processes. All of the regulatory components that act on NR—the kinases, phosphatases and 14-3-3 proteins—are likely to control many cellular processes. The remainder of this review shows how exploring questions about the wider cellular functions of one of these components, the 14-3-3s, led to the discovery that NR is just one of many 14-3-3-regulated plant proteins that seem to be coordinately controlled by a novel sugar-sensing signalling pathway.

Finding 14-3-3s as regulators of an enzyme in primary plant metabolism was intriguing because the majority of the known 14-3-3-binding proteins are enzymes of oncogenic signalling pathways and cell cycle regulators in mammalian cells [30]. We are curious to know whether this apparent disparity in the types of 14-3-3-binding proteins between the two Kingdoms is real or an artificial bias due to the different emphases of molecular studies in plant and mammalian cells. Indeed, it is striking that for the few well-defined cases, 14-3-3 binding to individual plant and mammalian targets contributes in different ways to cell protection and survival [47, 48]. For example, as discussed above, one proposal is that by binding to and inhibiting phosphorylated plant nitrate reductase in leaves in the dark [9, 49], 14-3-3s ensure that toxic nitrite is not produced under conditions where it cannot be reduced further, whereas binding of 14-3-3s to the mammalian protein BAD prevents BAD from binding to Bcl-X_L and thus blocks apoptosis [50]. But although 14-3-3s are being implicated repeatedly in regulating defined points in control of cell growth, metabolism and development, little is known of how the global phosphorylation-dependent interactions of diverse targets with 14-3-3s are actually regulated within a single cell. Because we felt it was important to put NR into a wider regulatory context by tackling questions of global control by 14-3-3s, a number of 14-3-3 affinity binding procedures and reagents were developed to purify and identify further 14-3-3-binding targets, and to determine how their phosphorylation and 14-3-3-binding status is regulated in response to extracellular stimuli.

Many proteins that bind to 14-3-3s in competition with a 14-3-3-binding phosphopeptide have now been purified, and several of them identified ([28, 51] and see [29, 52]). Those 14-3-3-binding proteins that have been purified from extracts of cauliflower curd and cultured *Arabidopsis* cells are depicted on figure 2. One of the proteins purified by these methods was NR [51], which was a reassuring validation of the specificity of the purification method.

The identities of the other target proteins suggest many implications. For example:

1. We can deduce that 14-3-3s are likely to have profound effects on cytosolic carbon partitioning among the pathways of sugars, amino acid, nucleotide and protein biosynthesis in plants. However, we do not yet know the functional effects of phosphorylation and 14-3-3s on the majority of these target proteins. In tackling this problem, it will be important to keep a very open mind, because several of the enzymes that have been identified to bind 14-3-3s have 'moonlighting' activities that are not obviously related to their well-known biosynthetic functions. For example, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was once known only as a 'constitutive, housekeeping' enzyme in glycolysis. However, in mammalian cells new roles for GAPDH in DNA replication, translational control of gene expression and endocytosis have been uncovered [53]. Trehalose is produced by cells as an antidessicant. However, in *S. cerevisiae* the pathway intermediate, trehalose-6-phosphate, is a potent inhibitor of hexokinase, and *S. cerevisiae* mutants that are deficient in trehalose-6-phosphate production are unable to grow on glucose because their high hexokinase activity exceeds the capacity of other enzymes of glycolysis, leading to ATP depletion [54, 55]. The classic family of aminoacyl transfer RNA (tRNA) synthetases is implicated in a broad repertoire of functions in addition to their well-defined place in protein synthesis, and extending to roles in cell survival, tRNA processing, RNA splicing, RNA trafficking, and transcriptional and translational regulation [56]. We must be prepared to investigate whether regulation by 14-3-3s modulates the activities, substrate specificities or subcellular locations of these enzymes, including NR.
2. It is interesting to find that 14-3-3s bind to at least one of the (phosphorylated) protein kinases that are capable of phosphorylating the 14-3-3-binding site of NR. There are precedents in mammalian cells for 14-3-3s interacting with multiple components of signalling pathways, but the functional relevance of these observations is unknown.
3. Finding that 14-3-3s bind to many enzymes of metabolism, vesicle trafficking and cellular signalling in plant cells emphasises the oddity of the current perception in the mammalian literature that 14-3-3s are predominantly associated with signalling molecules. It seems extremely unlikely that the highly conserved 14-3-3s have such distinct roles in plants and mammalian cells. Perhaps 14-3-3s only appear to have an exclusive link with signalling in mammalian cells because of the intensity of research in this field, compared with the 'less fashionable' area of mammalian metabolism.

