Functional Analysis of SNF1-related Protein Kinase from wheat

Yoshihisa Ikeda

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Contents

ABBREVIATIONS

ATA	aurin tricarboxylic acid
BA	N^6-benzylaminopurine
bp	base pair
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-N,N,N,N,-tetraacetic acid
E. coli	*Escherichia coli*
GST	glutathione S-transferase
IPTG	isopropyl thio-β -D-galactoside
kb	kilobase
kDa	kilodalton
nt	nucleotides
min	minute
mRNA	messenger RNA
PCR	polymerase chain reaction
PMSF	phenylmethyl sulfonyl fluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	polyacrylamide electrophoresis
Tris	tris(hydroxymetyl)methylglycine
UTR	untranslated region

Introduction

Plants have developed systems to quickly and accurately transmit stimuli from the external environment and to adjust their metabolic pathways by modulating expression of sets of genes. Activation and/or inactivation of appropriate genes in response to particular stimuli are mediated through well-tuned signal transduction systems, in which protein phosphorylation cascades play crucial roles (Crews and Erikson, 1993; Stone and Walker, 1995). Phytohormones are also thought to be indispensable in signaling pathways, which involve perception and transduction of external signals (Klee and Estelle, 1991). Cytokinins have attracted particular interest because of their diverse physiological functions in response to light (Miller, 1956; Su and Howell, 1995), nutrients (Yu et al., 1998), growth (Brzobohaty et al., 1994; Chaudhury et al., 1993; D'Agostino and Kieber, 1999) and even wounding (Sano et al., 1996; Sano et al., 1994; Simmons et al., 1992). Recent work has revealed that cytokinins enhance transcription of several genes that are involved in nutrient metabolism (Dominov et al., 1992; Lu et al., 1990) and self-defense (Sano et al., 1996; Simmons et al., 1992).

During photosynthesis, when carbohydrates are synthesized, expression of many genes is coordinately regulated by external factors including light, carbon dioxide, temperature, and by internal factors including phytohormones such as cytokinins. It has been suggested that feedback regulation of photosynthesis is due to repression of photosynthetic gene transcription by carbohydrates (Sheen, 1990). Recent works have suggested that hexokinase, which was postulated to play an important role in sugar sensing in yeast (Ma et al., 1989; Rose et al., 1991), could be responsible for sensing sugars to repress a number of plant genes (Graham et al., 1994; Jang and Sheen, 1994).

However, there are some examples of hexose-independent changes in gene expression (Dijkwel et al., 1997; Wenzler et al., 1989) and in proton-sucrose symporter activity (Chiou and Bush, 1998). Thus, mechanisms by which sugar is sensed and sugar signals are transduced are not completely clear yet.

The sugar-catabolic mechanism has been extensively investigated in yeast (*Saccharomyces cerevisiae*) (Carlson, 1987). A protein kinase, SNF1, functions as a regulator to derepress many glucose-repressed genes, such as *SUC2* encoding a secreted invertase that hydrolyzes sucrose to glucose and fructose. In higher organisms, a similar mechanism has been predicted to function in the sugar assimilation process, and indeed genes with a significant sequence similarity with the yeast *SNF1* gene have been identified in organisms ranging from mammals (Hardie et al., 1998) to plants (Halford and Hardie, 1998). To date, more than 20 genes have been identified from higher plants, and termed as SNF1-related protein kinases (SnRKs). Based on putative amino acid sequence similarities, they are classified into three distinct subgroups (SnRK1 through SnRK3) (Halford and Hardie, 1998). Those from rye (RKIN1) (Alderson et al., 1991), tobacco (NPK5) (Muranaka et al., 1994) and *Arabidopsis* (AKIN10, AKIN11) (Bhalerao et al., 1999), all of which belong to the SnRK1 group, have been shown to complement the yeast *snf1* mutant cells. Antisense expression of potato SnRK1 homolog, PKIN1, resulted in a loss of sugar-inducible expression of sucrose synthase gene in leaves and tubers (Purcell et al., 1998). In barley aleurone layers, SnRK2 homolog (PKABA) has been shown to mediate abscisic acid-supressed gene expression (Gomez-Cadenas et al., 1999). Arabidopsis homolog of PKABA, AAAK1 (ABA-activated serine-threonine protein kinase), was shown to mediate ABA-induced anion channel activation and stomata closure (Li et al., 2000). It was also shown that the Sac3

gene product of Chlamydomonas, which belongs to SnRK2 subfamily, positively and negatively regulate the responses of the cell to sulfur limitation (Davies et al., 1999). However, functional analysis of SnRK3 has not been performed.

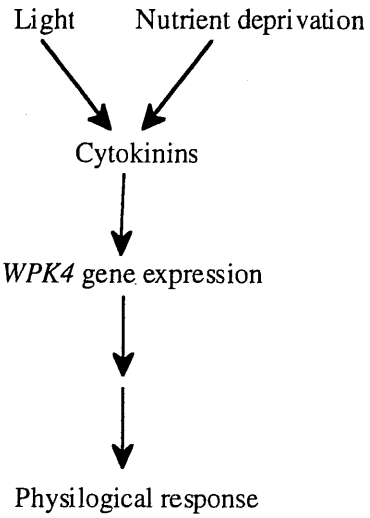

Figure 1 Hypothetical flowchart of signaling pathway of WPK4 transcripts accumulation. The transcript level of WPK4 is increased by both light and nutrition deprivation through the action of cytokinins.

WPK4, a gene encoding a SNF1-related protein kinase, was isolated from wheat (Sano and Youssefian, 1994). The deduced amino acid sequence of WPK4 suggested it to belong the SnRK3 subfamily (Halford and Hardie, 1998). The protein kinase catalytic domain is located in the N-terminal half and has 40-50% amino acid sequence identity with SnRK1s. However, beyond the kinase domain, it has little sequence similarity with other plant SnRKs. WPK4 also contains the proline-rich consensus sequence of XPYPPXP (X and Y refer to any residue and hydrophobic residues, respectively), which is predicted to be recognized by the SH3 domain present in cytoskeletal elements and signal transducing proteins (Fig. 2A) (Musacchio et al., 1994). The transcript level of *WPK4* is increased by both light and nutrition deprivation through the action of cytokinins (Sano and Youssefian, 1994). A hypothetical flowchart of signaling pathway of WPK4 is illustrated in Fig. 1.

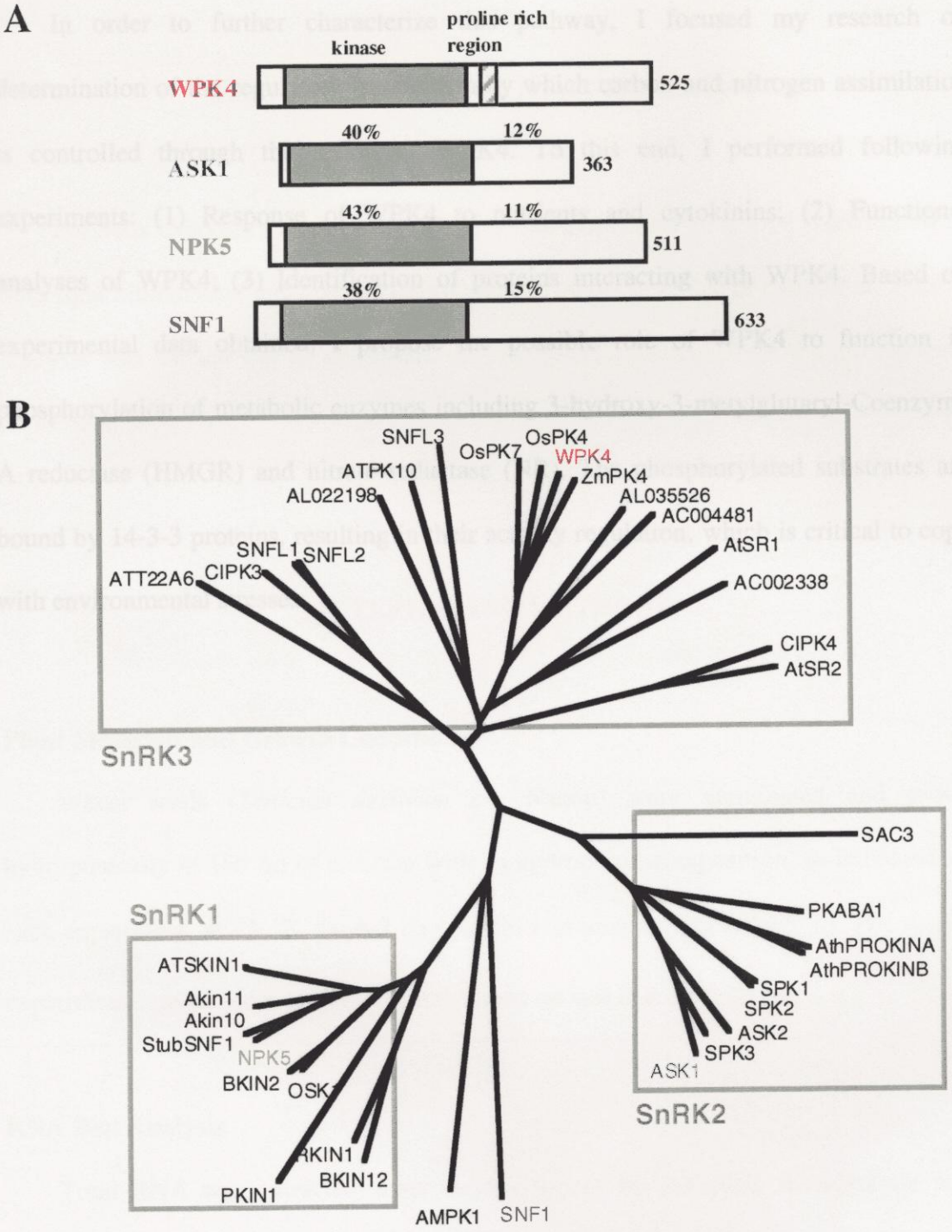

Figure 2. Structure and evolutional distance of plant SNF1-related protein kinases. (A) Domain structure of members of the SNF1 family of protein kinases. The N-terminal protein kinase catalytic domain is indicated as colour box and the proline rich region of WPK4 is hatched. Figures above N- and C-terminal domain give the degree of amino acid sequence identity with WPK4, isolated from wheat in our laboratory. Numbers at the right of each diagram give the number of amino acid residues in the protein. (B) Dendrogram showing relative evolutionary distances between members of the SNF1 protein kinase family. Alignments were produced using the clustal x software. The boxes superimposed over the Dendrogram show the division of the plant members of the family into subfamilies. OsPK4, OsPK7, ZmPK4, AtSR1 and AtSR2 were isolated and characterized in our labolatory.

In order to further characterize this pathway, I focused my research on determination of the regulatory mechanism by which carbon and nitrogen assimilation is controlled through the action of WPK4. To this end, I performed following experiments: (1) Response of WPK4 to nutrients and cytokinins; (2) Functional analyses of WPK4; (3) Identification of proteins interacting with WPK4. Based on experimental data obtained, I propose the possible role of WPK4 to function in phosphorylation of metabolic enzymes including 3-hydroxy-3-metylglutaryl-Coenzyme A reductase (HMGR) and nitrate reductase (NR). The phosphorylated substrates are bound by 14-3-3 proteins, resulting in their activity regulation, which is critical to cope with environmental stresses.

Chapter I

Expression pattern of *WPK4* transcripts

Previous experiments suggested that the transcript level of *WPK4* is increased by both light illumination and nutrient deprivation through the action of cytokinins. Further analysis was carried out to determine the regulatory mechanism of the *WPK4* gene expression.

MATERIAL AND METHODS

Plant Materials and Growth Conditions

Wheat seeds (*Triticum aestivum* cv. Nanbu) were germinated and grown hydroponically in 160 ml of solution with an appropriate composition, as indicated for each experiment, at 23 ℃ for 6-7 days under continuous light conditions. For further experiments, plants were grown and maintained on soil in a greenhouse.

RNA Blot Analysis

Total RNA was extracted from various tissues by the aurin tricarboxylic acid (ATA) method (Verwoerd et al., 1989). Aliquots of 36 μg per lane were denatured, fractionated by 1.0% formaldehyde/agarose gel electrophoresis, and transferred onto nylon membranes (Hybond-N, Amersham). Northern hybridizations were performed according to standard protocols (Sambrook et al., 1989). As the *WPK4*-specific probe, a

0.8-kb fragment was amplified by PCR using primers for FWD: 5'-CCTTACTAGCCTGATCATGCG-3' and for RV: 5'-TTGTTCCTGTCAGTTGCACC-3.' As the *CAB* (chlorophyll a/b binding protein) probe, a 0.4-kb fragment was amplified by PCR using primers for FWD: 5'- GCAATGGTGTGAAGTTCGG -3' and for RV: 5'-ACGCGTTGTTGTTGACAGG-3'.As the *RBCS* (ribulose-1, 5-bisphosphate carboxylase/oxygenase small subunit) probe, a 0.3-kb fragment was amplified by PCR using primers for FWD: 5'- TGTCTTACTTGCCACCCCTC-3' and for RV: 5'-AGGGTACTCCTTCTTGACCTCC-3'. As the *NR* (nitrate reductase) probe, a 0.5-kb fragment was amplified by PCR using primers for FWD: 5'-AAGCACATCTTCGTTTGCG-3' and for RV: 5'-CGATCACGTACCACACCTTG-3'. Probes were radioactively labeled using the *Bca*BEST labeling Kit (TAKARA). To normalize for the amount of RNA loaded, the membrane was stripped off the former probe by boiling in a 0.1% SDS solution for 1 min before rehybridization with a wheat actin probe prepared as described earlier. Each mRNA level was estimated by measuring signal intensity with NIH image software. The value was normalized with values for actin mRNA transcripts.

RESULTS

Accumulation of *WPK4* Transcripts in Green Tissues

The tissue specific accumulation of *WPK4* transcripts was examined with RNA blot hybridization using mature plants. The *WPK4* transcripts accumulated predominantly in leaf sheath and leaf blade and to a lesser extent in spike

(approximately two weeks after heading) but not in roots, indicating a link between its expression and photosynthesis (Fig. 3). Based on this observation, the following experiments were performed mostly with light-grown green seedlings.

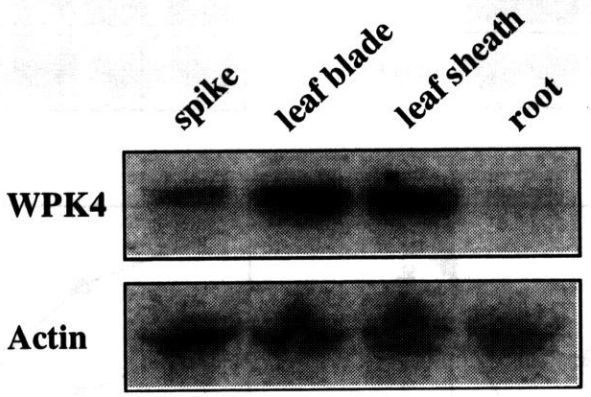

Figure 3. Tissue specific accumulation of *WPK4* transcripts. Total RNA was extracted from the indicated tissue of mature wheat plants, and subjected to RNA blot hybridization analysis. The hybridization probes were a 0.8 kb *WPK4* specific sequence (WPK4) and a 1.2 kb wheat actin cDNA (Actin) as the internal standard.

Effects of Inorganic Salts on *WPK4* Transcript Levels

Seven-day-old seedlings were exposed to various nutrient stresses and transcript accumulation of *RBCS*, *NR* and *WPK4* was examined by RNA blot hybridization. *RBCS* and *NR* were selected because transcript accumulation of the former is typically repressed by sugars, and that of the latter is rapidly induced by nitrates. When hydroponically grown seedlings were transferred to a 5-fold diluted MS medium, *WPK4* transcripts decreased within 3h, whereas transcripts for *RBCS* and *NR* accumulated in a time-dependent manner (Fig. 4A). When seedlings, which were initially grown in a 5-fold diluted MS medium, were transferred to a nutrient deprived medium, i.e., water, *WPK4* transcripts increased by 48 h (Fig. 4B). In contrast, transcripts for *RBCS* decreased by 48 h, and *NR* transcripts rapidly declined within 3 h (Fig. 4B).

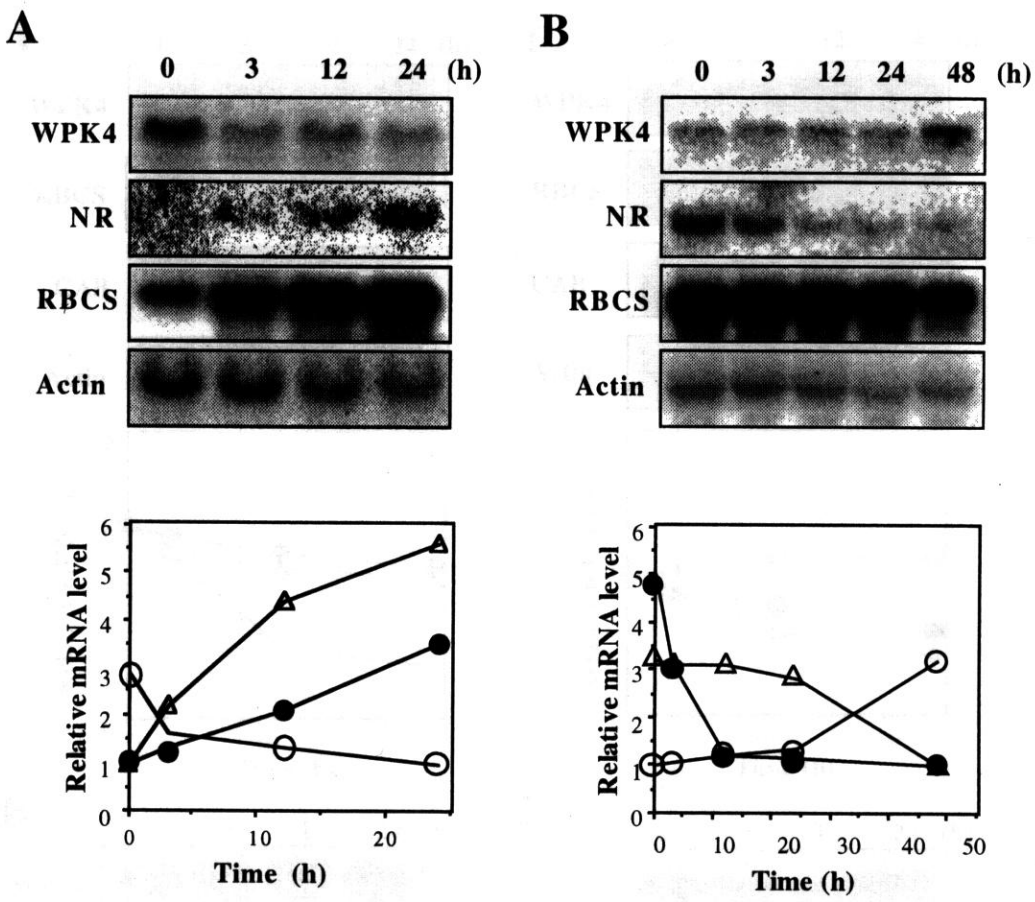

Figure 4. Effects of inorganic salt on *WPK4* transcript accumulation. Total RNA was isolated from indicated samples and analyzed by RNA blot hybridization. (A) Seven-day-old seedlings, grown in water, were transferred to 1/5 MS medium and harvested after the indicated time period. Relative mRNA levels were densitometrically estimated for *WPK4* (○), *NR* (●) and *RBCS* (△). (B) Seven-day-old seedlings, grown in 1/5 MS medium, were transferred to water and harvested after the indicated time period. Relative mRNA levels were densitometrically estimated for *WPK4* (○), *NR* (●) and *RBCS* (△). The hybridization probes were a 0.8 kb *WPK4* specific sequence (WPK4), a 0.5 kb wheat *NR* sequence (NR), a 0.6 kb wheat *RBCS* sequence (RBCS) and a 1.2 kb wheat actin cDNA (Actin).

