Isolation and functional characterization of *W55a* encoding a putative SnRK2 protein in wheat

Running title: A wheat SnRK2 kinase mediates multiple stress responses

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Abstract

Protein kinases play crucial roles in response to external environment stress signals. A putative protein kinase, W55a, belonging to SNF1-related protein kinase 2 (SnRK2) subfamily, was isolated from a cDNA library of drought-treated wheat seedlings. The entire length of W55a was obtained using rapid amplification of 5’ cDNA ends (5’-RACE) and reverse transcription-PCR (RT-PCR). It contains a 1,029 bp open reading frame (ORF) encoding 342 amino acids. The deduced amino acid sequence of W55a had eleven conserved catalytic subdomains and one Ser/Thr protein kinase active-site that characterize Ser/Thr protein kinases. Phylogenetic analysis showed that W55a was 90.38% homologous with rice SAPK1, a member of the SnRK2 family. Using nullisomic-tetrasomic and ditelocentric lines of Chinese Spring, W55a was located on chromosome 2BS. Expression pattern analysis revealed that W55a was upregulated by drought and salt, exogenous abscisic acid, salicylic acid, ethylene and methyl jasmonate, but was not responsive to cold stress. In addition, W55a transcripts were abundant in leaves, but not in roots or stems, under environmental stresses. Transgenic Arabidopsis plants overexpressing W55a exhibited higher tolerance to drought. Based on these findings, W55a encodes a novel dehydration-responsive protein kinase that is involved in multiple stress signal transduction.

Key words: chromosomal location, induction kinetics, SnRK2, stress response, transgenic Arabidopsis, wheat

Plants are continually exposed to unfavorable abiotic and biotic stresses from the environment, such as drought, soil salinity, low-temperature and diseases. In order to protect themselves, plants have evolved an ability to cope with stress. When they
perceive stress stimuli, defense mechanisms are immediately activated to trigger series
of physiological and biochemical responses to avoid or alleviate damage to cells
(Xiong et al. 2002). Protein kinases play vital roles in perceiving and transmitting
external stimuli.

Based on substrate specificity, all identified protein kinases were divided into three
major classes: Ser/Thr protein kinases, tyrosine protein kinases, and dual specificity
for Ser/Thr/Tyr protein kinases (Hanks et al. 1988; Lindberg et al. 1992). Protein
kinases related to adverse signal transmission are mainly calcium-dependent protein
kinases (CDPKs), mitogen-activated protein kinases (MAPKs), receptor-like kinases
(RLKs), ribosomal-protein kinases, or transcription-regulation proteins (Knetsch et al.

SNF1-related protein kinases (SnRKs) belonging to the Ser/Thr protein kinase class
were recently shown to be involved in stress responses. Based on sequence similarity,
cellular function, and domain structure, the SnRK family was divided into three
subfamilies, SnRK1, SnRK2 and SnRK3 (Halford and Hardie 1998; Hardie 1999;
Hrabak et al. 2003). Over the past several years, SnRK1 and SnRK3 were given more
attention (Chikano et al. 2001; Thelander et al. 2007; Ikeda et al. 2000); however, the
plant-specific SnRK2 subgroup is poorly reported. It was suggested that functioned in
sensing environmental changes (Holappa and Walker-Simmons 1995; Yoon et al. 1997;
Shen et al. 2001). At least three signaling pathways and different phosphorylation
mechanisms were involved in the activation of SnRK2 proteins under environmental
stresses (Boudsocq et al. 2007). Such results suggested that SnRK2 proteins initiate
multiple and complex signal transductions in response to environmental stresses, and
that the physiological roles of each SnRK2 protein might be different. However, most
studies on SnRK2 focus on their importance in water-deficient and hyperosmotic stress
signaling (Li et al. 2000; Mikolajczyk et al. 2000; Boudsocq et al. 2004; Kelner et al.
2004; Kobayashi et al. 2004; Umezawa et al. 2004; Chae et al. 2007), but the
mechanisms of exogenous hormone induction kinetics and signaling transduction are
still fragmentary.

Wheat (Triticum aestivum L.) is one of the world’s largest crops. However, little is
known about the functions of SnRK protein kinases in wheat. We isolated a cDNA fragment that encoded a drought-induced putative protein kinase belonging to the SnRK2 subfamily by phage hybridization in situ and designated the gene as W55a. Reverse transcription-PCR (RT-PCR) analysis showed that W55a was involved in response to abiotic stresses and exogenous hormones. Furthermore, overexpression of W55a improved the drought tolerance in Arabidopsis plants. The aim of the present study was to provide further information to determine the role of SnRK2 in the perception and transduction of stress signals.