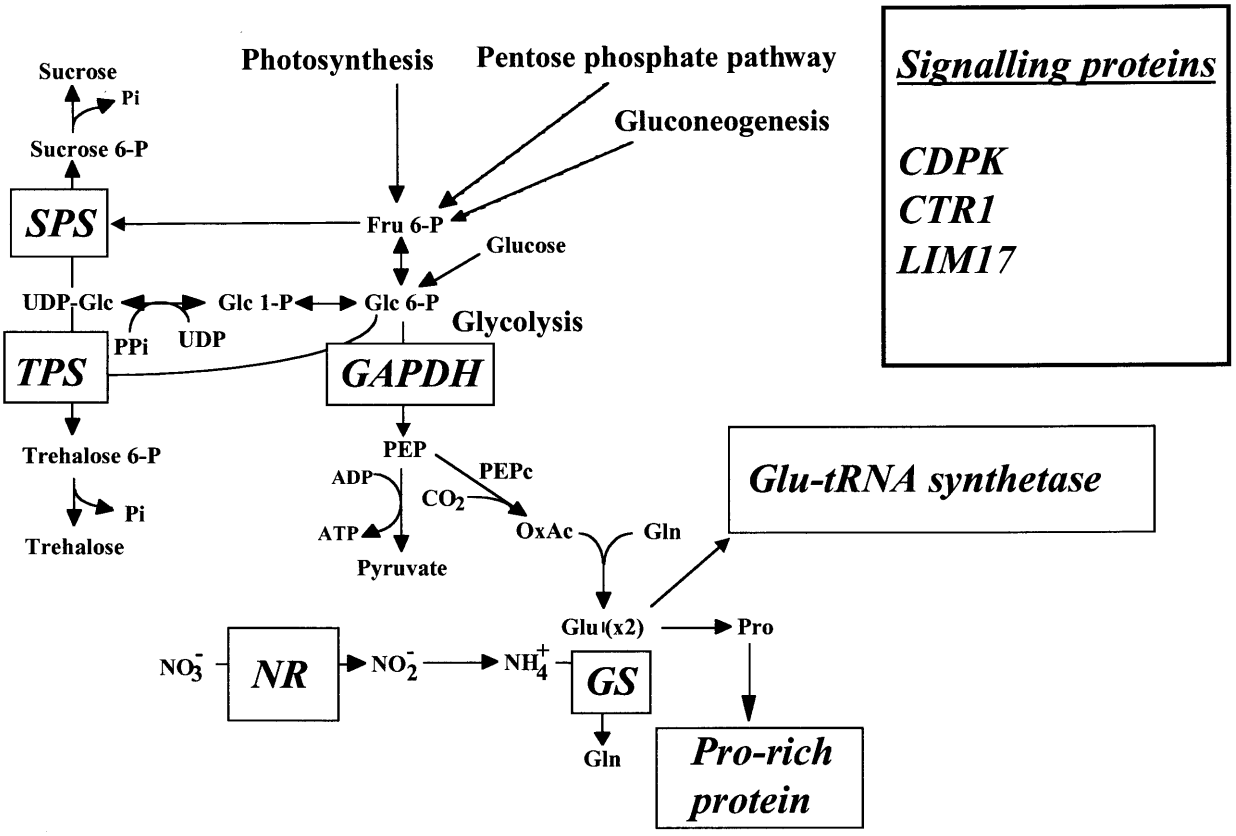

Figure 2. Enzymes of primary cytosolic plant metabolism and signalling that bind to 14-3-3s in competition with synthetic 14-3-3-binding phosphopeptides. CDPK, calcium-dependent protein kinase; CTR1, protein kinase whose mutation is responsible for a 'constitutive triple response' to ethylene phenotype in *Arabidopsis* seedlings; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glutamine synthetase (note that NH_4^+ substrate for cytosolic GS probably comes from protein degradation, rather than primary nitrate assimilation); LIM17, cauliflower protein with extensive sequence similarity to the lily LIM17 protein that is expressed early in floral development; NR, nitrate reductase; SPS, sucrose phosphate synthase; TPS, trehalose phosphate synthase.