Effects of Sugars on *WPK4* Transcript Levels

When experiments were performed with sucrose solution instead of MS medium, similar results were obtained. *CAB* gene was also selected because of the same reason of that of *RBCS*. Addition of 0.7% (w/v) sucrose to seedlings cultivated in water resulted in a decrease of *WPK4* transcripts 3h later as well as *RBCS* and *CAB* transcripts (Fig. 5A). When seedlings were cultured initially in 0.7% (w/v) sucrose and then transferred

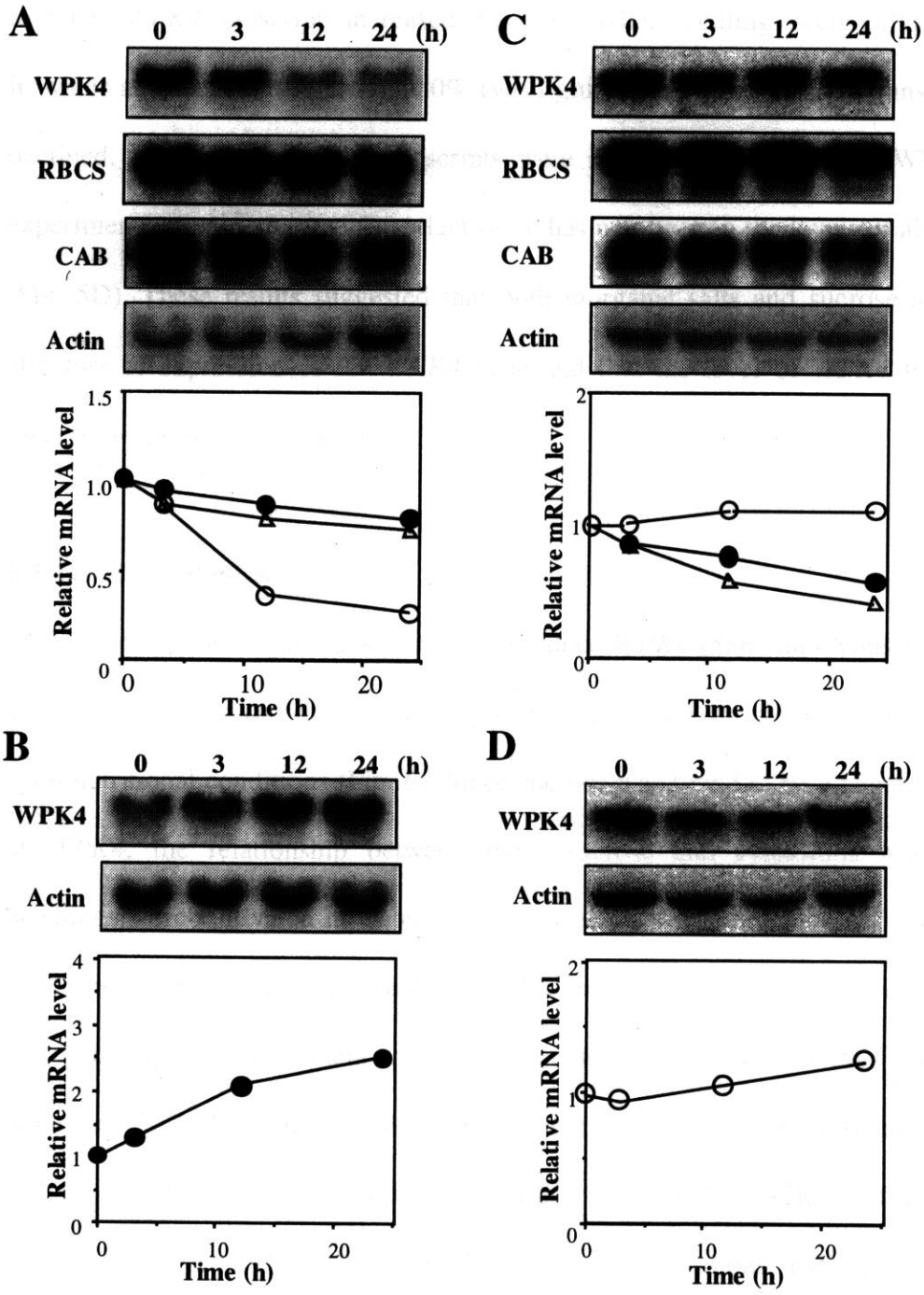

Figure 5. Effects of various sugars on *WPK4* transcript accumulation. Total RNA was isolated from indicated samples and analyzed by RNA blot hybridization. (A) Seven-day-old seedlings, grown in water, were transferred to water containing 0.7% (w/v) sucrose and harvested after the indicated time period. Relative mRNA levels were densitometrically estimated for *WPK4* (○), *RBCS* (●) and *CAB* (△). (B). Similarly, seven-day-old seedlings, grown in water containing 0.7% (w/v) sucrose, were transferred to water and harvested after the indicated time period. Relative mRNA levels were densitometrically estimated for sucrose removal(●). (C) Seven-day-old seedlings, grown in water, were transferred to water containing 1% (w/v) glucose and harvested after the indicated time period. Relative mRNA levels were densitometrically estimated for *WPK4* (○), *RBCS* (●) and *CAB* (△). (D) Seven-day-old seedlings, grown in water, were transferred to water containing 1% (w/v) galactose and harvested after the indicated time period. Relative mRNA levels were densitometrically estimated for galactose addition (○). The hybridization probes were a 0.8 kb *WPK4* specific sequence (WPK4) and a 1.2 kb wheat actin cDNA (Actin).

to water, *WPK4* transcripts increased (Fig. 5B). When seedlings were cultured initially in water and then transferred to 1.0% (w/v) glucose, *RBCS* and *CAB* transcripts were declined. In contrast, *WPK4* transcripts were not affected (Fig. 5C). When similar experiments were performed for galactose, it has no effect on the levels of all transcripts (Fig. 5D). These results suggested that both inorganic salts and sucrose are negative effectors of expression of the *WPK4* gene and that the level of *WPK4* transcripts is reversibly controlled by sucrose.

Effects of Cytokinins

Cytokinins were previously shown to induce *WPK4* (Sano and Youssefian, 1994), and this study showed the degree of positive regulation by cytokinin was dependent upon nutritional conditions (Fig. 6). Since sucrose was found to be a negative regulator of *WPK4*, the relationship between these sucrose and cytokinins was analyzed. Seedlings were cultivated in water, and then treated with BA (N^6-benzylaminopurine), or sucrose or both. Upon treatment with BA alone, *WPK4* transcripts were increased up to 3-fold (Fig. 7A). Treatment with 0.7% sucrose down-regulated *WPK4* transcripts by more than 50% (Fig. 7B). When seedlings were cultivated in the presence of both BA and sucrose, *WPK4* transcripts were temporarily increased for 12h, but then decreased to the initial level by 24h (Fig. 7C). These observations indicate that sucrose antagonizes the BA effect, and therefore may be involved in signal transduction pathways of WPK4.

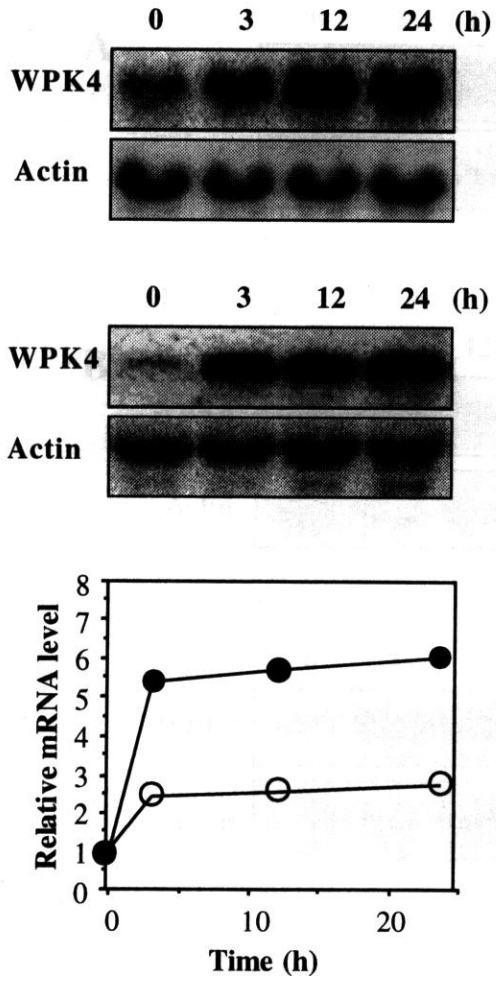

Figure 6. Effects of cytokinins on *WPK4* transcript accumulation. Total RNAs were isolated from indicated samples and subjected to RNA blot hybridization analysis. (A) Seven-day-old seedlings, grown in 1/2 MS medium were treated with 100 μ M BA for the indicated time period. (B) Seven-day-old seedlings, grown in 1/5 MS medium were treated with 100 μ M BA for the indicated time period. Relative mRNA levels were densitometrically estimated for samples extracted from seedlings grown in 1/2 MS (○) or in 1/5 MS (●) media.

Requirement of *de novo* protein synthesis for the induction of *WPK4* transcript accumulation

To examine whether *de novo* protein synthesis is required for the induction of *WPK4* transcript accumulation by cytokinins and nutrient deprivation, green seedlings were incubated with cycloheximide (20 μM) prior to the treatments. In neither case, did the drug block the induction (Fig. 8), suggesting that expression of *WPK4* gene does

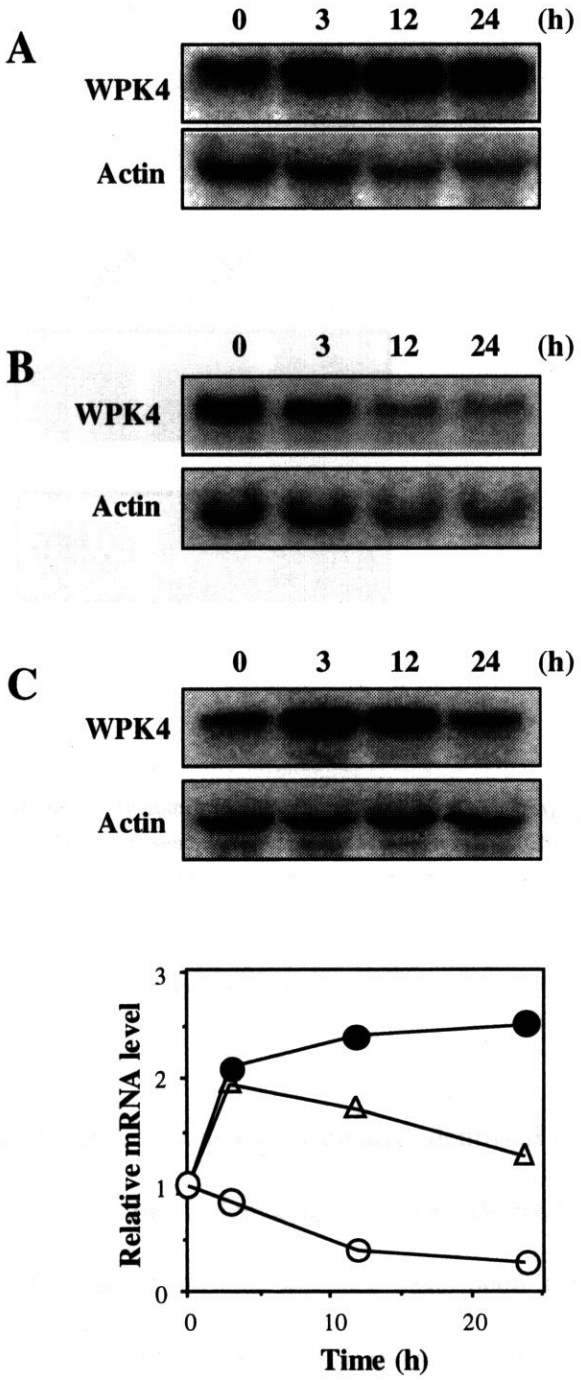

Figure 7. Antagonistic effects of cytokinins and sucrose. Total RNAs were isolated from indicated samples and subjected to RNA blot hybridization analysis. (A) Seven-day-old seedlings, grown in water were treated with 100 μM BA for the indicated time period. (B) Seven-day-old seedlings, grown in water were transferred to water containing 0.7% (w/v) sucrose and harvested after the indicated time period. (C) Seven-day-old seedlings, grown in water were transferred to water containing 0.7% (w/v) sucrose and 100 μM BA for the indicated time period. The hybridization probes were a 0.8 kb *WPK4* specific sequence (WPK4) and a 1.2 kb wheat actin cDNA (Actin). Relative mRNA levels were densitometrically estimated for samples extracted from seedlings grown in water treated with BA (●) or sucrose addition (○) or treated with BA and sucrose addition (△)

not require nascent peptide synthesis.

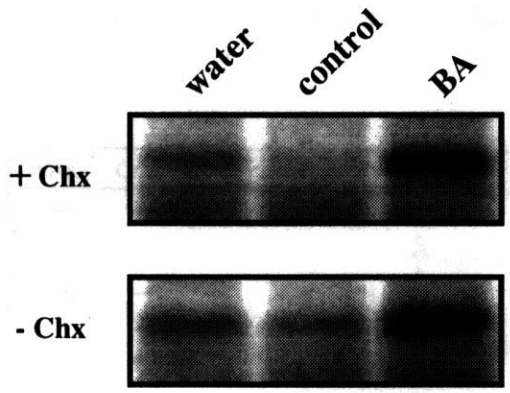

Figure 8. Effects of cycloheximide on the transcripts of *WPK4*. Seven-day-old seedlings grown in 1/5 MS medium were pretreated in the absence (-Chx) or presence (+Chx) of 20 μM cycloheximide (Chx) and transferred to water or treated with 10 μM BA for 24 hr. The hybridization probe was a 0.8kbp *WPK4* specific sequence.

DISCUSSION

The SNF1 protein kinase, which was originally identified in yeast cells, plays an essential role in glucose utilization (Celenza and Carlson, 1986). Since its discovery, many genes encoding SNF1-related protein kinases have been reported to exist in various organisms including higher plants (Stone and Walker; 1995; Halford and Hardie, 1998). Analyses of the tissue specific expression revealed that these genes were classified into two groups; those encoding NPK5 (tobacco), AKIN10 (*arabidopsis*), BKIN2 (barley) and PKIN1 (pea) appeared to be ubiquitously expressed in all tissues, whereas those encoding BKIN12 (barley) and RKIN1 (rye) are specifically expressed in

cereal seeds (Alderson et al., 1991). Transcripts of *WPK4* in mature wheat plants were found in this study to mostly accumulate in leaf sheaths and leaf blades, indicating that WPK4 mainly functions in photosynthetic tissues. These different expression patterns suggest different functions among structurally related protein kinases, although the

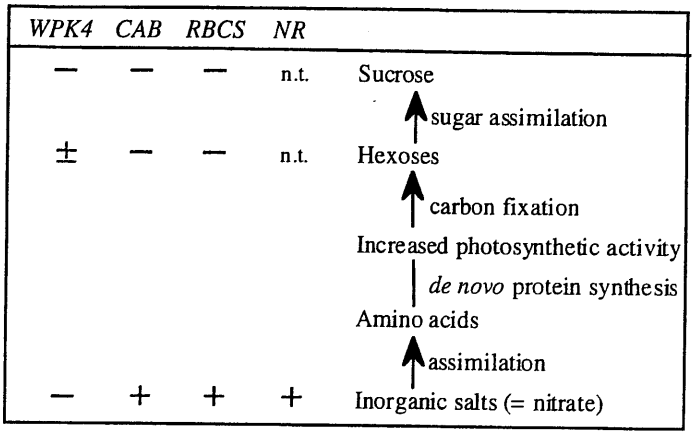

Figure 9. Summery of the findings for transcript accumulation.
Transcripts induced (+), repressed (-), not affected (±) and not tested (n.t.) are shown in the left. Arrows indicate the proposed assimilation steps and the resulting conpounds are shown in the right.

physiological roles of the majority of them have yet to be determined.

The level of *WPK4* transcripts was previously shown to increase upon exposure to an inorganic salt deficiency (Sano and Youssefian, 1994). Results from this study were summarized in Fig. 9 (left). Since the present kinetic analyses showed *WPK4* transcripts to be increased by sucrose depletion and decreased by sucrose application, the question arises as to whether inorganic salts or sucrose are primarily responsible for regulation of *WPK4* expression. To address this question, the transcript levels of *NR*, *RBCS* and *CAB* were simultaneously examined with those for *WPK4* upon salt or sugar treatments. The transcript level of *RBCS* fluctuated inversely, but consistently with that of *WPK4*, whereas *NR* transcripts responded differently. Although more experimental work is

necessary to determine the exact relationship between salts and sucrose, these observations suggest that the change of *WPK4* transcript level upon inorganic salt deprivation possibly results from decline of endogenous sucrose caused by decrease in photoassimilating activity. It has been considered that sucrose and hexose levels might be linked, depending on the sucrose-metabolizing enzymes. But a certain genes were known to be specifically induced by sucrose. This study revealed that transcripts of *WPK4* were reversibly regulated by sucrose but not by glucose (Fig. 5).

To my knowledge, *WPK4* is the only protein kinase gene whose transcription is up-regulated by cytokinins and down-regulated by sucrose. The fact that *WPK4* transcripts first increased and then gradually decreased when nutrient deprived seedlings were simultaneously exposed to both cytokinins and sucrose indicates that cytokinins predominate over sucrose. Thus, it is attractive to speculate that cytokinins initially induce production of WPK4, which triggers/activates sugar synthesis, and that accumulated sugars block a certain step in signal transduction pathways of WPK4 to shut-down cytokinin signals. This kind of feedback control system might best explain our present data. Further work is now necessary to substantiate this hypothesis.