Results

Isolation and sequence analysis of gene W55a

A cDNA fragment W55a (GenBank accession no. DQ343300) was isolated from a drought-induced cDNA library of wheat landrace Xiaobaimai by in situ phage hybridization. A W55a fragment, lacking the 5′ end, was 1,496 bp in length, but contained a putative Ser/Thr domain. Considering the important roles of Ser/Thr protein kinase (STK) motifs in signal transduction in cells, the 5′ end of the cDNA fragment was obtained using 5′ cDNA ends (5′-RACE) and the full-length cDNA was finally obtained using RT-PCR (Figure 1). Another clone, W55c (DQ343302), that was highly homologous with W55a, was isolated from the cDNA library (Figure 2). W55a was chosen for further analysis because both clones were almost identical (98.54%).

W55a, encodes a 342 amino acid protein and is 1635 bp in length, with a predicted molecular weight of 38.84 KD and a theoretical pI of 5.93 (Figure 1). Protein W55a has 11 subdomains within the highly conserved catalytic domain involved in kinase activity, suggesting that W55a encodes a putative protein kinase (Ali et al. 1994; Hanks and Hunter 1995; Rudrabhatla and Rajasekharan 2002; Jang et al. 2004). Gene W55a has a conserved nucleotide binding site, GSGNFG (Grant et al. 1998), at 10 residues downstream from the start Met, and a stretch of acidic amino acid residues at the C-terminal domain. Additionally, one potential N-myristoylated site, one Ser/Thr
protein kinase active-site, and one transmembrane spanning region are present in the catalytic domain (Figure 1). A conserved Thr residue between the DFG and APE conserved motifs was identified. Phosphorylation of this motif could activate many other protein kinases in these domains (Johnson et al. 1996). The protein most highly homologous with W55a in the GenBank library was SAPK1 (90.38%), a member of SnRK2 subfamily from rice (Figure 2 and Figure 3). Therefore, W55a gene likely encodes a novel SnRK2 protein in wheat.

**Chromosomal location of W55a gene**

Nullisomic-tetrasomic and ditelocentric lines of Chinese Spring were used to determine the location of W55a. As shown in Figure 4A, W55a was assigned to chromosome 2B because the PCR fragment was absent in the nullisomic-tetrasomic line lacking that chromosome. Gene W55c was located on 2A using the same method (data was not shown). Gene W55a was further located to the short arm of chromosome 2B (2BS), because the PCR fragment was absent in the ditelocentric line 2BL which lacks 2BS (Figure 4B).

**Transcript accumulation of W55a in various wheat organs**

Total RNAs, extracted from roots, stems and leaves of seedlings, were treated with water, salt, drought and exogenous abscisic acid (ABA) to analyze the target gene expression in various organs. W55a displayed specific tissue expression, and accumulated mainly in leaves, but not in roots and stems under salt, drought and ABA treatments (Figure 5).

**Expression patterns of the W55a gene**

To investigate the expression patterns of the W55a gene under stress conditions, RT-PCR was performed. Figure 6 shows the time profiles of W55a mRNA accumulation in wheat leaves exposed to different stresses. Following drought and salt treatments, mRNA levels were upregulated in a time-dependent manner, with peak levels being recorded after 1 and 2 h, respectively. As expected, transcription of W55a
was highly upregulated by ABA and reached the highest level at 6 h. However, W55a was not responsive to cold (LT) stress.

Exogenous stimuli related to defense signaling were developed to determine whether W55a was responsive to defense responses. After application exogenous salicylic acid (SA), the W55a transcript reached a maximum level at 2 h. Under ethylene (ET) and methyl jasmonate (MeJA) treatments, W55a was highly upregulated and reached the highest level both at 6 h; however, the amounts of transcript in both cases were not as high as with SA treatment (Figure 6). These results indicate that W55a is possibly regulated by drought and salt stresses in an ABA-dependent manner, and is also responsive to exogenous SA, ET, and MeJA.

Overexpression of W55a enhanced drought tolerance in Arabidopsis

To further investigate its function, the W55a gene under control of the CaMV35S promoter was transformed into Arabidopsis plants. Expression in three transgenic lines was markedly stronger than that of the wild type under normal conditions (Figure 7A). Three-weeks-old plants of these transgenics were used to assess the tolerance to drought stress. Under water-free conditions, all wild-type plants were dead after 3 weeks, whereas 30–45% of plants in the three transgenic lines survived, continued to grow after rewatering, and finally produced seeds (Figure 7B).