4. Does the identification of this bewildering array of 14-3-3 target processes make it any easier to interpret the significance of discovering 14-3-3 proteins in the context of regulation of nitrate assimilation? The answer to this question must surely depend on the extent of regulatory interactions among the diverse 14-3-3-binding proteins. For example, each 14-3-3 dimer that binds to NR has the potential to bind to another target protein simultaneously. Do 14-3-3s act as adapters that link NR physically to any other target proteins? The 14-3-3s only recognise and bind to phosphorylated sites within specific peptide sequences, which leads to the expectation that there may be a limited number of protein kinases that are capable of phosphorylating 14-3-3 binding sites on target proteins. Do the protein kinases that phosphorylate NR also phosphorylate 14-3-3 binding sites on other target proteins? In other words, is the global phosphorylation and 14-3-3-binding status of other target proteins in a cell coordinated with phosphorylation of NR, or are individual target-14-3-3 interactions regulated independently?

14-3-3s regulate the global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cell extracts

In order to tackle the question of global control by 14-3-3s, Moorhead and colleagues [28] developed sensitive 14-3-3 overlay assays to monitor how the phosphorylation- and 14-3-3-binding status of all the target proteins in any cell extract respond to extracellular stimuli. These assays are analogous to Western blots, but instead of probing cell extracts for proteins that bind to an antibody, the blots are overlayed with digoxygenin-labelled 14-3-3 proteins (DIG-14-3-3s). Proteins that contain 14-3-3-binding phosphopeptide

motifs will bind to the DIG-14-3-3s, which are in turn detected with an anti-DIG antibody. Using this technique, it has been found that extracts of plant tissues or cells contain many proteins that bind to the DIG-14-3-3s, and whose binding is specifically blocked by competition with synthetic 14-3-3-binding phosphopeptides. Importantly, all of these DIG-14-3-3-binding proteins are also coimmunoprecipitated from tissue and cell extracts using an anti-14-3-3 antibody, confirming that the native proteins bind to endogenous 14-3-3s in the plant extracts.

The sensitivity of these assays provided the opportunity to monitor how the 14-3-3-binding status of all target proteins in a cell changed in response to extracellular stimuli. An *Arabidopsis* cell suspension culture was chosen for these experiments: *Arabidopsis* because of the ease of identifying proteins by comparing mass fingerprints of protein digests with the sequence databases, and cell culture because compared with intact plants, it is relatively easy to grow bulk cultures and subject cells to diverse stimuli reproducibly and rapidly. Changes in the 14-3-3-binding status of NR and other individual target proteins was observed in response to hormones and changes in cell density in these cells. The most dramatic event, however, occurred in response to sugar starvation, and a current working model of what occurred is depicted in figure 3. In sugar-fed cells, many proteins were phosphorylated and bound to 14-3-3 proteins. In contrast, in extracts of sugar-starved cells, all 14-3-3 binding was lost. As 14-3-3 proteins generally bind to phosphorylated sites on their targets, it seemed likely that the complete lack of interactions in sugar-starved cells could have been caused by dephosphorylation of all the target proteins. However, remarkably, Western blotting revealed that the loss of 14-3-3-binding proteins in the overlay assays of sugar-starved cell extracts coincided with the selective and limited proteolytic cleavage of those proteins that had been phosphorylated and bound to 14-3-3s in the sugar-fed cell extracts. In dissecting the mechanisms underlying these

