African Journal of Biotechnology Vol. 8 (13), pp. 2959-2962, 6 July 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Expression, purification and kinase activity analysis of maize ZmSPK1, a member of plant SnRK2 subfamily

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Accepted 19 May, 2009

Kinase activity is essential for a protein kinase to perform its biological function. In previous study we have cloned a novel plant SnRK2 subfamily gene from maize and named it as ZmSPK1. In this study the cDNA of ZmSPK1 with dHA-His6 tag was amplified by PCR and was subcloned into the yeast expression vector p426GAL1. The constructed plasmid named as p426GAL1-SPK1-dHA-His6 was transformed into *Saccharomyces cerevisiae*. Under the induction of galactose the recombinant protein was expressed. The purified recombinant protein showed a single specific band by the analysis of the western-blot. In an *in vitro* kinase assay, the purified recombinant protein showed phosphorylation and autophosphorylation activity. This showed that ZmSPK1 encodes a functional protein kinase.

Key words: *Zea mays* L., ZmSPK1, kinase activity.

INTRODUCTION

The SNF1 protein kinase family currently comprises SNF-1 itself in the *Saccharomyces cerevisiae*, the AMP-activated protein kinases (AMPKs) in mammals, and the SNF1-related protein kinases (SnRKs) in higher plants (Halford and Hardie 1998). Mammalian AMPKs belong to a highly conserved serine/threonine protein kinase family. They have many and varied downstream targets indicating their wide variety of biological roles. AMPKs are firstly to be considered as a key regulator of cellular and whole body energy homeostasis and can be activated by a lot of pathological and physiological stimuli controlling cellular glucose level and/or AMP: ATP ratio (Hardie et al., 1998). For example, AMPKs can be activated by AMP in the higher AMP/ATP ratio and by its upstream protein kinase AMP-activated protein kinase (AMPKK). It was also found that AMPKs can be regulated by hormones and cytokines which can influence the whole body metabolism (Kahn et al., 2005). Besides these, recently AMPKs were found to regulate the cell proliferation and polarity (Williams and Brenman 2008) indicating their important roles in Mammalian cells.

In yeast, SNF1 has the highly conserved structure and function with Mammalian AMPKs. Both of them are heterotrimeric protein complexes, consisting of a catalytic activating subunit (α) and two regulatory subunits (β and γ) (Lu et al., 2007). SNF1 can also play central roles in the sugar signaling transduction pathway and metabolic stress responses (Hardie et al., 1998). The well known glucose repression is the phenomenon that when the glucose in the medium for *S. cerevisiae* is adequate, a large number of genes are switched off. The preferred carbon source for the yeast is glucose. The SNF1 gene is activated in response to low cellular glucose levels and is required for the derepression of genes that are repressed by glucose (Gancedo, 1998; Ronne, 1995; Rolland et al., 2006). Furthermore, SNF1 can also play important roles in sporulation, glycogen accumulation and peroxisome biogenesis (Hardy et al., 1994; Simon et al., 1992).

Plants contain three SnRK subfamilies, SnRK1, SnRK2 and SnRK3. Among them SnRK1s are the closest homologue of the yeast SNF1 and the mammalian AMPKs (Halford and Hardie 1998). Based on the amino acid sequence similarity and expression patterns, the SnRK1 family of cereals can be subdivided further into two groups SnRK1a and SnRK1b (Halford and Hardie 1998). Compared to yeast SNF1, SnRK1s have a highly conserved N-terminal catalytic domain (Halford and Hardie 1998). Some members of the SnRK1 subfamily can complement yeast snf1-defective mutants (Muranaka et al., 1994). Recent studies are providing more details about the important roles of SnRK1s in cellular carbohydrate metabolism, stress signaling and plant development (Baena-González

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et al., 2007; Baena-González and Sheen, 2008; Zhang et al., 2001; Lu et al., 2007). All these findings suggest the similar role for the plant SnRK1 subfamily in regulating the carbon and energy metabolism to that in the yeast SNF1 (Hardie et al., 1998).