Chapter II

Enzymatic activity of WPK4

The mammalian homolog of SNF1, AMPK was shown to regulates key enzymes of cholesterol and fatty acid biosynthesis such as 3-hydroxy-3-metylglutaryl-Coenzyme A reductase (HMGR) and acetyl-CoA carboxylase (Hardie et al., 1998). Similar to AMPK, plant SNF1 homologs were shown to phosphorylate not only HMGR but also plant specific enzymes such as nitrate reductase (NR) and sucrose phosphate synthase (SPS) (Hardie et al., 1998). It was known that HMGR is the overall rate-limiting step for the whole isoprenoid biosynthetic pathway and that the activity of HMGR was inactivated by phosphorylation. SAMS peptide, a synthetic peptide containing the primary phosphorylation site for AMPK of rat acetyl-CoA carboxylase (Davies et al., 1989), was also reported to be phosphorylated by plant extracts (Mackintosh et al., 1992). Whereas NR catalyzes the first step in the assimilation of nitrogen from nitrate into nitrogen-containing compounds such as amino acids, SPS catalyzes a key step in sucrose biosynthesis in source tissues. These observations indicate SnRK1 may be involved in nutrient signal transduction pathways. However, functional analysis of SnRK3 have so far been few. Since the deduced amino acid sequence of WPK4 suggested it to belong the SnRK3 subgroup, further characterization on protein level was carried out.

As an initial step, kinase activity of WPK4 was analyzed. Next, substrate specificity was examined whether WPK4 phosphorylates HMGR or NR because the kinase domain of WPK4 was similar to other SnRKs. Finally, yeast complementation test was carried out whether WPK4 can be functional homolog of SNF1 in yeast cells.

MATERIAL AND METHODS

Construction of Plasmids

The *WPK4* cDNA was digested with *Eco*RI, and ligated to pGEX-2T (Pharmacia) at the *Eco*RI site to fuse in frame the coding sequence for glutathione S-transferase (GST) with that for WPK4. The resulting construct was designated pGEX-WPK4. A *WPK4* mutant (WPKM4) in which the AAG codon (nucleotide positions 230-232) for a Lys was substituted for a GAT codon (Asp) was also prepared with the aid of the polymerase chain reaction technique.

A DNA fragment encoding peptides from *Oryza sativa* HMGR (corresponding to residues 471 to 576), *Nicotiana tabacum* Nia1, encoding nitrate reductase, (corresponding to residues 469 to 560) were amplified by PCR and introduced *Eco*RI site (underline) at the 5'-end or *Xho*I site at the 3'-end (double underline). Oligonucleotides used in PCR reactions were as follows.

*Os*HMGR.F: 5'-GAATTCTCCCAGTGCATCAC-3'

*Os*HMGR.R: 5'-TACCTCGAGCATGCCACATGG-3'

*Nt*Nia1.F: 5'-GAATTCTGGTTCCGAGTAAAG-3'

*Nt*Nia1.R: 5'-GAAACTCGAGGCGTCATAG-3'

The PCR reactions were performed using the *Pyro*Best DNA polymerase (TAKARA). The amplification products were cloned into pT7 Blue vector and confirmed by DNA sequencing. Plasmids were digested with *Eco*RI and *Xho*I and the resulting fragments were purified by Prep-A-Gene and cloned into pGEX4-T1 (Pharmacia) at *Eco*RI and

*Xho*I sites, to create pGEX-HMGR or pGEX-Nia1.

Expression and Purification of GST Fusion Proteins

Plasmids pGEX-WPK4, pGEX-WPKM4 were introduced into the *Escherichia coli* DH5α strain, and the transformants were grown in two-liters of 2xYT medium with vigorous shaking at 21 ℃ to an optical density of 0.6 at 600 nm. Isopropyl thio-β-D-galactoside (IPTG) was then added to a final concentration of 0.1 mM, and the cultures were further incubated at 16 ℃ for 6 h. Cells were pelleted and suspended in 20 mL of lysis buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 10 mM 2-mercaptoethanol, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 10% sucrose, 0.5% lauryl sarcosinate] and disrupted by sonication. After ultracentrifugation, the supernatants were applied to a 3 mL glutathione-Sepharose (Pharmacia) column. The columns were washed with twenty times of bed volumes of T buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 10 mM 2-mercaptoethanol], then further washed with five times of bed volumes of T buffer containing 3M NaCl. Absorbed proteins were eluted with 15 mL elution buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 10 mM 2-mercaptoethanol, 25 mM glutathione (reduced form)] and the fractions containing GST-WPK4, GST-WPKM4 were applied to a DEAE-Toyopearl column equilibrated with T buffer to remove nonspecific proteins. Proteins were eluted with 50 mL T buffer with 0 to 0.25 M NaCl linear gradient. One-milliliter fractions were collected, assayed for protein concentration and subjected to SDS-polyacrylamide gel electrophoresis. The gels were stained with Coomassie brilliant blue R250. Fractions that contained purified GST-WPK4, GST-WPKM4 fusion proteins were concentrated

by ultrafiltration with Centricon (Amicon). Purified fusion proteins were used in further experiments.

For GST-HMGR or GST-Nia1 proteins purification, DH5 α cells harboring pGEX-HMGR or pGEX-Nia1 plasmids were grown in one-liters of LB medium with vigorous shaking at 37 ℃ to an optical density of 0.7 at 600 nm. IPTG was then added to a final concentration of 0.1 mM, and the cultures were further incubated at 25 ℃ for 6 h. Cells were pelleted and suspended in 20 mL of lysis buffer and disrupted by sonication. After ultracentrifugation, pellets were washed twice by 10 mL of lysis buffer and pelleted by centrifugation. The pellets were dissolved in 25 mL of T buffer containing 8M urea, incubated at room temperature for an hour then separated by ultracentrifugation. After ultracentrifugation for an hour, supernatants were applied to a glutathione-Sepharose (Pharmacia) column and GST fusion proteins were purified described above.

Autophosphorylation Reaction of WPK4

To measure autophosphorylation, aliquots of 100 ng of GST-WPK4 or GST-K75D fusion proteins were incubated with 0.1 mM [γ-^{32}P] ATP (6000 Ci/mmol) in kinase buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1 mM PMSF, 20 mM $MgCl_2$ or 20 mM $MnCl_2$ in a final volume of 30 μL at room temperature for 30 min. The reaction was terminated by adding 1/4 volume of 5x Laemmli's sample buffer [200 mM Tris-HCl (pH 6.8), 50 mM dithiothreitol, 5% SDS, 50% glycerol, 0.1% bromophenol blue]. After heating at 95 ℃ for 5 min, the mixture was subjected to electrophoresis in 12.5% SDS-polyacrylamide gel, stained with Coomassie brilliant blue

R-250, dried, and autophotographed at –80 ℃ for 16h.

In vitro Kinase Assay

To measure kinase activity, aliquots of 20 ng of GST-WPK4 fusion protein was incubated with 0.1 mM [γ-^{32}P] ATP (6000 Ci/mmol) in kinase buffer. A kinase assay was conducted by adding to the kinase buffer with 1 μg of myelin basic proteins (MBP) or of GST-HMGR or GST-Nia1 as substrates. The reaction was terminated by adding 1/4 volume of 5x Laemmli's sample buffer [200 mM Tris-HCl (pH 6.8), 50 mM dithiothreitol, 5% SDS, 50% glycerol, 0.1% bromophenol blue]. After heating at 95 ℃ for 5 min, the mixture was subjected to electrophoresis in 12.5% SDS-polyacrylamide gel, stained with Coomassie brilliant blue R-250, dried, and autophotographed at –80 ℃ for 16h.

Yeast Complementation Test

A fragment containing the ADH1 promoter and CYC1 terminator was excised from pD2 and inserted into YEplac 195 (2μ, *URA3*) at *Sph*I and *Hin*dIII sites to generate pF0. An *Eco*RI fragment containing *WPK4* cDNA was ligated into the *Eco*RI site of pF0 in both orientation (pWPK4: sense oriented, pASWPK4: antisense oriented). An *Eco*RI fragment containing the entire coding region of SUC2 was prepared by PCR using yeast genomic DNA as a template and primers tagged with *Eco*RI recognition sites, then ligated into pF0 (pSUC2). The *WPKM4* construct was prepared with the aid of PCR, then ligated into the *Eco*RI site of pF0 to generate pWPKM4. The C-terminal truncated *WPK4* was constructed as follows: A 0.9kb 5' terminal fragment of *WPK4* was excised

from pGEX-2T by digestion with *Eco*RI and *Pvu*II, then inserted into pBluescript II KS (-) at *Eco*RI and *Sma*I sites to generate pWB1. A 5' terminal fragment of *WPK4* was excised by *Bam*HI and *Hin*dIII double digestion from pWB1, then ligated to the pKF19 Km vector digested with *Bam*HI and *Hin*dIII to generate pWK1. The 5' terminal fragment of *WPK4*, generated by digestion of pWK1 with *Eco*RI, was ligated into pF0 (pWPK4 C). The orientation of the ligated fragment was confirmed by appropriate restriction endonuclease digestion. Resulting constructs were transformed into the yeast strain MCY1846 (*lys 2-801, ura3-52, snf1 Δ10*) (provided by Marian Carlson). For examination of the effects of various carbon sources on growth of transformed cells, they were plated on medium lacking uracil and supplemented with 2% sucrose and cultured at 30 ℃ under anaerobic conditions.

RESULTS

Divalent Cation Requirement and Substrate Specificity

In order to characterize the gene product, a fusion protein of WPK4 with GST was produced in *Escherichia. coli* cells, and its enzymatic activity was assayed. In the presence of Mg^{2+}, GST-WPK4 fused protein exhibited autophosphorylation activity. The reaction, however, was not observed when Mg^{2+} was replaced with Mn^{2+} ions. Two protein fragments were visible when the GST-WPK4 proteins were purified, possibly due to a slight difference in size of expressed proteins. Both proteins have protein kinase activity (Fig.10). When the Lys at the amino acid position 75 of WPK4, which is

analogous to the Lys that is essential for ATP binding in most Ser/ Thr protein kinases, was replaced by Asp (WPKM4), autophosphorylation activity was completely abolished (Fig. 10).

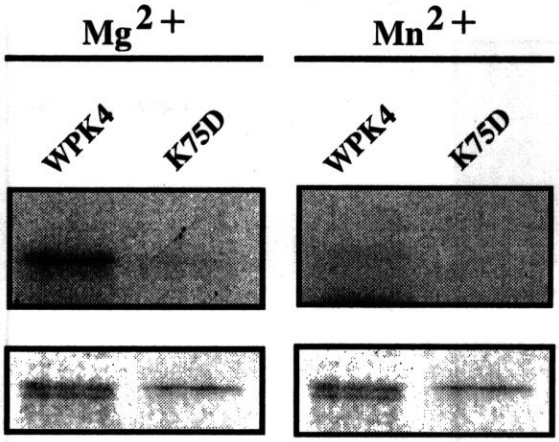

Figure 10. Autophosphorylation analysis of GST-WPK4 and GST-K75D fusion proteins. Samples were antophosphorylated in reaction mixtures containing either Mg^{2+} (left) or Mn^{2+} (right) and separated by SDS-PAGE. The autoradiogram and profiles of the gel stained with Coomassie brilliant blue are shown in the upper and lower panels, respectively.

WPK4 also phosphorylated myelin basic protein (MBP) (Fig. 11A), with the same divalent cation requirement. Since the several substrates of the SnRK1 have been identified, a peptide fragment of rice HMGR or tobacco Nia1 either containing the putative phosphorylation site was tested in place of the non-specific substrate, MBP. The results clearly showed WPK4 to be able to phosphorylate both GST-HMGR and GST-Nia1 peptide, but not GST *in vitro* (Fig. 11B).

Complementation of Yeast *snf1* Mutation

To examine whether or not WPK4 functions in catabolite repression in yeast cells, a yeast complementation test was carried out. cDNAs encoding full length WPK4,

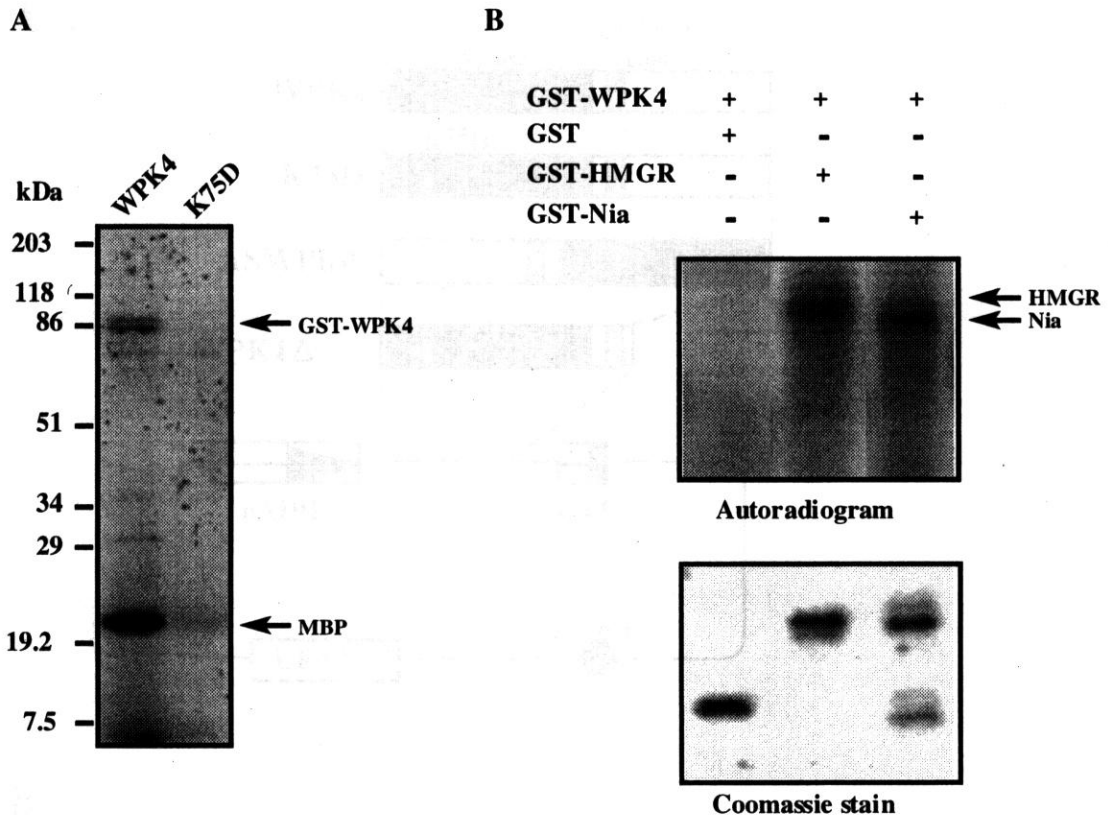

Figure 11. WPK4 functions as a protein kinase. (A) *In vitro* phosphorylation analysis of GST-WPK4 fusion proteins. Aliquots of 100 ng of GST-WPK4 or GST-K75D fusion proteins were incubated in the presence of 500 ng of myelin basic protein (A), or 1 μg of GST, GST-HMGR or GST-Nia fusion proteins (B) as the substrates. After the phosphorylation reaction, samples were separated by 12.5% SDS-PAGE, dried, and exposed for autoradiography. Relative molecular mass of the standard samples are indicated in left (in A). The autoradiogram and profiles of the gel stained with Coomassie brilliant blue are shown in the upper and lower panels, respectively (in B).

WPKM4, C terminal truncated WPK4 or antisense-oriented WPK4 were expressed in *snf1* mutant cells under the control of an ADH promoter. Mutant cells transformed with *SUC2*, which encodes a secreted invertase that hydrolyses sucrose to glucose and fructose, were used as a positive control. The *snf1* mutant cells, which carried the C-terminal truncated WPK4 containing the kinase domain but lacking the 3'-region beyond the kinase domain, grew on medium supplemented with sucrose. The growth rate was low in comparison with wild-type cells (Fig. 12). Cells containing the other constructs failed to grow on the medium. These results suggested that the C-terminal region functions as the regulatory domain, presumably by inhibiting the kinase activity.

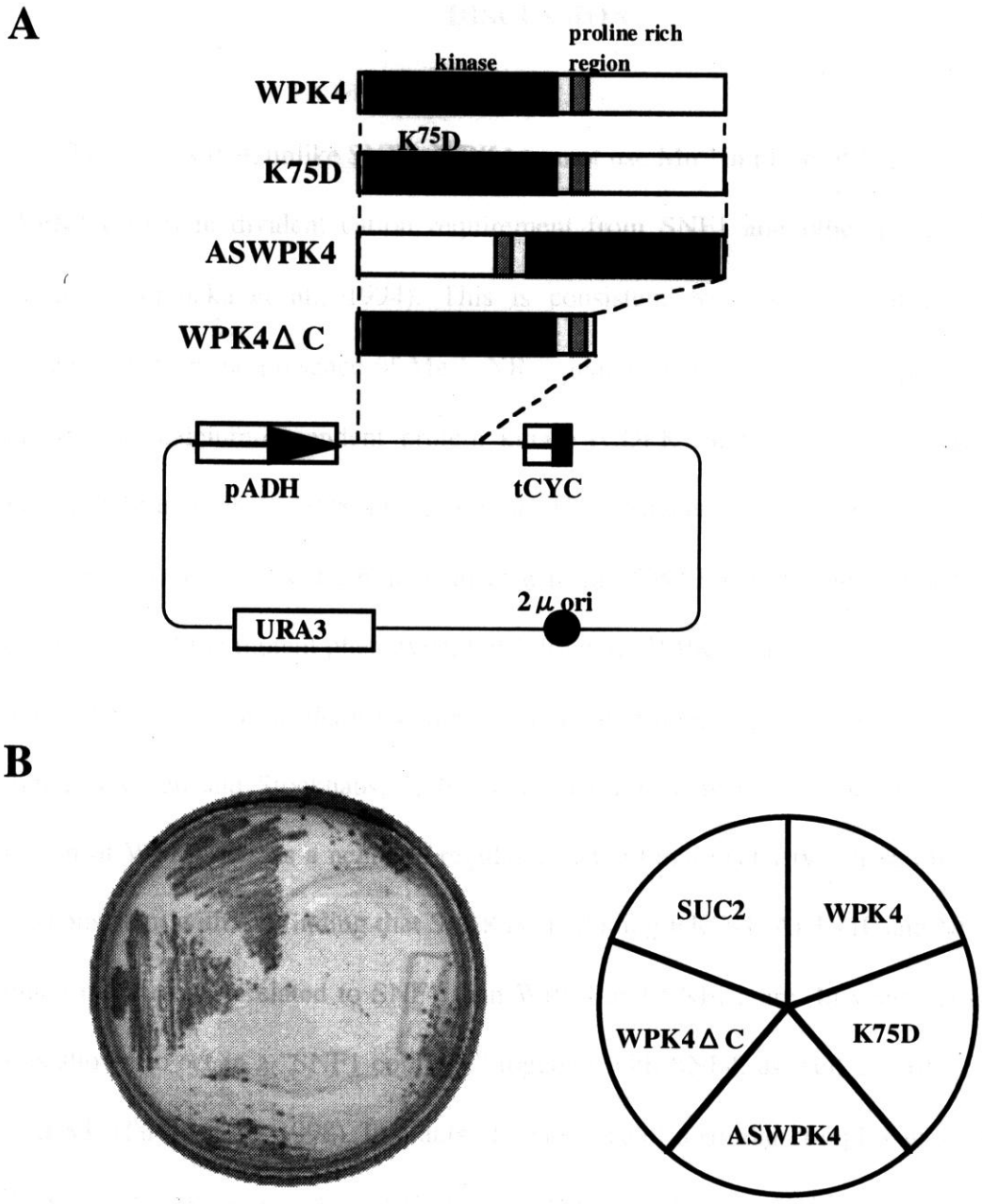

Figure 12. Complementation of *snf1* mutant cells by various WPK4 constructs. (A) Schematic representation of various *WPK4* constructs used in this assay. The N-terminal protein kinase catalytic domain is indicated as black box and the proline rich region of WPK4 is hatched(B) Cells of *S. cerevisiae* MCY1846 (*snf1*) harboring pWPK4, pASWPK4, pK75D, WPK4 C or pSUC2 were streaked on agar plates supplemented with 2% sucrose. Cells of MCY1846 were grown at 30℃ for 6 days.

