Specific Binding of a 14-3-3 Protein to Autophosphorylated WPK4, an SNF1-related Wheat Protein Kinase, and to WPK4-phosphorylated Nitrate Reductase*

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WPK4 is a wheat protein kinase related to the yeast protein kinase SNF1, which plays a role in catabolite repression. To identify proteins involved in signal transduction through WPK4, we performed yeast two-hybrid screens and isolated two cDNA clones designated as TaWIN1 and TaWIN2. Both encode 14-3-3 proteins that, upon autophosphorylation, bind the C-terminal regulatory domain of WPK4. Mutational analysis through amino acid substitution revealed that TaWIN1 and TaWIN2 primarily bind WPK4 through phosphoserines at the positions 388 and 418, both located in the C-terminal region. Mutations in the conserved residues of the TaWIN1 amphipathic groove impaired the ability of TaWIN1 to bind to WPK4. A screen for in vitro phosphorylation of proteins involved in nutrient metabolism revealed a putative WPK4 substrate, nitrate reductase; its hinge 1 region was efficiently phosphorylated by WPK4. Subsequent far Western blots showed that it specifically bound TaWIN1. Since nitrate reductase has been shown to be inactivated by phosphorylation upon 14-3-binding, the present findings strongly suggest that WPK4 is the protein kinase responsible for controlling the nitrogen metabolic pathway, assembling the nitrate reductase and 14-3-3 complex through its phosphorylation specificity.

Members of the SNF1-related protein kinases play a central role in phosphorylation cascades involved in carbon assimilation in animals, fungi, and plants (1). In Saccharomyces cerevisiae, the SNF1 complex is activated in response to glucose deprivation and inhibits the repression of many glucose-repressed genes, including genes involved in alternate carbon source utilization, respiration, and gluconeogenesis (1). In plants, more than 20 genes encoding the SNF1-related protein kinases (SnRKs) have been identified (2). Based on sequence similarity, they are classified into three distinct subgroups (SnRK1-SnRK3) of which SnRK1 has been the best characterized (2). For example, SnRK1s from rye (RKN1), tobacco (NPK5), and Arabidopsis thaliana (AKIN10, AKIN11) were shown to complement yeast snf1 mutant cells (2). Antisense expression of a potato SNF1 homolog resulted in a loss of sugar-inducible expression of sucrose synthase (3). These observations suggest that members of the SnRK1 subgroup are SNF1 orthologs that activate sugar-inducible gene expression in sugar signaling pathways (2). However, the physiological functions of the other two subgroups, SnRK2 and SnRK3, remain relatively poorly characterized.

One method of determining the physiological role of SnRKs is to identify proteins that interact with them. For example, SNF1 and its mammalian homolog AMPK, a master switch that regulates carbohydrate and fat metabolism (4), combine with other proteins to form a heterotrimer that includes both catalytic subunits, SNF1p and AMPKα, respectively, and non-catalytic subunits including SIP1p (SIP2p, GAL83p, AMPKβ) and SNF4p (AMPKγ) (4). In the case of SnRKs, a recent survey using yeast two-hybrid system also revealed that the A. thaliana SnRK, AKIN1a, interacted with non-catalytic subunits AKINα1/β2 and AKINα1γ (1). AKIN10 and AKIN11 were also shown to form complexes with the protein PRL1 (5). CIPK, a member of the SnRK3 subgroup, was found to interact with an EF-hand-type calcium-binding protein similar to yeast calcineurin B (6). These observations clearly indicate that SnRKs function through specific interaction with signaling protein factors involved in nutrient metabolism.

Previously we reported that WPK4, a gene encoding a wheat SnRK3, was up-regulated by light and cytokinins and down-regulated by nutrients (7). Primary sequence analysis revealed that C-terminal region of WPK4 is more distantly related than other SnRK subgroups, which suggests that WPK4 may possess another function in nutrient signal transduction pathways (8). To identify target proteins that interact with WPK4, we used the yeast two-hybrid system to identify two cDNA clones. Both encode 14-3-3 proteins that bound WPK4-phosphorylated nitrate reductase.