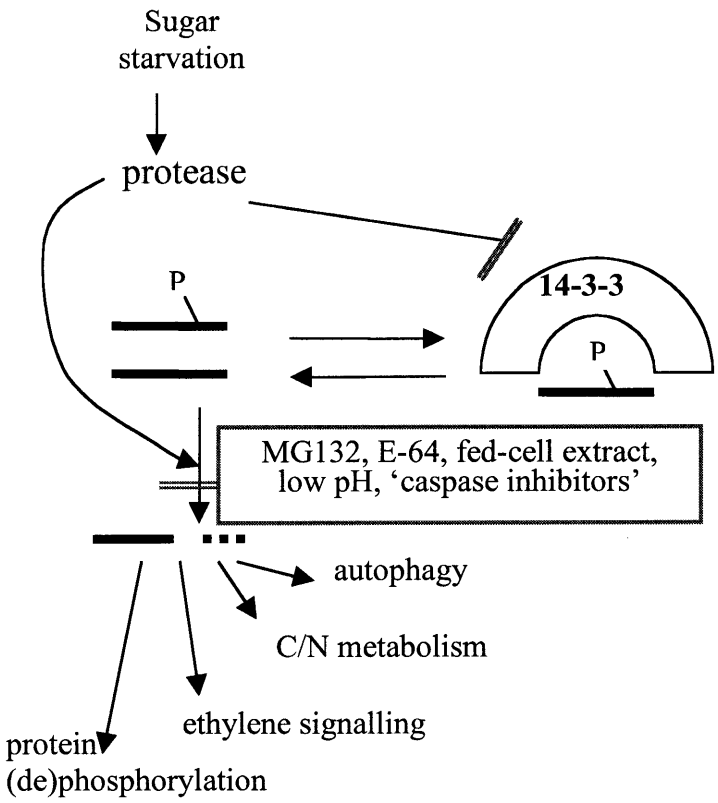

Figure 3. Global regulation of 14-3-3-binding proteins in *Arabidopsis* plant cells. This figure summarises our current working model of how the activity state of the *Arabidopsis* 14-3-3-binding targets is a function of the activity of protein kinases, protein phosphatases, 14-3-3 proteins and the MG132-sensitive protease (further details in text). In sugar-starved cell extracts, all of the target proteins that were tested were proteolytically cleaved. The properties of the protease identified in vitro are consistent with the hypothesis that 14-3-3s protect their target proteins from proteolysis. Because of the diversity of targets, we predict that their limited cleavage could have consequences for may cellular processes, several of which are indicated here. Parallel bars indicate inhibition.

events, a protease whose properties are consistent with it being the enzyme responsible for the observed cleavage events, was identified in extracts of sugar-starved cells, but not in extracts of sugar-fed cells. This protease was blocked in vitro and in vivo by the cysteine protease inhibitor, MG132, and was ATP independent, ruling out the proteasome. The protease was also completely inactive below pH 6.5, which is suggestive of a cytosolic enzyme, rather than a lysosomal or vacuolar protease. Importantly, this protease could only cleave target proteins in vitro when they were free of 14-3-3s. Substrate proteins were completely protected from proteolysis when they were phosphorylated and bound to 14-3-3s. This finding suggests that 14-3-3 proteins must be dissociated from the target proteins in sugar-starved cells (either by dephosphorylation of the target proteins, and/or by some other mechanism) prior to their cleavage by the starvation-induced protease [51].

Because of the diversity of plant 14-3-3-binding proteins (fig. 2), we would expect numerous cellular processes to be altered depending on the phosphorylation, the 14-3-3-binding status and the state of the cleavage of these targets. Cleavage of 14-3-3-binding partners may not necessarily lead to loss of function. Whereas NR activity in starved cell extracts was very low, suggesting that its cleavage had led to its inactivation, there were indications that the cleavage fragments of other 14-3-3-regulated proteins retained enzymatic activity, though their regulatory properties may be altered.

Does the dramatic and selective cleavage of 14-3-3-regulated protein in sugar-starved cell extracts explain the major shifts in metabolism that have been detailed in many physiological studies of sugar-starved plants [57, 58]? For example, is cleavage of NR a mechanism for blocking nitrate assimilation into amino acids when supplies of sugars are too low to supply the necessary carbon?