DISCUSSION

The results that, unlike SNF1, WPK4 cannot use Mn^{2+} in place of Mg^{2+} suggest that WPK4 differs in divalent cation requirement from SNF1 and other proteins of this family (Muranaka et al., 1994). This is consistent with biochemical experiments showing that, in the presence of Mg^{2+}, NR is inactivated by phosphorylation catalyzed *in vitro* by calcium-dependent protein kinase (CDPK) and by SNF1-related protein kinases (Douglas et al., 1998; Douglas et al., 1997; Sugden et al., 1999).

Expression of the C-terminal truncated form of WPK4 in the *snf1* mutant of yeast complemented the mutant phenotype, but full length WPK4 was not effective. The full-length SNFL1 from *Sorghum bicolor* also exhibited no complementation of this yeast mutant (Annen and Stockhaus, 1998). It is therefore conceivable that the C-terminal region of WPK4 acts as a negative regulator of the kinase activity. These observations are consistent with the finding that SnRK1s (including RKIN1, AKIN10 and NPK5) are much more closely related to SNF1 than WPK4 and SNFL1 are. In yeast cells, SNF1 was shown to act in a "SNF1 complex" together with SNF4, as well as SIP1, SIP2, or GAL83 (Hardie et al., 1998). In plants, the presence of a similar complex was reported. By yeast two-hybrid system, AKIN10 was shown to interact with PRL1 protein, which contains repetitive sequences characteristic of a family of regulatory proteins known as WD-40 repeat proteins (Bhalerao et al., 1999; Nemeth et al., 1998). StubSNF1 from potato interacted with yeast GAL83/SIP1/SIP2 ortholog (termed StubGAL83) and with SNF4 in the same system (Lakatos et al., 1999). Both PRL1 and StubGAL83 proteins bind at C-terminal region of SnRK1 proteins. Although the biochemical evidence showed that SnRK1 could phosphorylate HMGR, NR, and SPS *in vitro* (Halford and

Hardie, 1998), these observations suggested that SnRK1 proteins are orthologs of SNF1 and being required to activate relevant gene expression. Whether or not SnRK2 and SnRK3 functionally similar to SnRK1 has not been determined. Here I found that WPK4 can indeed share substrates with SnRK1 *in vitro*. Whether or not WPK4 phosphorylates NR or HMGR as the native substrate awaits to be determined.

Chapter III

Isolation of WPK4-interacting factors by yeast two-hybrid system

It was shown that, using yeast two-hybrid system, AKIN α 1, a member of SnRK1, interacted with noncatalytic subunits, AKIN β 1/β 2 and AKIN γ (Bouly et al., 1999). Also AKIN10 and AKIN11, both belonging to SnRK1, were found to form a complex with PRL1 protein (Bhalerao et al., 1999), which contains repetitive sequences characteristic of a family of regulatory proteins known as WD-40 repeat proteins (Nemeth et al., 1998). StubSNF1 from potato interacted with the yeast GAL83/SIP1/SIP2 ortholog (StubGAL83) and yeast SNF4 in the same system (Lakatos et al., 1999). These proteins were found to bind at noncatalytic regions located at the C-terminal region of SnRK1. The C-terminal region of SNF1 family proteins was proposed to be directly involved in interactions with signaling partners. These observations suggested that the C-terminal region of each protein play distinct roles among plant SNF1-related protein kinase subfamily. In order to identify proteins that interact with WPK4, yeast two-hybrid system was employed and several cDNA clones were identified.

MATERIAL AND METHODS

Construction of cDNA library

HybriZAP-2.1 Two-Hybrid Predigested Vector Kit (STRATAGENE) was used in

this study. The entire coding region of *WPK4* was digested with *Eco*RI and filled in Klenow fragment to make blunt end. After blunting, the DNA fragment was ligated in pGBT9 vector at *Sma*I site. The cloned vector was digested with *Eco*RI and *Pst*I, then ligated in pBD-Gal4 Cam vector to fuse to GAL4 DNA binding domain (GBD). The construct was confirmed by DNA sequencer. Wheat seedlings, grown in distilled water for 7 days, were treated for 24 hours with 100 μ M BA (N^6-benzylaminopurine), and poly $(A)^+$ mRNA was isolated according a protocol of The Poly A Tract mRNA isolation systems (Promega). The cDNA was synthesized using a ZAP-cDNA Synthesis Kit (STRATAGENE) and fused to GAL4 transactivation domain (GAD) in pAD-GAL4-2.1 phagemid vector. This library contains approximately 2×10^7 independent clones.

Yeast Two-Hybrid Screen

Yeast strain YRG-2 was transformed by the polyethylene glycol/lithium acetate method(Ito et al., 1983; Schiestl and Gietz, 1989). YRG2 was initially transformed with pBD-WPK4, then with library plasmids. Transformants (1×10^6) harboring bait and pray plasmids were screened on plates containing 20 mM 3-aminotriazole (3-AT) in synthetic dextrose (SD) medium without Trp, Leu and His.

RESULTS AND DISCUSSION

Isolation of WPK4-interaction factors by yeast-two hybrid system

In order to identify proteins which interact with WPK4, a cDNA library containing 2×10^7 independent clones was constructed from mRNAs isolated from wheat seedlings which were treated with N^6-benzylaminopurine (BA), and screened using yeast two-hybrid system. Prior to screening, we confirmed that the bait construct, containing GAL4-DB-WPK4 fusion protein, does not activate transcription of reporter genes by itself (data not shown). After screening 1×10^6 colonies, 9 clones which activated two reporter genes, *HIS3* and *lacZ* were obtained (Table 1). The intensity of the lacZ activity

Clone	Similarity	frame	insert size(kbp)
10J6A	14-3-3 protein (WIN1b)	+1	1.2
29M1C	14-3-3 protein (WIN1a)	+1	1.1
10J40A	14-3-3 protein (WIN1a)	+1	1.0
10J28A	14-3-3 protein (WIN2)	+1	1.1
10J22A	14-3-3 protein (WIN1a)	+1	1.1
29M1A	Ribosomal Protein (S15)	+3	1.1
29M14B	Bowman-Birk type Proteinase Inhibitors	+1	0.7
10J65A	Phospholipid Transfer Protein	+2	0.5
29M4C	Betaine Aldehyde Dehydrogenase	+2	1.0

Table 1 Clones isolated by yeast two-hybrid system as putative WPK4 interacting factors. Similarity, frame, and insert size are indicated. Putative protein expressed in frame with Gal4 activation domain is indicated as +1.

of these clones was described as follows. 10J6A >> 29M1C > 10J40A > 10J28A > 10J22A > 29M1A > 29M14B > 10J65A > 29M4C (data not shown). Sequence analysis revealed that 10J22A, 29M1C, and 10J40A were from the same gene but differed in the 5'-UTR length. Predicted coding sequence of all clones were correctly expressed in frame as a GAL4 activation domain (GAD) (Fig. 13). Sequence analysis showed that 10J6A was almost identical with 29M1C (Fig. 14). Subsequently 29M1C and 10J6A were designated as *TaWIN* (*Triticum aestivum* WPK4-interacting factor) *1a*, and *TaWIN1b* respectively. *TaWIN1a* and *TaWIN1b* encoded a polypeptide of 266 amino

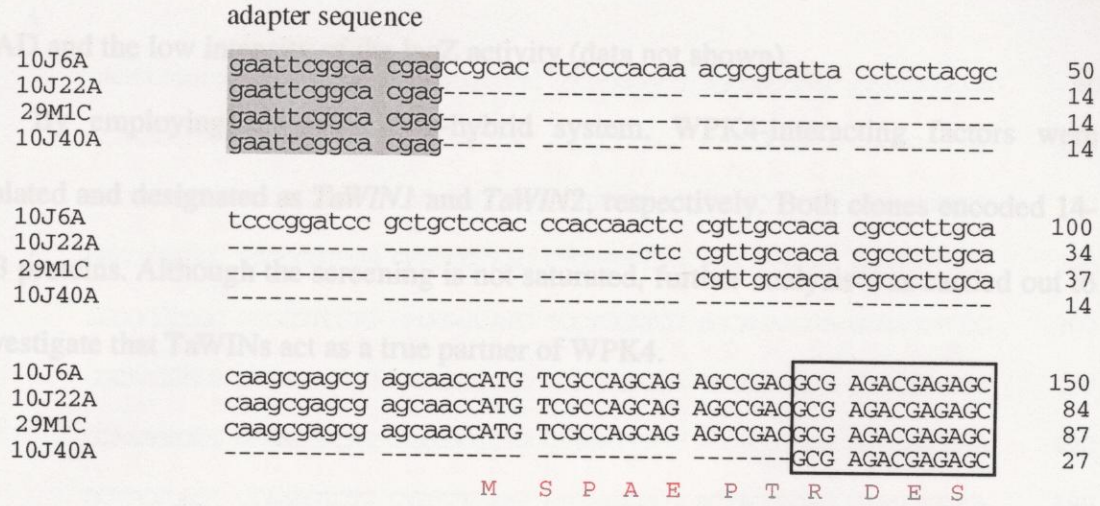

Figure 13. Nucleotide sequence alignment of WIN1 clones. 10J6A, 10J22A and 29M1C contains 103, 40, 37bp 5' untranspated region respectively. 10J40A lacks 20bp coding region. An open reading frame encoded by these clones were fused in-frame to the GAL4 transcriptional activation domain of pAD GAL4. Adapter sequence was shaded and identical sequences were boxed. Predicted amino acid sequences were shown in red.

acid residues with a relative molecular mass of 29.4 kDa. Since TaWIN1a and TaWIN1b differed in only three amino acids, we refer them as TaWIN1 hereafter. A search of GenBank indicated that the encoded polypeptide showed similarity with 14-3-3 protein and with the highest similarity to GF14d isolated from rice (Fig. 15B) (Schultz et al., 1998). The predicted coding sequence of the 10J28A clone was expressed in frame as GAD with the extra 8 amino acids as a translated product of the 5'UTR (Fig. 16A). 10J28A was closely related to the *TaWIN1* showing 70% similarity and designated as *TaWIN2*. *TaWIN2* encoded a polypeptide of 259 amino acid residues with a relative molecular mass of 28.6 kDa (Fig. 16B). 29M1A encoded ribosomal protein whose predicted amino acid is similar to rice ribosomal protein S15 but failed to express in frame as GAD. 29M14B encoded bowman-birk type proteinase inhibitor as a fusion with GAD. 10J65A encode phospholipid transfer protein but failed to express in frame as GAD. 29M4C encoded betaine aldehyde dehydrogenase but failed to express in flame as GAD. These clones (29M1A, 29M14B, 10J65A, and 29M4C) were thought

to be false positive clones because most of them were not expressed as fusion with GAD and the low intensity of the lacZ activity (data not shown).

By employing the yeast two-hybrid system, WPK4-interacting factors were isolated and designated as *TaWIN1* and *TaWIN2*, respectively. Both clones encoded 14-3-3 proteins. Although the screening is not saturated, further analysis was carried out to investigate that TaWINs act as a true partner of WPK4.

A

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gaattcggca cgagcgttgc cacacgccct tgcacaagcg agcgagcaac cATGTCGCCA      60
E F G T  S V S  S S P  C T S E  R A T  M S P
GCAGAGCCGA CGCGAGACGA GAGCGTCTAC ATGGCCAAGC TTGCGGAGCA GGCCGAGCGC     120
A E P T  R D E  S V Y  M A K L  A E Q  A E R
TACGAGGAGA TGGTGGAGTT CATGGAGCGC GTCGCGAAGG CCACCGGCGG GGCAGGGCCC     180
Y E E M  V E F  M E R  V A K A  T G G  A G P
GGGGAGGAGC TATCCGTCGA GGAGCGCAAC CTGCTCTCTG TGGCTTACAA GAATGTCATC     240
G E E L  S V E  E R N  L L S V  A Y K  N V I
GGGGCCCGGC GCGCGTCCTG GAGGATCATC TCCTCCATCG AGCAGAAGGA GGAAGGTCGG     300
G A R R  A S W  R I I  S S I E  Q K E  E G R
GGCAACGACG CGCACGCCGC CACCATCCGC TCCTACCGCA GCAAGATCGA GGCCGAGCTC     360
G N D A  H A A  T I R  S Y R S  K I E  A E L
GCAAAGATCT GCGACGGCAT CCTCGCCCTG CTTGATTCCC ACCTCGTGCC ATCCGCCGGA     420
A K I C  D G I  L A L  L D S H  L V P  S A G
GCTGCCGAGT CCAAAGTCTT CTATCTCAAG ATGAAGGGCG ACTACCACAG GTACCTTGCA     480
A A E S  K V F  Y L K  M K G D  Y H R  Y L A
GAGTTTAAGT CCGGTGGGGA GAGGAAGGAA GCCGCCGAGA GCACCATGAA CGCGTACAAA     540
E F K S  G G E  R K E  A A E S  T M N  A Y K
GCTGCTCAGG ATATCGCCCT AGCAGATTTG GCTCCAACCC ACCCCATCAG GCTTGGGCTT     600
A A Q D  I A L  A D L  A P T H  P I R  L G L
GCACTCAACT TTTCTGTGTT CTACTATGAG ATCTTGAACT CCCCTGACCG CGCCTGCAAC     660
A L N F  S V F  Y Y E  I L N S  P D R  A C N
CTTGCAAAAC AAGCCTTTGA TGAGGCTATA TCAGAGCTGG ACAGCTTAGG CGAGGAATCC     720
L A K Q  A F D  E A I  S E L D  S L G  E E S
TACAAGGACA GCACTTTAAT CATGCAGCTC CTGCGTGACA ATTTGACTCT ATGGACATCC     780
Y K D S  T L I  M Q L  L R D N  L T L  W T S
GACACCAATG AGGATGACGT TGATGAGATT AAGGAAGCCC CAGCTCCAAA AGAATCTGGA     840
D T N E  D D V  D E I  K E A P  A P K  E S G
GACGGACAGT GAggtgaatg aaaacaacag tctgtgcatt gttgggggca atggaagtcg     900
D G Q  .
gtcttggttc tagtgctcat actgttgtca tcactacctc tgtttaatta tgcacttaag     960
aagtttctgg gctatgtttt tctggtgctg ataaatgctg gctggattcg tgttttcttt    1020
ttaaatctcg ccacaagcaa aagacctgct ctttttcacc attagaatgt ttgctatatc    1080
taaaaaaaaa aaaaaaaaaa aaaaaaaa                                       1108
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B

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29M1C   MSPAEPTRDE SVVMAKLAEQ AERYEEMVEF MERVAKATGG AGPGEELSVE ERNLLSVAYK  60
OsGF14d MSPAEPTRDE SVVKAKLAEQ AERYEEMVEY MERVARAAGG ASQGEELTVE ERNLLSVAYK  60

29M1C   NVIGARRASW RIISSIEQKE EGRGNDAHAA TIRSYRSKIE AELAKICDGI LALLDSHLVP 120
OsGF14d NVIGARRASW RIISSIEQKE EGRGNDAHAA TIRSYRGKIE AELAFICDGI LALLDSHLVP 120

29M1C   SAGAAESKVF YLKMKGDYHR YLAEFKSGDE RKEAAESTMN AYKAAQDIAL ADLAPTHPIR 180
OsGF14d SAGAAESKVF YLKMKGDYHR YLAEFKSGDE RKQAAESTMN AYKAAQDIAL ADLAPTHPIR 180

29M1C   LGLALNFSVF YYEILNSPDR ACNLAKQAFD EAISELDSLG EESYKDSTLI MQLLRDNLTL 240
OsGF14d LGLALNFSVF YYEILNSPDR ACNLAKQAFD EAISELDSLG EESYKDSTLI MQLLRDNLTL 240

29M1C   WTSDTNEDDV DEIKEAEAPK ESGDGQ                                     266
OsGF14d WTSDANDDGG DEIKEAAAPK EEGDQ-                                     265
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Figure 15. Nucleotide and deduced amino acid alignment of 29M1C clone. (A) Nucleotide sequence of 29M1C clone isolated yeast two-hybrid system. Putative open reading frame was shown in a capital letter and polypeptides expressed as a GAD was shown in red. Untranslated region was shown in small letter. (B) Sequence alignment fo predicted protein sequences of 29M1C and rice GF14d Gaps were introduced for optimal alignment and are shown by dashes. Identical amino acid residues are boxed. Amino acid positions are shown on the right.