Root growth assays also directly reflected the seedling response to various stresses. Five-days-old seedlings of the transgenics and wild type plants transferred to MS media containing 2% PEG (mock drought) were grown for 8 days in vertical orientation before recording root length. As shown in Figure 7C, there were statistically significant differences in root growth between the transgenic and wild-type plants. The root lengths of the transgenic lines were more than twofold those of the wild-type when grown on the PEG supplemented medium. The results clearly indicated that W55a improved drought tolerance in transgenic plants.

Discussion
Protein kinases play vital roles in signal perception and transduction under abiotic and biotic stress conditions. There are more than 600 genes that encode protein kinases in the *Arabidopsis* genome (Morris and Walker 2003). We isolated a novel gene, *W55a*, from wheat. In *W55a*, 11 typical catalytic domains of protein kinase and a Ser/Thr protein kinase active-site were present. Phylogenetic analysis further showed that *W55a* was a member of the SnRK2 subfamily of protein kinases.

Myristoylation, an irreversible and post-translational protein modification, is crucial in regulating conformational stability and interacting with membranes or the hydrophobic domains of other proteins (Zheng et al. 1993; Resh 1999; Olsen and Kaarsholm 2000). In eukaryotic cells, N-myristoylation occurs mainly on cytoplasmic or nucleoplasmic proteins. The eukaryotic transmembrane protein, B96Bom, can be N-myristoylated and the N-myristoylated protein can be translocated across the membrane (Utsumi et al. 2005). In addition, N-myristoylation plays a vital role in signal transduction in plant responses to environmental stresses (Podell and Grinskov 2004). Ishitani et al. (2000) reported that SOS3 function in plant salt tolerance require for both N-myristoylation and calcium binding. The catalytic domain of *W55a* contains one potential N-myristoylation site and one potential transmembrane spanning region. This suggests that *W55a* may be involved in N-myristoylation response and located on the cell-membrane.

The transcripts of *W55a* accumulated in the seedling leaves under drought and salt stress treatments, suggesting that *W55a* is involved in response to abiotic stresses. There are at least two independent signal transduction pathways in plants under abiotic stresses, ABA-independent and ABA-dependent signal transduction cascades (Shinozaki and Yamaguchi-Shinozaki 1996; Bray 1997). Expression pattern analysis showed that *W55a* was involved in an ABA-dependent signal transduction pathway. ABA functions in water balance and cell resistance by regulating guard cell stomatal opening and closure and inducing relate genes expression during drought and high salinity stresses. AAPK, a faba bean member of the SnRK2 family was shown to be induced by ABA and to have enhanced tolerance to drought due to ABA regulation of stomates (Li and Assmann 1996; Li et al. 2002). Similarly, *Arabidopsis* OST1,
activated by ABA, regulated stomatal apertures under water deficit conditions (Mustilli et al. 2002). Overexpression of \textit{W55a} improved the survival rate of transgenic \textit{Arabidopsis} plants, and obviously permitted greater root length development under mock drought conditions. Therefore, \textit{W55a} may be involved in enhancement of drought tolerance by regulating root development and growth.

Plant defense in response to pathogen attack is regulated through a complex network of signaling pathways that include three signaling molecules: SA, JA and ET. These are divided into two major pathogen defense signaling pathways that are either SA-dependent, or an SA-independent pathway that involves JA and ET. These pathways do not function independently, but interact with each other through an intricate network (Kunkel and Brooks 2002). Salicylic acid plays a key role in plant defense against pathogens, signaling the induction of \textit{pathogenesis-related (PR)} protein genes. Exogenous application of SA can enhance resistance to pathogen attack (Ryals 1996; Durner et al. 1997; Iwai et al. 2007). Ethylene accumulation may rapidly increase when seeding plants suffer wounding or attack from pathogens (Broekaert et al. 2006). Subsequently, certain defense responses are induced. Treatments with exogenous JA or MeJA can induce the expression of many wound-responsive and defense-responsive genes including basic PR and protein kinases (Penninckx et al. 1998). Several reports indicate a positive interaction between the ET and JA/MeJA signaling pathways (Schenk et al. 2000), but an antagonistic relationship between SA and JA/MeJA at the biosynthetic and signaling levels (Dong 1998; Reymond and Farmer 1998; Spoel et al. 2003). In fact, SA and JA do not always act antagonistically; they can cooperate in the regulation of different stress responses (Mur et al. 2006). For example, combinations of ET and MeJA synergistically activated defense genes, such as \textit{PRI} and \textit{PR5} (Xu et al. 1994). In addition, both positive and negative regulatory interactions exist between SA and ET signaling pathways. Salicylic acid and ethylene may work together to coordinately induce several defense-related genes in \textit{Arabidopsis} (Schenk et al. 2000). Gene \textit{W55a} was markedly accumulated when wheat plants were treated by exogenous hormones, including SA, ET and MeJA. Therefore, \textit{W55a} may be involved in defense responses, and function as an integrator of multiple defense
Recent studies revealed that several molecules, including kinases and transcription factors, are involved in crosstalk among stress signaling pathways (Mizoguchi et al., 1996; Xiong and Yang 2003; Jung et al. 2007). In the present work, we isolated and characterized wheat protein kinase gene W55a that is responsive to multiple stress signals. To our knowledge, this is the first report on the induction kinetics of a wheat SnRK2 protein kinase after abiotic stress and exogenous hormone treatments. The results suggest that W55a plays an important role as a crosstalk node among multiple stresses. However, further investigations of physiological and biochemical functions of stress resistance are needed to reveal its function.