Sugar starvation is a normal part of a plant's lifestyle. The supply of sugars generated by photosynthesis is not continuous and stops partially or completely in the dark, during winter and when leaves are shed. Starvation is in fact one of the essential signals for plant development, and 'sugar-sensing' pathways regulate processes from germination through photomorphogenesis, flowering and senescence. For example, sugars inhibit the red-light-triggered developmental transition that converts a starving etiolated seedling into a vegetative plant that is fully capable of photosynthesis. It seems likely that the finding that 14-3-3s participate in a sugar-sensing pathway controlling cleavage of many targets, including NR, may underlie effects of sugars on plant metabolism and development. Furthermore, there may be links between these sugar-regulated biochemical events and the striking developmental phenotypes of the transgenic potatoes developed by Jan Szopa's group. They found that transgenic potatoes overexpressing 14-3-3s displayed a prolonged stay-green phenotype with delayed leaf senescence, and antisense 14-3-3 plants showed early senescence. It would be very interesting to examine how the activity state and starvation-induced proteolysis of NR, and other 14-3-3-regulated proteins, is affected in these transgenic plants [59, 60].

How is posttranslational regulation of NR by phosphorylation and 14-3-3s connected to NR degradation in planta?

Once the sugar-regulated protease is identified at the molecular level, it will be important to determine how it is regulated in planta. In particular, there is a puzzle about the relationship between 14-3-3 binding and proteolysis of NR in green leaves. As described earlier, it is well known that NR in plant leaves is rapidly inhibited by phosphorylation and 14-3-3 binding in response to darkness. In addition, however, leaf NR is degraded when plants are left in the dark for a prolonged period of several hours (the exact time depending on species). These findings led to the expectation that binding to 14-3-3s might be a prerequisite for degradation. In line with this speculation, Weiner and Kaiser [16] have identified a phenylmethylsulphonyl fluoride (PMSF)-sensitive protease that is present in extracts of leaves harvested from leaves in the dark, but not in extracts of illuminated leaves. Furthermore, immunodepletion of 14-3-3s from an extract of leaves harvested in the dark prevented NR degradation by the PMSF-sensitive protease, which led to the proposal that 14-3-3s target NR for proteolysis in the dark.

In contrast, the previous section outlined the identification of a protease that is induced by sugar starvation of *Arabidopsis* cells. The starvation-induced protease is inhibited by the cysteine protease inhibitor, MG132, but not by PMSF, and catalyses limited cleavage of several plant 14-3-3-binding proteins in vitro, but only when these protein substrates are in their unbound state. Phosphorylation and 14-3-3 binding completely protects these target proteins from cleavage. There are several possibilities to reconcile the apparent discrepancies. One point is that we do not yet know whether the MG132-sensitive protease is active in leaves, and do not know whether this enzyme is responsible for degrading NR in prolonged darkness. However, if the MG132-sensitive protease were responsible for degradation of NR in prolonged darkness, first, the protease would have to be induced in the dark and, second, NR would somehow have to be 'deprotected' from 14-3-3s during prolonged darkness in order to become a substrate for this enzyme. Consistent with the latter scenario, the relative abundance of 14-3-3 proteins has been reported to decline gradually during prolonged darkness [59]. In

addition, and in contrast to other work [16, 40], the unphosphorylated form of NR has been reported previously to be the least stable form in leaf extracts [1]. Solving the puzzles of which protease is relevant under which circumstance and whether or not 14-3-3s target NR for degradation or protect NR from degradation requires further analytical tools. Antibodies and assays will be needed to follow the activation of both the PMSF-sensitive and MG132-sensitive proteases in planta, and we must have a knowledge of the in vivo cleavage site(s) on NR.

Future prospects

The basics of a mechanism for rapid control of NR in response to changes in photosynthesis has been deduced, and several of the relevant regulatory components identified at the molecular level. There are tantalising hints of further levels of control to explore, particularly arising from the finding that NR is just one of many 14-3-3-regulated proteins that are selectively cleaved in response to starvation by a novel sugar-regulated protease. Many challenges and opportunities are presented by the recognition that NR is controlled as one of many targets of extensive regulatory networks that are likely to influence the growth and development of the whole plant. It would be exciting to think that manipulating these signalling pathways and networks may provide means to altering biosynthesis, metabolite partitioning, cell survival and development, ultimately leading to genetic strategies for channelling yield and nutritional value to seeds, flowers and tubers, while reducing dependency on chemical fertilisers.