SnRK2 and SnRK3 subfamilies are unique to plants with less sequence similar to SNF1 and AMPK than SnRK1. Compared to members of the SnRK1 and SnRK2 subfamilies, members of the SnRK3 subfamily do not show similarity in the C-terminal domains (Halford and Hardie 1998). Studies show that members of the SnRK3 subfamily may response to cytokinins, high intensity light, insufficient of nutrients (Ohba et al., 2000). Furthermore, SOS2, as a well known member of SnRK3 subfamily, is involved in conferring salt tolerance (Halfter et al., 2000; Liu et al., 2000). The N-terminal catalytic domain of SnRK2 is also very similar to SNF1 and the C-terminal conserved domains of SnRK2 is relatively shorter than that of SnRK1 (Halford and Hardie 1998). SnRK2 subfamily are considered to be involved in osmotic signaling and some of them can be activated by ABA, saline and hyperosmotic stresses (Monks et al., 2001; Yoon et al., 1997; Kobayashi et al., 2004; Boudsocq et al., 2004).

In previous study, we have cloned a maize SnRK2 subfamily gene and named it as ZmSPK1. Semi-Quantitative RT-PCR analysis showed that the ZmSPK1 expression is induced by mannitol, salt and abscisic acid (ABA). Furthermore, the ZmSPK1 is most abundant in reproductive organs among different tissues. These results suggest that ZmSPK1 might play multiple roles in abiotic stress resistance pathways, as well as in plant reproductive development (Zou et al., 2006). In this study we constructed the yeast expression vector to express ZmSPK1 protein and analyzed its kinase activity. This will provide more information about its biochemical properties and more hints to its biological functions for further study.

MATERIALS AND METHODS

Escherichia coli and yeast strains and construction of vectors

Escherichia coli strain DH5α, *S. cerevisiae* strain W303-1A (MATa, leu2, his3, ade2, trp1 and ura3) are both stored in our laboratory. The ZmSPK1 in pGEM-T was amplified by PCR with adding BamH I and Stu I sequences into 5' and 3' primers respectively. The PCR product was digested with BamH I/Stu I. The clone vector pBKS-CBF3-dHA-His6 (stored in our laboratory) was digested with BamH I/Stu I to release the *CBF3*. The digested products were ligated with T4 DNA ligase (TaKaRa, Dalian, China). The ligation product was pBKS-ZmSPK1-dHA-His6. The segment ZmSPK1-dHA-His6 was obtained by digesting pBKS-ZmSPK1-dHA-His6 with BamH I/Cla I, and subcloned into the yeast expression vector p426GAL1 (stored in our laboratory) digested with BamH I/Cla I. The ligation product was p426GAL1-ZmSPK1-dHA-His6.

Expression, purification and Western-blot analysis of ZmSPK1-dHA-His6

The p426GAL1-ZmSPK1-dHA-His6 construct was transformed into yeast strain W303-1A (MATa, leu2, his3, ade2, trp1 and ura3) and then selected on SC-URA medium (Liang et al., 1997). The fusion protein was induced with 2% galactose in SC-URA medium. The induced protein was purified via ProBond resin (Invitrogen, USA) according to the procedure recommended by the manufacturer. The purified fusion protein was confirmed by 12% SDS-PAGE gel and Western-blot using rat monoclonal antibody anti-HA (3f10, Roche Applied Science, Germany) as first antibody and peroxidase-conjugated goat anti-rat IgG (H + L) (Zhongshan Goldenbridge Biotechnology Co. Ltd., China) as second antibody.

Protein kinase assays in solution

About 1 μg of the native purified fusion protein and 2 μg of myelin basic protein (MBP) (Upstate, USA) were incubated with 5 μCi of [γ-^{32}P] ATP (about 185 TBq mmol $^{-1}$) in kinase buffer (25 mM Tris-HCl, pH 7.0, 1 mM DTT, 10 mM $MnCl_2$ and 20 μM ATP). The final volume of an incubation sample was 25 μl. After 30 min of incubation at 30℃, the reaction was terminated by adding 2 ×gel loading buffer and incubation at 95℃ for 5 min. The phosphorylated protein was separated by 12% SDS-PAGE gel. Then the gel was dried and exposed to a PhosphoImaging cassette with subsequent analysis on a PhosphoImager (typhoon 8600, Amersham Biosciences, USA). As a negative control, about 1 μg denatured purified recombinant protein (treated at 95℃ for 5 min) was also assayed as described above.