Materials and Methods

Plant materials and stress treatments

Seedlings of Chinese wheat landrace Xiaobaimai grown hydroponically at 20–25°C for 10 days were subjected to various abiotic stresses and exogenous hormone treatments. Leaves, stems, and roots were separately collected, immediately immersed in liquid nitrogen, and stored at -80°C for RNA extraction.

All environmental and hormonal treatments were carried out as follows. Materials were collected at 0, 0.5, 1, 2, 6, 12, 24 or 48 h after treatment. For drought treatment, intact plants were carefully removed from the soil, gently washed with distilled water, and placed in filter papers for 0, 5 min, 0.5, 1, 2, 6, 12 or 24 h, respectively. For salinity treatment, growing plants were irrigated once with 100 mmol/L NaCl. For low-temperature treatment, plants were placed in a 4°C environment. For ABA, SA, MeJA treatments, 100 µmol/L ABA, 50 µmol/L SA or 50 µmol/L MeJA were sprayed onto the leaves. For ET treatment, plants were placed in a sealed plexiglass chamber into which 200 µl/L gaseous ethylene was injected.

Construction and screening of the cDNA library

A cDNA library was constructed following guidelines of the manufactures (ZAP
Express® Predigested Gigapack® III Gold Cloning Kit, Stratagene, La Jolla, CA, USA). RNA was isolated from the seedlings of Xiaobaimai wheat that had been dehydrated for 2 h using the Quickprep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden). The cDNA library was screened following the plaque hybridization method as described by Xu et al. (2007).

**Isolation of W55a full-length cDNA**

Rapid amplifications of 5′-RACE (TaKaRa, Kyoto, Japan) and RT-PCR (Invitrogen, Carlsbad, CA, USA) were performed to obtain the 5′ end and the full-length cDNA of the gene W55a. Primers for 5′-RACE were designed according to the manufacturer’s instructions (TaKaRa).

**Total RNA extraction and cDNA synthesis**

Total RNA was isolated from wheat tissues after various hormonal and environmental treatments using the Trizol method (TianGen, Beijing, China) according to the manufacturer’s instruction. cDNA was synthesized by reverse transcriptase XL (AMV) (TaKaRa).

**Chromosomal location of gene W55a**

Nullisomic-tetrasomic and ditelocentric lines of Chinese Spring wheat were used to determine the chromosome location of W55a. The specific pair of primers for detecting W55a was 5′-TCATATCGGTCTTCTCCTCAGCTG-3′ and 5′-GAACCAC TCATGAGCGTCAAC-3′. The thermal cycle conditions were 4 min of initial denaturation followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s, extension at 72°C for 40 s, and a final extension at 72°C for 10 min. The PCR products (20 µL) were separated on 1.5% agarose gels and visualized with the Gel Doc EQ System (Bio-Rad, Hercules, CA, USA).

**Semi-quantitative RT-PCR**

One-step RT-PCR was carried out according to the instructions of the manufacture...
Total RNA as a template was amplified in a volume of 20 µl. The actin gene, used as an internal control for RT-PCR was run under the same conditions to normalize the amount of added template. The primers pair for amplifying W55a was 5'-GGTCTTGTGGAGTAACGCTCTATGTG-3' and 5'-GAACCACTCATGAGCGTCACAAC-3'. The thermal cycle conditions both actin and the W55a gene involved 4 min of initial denaturation followed by 27 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 40 s, and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR product (20 µL) was separated on a 1.5% agarose gel and visualized with the Gel Doc EQ System (Bio-Rad). Amplification was repeated three times.

**Drought treatment of transgenic Arabidopsis plants**

*Arabidopsis* (Col-0) plants used for transformation were grown in a growth chamber at 22°C under a 16 h light and 8 h dark photoperiod. Three T3 independent transgenic lines were used for analysis of drought tolerance. For drought stress treatment, sixty 3-weeks-old plants in each line were grown at 22°C without watering for 3 weeks. Survival rates were recorded after rewatering for two weeks.