1 Lillo C. (1983) Diurnal variations of nitrate reductase activity and stability in barley leaves. Physiologia Plantarum **58:** 184–188
2 Kaiser W. M. and Spill D. (1991) Rapid modulation of spinach leaf nitrate reductase by photosynthesis. II. In vitro modulation by ATP and AMP. Plant Physiol. **96:** 368–375
3 Huber J. L., Huber S. C., Campbell W. H. and Redinbaugh M. G. (1992) Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Arch. Biochem. Biophys. **296:** 58–65
4 MacKintosh C. (1992) Regulation of spinach-leaf nitrate reductase by reversible phosphorylation. Biochim. Biophys. Acta **1137:** 121–126
5 Spill D. and Kaiser W. M. (1994) Partial purification of 2 proteins (100 kDa and 67 kDa) cooperating in the ATP-dependent inactivation of spinach leaf nitrate reductase. Planta **192:** 183–188
6 MacKintosh C., Douglas P. and Lillo C. (1995) Identification of a protein that inhibits the phosphorylated form of nitrate reductase from spinach (*Spinacea oleracea*) leaves. Plant Physiol. **101:** 451–457
7 Glaab J. and Kaiser W. M. (1995) Inactivation of nitrate reductase involves NR-protein phosphorylation and subsequent binding of an inhibitor protein. Planta **195:** 514–518
8 Glaab J. and Kaiser W. M. (1996) The protein kinase, protein phosphatase and inhibitor protein of nitrate reductase are ubiquitous in higher plants and independentof nitrate reductase expression and turnover. Planta **199:** 57–63
9 Moorhead G., Douglas P., Morrice N., Scarabel M., Aitken A. and MacKintosh C. (1996) Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. Curr. Biol. **6:** 1104–1113
10 Douglas P., Morrice N. and MacKintosh C. (1995) Identification of a regulatory phosphorylation site in the hinge 1 region of nitrate reductase from spinach (*Spinacea oleracea*) leaves. FEBS Lett. **377:** 113–117
11 Muslin A. J., Tanner J. W., Allen P. M. and Shaw A. S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. Cell **84:** 889–897
12 Yaffe M. B., Rittinger K., Volinia S., Caron P. R., Aitken A., Leffers H. et al. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. Cell **91:** 961–971
13 Su W., Huber S. C. and Crawford N. M. (1996) Identification in vitro of a post-translational regulatory site in the hinge 1 region of *Arabidopsis* nitrate reductase. Plant Cell **8:** 519–527
14 Bachmann M., Huber J. L., Athwal G. S., Wu K., Ferl R. J. and Huber S. C. (1996) 14-3-3 proteins associate with the regulatory phosphorylation site of spinach leaf nitrate reductase in an isoform-specific manner and reduce dephosphorylation of Ser-543 by endogenous protein phosphatases. FEBS Lett. **398:** 26–30
15 Bachmann M., Shiraishi N., Campbell W. H., Yoo B. C., Harmon A. C. and Huber S. C. (1996) Identification of Ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. Plant Cell **8:** 505–517
16 Weiner H. and Kaiser W. M. (1999) 14-3-3 proteins control proteolysis of nitrate reductase in spinach leaves. FEBS Lett. **455:** 75–78
17 Kanamaru K., Wang R., Su W. and Crawford N. M. (1999) Ser-534 in the hinge 1 region of *Arabidopsis* nitrate reductase is conditionally required for binding of 14-3-3 proteins and in vitro inhibition. J. Biol. Chem. **274:** 4160–4165
18 Nussaume L., Vincentz M., Meyer C., Boutin J. P. and Caboche M. (1995) Post-transcriptional regulation of nitrate reductase by light is abolished by an N-terminal deletion. Plant Cell **7:** 611–621
19 Lillo C., Kazazaic S., Ruoff P. and Meyer C. (1997) Characterization of nitrate reductase from light- and dark-exposed leaves. Plant Physiol. **114:** 1377–1383
20 Pigaglio E., Durand N. and Meyer C. (1999) A conserved acidic motif in the N-terminal domain of nitrate reductase is necessary for the inactivation of the enzyme in the dark by phosphorylation and 14-3-3 binding. Plant Physiol. **119:** 219–230
21 Douglas P., Pigaglio E., Ferrer A., Halfords N. G. and MacKintosh C. (1997) Three spinach leaf nitrate reductase-3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca2+ ions. Biochem. J. **325:** 101–109
22 Sugden C., Donaghy P. G., Halford N. G. and Hardie D. G. (1999) Two SNF1-related protein kinases from spinach leaf phosphorylate and inactivate 3-hydroxy-3-methylglutaryl-coenzyme A reductase, nitrate reductase and sucrose phosphate synthase in vitro. Plant Physiol. **120:** 257–274
23 Sugden C., Crawford R. M., Halford N. G. and Hardie D. G. (1999) Regulation of spinach SNF1-related (SnRK1) kinases by protein kinases and phosphatases is associated with phosphorylation of the T loop and is regulated by 5′-AMP. Plant J. **19:** 433–439
24 Hardie D. G., Carling D. and Carlson M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cell? Annu. Rev. Biochem. **67:** 821–855
25 Douglas P., Moorhead G., Hong Y., Morrice N. and MacKintosh C. (1998) Purification of a nitrate reductase kinase from *Spinacea oleracea* leaves, and its identification as a calmodulin-domain protein kinase. Planta **206:** 435–442
26 Miller A. J. and Sanders D. (1987) Depletion of cytosolic free calcium induced by photosynthesis. Nature **326:** 397–400