RESULTS AND DISCUSSION

Expression of recombinant protein in *Saccharomyces cerevisiae*

The yeast expression vector p426GAL1 is the shuttle plasmid and can self-replicate in both *S. cerevisiae* and *E. coli.* cells, which can make our work more easier. So the plasmid of p426GAL1 was used in our study. After a series of restriction endonucleases digest, ligation and transformation work as described in materials and methods the fragment of ZmSPK1-dHA-His6 was eventually ligated into the p426GAL1. The recombinant plasmid was named as p426GAL1-ZmSPK1-dHA-His6 (Figure 1).

Induction of yeast cells containing recombinant plasmids (Figure 1) with 2% galactose in SC-URA medium resulted in the expression of the recombinant protein. As shown in Figure 2, the recombinant protein was successfully purified by ProBond resin. According to the SDS-PAGE gel analysis the molecular mass of the purified protein was about 43 KD which was similar to the estimated molecular mass of ZmSPK1 suggesting it might be the expected recombinant protein. In this study we could not find the obvious band of recombinant protein in the SDS-PAGE analysis of the crude extracts from yeast cells induced by galactose. The possible reason might be that the heterologous protein could cause effects that were deleterious to the host cells. In addition, the protease in yeast cells might degrade some of the heterologous proteins. However, we could get the purified recombinant protein via ProBond resin, which was enough for downstream studies.

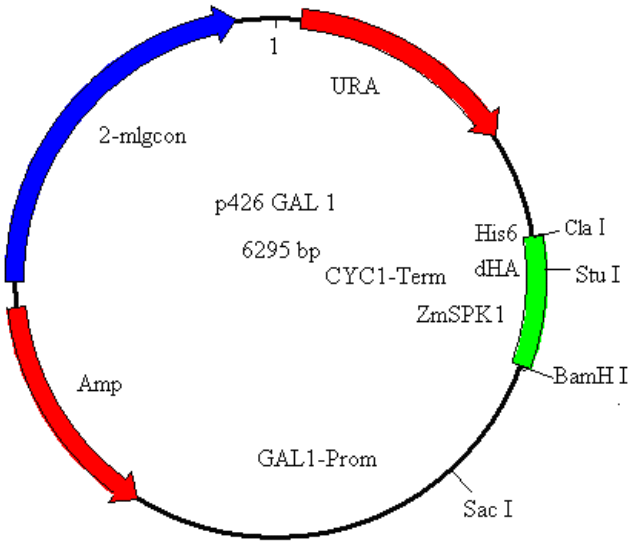

Figure 1. Construct (p426GAL1-ZmSPK1-dHA-His6) used for expression of ZmSPK1 in *Saccharomyces cerevisiae*.

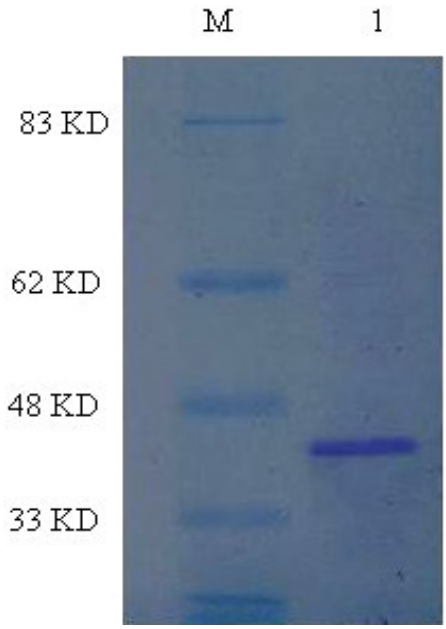

Figure 2. Expression of the recombinant ZmSPK1 protein in *Saccharomyces cerevisiae*. M; prestained protein marker (New England Biolabs, USA). 1; Purified ZmSPK1 protein.