A

```
gaattcggca cgaggtcgac agcgATGGCA AAGGCAGCGG CAACGAGGGA GGAGATGGTC   60
A  F  G  T  R  S  T  A  M  A  K  A  A  A  T  R  E  E  M  V
TACTTGGCGA AGCTGGCGGA GCAGGCCGAG CGGTACGAGG AGATGGTCGA GTTCATGGAG  120
Y  L  A  K  L  A  E  Q  A  E  R  Y  E  E  M  V  E  F  M  E
AAGGTCGTGG CGGCGGCGGG GACCGTCGAG CTCACCATCG AGGAGAGGAA CCTGCTCTCA  180
K  V  V  A  A  A  G  T  V  E  L  T  I  E  E  R  N  L  L  S
GTCGCGTACA AGAACGTCAT CGGGGCGCGT CGTGCTTCCT GGCGCATCGT GTCCTCCATC  240
V  A  Y  K  N  V  I  G  A  R  R  A  S  W  R  I  V  S  S  I
GAGCAGAAGG AGGAAGGGCG TGGAGCGGCG GGGCACGCCG CCGCGGCGCG CGGGTACCGC  300
E  Q  K  E  E  G  R  G  A  A  G  H  A  A  A  A  R  G  Y  R
GCACTTGTCG AGGCCGAGCT CTCCAACATC TGCGCGGGGA TCCTCCGTCT CCTCGACGAA  360
A  L  V  E  A  E  L  S  N  I  C  A  G  I  L  R  L  L  D  E
CGCCTAGTCC CCGCCGCTGC CGCCGTCGAC GCCAAGGTAT TCTACCTGAA GATGAAGGGA  420
R  L  V  P  A  A  A  A  V  D  A  K  V  F  Y  L  K  M  K  G
GACTACCATC GCTACCTCGC GGAGTTCAAG TCGGCCGCCG AGCGCAAGGA TGCCGCTGAC  480
D  Y  H  R  Y  L  A  E  F  K  S  A  A  E  R  K  D  A  A  D
TCCACCCTCG GTGCCTACCA GGCCGCTCAG GACATAGCCA TGAAGGAGCT GCCACCGACT  540
S  T  L  G  A  Y  Q  A  A  Q  D  I  A  M  K  E  L  P  P  T
CACCCCATCA GGCTGGGCCT CGCGCTCAAC TTCTCCGTGT TCTACTATGA GATCCTCAAC  600
H  P  I  R  L  G  L  A  L  N  F  S  V  F  Y  Y  E  I  L  N
TCGCCTGATC GTGCGTGCTC GCTCGCCAAA CAGGCCTTCG ATGAAGCCAT CGCTGAGCTG  660
S  P  D  R  A  C  S  L  A  K  Q  A  F  D  E  A  I  A  E  L
GATTCCCTCG GAGAAGATTC CTACAAGGAC AGCACCCTGA TCATGCAACT TCTCCGTGAC  720
D  S  L  G  E  D  S  Y  K  D  S  T  L  I  M  Q  L  L  R  D
AATCTCACTT TGTGGACCTC TGATATGCAG GATGACGCTG GCGATGAAAT GAGGGATTCA  780
N  L  T  L  W  T  S  D  M  Q  D  D  A  G  D  E  M  R  D  S
AGCAAGCCTG AGGATGAGCA GTAGtgaagg gtcctagcta ctgccctctt tctctcttaa  840
S  K  P  E  D  E  Q  .
ccttcgggta gttatgatgc ttgtacaagg accattttcg tgtgtccttt gttgctgggt  900
cttttatcta ccctttcttt cgttatattg ttatacatcg ccgtcctcgt gcattatttt  960
actttacaac ttgaatccaa aaaaaaaaaa aaaaaaaa                          998
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B

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10J28A      MAKAAATREE NVYLAKLAEQ AERYEEMVEF MEKVAAAGT- ----VELTIE ERNLLSVAYK   56
AT14omega   M---ASGREE LVYMAKLAEQ AERYEEMVEF MEKVSAAVDG ----DELTVE ERNLLSVAYK   53
29M1C       MSPAEPTHDE SVYMAKLAEQ AERYEEMVEF MERVAKTGG AGPGEELSVE ERNLLSVAYK   60

10J28A      NVIGARRASW RIVSSIEQKE EGRGAAGHAA AARGYRALVE AELSNICAGI LRLLDERLVP  116
AT14omega   NVIGARRASW RIISSIEQKE ESRGNDDHVT AIREYRSKIE TELSGICDGI LKLLDSRLIP  113
29M1C       NVIGARRASW RIISSIEQKE EGRGNDAHAA TIRSYRSKIE AELAKICDGI LALLDSHLVP  120

10J28A      AAAAVDAKVF YLKMKGDYHR YLAEFKSAAE RKDAADSTLG AYQAAQDIAM KELPPTHPIR  176
AT14omega   AAASGDSKVF YLKMKGDYHR YLAEFKTGQE RKDAAEHTLA AYKSAQDIAN AELAPTHPIR  173
29M1C       SAGAAESKVF YLKMKGDYHR YLAEFKSGGE RKEAAESTMN AYKAAQDIAL ADLAPTHPIR  180

10J28A      LGLALNFSVF YYEILNSPDR ACSLAKQAFD EAIAELDSLG EDSYKDSTLI MQLLRDNLTL  236
AT14omega   LGLALNFSVF YYEILNSPDR ACNLAKQAFD EAIAELDTLG EESYKDSTLI MQLLRDNLTL  233
29M1C       LGLALNFSVF YYEILNSPDR ACNLAKQAFD EAISELDSLG EESYKDSTLI MQLLRDNLTL  240

10J28A      WTSDMQDDAG DEMRD--SSK P-EDEQ                                      259
AT14omega   WTSDMQDDAA DEIKEAAAPK PTEEQQ                                      259
29M1C       WTSDTNEDDV DEIKEAPAPK ESGDGQ                                      266
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Figure 16. Nucleotide and deduced amino acid sequence alignment of 10J28A clone. (A) Nucleotide sequence of 10J6A clone isolated yeast two-hybrid system. Putative open reading frame was shown in a capital letter and polypeptides expressed as a GAD was shown in red. Untranslated region was shown in small letter. (B) Sequence alignment of predicted protein sequences of 10J28A, Arabidopsis omega type 14-3-3 protein and 29M1C. Gaps were introduced for optimal alignment and are shown by dashes. Identical amino acid residues are boxed. Amino acid positions are shown on the right.

Chapter IV

Characterization of WPK4-interacting factors as a 14-3-3 protein

Chapter III described isolation of WPK4 interacting factors by yeast-two hybrid system. Sequence analysis revealed that most of the clones encode distinct isoforms of 14-3-3 proteins which are ubiquitously found in eukaryote cells and thought to play important roles in signal transduction pathways by regulating a variety of intracellular signaling molecules (Aitken, 1996). The crystal structure analysis revealed that 14-3-3 protein consists of a bundle of nine α-helices organized in an antiparallel fashion, with the four N-terminal helices participating in dimer formation (Liu et al., 1995; Xiao et al., 1995). The dimeric molecule has a cup-like shape with a conserved inner surface and a variable outer surface. 14-3-3 proteins bind ligands such as phosphorylated consensus motif, RSXpSXP, found in Raf (Thorson et al., 1998; Tzivion et al., 1998), Bad (Zha et al., 1996) and CDC25 proteins (Peng et al., 1997; Zeng et al., 1998). However, phosphoserine recognition alone cannot account for all 14-3-3-ligand interactions (Petosa et al., 1998; Zeng et al., 1998). For example, unphosphorylated peptide was shown to bind to 14-3-3 proteins by random peptide phage display libraries as well. In this chapter, detailed mode of binding of TaWIN1 and TaWIN2 to WPK4 are presented.

MATERIAL AND METHODS

Quantitative β -galactosidase activity assay

Assays of β -galactosidase activity were according to the Yeast Protocols Handbook (Clontech) and performed in triplicate by using ONPG (o-nitrophenyl β -D-galactopyranoside as the substrate. Yeast strain Y190 were used for this assay. The β -galactosidase activity was calculated from the following equation:

Units of β -galactosidase activity = $OD_{420}/(V \times T \times OD_{600})$

Where V is the volume of the culture (mL), T is the reaction time (min), and OD_{600} is the optical density of the yeast cells at 600nm.

Construction of Plasmids

To express TaWIN1 or TaWIN2 as hexahistidine fusion proteins, the pAD-WIN1 or pAD-WIN2 plasmids were digested with *Eco*RI and *Xho*I to excise *TaWIN1* or *TaWIN2* cDNA, respectively, then ligated to pET32-a at the *Eco*RI and *Sal* I sites. The resulting construct was designated pET-WIN1 or pET-WIN2, respectively. Subsequently, *Eco*RI/*Sal* I fragment of *TaWIN1* was ligated to pGEX-4T1 at the *Eco*RI and *Sal*I site to fuse in frame the coding sequence for glutathione S-transferase (GST) with that for WIN1. The resulting construct was designated pGEX-WIN1 or pGEX-WIN2, respectively.

Site-directed Mutagenesis

A series of *WPK4* mutants (T204A, T204E, S388A, S418A and S388A/S418A)

were performed using the Mutan Km express Kit (TAKARA). pBD-WPK4 was digested with *Eco*RI and *Pst*I, ligated to pKF18k vector at the same endonuclease sites and used as a PCR template. The PCR reactions were performed using the *Pyro*Best DNA polymerase (TAKARA). Oligonucleotides used in PCR reactions were as follows (nucleotides corresponding to the mutagenised sequences were shown in underline).

T204A: 5'-TCTCCTCCACGCATTCTGCG-3'

T204E: 5'-TCTCCTCCACGAGTTCTG -3'

S388A: 5'-ACGCCCCGCAGCGCTTAATGC-3'

S418A: 5'-AGATTCATCGCGGGTGAACC-3'

PCR reactions were performed according to the ODA (Oligonucleotide-directed Dual Amber) method as a principle. After PCRs, the amplified DNA fragments were concentrated by EtOH precipitation, then dissolved in TE buffer and transformed in *E. coli* strain MV1184. For the S388/418A double mutation, mutagenised S418A clone was initially constructed then digested with *Eco*RI and *Pst*I. *Eco*RI/ *Pst*I fragments were cloned into the pKF18k vector again, then used as a template for the S388A mutation. The aimed constructs were selected from the kanamycin resistant clones. To verify the amino acid exchanges and to exclude additional mutations, all constructs were subjected to sequence analysis. For β -galactosidase activity assay, mutagenised inserts were subcloned into the pBD-Gal4 Cam vector at the *Eco*RI and *Pst*I sites.

A series of *TaWIN1* mutants (K60D, R67E, L185D, V189E, G221S and L233D) were performed using the same Kit (TAKARA). Oligonucleotides used in PCR reactions were as follows (nucleotides corresponding to the mutagenised sequences were shown in underline).

K60D: 5'-GGCTTACGACAATGTCATCG-3'

R67E: 5'-GCCCGGGAAGCGTCCTGGAGG -3'

L185D: 5'-GGCTTGCAGACAACTTTTCTG-3'

V189E: 5'-ACTTTTCTGAATTCTACTATG-3'

G221S: 5'-ACAGCTTAAGCGAGGAATCC-3'

L233D: 5'-TCATGCAGGATCTGCGTGAC-3'

The following steps were carried out as described above.

Expression and Purification of Recombinant Proteins

pGEX-WIN1, pGEX-WIN2, and pGEX-4T-1 vectors were transformed into *E. coli* DH5 α strain, and used for purification of glutathione S-transferase (GST), GST-WIN1 and GST-WIN2 fusion proteins. pET-WIN1 and pET-WIN2 contain cDNAs encoding WIN1 and WIN2 cloned into pET32a (Novagen), respectively. Expression and purification of GST-WPK4 and GST-K75D (which correspond to GST-WPKM4) have been described in the chapter II. The *E. coli* strain BL21 (DE3) carrying pET-WIN1 or pET-WIN2 was grown (OD_{600}= 0.7) in LB medium containing ampicillin (1 liter) and induced by isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 0.5 mM to express His-tagged WIN1 or WIN2. The cultures were further incubated at 24 ℃ for 6 h. Cells were pelleted by centrifugation and suspended in 40 mL of protein lysis buffer [20 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl, 10 mM 2-mercaptoethanol, 0.1% Tween 20, 40 mM imidazole] and disrupted by sonication. Ni-NTA superflow resin (Qiagen) was added to the supernatants and incubated for 1 hr at 4℃. The mix was loaded into the column and washed with lysis buffer. After washing, the protein was eluted in elution buffer [20 mM Na_2HPO_4 (pH 8.0), 300 mM NaCl, 10 mM 2-

mercaptoethanol, 0.1% Tween 20, 500mM imidazole]. The eluted protein solution was dialyzed against 40mM Tris-HCl (pH 7.5), 20 mM 2-mercaptoethanol at 4℃. After the dialysis, equal volume of 100% glycerol was added to the protein solution and stored at –30℃ until use.

Gel Filtration Analysis

GST-WIN1 fusion protein was expressed in *E. coli* DH5 α strain harboring pGEX-WIN1 and attached to glutathione Sepharose 4B resin, and then the fusion protein bound-beads were incubated with T Buffer containing 100 units of thrombin (Amersham Pharmacia Biotech) for 24 hours at 4℃. After collecting supernatant, TaWIN1 protein was further purified on DEAE-Toyopearl column using gradient of NaCl to remove thrombin. One μ g of purified proteins was applied to high resolution gel filtration chromatography previously equilibrated with 2×PBS (phosphate buffered sanile) at a flow rate of 1ml / min. For the standard proteins, albumin, ovalbumin, chymotrypsinogen A were used (LMW Gel Filtration Calibration Kit, Pharmacia Biotech).

***In vitro* Binding Assays**

His-tagged WIN1 protein was dissolved in protein binding buffer [20 mM Tris-HCl (pH 7.5), 140 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM EDTA (pH 8.0), 5 mM $MgCl_2$ 0.5% Tween 20] to the final concentration of 0.05%. 20 μl of glutathione-Sepharose beads with bound GST alone, GST-WPK4 or GST-K75D was added to 50 μl of protein binding buffer containing His-tagged WIN1 protein then incubated for 1 h at

4℃. The beads were washed with 10 times of bed volumes of protein binding buffer three times. Bound protein was eluted with 1×Laemmli's sample buffer and were resolved by 12.5% SDS-PAGE, transferred to PVDF membrane. Immunoblot analysis was performed by using monoclonal antibody raised against penta His-tag as the primary antibody (Novagen). The secondary antibody used was horseradish peroxidase-conjugated rabbit antiserum to mouse IgG, and immune complexes were detected by using the enhanced chemiluminescence kit (Amersham).

RNA Blot Hybridization Analysis

Total RNA was extracted by the aurin tricarboxylic acid (ATA) method. Aliquots of 36 μg per lane were denatured, fractionated by formaldehyde/1.0% agarose gel electrophoresis, and transferred onto nylon membranes (Hybond-N, Amersham) for hybridization with a ^{32}P-labelled probes at 42°C. The 5' region (0.3 kb) of *TaWIN1* cDNA and 3' non-coding region (0.8 kb) of *WPK4*-cDNA were used as a probe. Sheets were washed three times in a 0.1x SSC solution containing 0.1% SDS at 65°C for 15 min each. To normalize for the amount of RNA loaded, the membrane was stripped off the former probe by boiling in a 0.1% SDS solution for 1 min before rehybridization with a wheat actin probe prepared as described (Sano and Youssefian, 1994).

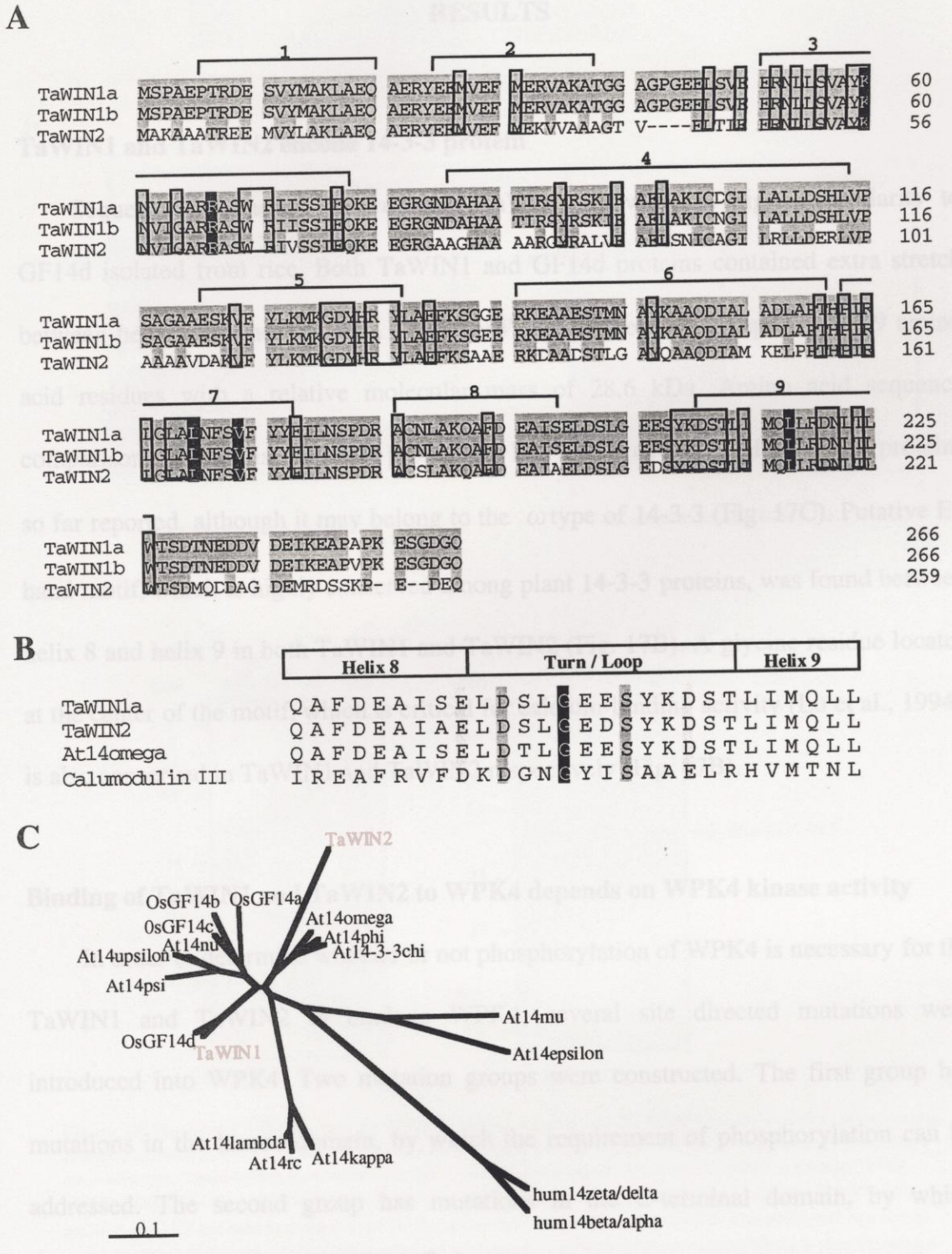