27 Aitken A. (1996) 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trends Cell Biol. **6:** 341–347
28 Moorhead G., Douglas P., Cotelle V., Harthill J., Morrice N., Meek S. et al. (1999) Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. Plant J. **18:** 1–12
29 Toroser D., Athwal G. S. and Huber S. C. (1998) Site-specific regulatory interaction between spinach leaf sucrose- phosphate synthase and 14-3-3 proteins. FEBS Lett. **435:** 110–114
30 Lopez-Girona A., Furnari B., Mondesert O. and Russell P. (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. Nature **397:** 172–175
31 Brunet A., Bonni A., Zigmond M. J., Lin M. Z., Juo P., Hu L. S. et al. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell **96:** 857–868
32 May T. and Soll J. (2000) 14-3-3 proteins form a guidance complex with chloroplast precursor proteins in plants. Plant Cell **12:** 53–64
33 Van Der Hoeven P. C., Van Der Wal J. C., Ruurs P., Van Dijk M. C. and Van Blitterswijk J. (2000) 14-3-3 isotypes facilitate coupling of protein kinase C-zeta to Raf-1: negative regulation by 14-3-3 phosphorylation. Biochem. J. **345 Pt 2:** 297–306
34 Baunsgaard L., Fuglsang A. T., Jahn T., Korthout H. A., de Boer A. H. and Palmgren M. G. (1998) The 14-3-3 proteins associate with the plant plasma membrane H(+)-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. Plant J. **13:** 661–671
35 Meek S., Morrice N. and MacKintosh C. (1999) Microcystin affinity purification of plant protein phosphatases: PP1C, PP5 and a regulatory A-subunit of PP2A. FEBS Lett. **457:** 494–498
36 Kaiser W. M., Spill D. and Brendle-Behnisch E. (1992) Adenine nucleotides are apparently involved in the light-dark modulation of spinach-leaf nitrate reductase. Planta **186:** 236–240
37 Aguera E., Poblete L., delaHaba P. and Maldonado J. M. (1999) Light modulation and in vitro effects of adenine nucleotides on leaf nitrate reductase activity in cucumber (*Cucumis sativus*). Physiologia Plantarum **105:** 218–223
38 Athwal G. S., Huber J. L. and Huber S. C. (1998) Biological significance of divalent metal ion binding to 14-3-3 proteins in relationship to nitrate reductase inactivation. Plant Cell Physiol. **39:** 1065–1072
39 Huber S. C. and Kaiser W. M. (1996) 5-aminoimidazole-4-carboxamide riboside activates nitrate reductase in darkened spinach and pea leaves. Physiologia Plantarum **98:** 833–837
40 Kaiser W. M. and Huber S. C. (1997) Correlation between apparent activation state of nitrate reductase (NR), NR hysteresis and degradation of NR protein. J. Exp. Botany **48:** 1367–1374
41 Botrel A. and Kaiser W. M. (1997) Nitrate reductase activation state in barley roots in relation to the energy and carbohydrate status. Planta **201:** 496–501
42 Botrel A., Magne C. and Kaiser W. M. (1996) Nitrate reduction, nitrite reduction and ammonium assimilation in barley roots in response to anoxia. Plant Physiol. Biochem. **34:** 645–652
43 Provan F. and Lillo C. (1999) Photosynthetic post-translational activation of nitrate reductase. J. Plant Physiol. **154:** 605–609