EXPERIMENTAL PROCEDURES

Yeast Two-Hybrid Screen and Quantitative β-Galactosidase Assays—Yeast two-hybrid screening was conducted using a HybriZAP-2.1 two-hybrid vector system (Stratagene). The cDNA library was prepared from 7-day-old wheat seedlings grown in water and treated with 100 μM N6-benzylamino purine for 24 h using a ZAP-cDNA synthesis kit (Stratagene). A HybriZAP two-hybrid vector was used to construct a GAL4 activation domain fusion cDNA library (2 × 10^6 plaque-forming units) that was converted to a yeast plasmid library by in vivo excision. pBD-WPK4, in which WPK4 cDNA was inserted in-frame with a right orientation into a pBD-GAL4 Cam vector, was used as the bait plasmid. The yeast strain YRG2 (Stratagene), which carries pBD-WPK4, was
subsequently transformed with the library plasmids.

**Construction of Plasmids**—The K75D-WPK4 (8) and ΔC-WPK4 genes were cloned into a pBd-Gal4 Cam vector. A series of WPK4 mutants (T204A, T204E, S288A, S418A, and S388A/S418A) were constructed using the Mutan-Express KIT (TAKARA) and appropriate oligonucleotides. For the S388A/S418A double mutant, the S339A mutation was generated on the S418A clone. A series of TaWIN1 mutants (K60D, R67E, L185D, V189E, G221S, and L233D) were similarly constructed. All mutagenized inserts were subcloned into the pAD Gal4 vector. The ΔN-WPK4 gene containing the 3′-distal 1-kilobase fragment of WPK4 with the double mutation S388A/S418A was cloned into a pGEX-2T vector (Amersham Pharmacia Biotech). The entire coding regions of TaWIN1 and TaWIN2 were cloned in-frame into pGEX-IT-1 (Amersham Pharmacia Biotech), yielding pGEX-TaWIN1 and pGEX-TaWIN2, respectively. These were also cloned in-frame into pET32a (Novagen), yielding pET-TaWIN1 and pET-TaWIN2. A DNA fragment encoding a peptide corresponding to the residues 469–560 of tobacco Nia1 (nitrate reductase) was amplified by polymerase chain reaction and cloned into pGEX4-T1 (Amersham Pharmacia Biotech). TaWIN1 was prepared as described (8).

**Expression and Purification of Recombinant Proteins**—Glutathione S-transferase (GST) and its fusion derivatives were expressed in **E. coli** DH5α cells and purified as described (8). His-tagged proteins were expressed in the E. coli strain BL21 (DE3) and purified by Ni-NTA superficil resin (Qiagen). Purified proteins were dialyzed and stored in 30% glycerol until use.

**Gel Filtration Analysis**—GST-TaWIN1 fusion proteins were retained in a glutathione-Sepharose 4B resin. Beads were then incubated with T buffer containing 100 units of thrombin (Amersham Pharmacia Biotech) at 4 °C for 24 h. TaWIN1, purified with a DEA-Toyopearl column, was put through high resolution gel filtration chromatography and equilibrated with 20 mM phosphate-buffered saline.

**Far Western Blot Analysis**—A previously described protocol for far Western blotting was employed, with modifications (9). GST, GST-K75D were incubated in a kinase buffer (20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 20 mM MgCl2, and 20 μM ATP) at room temperature for 1 h and washed with the protein binding buffer. 20 μl of each sample of glutathione-Sepharose beads was added to 50 μl of the Histagged TaWIN1 protein solution, then incubated at 4 °C for 1 h. Proteins were released from the resin by boiling in Laemmli’s sample buffer and separated by 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. His-TaWIN1 was detected using an anti-(His) monoclonal antibody (Novagen) and chemiluminescence (ECL, Amersham Pharmacia Biotech).

**RESULTS**

**Isolation of WPK4-interacting Factors**—Screening 1 × 10^6 clones by the yeast two-hybrid system, isolated three clones that activated two reporter genes, HIS3 and lacZ. Sequence analysis revealed that two were derived from the same gene and were designated as TaWIN1 (Triticum aestivum WPK4-interacting factor 1). The third showed a 70% similarity to TaWIN2 (Triticum aestivum WPK4-interacting factor 2). All three encoded 14-3-3 proteins (Fig. 1A). TaWIN1 encoded a polypeptide consisting of 266 amino acid residues with a relative molecular mass of 29.4 kDa, TaWIN1 showed the highest similarity to GF14d, isolated from rice (10). TaWIN2 encoded a polypeptide consisting of 259 amino acid residues with a relative molecular mass of 28.6 kDa. TaWIN2 belonged to the omega-type 14-3-3 proteins but is more distantly related (Fig. 1C). A putative EF-hand motif is also found in both TaWIN1 and TaWIN2