Figure 17. *TaWIN1* and *TaWIN2* (WPK4-interacting factor 1, 2) encode a 14-3-3 protein. (A) Sequence alignment of predicted protein sequences of TaWIN1a, TaWIN1b and TaWIN2 with α-helical segments indicated. Identical amino acids residues are shaded. Residues that are invariant across 84 sequences and 30 species are boxed. Residues that are necessary to engage a cluster with phosphorylated consensus motif are shown in reverse contrast. (B) Amino acid alignment of the potential EF hand motif. The expanded region of TaWIN1 protein is from the start of the C-terminal domain at Glu-207 to Leu-234. Shaded amino acid residues indicate conserved or similar residues between TaWINs and domain III of calmodulin. (C) Dendrogram showing amino acid sequence relationships among *Arabidopsis*, *Oryza sativa* and human 14-3-3 proteins.

RESULTS

TaWIN1 and TaWIN2 encode 14-3-3 protein

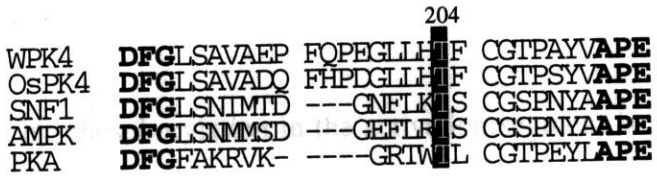
Sequence comparison showed that TaWIN1 showed the highest similarity to GF14d isolated from rice. Both TaWIN1 and GF14d proteins contained extra stretch between helix 2 and helix 3 (Fig. 17A). *TaWIN2* encoded a polypeptide of 259 amino acid residues with a relative molecular mass of 28.6 kDa. Amino acid sequence comparison showed that TaWIN2 protein has less similarity to any plant 14-3-3 proteins so far reported, although it may belong to the ω type of 14-3-3 (Fig. 17C). Putative EF hand motif, which is highly conserved among plant 14-3-3 proteins, was found between helix 8 and helix 9 in both TaWIN1 and TaWIN2 (Fig. 17B). A glycine residue located at the center of the motif, which is critical for calcium-binding activity (Lu et al., 1994), is also conserved in TaWIN1 and TaWIN2 respectively (Fig. 17B).

Binding of TaWIN1 and TaWIN2 to WPK4 depends on WPK4 kinase activity

In order to determine whether or not phosphorylation of WPK4 is necessary for the TaWIN1 and TaWIN2 to bind to WPK4, several site directed mutations were introduced into WPK4. Two mutation groups were constructed. The first group has mutations in the kinase domain, by which the requirement of phosphorylation can be addressed. The second group has mutations in the C-terminal domain, by which interacting amino acids can be identified.

Substitution of the Lys at the position 75 in the domain II into Asp (K75D) results in loss of kinase activity. This was the case for WPK4 in Chapter II. As more and more kinases have been discovered and determined the crystal structures, it is recognized that

A



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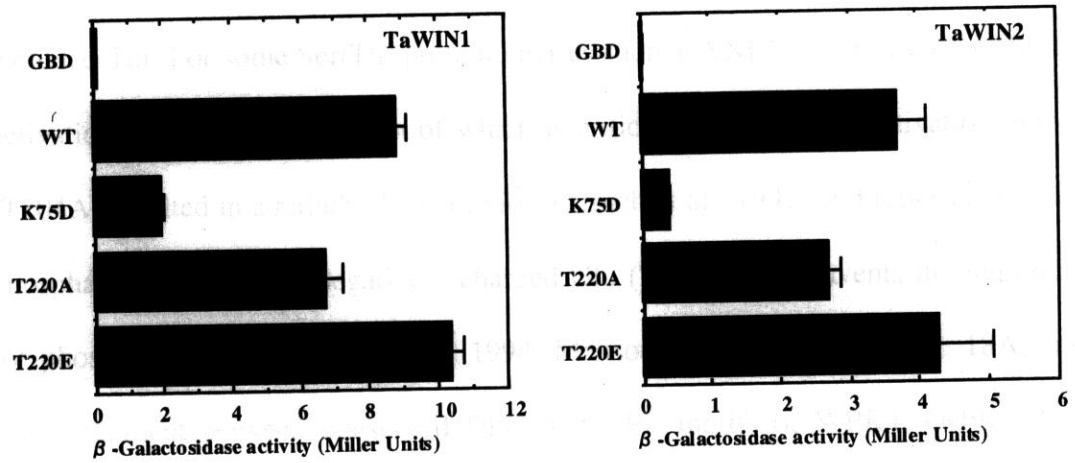

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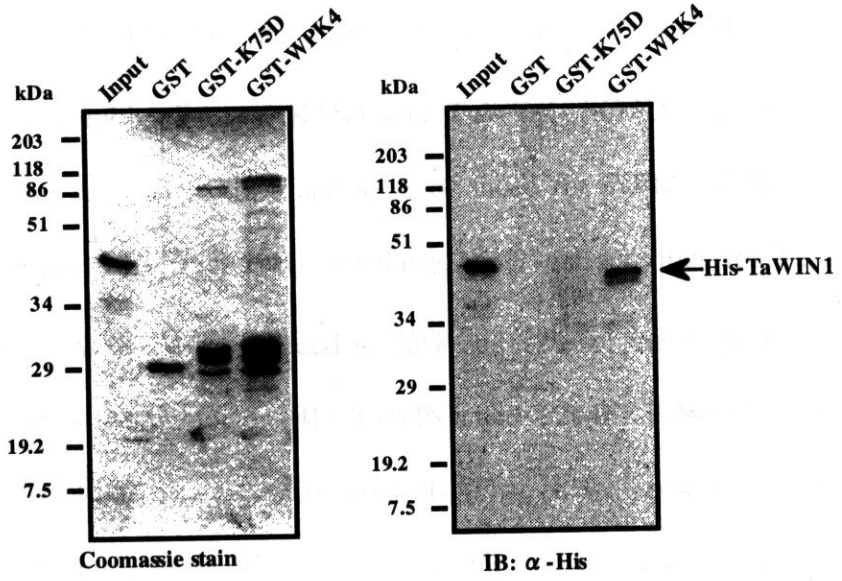

Figure 18. Binding of TaWIN1 and TaWIN2 to WPK4 depends on the WPK4 kinase activity. (A) Alignment of sequences in the activation loop of a number of protein kinases. The Asp-Phe-Gly (DFG) and Ala-Pro-Glu (APE) motifs are in bold type. Conserved threonine residue are shown in reverse contrast. (B) Interaction of WPK4 and mutant WPK4 (K75D, T220A, T220E) with TaWIN1 and TaWIN2. Y190 cells were cotransformed with the bait and pray combinations depicted to the left and quantitatively assayed for β-galactosidase activity. Three experiments were performed on a culture grown from each colony. The mean ±SD is presented. (C)Interaction of His-TaWIN1 with GST-WPK4 *in vitro*. His-tagged TaWIN1 was incubated with immobilized GST, GST-K75D and GST-WPK4. Profiles of the gel stained with Coomassie Brilliant Bluee was shown (left) and bound proteins were detected by western blotting using anti Penta-His antibody (right). Relative molecular masses of the standard samples are indicated in left.

control by phosphorylation in the activation segment is a property of most, but not all, protein kinases (Johnson et al., 1996). The activation loop was defined as the region spanning conserved sequences DFG (Asp-Phe-Gly) and APE (Ala-Pro-Glu) and included Thr. For some Ser/Thr protein kinases such as SNF1, substitution of Thr in the activation loop, phosphorylation of which is critical for enzymatic activation, into Ala (T204A) resulted in a reduction of activity (Estruch et al., 1992) and replacement of this phosphate acceptor by a negatively charged Glu (T204E) circumvents the requirement for phosphorylation (Cowley et al., 1994; Mansour et al., 1994). In Fig. 18A, amino acid alignment between conserved DFG and APE motifs of WPK4, OsPK4, SNF1, AMPK and PKA was shown. The three mutations described above were examined for binding ability to TaWIN1 and TaWIN2 by quantitative liquid assay. The β-gal activities for combinations of K75D and TaWIN1 (K75D: TaWIN1), and K75D: TaWIN2 were reduced to 22% and 11% of those for WPK4: TaWIN1 and WPK4: TaWIN2, respectively (Fig. 18B). Similarly, the β-gal activities for T204A: TaWIN1 and T204A: TaWIN2 were reduced to 76% and 72% of the respective controls (Fig. 18B). In contrast, those for T204E: TaWIN1 and T204E: TaWIN2 were increased to 118% and 115% of the respective controls (Fig. 18B). These results suggested that autophosphorylation of WPK4 is indispensable for interaction between WPK4 and TaWIN1 and TaWIN2. Results also indicated that one of the target sites for phosphorylation is the Thr residue in the activation loop of WPK4. But whether or not such a phosphorylation occurred by own remained unclear.

Direct interaction between WPK4 and TaWIN1 was examined by *in vitro* binding assays. Intact WPK4 and its K75D mutant were produced in *E. coli* as fusion proteins

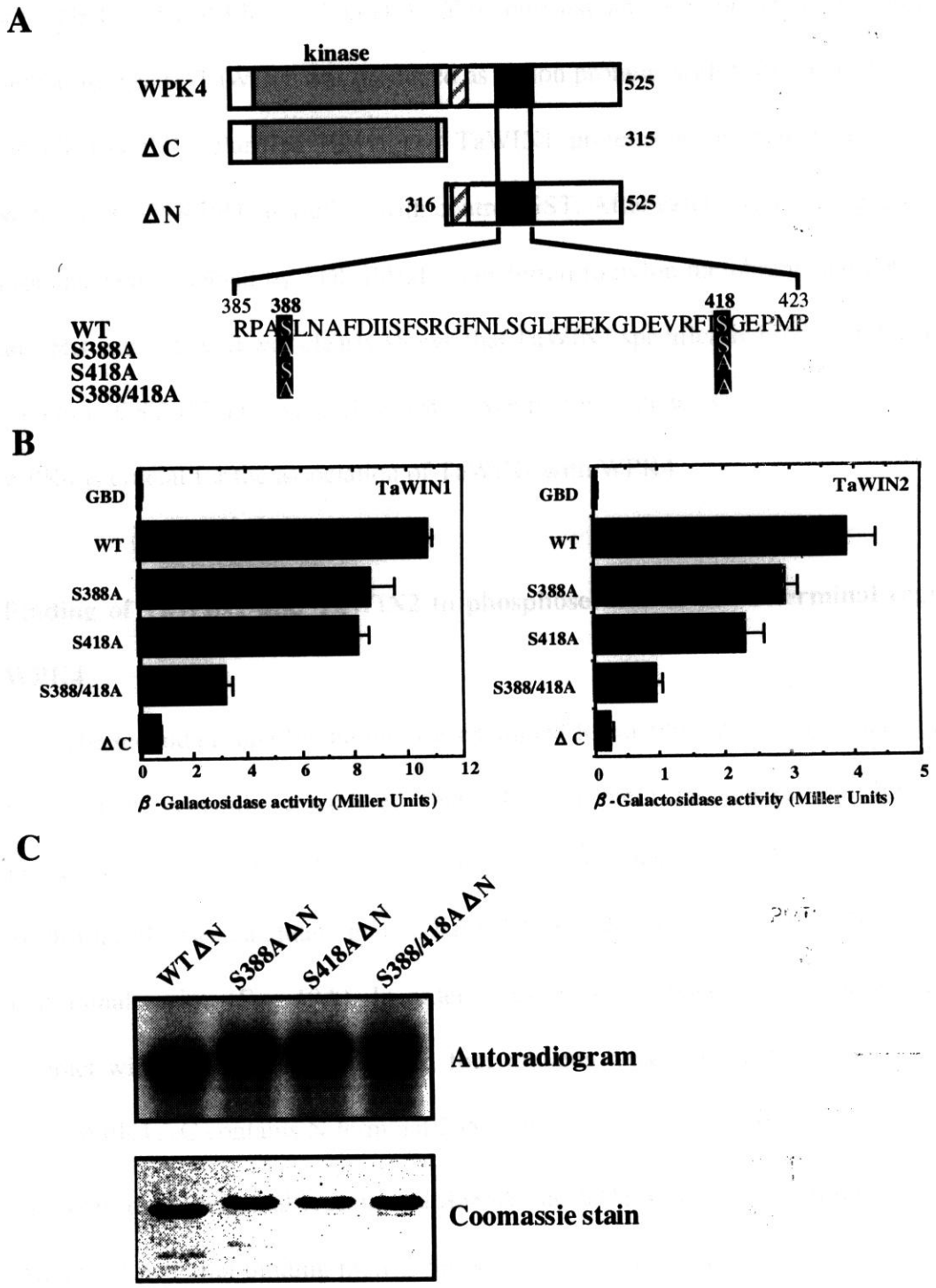

Figure 19. Autophosphorylation of both Ser-388 and Ser-418 mediate TaWIN1 and TaWIN2 binding. (A) Schematic representation of mutant constructs used in this assay. (B) Interaction of WPK4 and mutant WPK4 (S388A, S418A, S388/418A) with TaWIN1 and TaWIN2. Y190 cells were cotransformed with the bait and pray combinations depicted to the left and quantitatively assayed for β-galactosidase activity. Three experiments were performed on a culture grown from each colony. The mean$\pm$SD is presented. (C)*In vitro* phosphorylation analysis. 50ng of GST-WPK4 fusion proteins were incubated in the presence of 1 μg of GST-ΔN or indicated mutant forms of GST-ΔN constructs as the substrates. After the phosphorylation reaction, samples were separated by 12.5% SDS-PAGE, dried and exposed for autoradiography. The autoradiogram and profiles of the gel stained with Coomassie Brilliant Blue are shown in the top and the bottom, respectively.

with GST (GST-WPK4 and GST-K75D), purified and immobilized on glutathione Sepharose beads. TaWIN1 was produced as fusion proteins with histidine residues (His-tagged TaWIN1). Purified His-tagged TaWIN1 proteins were incubated with GST-WPK4 or GST-K75D, as well as with control GST. After extensive washing, the bound proteins were resolved by SDS-PAGE, transferred to nylon membrane and detected by anti-His antibodies. It was clearly shown that TaWIN1 specifically binds to GST-WPK4, but not to GST-K75D *in vitro* (Fig. 18C). Again, it was shown that the kinase activity of WPK4 is crucial for the association of TaWINs with WPK4.

Binding of TaWIN1 and TaWIN2 to phosphoserines in the C-terminal region of WPK4

The second group of mutation was examined for identification of interacting amino acids. It is generally considered that 14-3-3 protein recognizes and binds to a phosphorylated serine residue in the consensus motif, RSXpSXP (Muslin et al., 1996). WPK4 has the two candidate motifs, RPASLN and RFISGEP, for 14-3-3 binding in the C-terminal region (Fig. 19A). In order to examine the possibility of these motifs to interact with TaWIN1 and TaWIN2, four mutants were constructed as shown in Fig. 19A. WPK4 Δ C contains N-terminal 265 amino acid region but lacks both two potential motifs in the C-terminal region, and S388A and S418A contain substitutions of Ser to Ala in the potential binding motifs. S388A/S418A double mutant was also constructed. Quantitative liquid assay showed that the WPK4 Δ C mutation completely lost the ability to bind to TaWIN1 and TaWIN2 (Fig. 19B). Bindings of S388A with TaWIN1 and TaWIN2 (S388A: TaWIN1 and S388A: TaWIN2) were reduced to 80% and 76% of

the controls, respectively (Fig. 19B). Similarly, bindings of S418A: TaWIN1 and S418A: TaWIN2 were reduced to 76% and 59%, respectively (Fig. 19B). Bindings of the double mutant (S388A/S418A: TaWIN1 and S388A/S418A: TaWIN2) were additively reduced to 30% and 24%, respectively (Fig. 19B). These results indicated that binding of TaWIN1 and TaWIN2 to WPK4 is mediated by phosphorylated Ser residues at the positions 388 and 418 located in the C-terminal nonkinase region. Since these two sites are necessary for effective binding, the dimeric form of TaWIN1 or TaWIN2 is predicted to bind to a single WPK4 polypeptide.

To obtain the direct evidence that these two Ser residues (S388 and S418) are really an autophosphorylation sites, various mutants were constructed as shown in Fig. 19C and kinase assay was carried out. A series of Δ N constructs contain C-terminal 165 amino acid region but lack kinase catalytic domain. Although the clear difference between WT Δ N and two single mutants (S388A Δ N and S418A Δ N) was not observed, phosphorylation of double mutant was reduced compared to the other mutants (Fig. 19C). The results also showed that other phosphorylation sites exists in the C-terminal region, which does not confer binding of TaWINs.

Conserved amino acid residues in the amphipathic groove of TaWIN1 is necessary for WPK4 binding

The crystal structure of 14-3-3 proteins in a complex with phosphopeptide ligands was shown to form dimeric molecule like a cup shape with conserved inner surface and variable outer surface (Liu et al., 1995; Xiao et al., 1995). The inner surface forms an amphipathic groove to afford a principle binding site for 14-3-3 ligands (Petosa et al.,

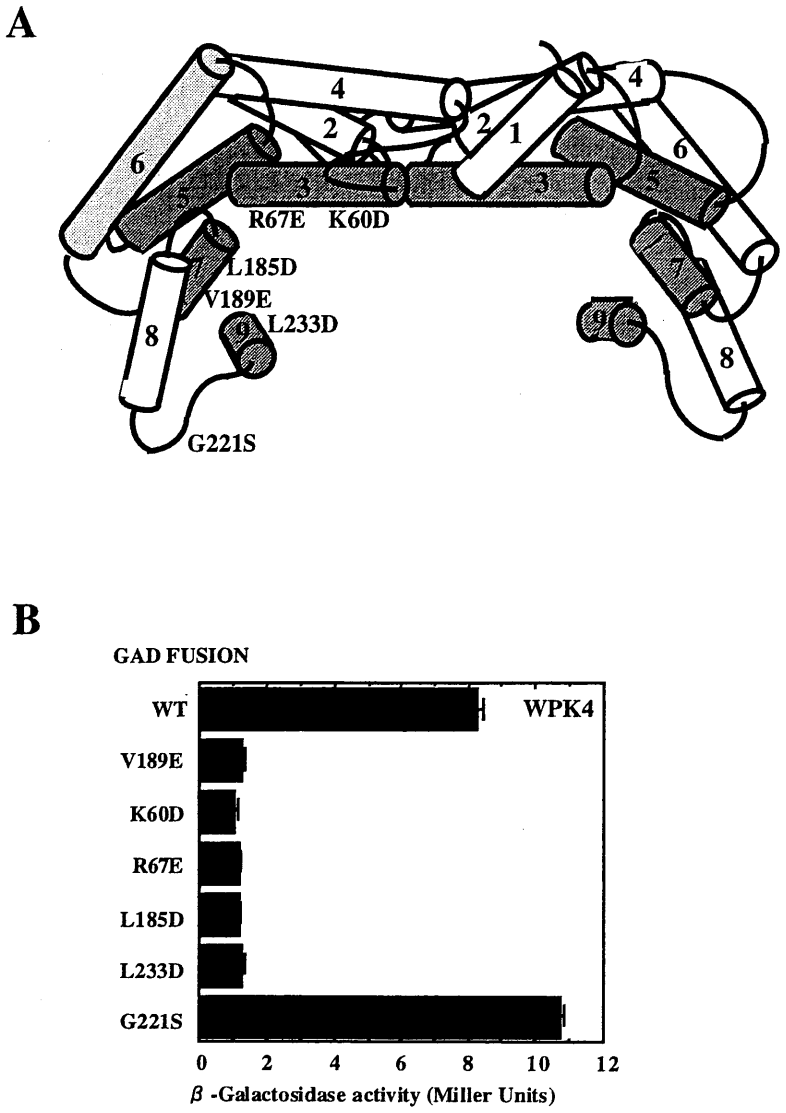

Figure 20. Residues exposed in the amphipathic groove of TaWIN1 are necessary for the binding to WPK4. (A) Schematic representation of the helices comprised by a 14-3-3 dimer, with the relative locations of amino acid substitutions introduced into TaWIN1 protein by site directed mutagenesis, is shown. Helixes forming the amphipathic groove are shown in gray. This figure was designated from the notion of crystal structure of human 14-3-3ζ isoform. Amino acids numbers were derived from TaWIN1 protein. (B) Y190 cells were cotransformed with the bait and pray combinations depicted to the left and quantitatively assayed for β -galactosidase activity. Three experiments were performed on a culture grown from each colony. The mean ± SD is presented.

1998). According to this information, site-directed mutagenesis was performed by replacing conserved basic and hydrophobic amino acid residues located in the inner surface of TaWIN1 with other amino acids. Basic amino acids (K60, R67) within the

helix 3, and hydrophobic amino acids (L185, V189, L233) within the helices 7 and 9 were substituted to acidic amino acids (K60D, R67E, L185D, V189E, L233D), respectively (Fig. 20A). These mutated proteins were then assayed for binding with WPK4. The β-gal activity for combinations of WPK4 and each of mutants (K60D, R67E, L185D, V189E and L233D) was decreased to 13-15% of that for WPK4/TaWIN1 (Fig. 20B), clearly indicating that these amino acids are essential for WPK4/TaWIN1 interaction.

Arabidopsis 14-3-3 protein, GF14 ω, was shown to bind calcium in the putative EF hand motif. For calcium binding, central Gly in the EF hand was critical (Lu et al., 1994). When Gly in the putative EF hand motif of TaWIN1 was substituted to Ser (G221S), which naturally presents in some plant 14-3-3 proteins, the β-gal activity was increased to 129% of the control (Fig. 20B). This suggests that, at least in yeast cells, the presence of calcium ion may confer inhibitory effects on WPK4/TaWIN1 interaction.

Next, the ability of TaWIN1 to form dimer was confirmed by gel-filtration chromatography, which indicated the native molecular masses of TaWIN1 to be approximately 64kDa (Fig. 21A left). Resolution of this fraction by SDS-PAGE showed a single fragment with 32kDa, an expected size for monomeric TaWIN1 (Fig. 21A right). Heterodimer formation between TaWIN1 and TaWIN2 was then examined by *in vitro* binding assays. Recombinant TaWIN1 protein was produced in *E. coli* as fusion proteins with GST (GST-TaWIN1), purified and immobilized on glutathione-Sepharose beads. TaWIN1 and TaWIN2 were also produced as fusion proteins with histidine residues (His-TaWIN1 and His-TaWIN2). Purified His-tagged proteins were incubated

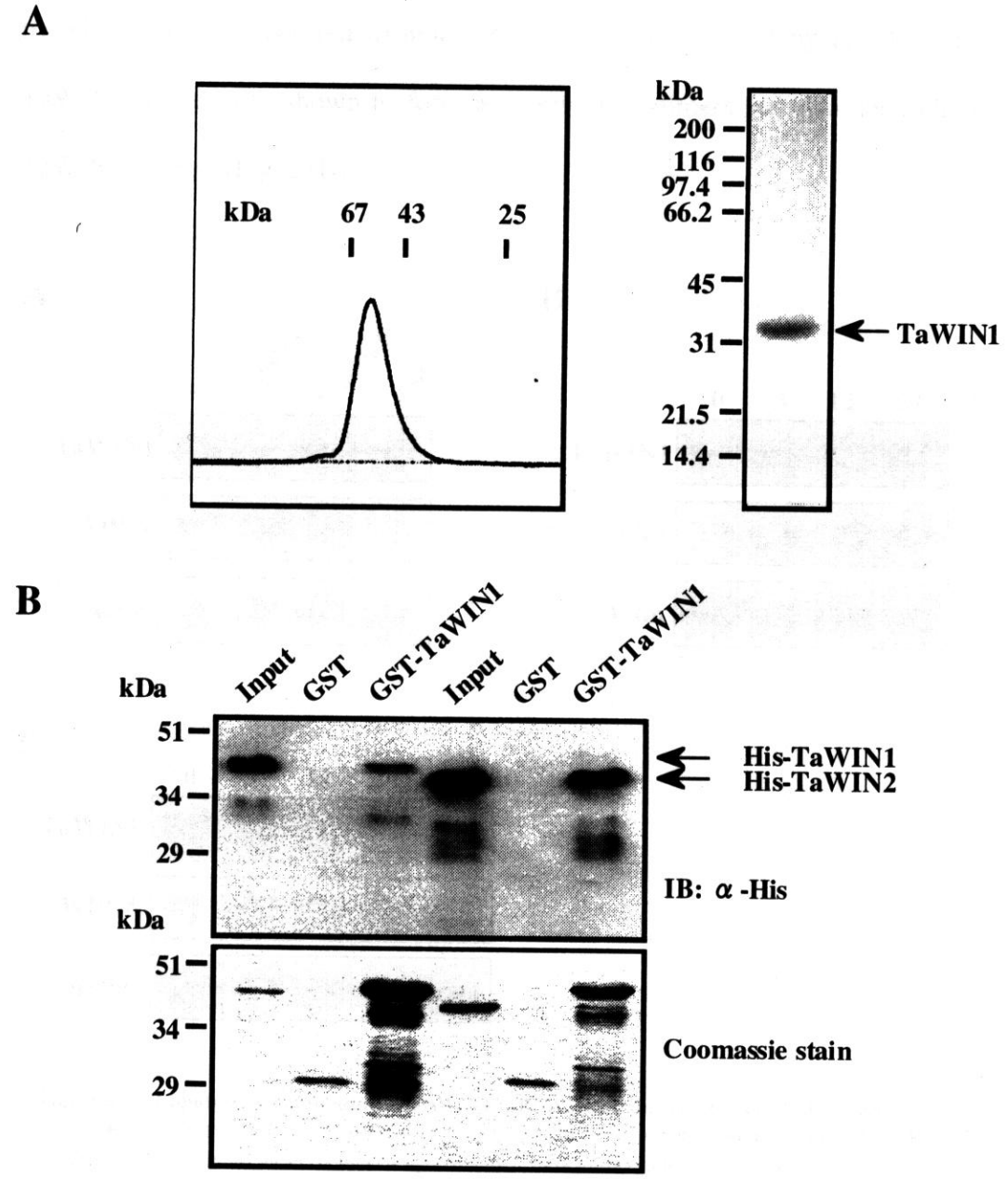