Western-blot analysis of recombinant protein

For further conformation, western-blot was performed with the sample of the fusion protein. To exclude the non-specific reaction, the protein extracts from yeast cells expressing empty vector was set as a negative control. As shown in Figure 3 the sample from the yeast transformed

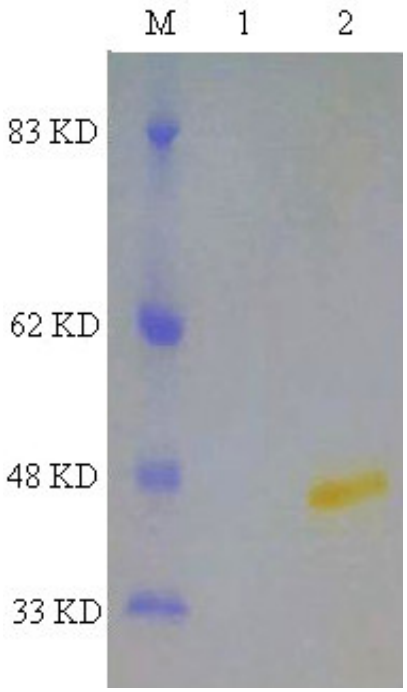

Figure 3. Western-blot analysis of purified recombinant ZmSPK1 protein. M; Prestained protein marker. 1; Protein extracts from yeast cells expressing empty vector. 2; Purified recombinant ZmSPK1 protein.

with an empty vector gave no specific HA-reactive bands in the immunoblot analysis. While the sample from the yeast transformed with recombinant plasmid gave only one specific HA-reactive band. Furthermore, the molecular mass shown in Figure 3 was about the same as that shown in Figure 2. This showed that the fusion ZmSPK1 protein was successfully expressed in yeast cells.

Kinase activity analysis of ZmSPK1 protein

Phosphorylation activity is the basic function of a protein kinase and is essential for performing its biological function. In previous study, via bioinformatic analysis we had found that the ZmSPK1 protein has a protein kinase catalytic domain and a protein kinase ATP-binding region signature. A Ser/Thr protein kinase active-site signature was also found in ZmSPK1 protein sequence (Zou et al., 2006). All of these strongly suggested that ZmSPK1 might be a functional protein kinase. In this study an *in vitro* phosphorylation activity of the fusion ZmSPK1 protein was checked. Figure 4 showed that the lane with samples of native ZmSPK1 protein and native MBP showed two strong radioactive bands corresponding to the fusion ZmSPK1 protein and MBP, respectively, while the lane with samples of denatured ZmSPK1 protein and native MBP showed no signals. These results revealed that Zm-

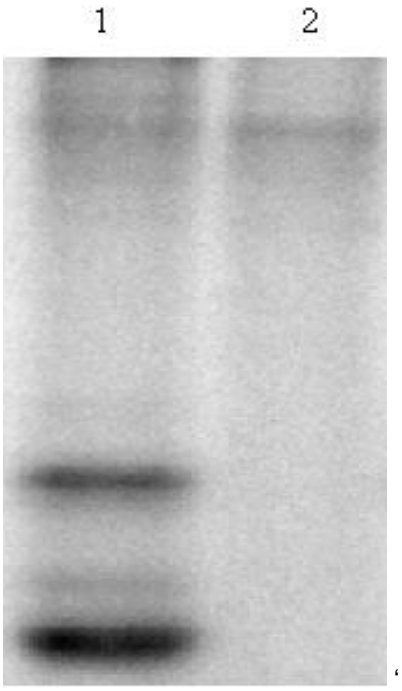

Figure 4. Phosphorylation activity assay of recombinant ZmSPK1 protein. 1; Samples of native ZmSPK1 and native MBP. 2; Samples of denatured ZmSPK1 and native MBP.

SPK1 not only had the autophosphorylation activity but also could phosphorylate other substrate protein. Based on the results, we can make a conclusion that ZmSPK1 may encode a functional protein kinase. This result could not only provide us an opportunity to understand its biochemical properties but also give us more hints for further studies. During the preparation of this manuscript, the work of transformation ZmSPK1 gene into *Arabidopsis* is being done.

ACKNOWLEDGMENTS

This work was financially supported by grants from National Natural Science Foundation of China (No. 30700 433) and National Key Technology R&D Program (2006 BAD02A13).

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