![Fig. 1](https://example.com/f1.png)
protein kinase A (Fig. 2A). Substitution with Ala (T204A) reduced interaction with TaWIN1 and TaWIN2 to 76 and 72% that of wild type, respectively (Fig. 2B). By contrast, substitution with Glu (T204E) strengthened the interaction to 118 and 115%, respectively (Fig. 2B). The mutant lacking phosphorylation activity (K75D) (8) showed essentially no interaction with either TaWIN1 or TaWIN2 (Fig. 2B). The results indicate the necessity of kinase activity for the binding of WPK4 to TaWIN1 and TaWIN2 as well as the crucial role of the Thr in the activation loop. A GST pull-down assay showed that TaWIN1 specifically binds GST-WPK4 but not GST-K75D in vitro, indicating a direct interaction between WPK4 and TaWIN1 (Fig. 2C).

Binding of TaWINs to the C-terminal Region of WPK4—There are two potential 14-3-3 binding motifs, RPASLN (with S388) and RFISGEP (with S418) in the C-terminal region of WPK4. To determine whether these motifs are indeed binding sites, we constructed mutants in which one or both of the two Ser residues were replaced with Ala (Fig. 3A). A third mutant lacking the C-terminal region was also constructed (D). A quantitative liquid assay showed that the DC mutation completely abolished the binding of WPK4 to TaWIN1 and TaWIN2 (Fig. 3A). Yeast Y190 cells were cotransformed with the bait and prey combinations and quantitatively assayed for b-galactosidase activity. Bars indicate the mean of three independent assays. GBD, GAL4 DNA binding domain. WT, wild type. C, GST pull-down assay for interaction between TaWIN1 and WPK4. His-tagged TaWIN1 was incubated with immobilized GST, GST-K75D, or GST-WPK4. Proteins bound to the Sepharose beads were pelleted, washed, subjected to SDS-PAGE, stained with Coomassie Brilliant Blue (CBB, left), and detected by Western blotting using an anti-Penta-His antibody (right). Relative molecular masses of the standard samples are indicated at the left. The arrow indicates His-TaWIN1.
TaWIN2, thereby demonstrating that the C-terminal region is critical for interaction (Fig. 3B). Binding of the S388A mutant to TaWIN1 and TaWIN2 was reduced to 80 and 76% of the control, respectively, whereas that of S418A reduced binding to 76 and 59%, respectively (Fig. 3B). The S388A/S418A double mutant had even further reduced binding, with only 25% of the control (Fig. 3B). These results show that the binding of TaWIN1 and TaWIN2 to WPK4 is primarily mediated by the phosphorylated Ser-388 and Ser-418 of WPK4. To determine whether these two Ser residues are sites of autophosphorylation, two N-terminal-truncated mutations (ΔN) containing only the 165-amino acid C-terminal region were constructed (Fig. 3A) and subjected to phosphorylation by GST-WPK4 (Fig. 3C). The results showed no clear difference between ΔN and Ser-388/418A ΔN in phosphate receptor function, suggesting the presence of another phosphorylation site(s) in the C-terminal region. Far Western blotting analysis showed that phosphorylated Ser-388/418A ΔN was able to bind TaWIN1, although the efficiency was low (Fig. 3C). These results showed that, in addition to two Ser-containing regions described above, there is an additional binding site(s) for TaWIN1 in the C-terminal region of WPK4.

Molecular Properties of TaWIN1—In general, 14-3-3 proteins form dimers and their inner surfaces form amphipathic grooves that serve as the principle binding site for 14-3-3 ligands (12). Site-directed mutagenesis was used to change the conserved basic and hydrophobic residues located in the inner surface of TaWIN1. Basic residues within helix 3 (Lys-60, Arg-67) and hydrophobic residues within helices 7 and 9 (Leu-185, Val-189, Leu-233) were substituted with acidic residues (K60D, R67E, L185D, V189E, L233D) (Fig. 4A). Binding assays showed all of these residues to be critical for interaction between TaWIN1 and WPK4 (Fig. 4A). However, when the Gly in the putative EF-hand motif of TaWIN1 was substituted by Ser (G221S), no loss of binding activity was observed (Fig. 4A). This suggests that, at least in yeast cells, calcium ions are not required for WPK4/TaWIN1 interaction. The capacity for TaWIN1-TaWIN2 interaction.