44 Yamasaki H. and Sakihama Y. (2000) Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. FEBS Lett. **468:** 89–92
45 Scheible W. R., Gonzalez-Fontes A., Morcuende R., Lauerer M., Geiger M., Glaab J. et al. (1997) Tobacco mutants with a decreased number of functional nia genes compensate by modifying the diurnal regulation of transcription, post-translational modification and turnover of nitrate reductase. Planta **203:** 304–319
46 Man H. M., Abd-El Baki G. K., Stegmann P., Weiner H. and Kaiser W. M. (1999) The activation state of nitrate reductase is not always correlated with total nitrate reductase activity in leaves [In Process Citation]. Planta **209:** 462–468
47 MacKintosh C. (1998) Regulation of cytosolic enzymes in primary metabolism by reversible protein phosphorylation. Curr. Opin. Plant Biol. **1:** 224–229
48 Xing H., Zhang S., Weinheimer C., Kovacs A. and Muslin A. J. (2000) 14-3-3 proteins block apoptosis and differentially regulate MAPK cascades. EMBO J. **19:** 349–358
49 Bachmann M., Huber J. L., Liao P. C., Gage D. A. and Huber S. C. (1996) The inhibitor protein of phosphorylated nitrate reductase from spinach (*Spinacia oleracea*) leaves is a 14-3-3 protein. FEBS Lett. **387:** 127–131
50 Zha J., Harada H., Yang E., Jockel J. and Korsmeyer S. J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). Cell **87:** 619–628
51 Cotelle V., Meek S. E. M., Provan F., Milne F. C., Morrice N. and MacKintosh C. (2000) 14-3-3s regulate global cleavage of their diverse binding partners in sugar-starved *Arabidopsis* cells. EMBO J. **19:** 2869–2876
52 Camoni L., Harper J. F. and Palmgren M. G. (1998) 14-3-3 proteins activate a plant calcium-dependent protein kinase (CDPK). FEBS Lett. **430:** 381–384
53 Sirover M. A. (1999) New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim. Biophys. Acta **1432:** 159–184
54 Hohmann S., Bell W., Neves M. J., Valckx D. and Thevelein J. M. (1996) Evidence for trehalose-6-phosphate-dependent and -independent mechanisms in the control of sugar influx into yeast glycolysis. Mol. Microbiol. **20:** 981–991
55 Goddijn O. and Smeekens S. (1998) Sensing trehalose biosynthesis in plants. Plant J. **14:** 143–146
56 Martinis S. A., Plateau P., Cavarelli J. and Florentz C. (1999) Aminoacyl-tRNA synthetases: a new image for a classical family. Biochimie **81:** 683–700
57 Koch K. E. (1996) Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47:** 509–540
58 Morcuende R., Krapp A., Hurry V. and Stitt M. (1998) Sucrose-feeding leads to increased rates of nitrate assimilation, increased rates of alpha-oxoglutarate synthesis and increased synthesis of a wide spectrum of amino acids in tobacco leaves. Planta **206:** 394–409
59 Markiewicz E., Wilczy′nski G., Rzepecki R., Kulma A. and Szopa J. (1996) The 14-3-3 protein binds to the nuclear matrix endonuclease and has a possible function in the control of plant senescence. Cell. Mol. Biol. Lett. **1:** 391–415
60 Wilczynski G., Kulma A. and Szopa J. (1998) The expression of 14-3-3 isoforms in potato is developmentally regulated. J. Plant Physiol. **153:** 118–126