Figure 21. Homo and heterodimer formation of TaWIN1 protein. (A) Elution profile of recombinant TaWIN1 proteins by high resolution gel filtration chromatography. The elution peaks for Albumin (67 kDa), Ovalbumin (43 kDa), Chymotripsinogen A (25 kDa) are shown above the A280 trace for TaWIN1 elution (left). SDS-PAGE analysis of the peak fraction. Arrow indicated TaWIN1 protein. Protein molecular mass markers are indicated (left). (B) Interaction of His-TaWIN1 or His-TaWIN2 with GST-TaWIN1 *in vitro*. His-tagged TaWIN1 or His-tagged TaWIN2 were incubated with immobilized GST and GST-TaWIN1, respectively. Bound proteins were detected by western blotting using anti Penta-His antibody (upper) and the profiles of the gel stained with Coomassie Brilliant Bluee was shown (lower). Relative molecular masses of the standard samples are indicated in left. Arrows indicated His-TaWIN1 or His-TaWIN2 proteins, respectively.

with GST or GST-TaWIN1, and after extensive washing, bound proteins were resolved by SDS-PAGE, transferred to nylon membrane and detected by anti-His antibodies. TaWIN1 was clearly shown to form not only homodimers but also heterodimers with TaWIN2 *in vitro* (Fig. 21B).

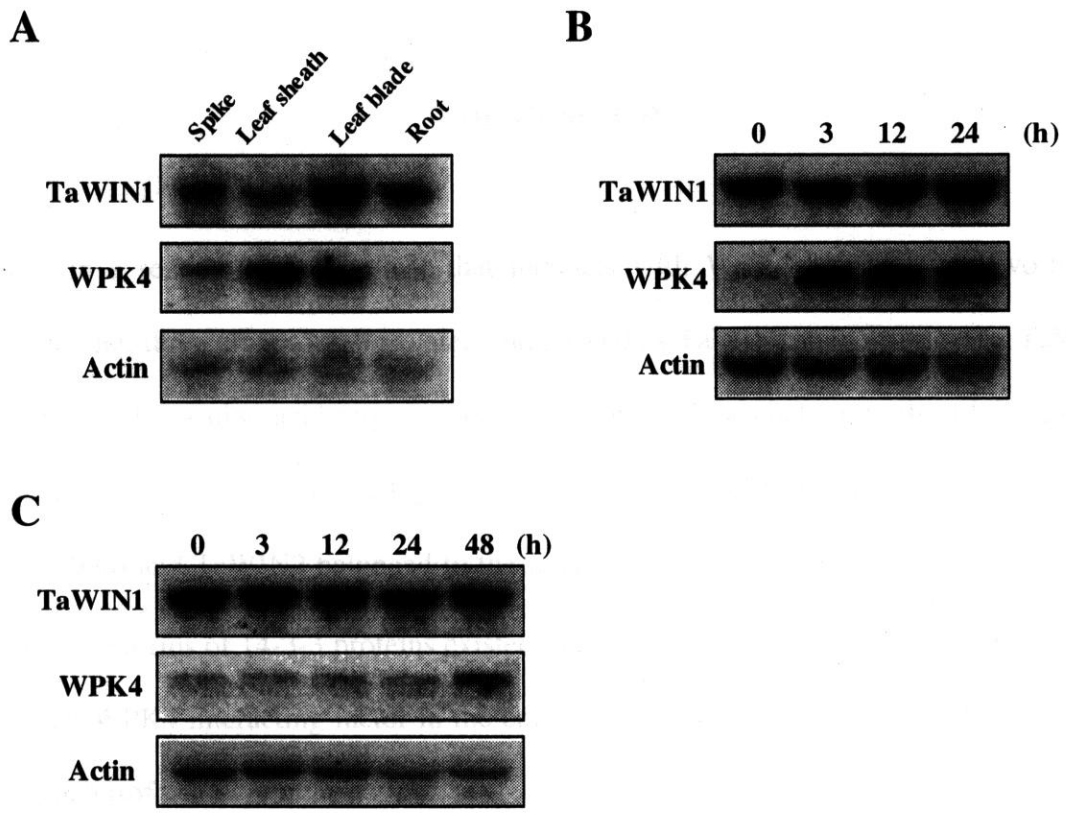

Figure 22. Ubiquitous accumulation of *TaWIN1* mRNA. (A) Tissue specificity of *TaWIN1* transcripts. Total RNA was extracted from spike, leaf sheath, leaf blade and root. (B) Effect of cytokinin application of *TaWIN1* mRNA. Six-day-old green seedlings, grown under continuous light condition, were treated with 100μ M BA for the indicated time period. (C) Effect of inorganic salts deprivation on *TaWIN1* mRNA levels. Six-day-old green seedlings, grown under continuous light condition in 1:5 MS medium, were transferred in water for the indicated time period. The hybridization probes were 0.3kb 5' UTR region of *TaWIN1* cDNA, 0.8kb 3' regions of *WPK4* cDNA and 1.2kb wheat actin cDNA, respectively.

Ubiquitous accumulation of *TaWIN1* transcripts

Transcripts of *WPK4* have shown to be accumulated in response to cytokinins and nutrient deprivation. To determine the regulation of *TaWIN1* at the transcriptional level, RNA blot analysis was carried out. In mature wheat plants, transcripts of *TaWIN1*

ubiquitously accumulated in all tissues tested (Fig. 22A). Neither treatment of cytokinins nor the transfer to the nutrient deprivated medium affected the levels of *TaWIN1* transcripts (Fig. 22B and C). These data suggest that the *TaWIN1* gene is constitutively expressed and its expression does not respond to the above conditions.

DISCUSSSION

In a search for the partner that interacts with WPK4 by the yeast two-hybrid screening, three clones were isolated and termed as TaWIN1a, TaWIN1b and TaWIN2 respectively. Amino acid sequence revealed that all clones belong to the 14-3-3 protein family. TaWIN1 showed the highest similarity to GF14d isolated from rice(Schultz et al., 1998) and TaWIN2 belonged to the ω type of 14-3-3. It should be noted that, while many isoforms of 14-3-3 proteins existed in plant cells, only two isoforms were isolated as the WPK4 interacting factor in the current study. This may imply the divergent role of each isoform *in planta.*

The crystal structure analysis revealed that 14-3-3 protein consists of a bundle of nine α-helices organized in an antiparallel fashion, with four N-terminal helices participating in dimer formation (Fig. 24 A) (Liu et al., 1995; Xiao et al., 1995). The dimeric molecule has a cup-like shape with a conserved inner surface and a variable outer surface. The inner surface is amphipathic with a cluster of basic and polar residues on one side (helices 3 and helices 5) and a cluster of hydrophobic residues on the other (helices 7 and helices 9) and this amphipathic groove would form the principal binding

site for 14-3-3 ligands (Petosa et al., 1998). In this study, yeast two-hybrid assay showed that the basic (K60 and R67 at helices 3) and hydrophobic (L185 and V189 at helices 7 and L233 at helices 9) residues of TaWIN1 were necessary for WPK4 binding (Fig. 20). 14-3-3 proteins bind the ligands containing phosphorylated consensus motif, RSXpSXP, found in Raf, Bad and CDC25. However, phosphoserine recognition cannot account for all 14-3-3-ligand interactions. Unphosphorylated peptide was shown to bind to 14-3-3 proteins by random peptide phage display libraries as well (Petosa et al., 1998; Zeng et al., 1998). The fact that the binding of TaWIN1 or TaWIN2 to WPK4 is dependent on WPK4 autophosphorylation suggests that phosphorylation of serine residues are essential. The C-terminal region of WPK4 was found to act as a pseudosubstrate region (internal sequences resembling substrates), and the resulting autophosphorylation was necessary for TaWINs binding. Further analysis revealed that autophosphorylation of both Ser-388 and Ser-418 to be critical for TaWIN1 and TaWIN2 binding (Fig. 19B). This mode of binding reminds us that of Raf/14-3-3 binding. It was shown that the phosphoserine-dependent binding of 14-3-3 to Raf is mediated by two Ser residues of Raf and that dimeric 14-3-3 proteins are the essential cofactor for Raf kinase activity (Tzivion et al., 1998).

It was shown that Arabidopsis 14-3-3 protein, GF14ω, bound calcium at the potential EF hand motif located between helix 7 and helix 8 at low affinity (Lu et al., 1994). The resulting calcium binding induced a conformational change of GF14ω protein. The Gly residue located between the helixes was found to be critical for calcium binding activity (Lu et al., 1994). Amino acid sequences analysis of plant 14-3-3 proteins revealed that about 30% of them have a substitution for the conserved Gly

residue (usually Ser residue), whereas all of others have the conserved Gly. This Gly residue was conserved in both TaWIN1 and TaWIN2 as well as Arabidopsis GF14ω, suggesting that TaWIN1 and TaWIN2 may bind calcium under physiological condition. Potential role of calcium on TaWIN1: WPK4 binding was indirectly analyzed by substituting the Gly residue to Ser residue (G221S). Results showed that binding of G221S to WPK4 was slightly increased in comparison with that of wild type (Fig. 20B) in yeast cells. Conformational change of TaWIN1 and the competition among 14-3-3 proteins against WPK4 binding might have occured when the cytosolic calcium concentrations were increased.

Cytosolic enzymes and the plasma membrane H^+-ATPase were shown to be regulated by a reversible phosphorylation (Assmann and Shimazaki, 1999) and , in some case, phosphorylation creates a phosphopeptide motif that is recognized by and bound to 14-3-3 proteins. Binding of 14-3-3 proteins changes activity of several enzymes and/or ion pumps (MacKintosh, 1998). For example, activity of nitrate reductase (NR) is controlled by a two-step mechanism (MacKintosh, 1998). The Ser residue at hinge I region is phosphorylated (Bachmann et al., 1996; Su et al., 1996). This is catalyzed *in vitro* by a calcium-dependent protein kinase (CDPK) or a SNF1-related protein kinase (Douglas et al., 1998; Douglas et al., 1997; Halford and Hardie, 1998). The phosphorylation alone has no direct effect on NR activity, but it creates the phosphopeptide motif that is recognized and bound by several isoforms of 14-3-3 proteins. This interaction is responsible for inactivation of NR activity (MacKintosh, 1998). Another enzyme, sucrose phosphate synthase (SPS) can be either activated or inactivated by distinct phosphorylation. Reversible phosphorylation of Ser-158 and Ser-

424 of spinach SPS was thought to be responsible for light/dark modulation and osmotic stress activation (McMichael et al., 1993; Toroser and Huber, 1997), respectively. Moreover, it was shown that SPS is associated with 14-3-3 proteins *in vivo* and that phosphorylated Ser-229 is necessary for 14-3-3 binding (Toroser et al., 1998). But the effect of 14-3-3 proteins on SPS activity remains unclear (Moorhead et al., 1999; Toroser et al., 1998).

It was reported that bacterially expressed Arabidopsis CDPK is bound to and activated by 14-3-3 proteins (Camoni et al., 1998). However, neither cauliflower CDPK nor CDPK that was purified by 14-3-3 affinity chromatography from an Arabidopsis extract were activated by adding 14-3-3 proteins *in vitro* (Moorhead et al., 1999). This resembles to our case that TaWINs did not show regulatory effects on WPK4 kinase activity *in vitro*. It could be possible that binding of TaWINs to WPK4 causes changes in their subcellular localization, or that additional WPK4 interacting factor that regulates WPK4 kinase activity may exist.

Very recently, Arabidopsis SNFL homolog (CIPK1) that belongs to SnRK3 was identified as a calcineurin B-like calcium sensor binding protein (Shi et al., 1999). Members of the Arabidopsis calcineurin B-like calcium binding protein (AtCBL) family were found to be differentially regulated by stress conditions (Kudla et al., 1999). One member of the *AtCBL* gene family, *AtCBL* was highly inducible by stress signals, including drought, cold and wounding. Another member, *SALT OVERLY SENSITIVE 3* (*SOS3*), played a role in the salt resistance of Arabidopsis (Liu and Zhu, 1998). Interaction of AtCBLs with CIPK1 was required for calcium and for C-terminal region of CIPK1, which was similar to that of WPK4 (Kudla et al., 1999). Cold stress also increased *WPK4* mRNA levels (data not shown). It is predicted that, under various

stress conditions, individual WPK4 interacting factors (TaWINs) are alternatively bind to WPK4 and possibly regulates WPK4 kinase activity or subcellular localization and thereby several enzyme activities are coordinately modulated to adjust environmental changes.

Concluding remarks

During studying the cytokinin signal transduction network in plant cells, a gene encoding a SNF1-related protein kinase was isolated from wheat and designated as *WPK4*. *WPK4* transcripts were shown to be regulated by both light and nutrient deprivation through the action of cytokinins.

With a hope to determine the relationship among WPK4, and nutrient and cytokinins, I obtained the following experimental results. (1) Results from RNA blot analysis showed that *WPK4* transcript levels were primarily controlled by sucrose but not inorganic salts. Cytokinins affected antagonistically sucrose-repressing process; (2) Substrate specificity of WPK4 was determined and found that WPK4 phosphorylate hinge I region of NR and C-terminal region of HMGR *in vitro*; (3) Two types of 14-3-3 proteins were obtained to interact with WPK4. Two Ser residues located in the C-terminal region of WPK4 to be phosphorylated were needed for 14-3-3 proteins interaction; (4) Conserved amino acid residues in the amphipathic groove of 14-3-3 protein is necessary for WPK4 binding.

Based on these results, I propose following hypothesis. Levels of endogenous cytokinins are known to fluctuate depending on stresses. When endogenous cytokinin levels are elevated, WPK4 is newly synthesized and WPK4 phosphorylates C-terminal region of HMGR. This results in inactivation of HMGR and alters the carbon metabolism by limiting the isoprenoid biosynthetic pathways. Alternatively, during nutritionally starvation, when the substrates for NR and SPS are exhausted and therefore the relevant enzyme activities are no longer necessary, WPK4 is activated by some unknown mechanism and phosphorylates these enzymes. Then 14-3-3 proteins bind to

them, resulting in their enzymatic inactivation (Fig. 23). Thus, I propose that WPK4 functions as the metabolic modulator by directly phosphorylating the metabolic key enzymes and by creating 14-3-3 ligands.

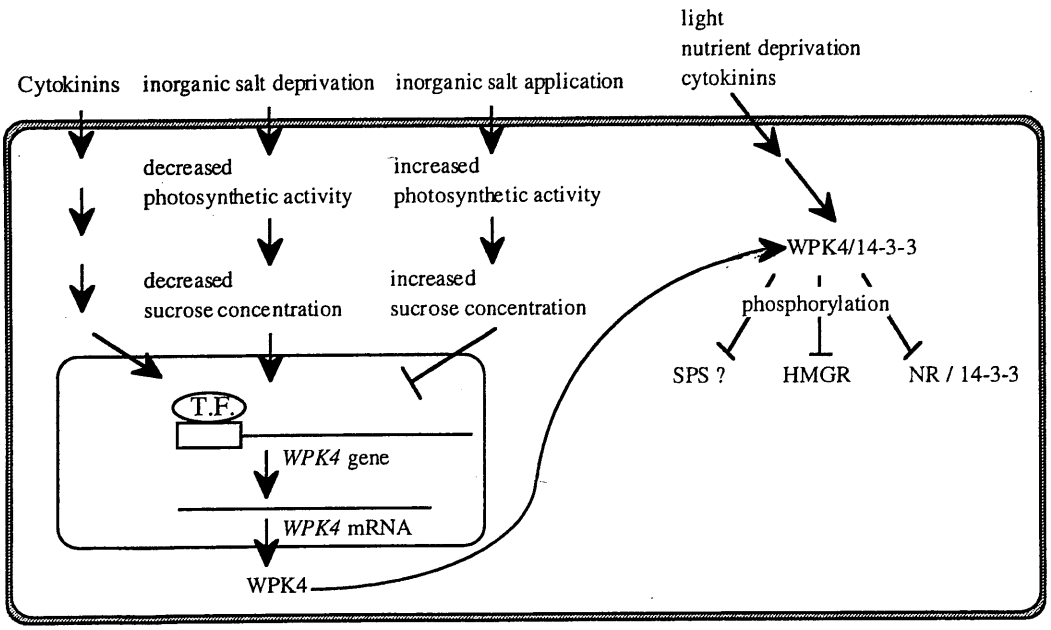

Figure 23. Proposed hypothesis of the regulatory mechanism of WPK4. Changes of *WPK4* transcripts level upon inorganic salt deprivation possibly results from decline of endogenous sucrose caused by decreases in photoassimilating activity. During nutritional starvation, when the substrates for nitrate reductase (NR) and sucrose phosphate synthase (SPS) are exhausted, and therefore the relevant enzyme activities are no longer necessary, WPK4 is activated by some unknown mechanism and phosphorylates these enzymes. Then 14-3-3 proteins bind to them, resulting in their enzymatic inactivation. When the endogenous cytokinin levels are elevated, WPK4 may newly be synthesized and phosphorylates HMGR. This results in inactivation of HMGR and alters carbon metabolism by limiting the isoprenoid biosynthetic pathway. T.F. means transcriptional factor.

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References

Aitken, A. (1996). 14-3-3 and its possible role in co-ordinating multiple signalling pathways. Trend Cell Biol. *6*, 341-347.

Alderson, A., Sabelli, P., Dickinson, J., Cole, D., Richardson, M., Kreis, M., Shewry, P., and Halford, N. (1991). Complementation of *snf1*, a mutation affecting global regulation of carbon metabolism in yeast, by a plant protein kinase cDNA. Proc. Natl. Acad. Sci. USA *88*, 8602-8605.

Annen, F., and Stockhaus, J. (1998). Characterization of a Sorghum bicolor gene family encoding putative protein kinases with a high similarity to the yeast SNF1 protein kinase. Plant Mol Biol *36*, 529-39.