FIG. 4. Effects of amino acid substitution and dimer formation on TaWIN1 function. A, Y190 cells were cotransformed with the bait and prey combinations shown at the left and quantitatively assayed for β-galactosidase activity. Mean values of three independent assays are indicated by bars. WT, wild type. B, GST pull-down assay showing the association of TaWIN1 with TaWIN2. Histidine-tagged TaWIN2 was incubated with glutathione-Sepharose-immobilized GST or GST-TaWIN1. Proteins bound to the Sepharose beads were pelleted, washed, subjected to electrophoresis, stained with Coomassie Brilliant Blue (CBB; left), and detected by Western blotting using an anti-Penta-His antibody (right). Relative molecular masses of the standard proteins are indicated at the left. The arrow indicates His-TaWIN1. C, elution profile of recombinant TaWIN1 proteins by high resolution gel filtration chromatography (left). The elution positions for albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa) are shown above the A280 trace for TaWIN1 elution. SDS-PAGE analysis of the peak fraction is shown in the right panel. Relative molecular masses are indicated in left.
under continuous light conditions, were treated with 100 μM WPK4 transcript accumulation. Six-day-old green seedlings, grown with probes for a 0.3-kilobase 5′-untranslated region of TaWIN1 transcript accumulation. Six-day-old green seedlings, grown under continuous light conditions, were treated with 100 μM N6-benzylaminopurine for the time period indicated; extracted RNAs were used for hybridization assays. Hybridization was performed (8) with probes for a 0.3-kilobase 5′-untranslated region of TaWIN1 cDNA, a 0.8-kilobase 3′ region of WPK4 cDNA, and a 1.2-kilobase wheat actin cDNA, respectively.

dimer formation was then analyzed by a GST pull-down assay, which clearly showed that TaWIN1 was able to bind TaWIN2 (Fig. 4B). This indicates that each are able to form heteromeric dimers. Purified recombinant TaWIN1 protein was subjected to gel filtration followed by SDS-PAGE, whereupon the relative molecular mass of each fraction after each step was estimated to be 64 and 32 kDa, respectively (Fig. 4C).

Binding of TaWIN1 to WPK-phosphorylated Nitrate Reductase—NR is not activated by phosphorylation followed by 14-3-3 protein binding (13). A peptide from the hinge 1 region of tobacco NR was expressed as a GST fusion protein and subjected to a WPK4 phosphorylation assay (Fig. 5). The results showed that WPK4 phosphorylates GST-NR and GST-HMGR, but not GST (Fig. 5B). TaWIN1 was subsequently examined by far Western blotting for binding phosphorylated NR. It is clear that TaWIN1 efficiently binds phosphorylated NR, but not HMGR in this system (Fig. 5C). TaWIN1 was not found to bind unphosphorylated NR (Fig. 5C).

Ubiquitous Accumulation of TaWIN1 Transcripts—The localization of TaWIN1 in wheat tissues was examined by RNA blot analysis. Although WPK4 transcripts were detected in upper-ground tissues, especially in leaves, those of TaWIN1 were found in all tissues examined (Fig. 6A). The induction profiles of these transcripts were also examined. Although

**DISCUSSION**

The present paper provides evidence that TaWINs, different isoforms of 14-3-3 proteins, interact with WPK4, and that TaWIN1 directly binds WPK4-phosphorylated NR. Yeast two-hybrid screens were performed to find proteins that interact with WPK4, and distinct clones termed TaWINs were isolated. The primary sequence indicated that they belong to the 14-3-3 protein family. In general, 14-3-3 proteins bind ligands containing phosphorylated consensus motif RSXpSXP (pS is phosphoserine), found in Raf (14), Bad (15), and CDC25 (16), although unphosphorylated peptides have also been shown to be the target of 14-3-3 proteins (17). Our finding that TaWINs bind only autophosphorylated WPK4 suggests that its C-terminal region is only a pseudosubstrate region; this was directly shown for the case of Ser-388 and Ser-418 autophosphorylation, which occurs in the C-terminal region.

Plant 14-3-3 proteins are involved in many cellular events, including enzyme regulation, gene expression, and protein translocation (18). Despite the diversity of these physiological functions, few reports have yet been presented on their interaction with protein kinases. One notable study found that they bind calcium-dependent protein kinase, resulting in enzymatic activation in vitro (19). By contrast, the binding of TaWINs to WPK4 did not influence kinase activity under our experimental conditions. Nevertheless, these findings suggest that some protein kinases may generally interact with 14-3-3 proteins, which assist in the transduction of signals that the kinases receive.