For determining the root under mock drought conditions, five-days-old T3 transgenic *Arabidopsis* plants were transferred to MS media supplemented with 2% PEG. The plates were arranged in vertical orientation with roots pointing downwards. Root lengths were measured after 8 days growth. The experiment was repeated three times.

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Reference


**Johnson LN, Noble ME, Owen DJ** (1996). Active and inactive protein kinases,


Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD


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**Figure Legends**

**Figure 1.** The nucleotide and deduced amino acid sequences of *W55a*.

Roman numerals designate locations of Ser/Thr kinase catalytic subdomains.
Nucleotides from 1 to 1635 are shown on the left and of amino acids 1 to 342 are shown on the right. The Gly-rich (GSGNFG) nucleotide binding site is underlined, and a potential N-myristoylation domain is in bold and italic script. A unique stretch of amino acids is boxed in frame at the C-terminus. The Ser/Thr protein kinase active-site signature is indicated in bold. The potential transmembrane spanning region is underlined in italic script.

Figure 2. Sequence alignment of W55a with other SnRK2 kinases.
Amino acid sequence alignment of W55a (accession No. DQ343300) and W55c (DQ343302) with other highly homologous SnRK2 kinases, including rice SAPK1 (AB1253032), maize ZmSPK1 (AY722708), and tobacco NtOSAK (AY081175). DFG and APE conserved motifs are underlined, and the conserved Thr is shown with a dot.

Figure 3. Phylogenetic analysis of wheat W55a and W55c with other reported plant SnRKs.
The rooted phylogenetic tree is based on the amino acid sequence alignment of the following SnRK genes: rice SAPK1 (AB1253032) and OSK3 (D82038), grape GDBrPK (AF178575), maize ZmSPK1 (AY722708), faba bean AAPK (AF186020), wheat WPK4 (ABO11670), Arabidopsis AtSRI (ABO35147), potato PKIN1 (X95996), rye RKIN1 (M74113), maize SnRK1 (AY486125), and moss PpSNF1a (AY347743).

Figure 4. Chromosomal localization of W55a gene.
(A). W55a gene was located on chromosome 2B using a nullisomic-tetrasomic series of Chinese Spring. 1A: NT 1A/1B, nullisomic 1A tetrasomic 1B; 1B: NT 1B/1D; 1D: NT 1D/1A; 2A: NT 2A/2B; 2B: NT 2B/2D; 2D: NT 2D/ 2A; 3A: NT 3A/3B; 3B: NT 3B/3A; 3D: NT 3D3B; 4A: NT 4A/4B; 4B: NT 4B/4A; 4D: NT 4D/4B; 5A: NT 5A/5B; 5B: NT 5B/5D; 5D: NT 5D/5B; 6A: NT 6A/6B; 6B: NT 6B/6A; 6D: NT 6D/6A; 7A: NT 7A/7B; 7B: NT 7B/7A; 7D: NT 7D/7B. The band at the arrow is absent in the aneuploid line lacking chromosome 2B. (B). The W55a gene was further locatized to chromosome 2BS using the 2BL ditelocentric line. M: DL2000 DNA ladder.
Figure 5. Tissue-specific expression of W55α under various stresses using RT-PCR. RNA was extracted from roots, stems and leaves treated by drought, NaCl (100 mmol/L), ABA (100 µmol/L) and water. Control reactions using actin primers were carried out to normalize the amounts of added template. Experiments were repeated three times.

Figure 6. Expression patterns of W55α in leaves in response to various treatments. Drought, salt, low temperature (LT), SA, ET, MeJA and ABA were applied to 8- to 10-day-old wheat seedlings. The actin gene was used as a control to normalize the amount of template (bottom panel). The experiment was repeated three times.

Figure 7. Effects of drought stress on transgenic Arabidopsis plants. (A): Expression of W55α of three transgenic lines and wild type (WT) Arabidopsis plants under normal conditions. (B): Survival rates of WT and CaMV35S::W55α transgenics (lines TL-1, TL-2, and TL-3) were estimated after drought stress treatment. Stress treatments were applied to 3-weeks-old transgenics and WT plants under similar conditions. Results are averages of three replicates ± SD. (C): Root growth of transgenic and wild type plants under PEG stress. Five-day-old seedlings were transferred and grown vertically on MS media containing 2% PEG for 8 days at 22°C. The results are averages of three replicates ± S.D.
Figure 4
Figure 5
Figure 6
Figure 7