Assmann, S. M., and Shimazaki, K. (1999). The multisensory guard cell. Stomatal responses to blue light and abscisic acid. Plant Physiol *119*, 809-16.

Bachmann, M., Shiraishi, N., Campbell, W., Yoo, B.-C., Harmon, A., and Huber, S. (1996). Identification of Ser-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. Plant Cell *8*, 505-517.

Bhalerao, R. P., Salchert, K., Bako, L., Okresz, L., Szabados, L., Muranaka, T., Machida, Y., Schell, J., and Koncz, C. (1999). Regulatory interaction of PRL1 WD protein with Arabidopsis SNF1-like protein kinases. Proc Natl Acad Sci U S A *96*, 5322-7.

Bouly, J. P., Gissot, L., Lessard, P., Kreis, M., and Thomas, M. (1999). Arabidopsis thaliana proteins related to the yeast SIP and SNF4 interact with AKINalpha1, an SNF1-like protein kinase. Plant J *18*, 541-550.

Brzobohaty, B., Moore, I., and Palme, K. (1994). Cytokinin metabolism: implications for regulation of plant growth and development. Plant Mol Biol *26*, 1483-97.

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*, 381-4.

Carlson, M. (1987). Regulation of sugar utilization in *Saccharomyces cerevisiae* species. J. Bacteriol. *169*, 4873-4877.

Celenza, J. L., and Carlson, M. (1986). A yeast gene that is essential for release from glucose repression encodes a protein kinase. Science *233*, 1175-80.

Chaudhury, A., Leetham, S., Craig, S., and Dennis, E. (1993). *amp1*-a mutant with high

cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. Plant J. *4*, 907-916.

Chiou, T.-J., and Bush, D. (1998). Sucrose is a signal molecule in assimilate partitioning. Proc. Natl. Acad. Sci. USA *95*, 4784-4788.

Cowley, S., Paterson, H., Kemp, P., and Marshall, C. J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell *77*, 841-52.

Crews, C. M., and Erikson, R. L. (1993). Extracellular signals and reversible protein phosphorylation: what to Mek of it all. Cell *74*, 215-7.

D'Agostino, I. B., and Kieber, J. J. (1999). Molecular mechanisms of cytokinin action. Curr Opin Plant Biol *2*, 359-64.

Davies, J. P., Yildiz, F. H., and Grossman, A. R. (1999). Sac3, an Snf1-like serine/threonine kinase that positively and negatively regulates the responses of Chlamydomonas to sulfur limitation. Plant Cell *11*, 1179-90.

Davies, S. P., Carling, D., and Hardie, D. G. (1989). Tissue distribution of the AMP-activated protein kinase, and lack of activation by cyclic AMP-dependent protein kinase, studied using a specific and sensitive peptide assay. Eur J Biochem *186*, 123-8.

Dijkwel, P., Huijser, C., Weisbeek, P., Chua, N.-H., and Smeekens, S. (1997). Sucrose control of phytochrome A signaling in Arabidopsis. Plant Cell *9*, 583-595.

Dominov, J. A., Stenzler, L., Lee, S., Schwarz, J. J., Leisner, S., and Howell, S. H. (1992). Cytokinins and auxins control the expression of a gene in Nicotiana plumbaginifolia cells by feedback regulation. Plant Cell *4*, 451-61.

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*, 435-42.

Douglas, P., Pigaglio, E., Ferrer, A., Halford, N., and MacKintosh, C. (1997). Three spinach leaf nitrate reductase-3-hydroxy-3-methylglutaryl-CoA reductase kinases that are regulated by reversible phosphorylation and/or Ca^{2+} ions. Biochem. J. *325*, 101-109.

Estruch, F., Treitel, M. A., Yang, X., and Carlson, M. (1992). N-terminal mutations modulate yeast SNF1 protein kinase function. Genetics *132*, 639-50.

Gomez-Cadenas, A., Verhey, S. D., Holappa, L. D., Shen, Q., Ho, T. H., and Walker-Simmons, M. K. (1999). An abscisic acid-induced protein kinase, PKABA1, mediates abscisic acid- suppressed gene expression in barley aleurone layers. Proc Natl Acad Sci U S A *96*, 1767-72.

Graham, I., Denby, K., and Leaver, C. (1994). Carbon catabolite repression regulates glyoxylate cycle gene expression in cucumber. Plant Cell *6*, 761-772.

Halford, N. G., and Hardie, D. G. (1998). SNF1-related protein kinases: global regulators of carbon metabolism in plants? Plant Mol Biol *37*, 735-48.

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*, 821-55.

Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. J Bacteriol *153*, 163-8.

Jang, J.-C., and Sheen, J. (1994). Sugar sensing in higher plants. Plant Cell *6*, 1665-1679.

Johnson, L. N., Noble, M. E., and Owen, D. J. (1996). Active and inactive protein kinases: structural basis for regulation. Cell *85*, 149-58.

Klee, H., and Estelle, M. (1991). Molecular Genetic Approaches To Plant Hormone Biology. Annu. Rev. Plant Physiol. Plant Mol. Biol. *42*, 529-551.

Kudla, J., Xu, Q., Harter, K., Gruissem, W., and Luan, S. (1999). Genes for calcineurin B-like proteins in Arabidopsis are differentially regulated by stress signals. Proc Natl Acad Sci U S A *96*, 4718-23.

Lakatos, L., Klein, M., Hofgen, R., and Banfalvi, Z. (1999). Potato StubSNF1 interacts with StubGAL83: a plant protein kinase complex with yeast and mammalian counterparts. Plant J *17*, 569-74.

Li, J., Wang, X. Q., Watson, M. B., and Assmann, S. M. (2000). Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science *287*, 300-3.

Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995). Crystal structure of the zeta isoform of the 14-3-3 protein. Nature *376*, 191-4.

Liu, J., and Zhu, J. K. (1998). A calcium sensor homolog required for plant salt tolerance. Science *280*, 1943-5.

Lu, G., Sehnke, P. C., and Ferl, R. J. (1994). Phosphorylation and calcium binding properties of an Arabidopsis GF14 brain protein homolog. Plant Cell *6*, 501-10.

Lu, J. L., Ertl, J. R., and Chen, C. M. (1990). Cytokinin enhancement of the light induction of nitrate reductase transcript levels in etiolated barley leaves. Plant Mol Biol *14*, 585-94.

Ma, H., Bloom, L. M., Walsh, C. T., and Botstein, D. (1989). The residual enzymatic phosphorylation activity of hexokinase II mutants is correlated with glucose repression in Saccharomyces cerevisiae. Mol Cell Biol *9*, 5643-9.

MacKintosh, C. (1998). Regulation of cytosolic enzymes in primary metabolism by reversible protein phosphorylation. Curr Opin Plant Biol *1*, 224-9.

Mackintosh, R. W., Davies, S. P., Clarke, P. R., Weekes, J., Gillespie, J. G., Gibb, B. J., and Hardie, D. G. (1992). Evidence for a protein kinase cascade in higher plants. 3-Hydroxy-3- methylglutaryl-CoA reductase kinase. Eur J Biochem *209*, 923-31.

Mansour, S. J., Matten, W. T., Hermann, A. S., Candia, J. M., Rong, S., Fukasawa, K., Vande Woude, G. F., and Ahn, N. G. (1994). Transformation of mammalian cells by constitutively active MAP kinase kinase. Science *265*, 966-70.

McMichael, R. W., Jr., Klein, R. R., Salvucci, M. E., and Huber, S. C. (1993). Identification of the major regulatory phosphorylation site in sucrose- phosphate synthase. Arch Biochem Biophys *307*, 248-52.

Miller, C. (1956). Similarity of kinetin and red light effects. Plant Physiol. *31*, 318-319.

Moorhead, G., Douglas, P., Cotelle, V., Harthill, J., Morrice, N., Meek, S., Deiting, U., Stitt, M., Scarabel, M., Aitken, A., and MacKintosh, C. (1999). Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. Plant J *18*, 1-12.

Muranaka, T., Banno, H., and Machida, Y. (1994). Characterization of tobacco protein kinase NPK5, a homolog of Saccharomyces cerevisiae SNF1 that constitutively activates expression of the glucose-repressible SUC2 gene for a secreted invertase of S. cerevisiae. Mol Cell Biol *14*, 2958-65.

Musacchio, A., Wilmanns, M., and Saraste, M. (1994). Structure and function of the SH3 domain. Prog Biophys Mol Biol *61*, 283-97.

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*, 889-97.

Nemeth, K., Salchert, K., Putnoky, P., Bhalerao, R., Koncz-Kalman, Z., Stankovic-Stangeland, B., Bako, L., Mathur, J., Okresz, L., Stabel, S., Geigenberger, P., Stitt, M., Redei, G. P., Schell, J., and Koncz, C. (1998). Pleiotropic control of glucose and hormone responses by PRL1, a nuclear WD protein, in Arabidopsis. Genes Dev *12*, 3059-73.

Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by

phosphorylation of Cdc25C on serine-216. Science *277*, 1501-5.

Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H., and Liddington, R. C. (1998). 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphipathic groove. J Biol Chem *273*, 16305-10.

Purcell, P. C., Smith, A. M., and Halford, N. G. (1998). Antisense expression of a sucrose non-fermenting-1-related protein kinase sequence in potato results in decreased expression of sucrose synthase in tubers and loss of sucrose-inducibility of sucrose synthase transcripts in leaves. Plant Journal *14*, 195-202.

Rose, M., Albig, W., and Entian, K. D. (1991). Glucose repression in Saccharomyces cerevisiae is directly associated with hexose phosphorylation by hexokinases PI and PII. Eur J Biochem *199*, 511-8.

Sambrook, J., Frisch, E., and Maniatis, T. (1989). Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY).

Sano, H., Seo, S., Koizumi, N., Niki, T., Iwamura, H., and Ohashi, Y. (1996). Regulation by cytokinins of endogenous levels of jasmonic acid and salicylic acids in mechanically wounded tobacco plants. Plant Cell Physiol *37*, 762-769.

Sano, H., Seo, S., Orudgev, E., Youssefian, S., Ishizuka, K., and Ohashi, Y. (1994). Expression of the gene for a small GTP binding protein in transgenic tobacco elevates endogenous cytokinin levels, abnormally induces salicylic acid in response to wounding, and increases resistance to tobacco mosaic virus infection. Proc. Natl. Acad. Sci. U S A *91*, 10556-10560.

Sano, H., and Youssefian, S. (1994). Light and nutritional regulation of transcripts encoding a wheat protein kinase homolog is mediated by cytokinins. Proc. Natl. Acad. Sci. U S A *91*, 2582-6.

Schiestl, R. H., and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr Genet *16*, 339-46.

Schultz, T. F., Medina, J., Hill, A., and Quatrano, R. S. (1998). 14-3-3 proteins are part of an abscisic acid-VIVIPAROUS1 (VP1) response complex in the Em promoter and interact with VP1 and EmBP1. Plant Cell *10*, 837-47.

Sheen, J. (1990). Metabolic repression of transcription in higher plants. Plant Cell *2*, 1027-1038.

Shi, J., Kim, K. N., Ritz, O., Albrecht, V., Gupta, R., Harter, K., Luan, S., and Kudla, J. (1999). Novel Protein Kinases Associated with Calcineurin B-like Calcium Sensors in Arabidopsis. Plant Cell *11*, 2393-2406.

Simmons, C. R., Litts, J. C., Huang, N., and Rodriguez, R. L. (1992). Structure of a rice

beta-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. Plant Mol Biol *18*, 33-45.

Stone, J., and Walker, J. (1995). Plant protein kinase families and signal transduction. Plant Physiol. *108*, 451-457.

Su, W., and Howell, S. (1995). The effects of cytokinin and light on hypocotyl elongation in Arabidopsis seedlings are independent and additive. Plant Physiol. *108*, 1423-1430.

Su, W., Huber, S., and Craford, N. (1996). Identification in vitro of a post-translational regulatory site in the hinge 1 region of Arabidopsis nitrate reductase. Plant Cell *8*, 519-527.

Sugden, C., Donaghy, P. G., Halford, N. G., and Grahame Hardie, D. (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.

Thorson, J. A., Yu, L. W., Hsu, A. L., Shih, N. Y., Graves, P. R., Tanner, J. W., Allen, P. M., Piwnica-Worms, H., and Shaw, A. S. (1998). 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. Mol Cell Biol *18*, 5229-38.

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*, 110-4.

Toroser, D., and Huber, S. C. (1997). Protein phosphorylation as a mechanism for osmotic-stress activation of sucrose-phosphate synthase in spinach leaves. Plant Physiol *114*, 947-55.

Tzivion, G., Luo, Z., and Avruch, J. (1998). A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. Nature *394*, 88-92.

Verwoerd, T., Dekker, B., and Hoekema, A. (1989). A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res *17*, 2362.

Wenzler, H., Mignery, G., Fisher, L., and Park, W. (1989). Sucrose-regulated expression of a chimeric potato tuber gene in leaves of transgenic tobacco plants. Plant Mol Biol *13*, 347-54.

Xiao, B., Smerdon, S., Jones, D., Dodson, G., Soneji, Y., Aitken, A., and Gamblin, S. (1995). Structure of a 14-3-3 protein and implications for coodination of multiple signalling pathways. Nature *376*, 188-191.

Yu, X., Sukumaran, S., and Mrton, L. (1998). Differential expression of the arabidopsis nia1 and nia2 genes. cytokinin-induced nitrate reductase activity is correlated with

increased nia1 transcription and mrna levels. Plant Physiol *116*, 1091-6.

Zeng, Y., Forbes, K. C., Wu, Z., Moreno, S., Piwnica-Worms, H., and Enoch, T. (1998). Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature *395*, 507-10.

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*, 619-28.

List of publications

Ikeda, Y., Koizumi, N., Kusano, T., Sano, H. (1999) Sucrose and cytokinin modulation of *WPK4*, a gene encoding a SNF1-related protein kinase from wheat. Plant Physiol. 121, 813-820