The activity of NR, the key enzyme in plant nitrogen assimilation pathways, is both transcriptionally and post-translationally regulated (20). Post-translational regulation is performed by reversible phosphorylation with subsequent binding of inhibitor proteins (20, 21). In previous studies, three protein kinases responsible for NR phosphorylation at Ser-543 were
isolated from spinach leaves and designated PKI, PKII, and PKIII (22). PKII was shown to be a calcium-dependent protein kinase, and PKIII was shown to be an SNF1-related kinase (23). NR, which is reversibly phosphorylated at Ser-543, has been shown elsewhere to interact with 14-3-3 proteins, which results in its enzymatic inactivation (13, 20). However, mechanisms by which NR kinase is activated and by which 14-3-3 proteins are recruited remain to be determined. In the present study we suggest that one of the NR kinases is WPK4 and propose the following working hypothesis. Upon external stimulation, WPK4 autophosphorylates its regulatory domain, to which the 14-3-3 protein (TaWIN) binds. WPK4 simultaneously phosphorylates NR, to which TaWIN is transferred; this forms the enzymatically inactive NR/TaWIN complex (Fig. 7). Since WPK4 is up-regulated by nutrient deprivation (8), our model may partly explain the complex regulation of NR; the reduction of NR activity by reduced nutrient assimilation (24) could conceivably be mediated through WPK4 system.

REFERENCES
1. Hardie, D. G., Carling, D., and Carlson, M. (1998) Annu. Rev. Biochem. 67, 821–855
2. Halford, N. G., and Hardie, D. G. (1998) Plant Mol. Biol. 37, 735–748
3. Purcell, P. Smith, A., and Halford, N. (1998) Plant J. 14, 195–202
4. Bouly, J. P., Gissot, L., Lessard, P., Kreis, M., and Thomas, M. (1999) Plant J. 18, 541–550
5. Bhalaria, R. P., Salchert, K., Bako, L., Okresz, L., Szabados, L., Muranaka, T., Machida, Y., Schell, J., and Koncz, C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 5322–5327
6. Shi, J., Kim, K. N., Ritz, O., Allbrecht, V., Gupta, R., Harter, K., Luan, S., and Kudla, J. (1999) Plant Cell 11, 2393–2406
7. Sano, H., and Youssifian, S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2582–2586
8. Ikeda, Y., Koizumi, N., Kusano, T., and Sano, H. (1999) Plant Physiol. 121, 813–820
9. Kinoshiita, T., and Shimazaki, K. (1999) EMBO J. 18, 5548–5558
10. Schultz, T. F., Medina, J., Hill, A., and Quatrano, R. S. (1998) Plant Cell 10, 837–847
11. Lu, G., Schneke, P. C., and Fehr, R. J. (1994) Plant Cell 6, 501–510
12. Liu, D., Bienkowska, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) Nature 376, 191–194
13. Bachmann, M., Huber, J. L., Liao, P. C., Gage, D. A., and Huber, S. C. (1996) FEBS Lett. 387, 127–131
14. Fanti, W. J., Muslin, A. J., Kikuchi, A., Martin, J. A., MacNicol, A. M., Gross, R. W., and Williams, L. T. (1994) Nature 371, 612–614
15. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–624
16. Conklin, D. S., Galaktionov, K., and Beach, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7892–7896
17. Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H., and Liddington, R. C. (1998) J. Biol. Chem. 273, 16305–16310
18. Finnie, C., Borch, J., and Collinge, D. B. (1999) Plant Mol. Biol. 40, 545–554
19. Camoni, L., Harper, J. F., and Palmgren, M. G. (1998) FEBS Lett. 430, 361–364
20. Kaiser, W. M., Weiner, H., and Huber, S. C. (1999) Physiol. Plant. 105, 385–390
21. Bachmann, M., McMichael, R. W., Huber, J. L., Kaiser, W. M., and Huber, S. C. (1995) Plant Physiol. 108, 1083–1091
22. Douglas, P., Pigaglio, E., Ferrer, A., Halford, N. G., and MacIntosh, C. (1997) Biochem. J. 325, 101–109
23. Douglas, P., Morrice, N., and MacIntosh, C. (1997) FEBS Lett. 377, 113–117
24. Crawford, N. M. (1995) Plant Cell 7, 859–868