Molecular Cloning and Expression of Sequence Variants of Manganese Superoxide Dismutase Genes from Wheat

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INTRODUCTION

Reactive oxygen species (ROS) are very harmful to living organisms due to the potential oxidation of membrane lipids, DNA, proteins, and carbohydrates. transformed E. coli strain QC 871, superoxide dismutase (SOD) double-mutant, with three sequence variant MnSOD1, MnSOD2, and MnSOD3 manganese superoxide dismutase (MnSOD) gene isolated from wheat. Although all QC 871 transformants grown at 37°C expressed mRNA of MnSOD variants, only MnSOD2 transformant had functional SOD activity. MnSOD3 expressed active protein when grown at 22°C, however, MnSOD1 did not express functional protein at any growing and induction conditions. The sequence comparison of the wheat MnSOD variants revealed that the only amino acid difference between the sequence MnSOD2 and sequences MnSOD1 and 3 is phenylalanine/serine at position 58 amino acid. We made MnSOD2S58F gene, which was made by altering the phenylalanine to serine at position 58 in MnSOD2. The expressed MnSOD2S58F protein had functional SOD activity, even at higher levels than the original MnSOD2 at all observed temperatures. These data suggest that amino acid variation can result in highly active forms of MnSOD and the MnSOD2S58F gene can be an ideal target used for transforming crops to increase tolerance to environmental stresses.

Key Words: Environmental stress, Escherichia coli, Expression, Manganese superoxide dismutase, Multigene family

and Gutteridge, 2007). ROS can attack all basic molecules of living organisms, such as lipids, carbohydrates, proteins, and nucleic acids, resulting in the peroxidation of membranes (Kendall and McKersie, 1989), breakage of polysaccharides (Sato et al., 1993), inactivation of enzymes (Fucci et al., 1983), and "nicking", cross-linkage and scission of DNA strands (Houot et al., 2001). Very efficient antioxidant enzyme systems have evolved in plants to scavenge ROS and resulting in protection from oxidation injury (Allen, 1995; Halliwell and Gutteridge, 2007). Up-regulated ROS scavenging systems usually manifest as increased activities of antioxidant enzymes from de novo synthesis (Allen, 1995). One of the most effective antioxidant enzymes is superoxide dismutase (SOD, EC. 1.15.1.1), which catalyzes the dismutation of two superoxide radicals (O₂⁻) resulting in the pro-
duction of H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2} (Halliwell and Gutteridge, 2007). Typically, H\textsubscript{2}O\textsubscript{2} is then further broken down by catalase. SOD has several isozymes, which can be classified by their cellular location and specific catalytic metals in plants (Halliwell and Gutteridge, 2007; Zelko et al., 2002). MnSOD has Mn (III) at the active site and is localized to mitochondria. FeSOD has Fe(III) at the active site and is found in chloroplasts. Copper-zinc superoxide dismutase has Cu (II) plus Zn (II) at the active site and is generally found in the cytosol and chloroplasts in plants.

Among these isozymes, MnSOD is the only form of SOD essential for the survival of aerobic life (Carlioz and Touati, 1986). Furthermore, increase of MnSOD activity can confer more tolerance to many environmental stresses. Tolerance of chilling stress in Zea mays increased significantly when a tobacco MnSOD gene was expressed in the chloroplasts (Breuseman et al., 1999). Both freezing tolerance and recovery from freezing stress were enhanced in Medicago sativa transformed with a tobacco MnSOD gene targeted to the chloroplasts or mitochondria (McKersie et al., 1993). MnSOD transcripts increased significantly in both spring and winter wheat seedlings exposed to 2°C, presumably leading to more MnSOD enzyme production in the mitochondria (Wu et al., 1999; Baek and Skinner, 2003). Transgenic Brassica plants overexpressing a wheat MnSOD gene were more tolerant of both oxidative stress and aluminum toxicity (Basu et al., 2001). Ectopic expression of MnSOD gene from Tamarix androssowii in poplar plants enhanced salt tolerance (Wang et al., 2010). MnSOD enzyme can be used for several beneficial applications, such as an agent treating oxidative damage (McCord et al., 2001), and controlling dermal inflammatory reactions associated with chemical irritation and acne (Wilder et al., 1990), prolonged in vivo cell cycle progression and up-regulation of mitochondrial thioredoxin (Kim et al., 2010).

The mass of the MnSOD holoenzyme composed of four subunits in plants is estimated to be around 91 kDa (Streller et al., 1994). Many plants have several copies of MnSOD genes (Miao and Gaynor, 1993; Zhu and Scandalios, 1993; Streller et al., 1994 Baek and Skinner, 2006). Members of the MnSOD multigene family have slightly different genomic and amino acid sequences, however, the end products, MnSOD enzymes, have the same function (Zhu and Scandalios, 1993). DNA sequence information on wheat MnSOD genes in GenBank (http://www.ncbi.nlm.nih.gov/), TIGR (www.tigr.org), and GrainGenes (http://wheat.pw.usda.gov/GG2/index.shtml) and as well as sequence information obtained in our laboratory indicated that MnSOD in wheat comprised a multigene family with many members differing in nucleotide and amino acid sequence, as well as the 3’untranslated regions. Because both high and low temperature stress can result in ROS formation, MnSOD protein products from some of the sequence variants may be more active than others at specific temperatures.

In this study, we investigated the enzymatic activities of amino acid variants of wheat MnSOD genes expressed in SOD - E. coli. E. coli strain QC 871 (sod\textsubscript{A}, sod\textsubscript{B} double mutant Carlioz and Touati, 1986) does not contain any MnSOD gene, therefore, there is no chance of making a complex with E.coli original MnSOD with expressed wheat MnSOD in the transformed QC 871 and detected SOD activity in transformed QC 871 solely comes from the newly expressed wheat MnSOD.

**Materials and Methods**

Molecular cloning of multiple MnSOD genes

Total RNA from the leaves of winter wheat 442 (Storlie et al., 1998) was extracted by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). About 200 mg of whole leaves collected from 2 week old wheat plants were ground in liquid nitrogen with a mortar and a pestle; 2 ml Trizol solution was added to the mortar, which froze to the mortar surface. After thawing, the total RNA was extracted according to the instructions provided with the Trizol reagent. The RNA was quantified with a spectrophotometer (Jasco V-530, Jasco Inc, MD, USA) and quality was assessed by running 2 mg on a 1.2% agarose gel under 1 x modified TAE buffer (100 mM Tris-acetate, 1 mM Na2EDTA). RNA was stored at ~80°C before being used for RT-PCR.

Complementary DNA was made by using the total RNA and oligo dT primers with the ThermoScript kit (Invitrogen, San Diego, USA) according to the manufacturer's instructions. PCR was carried out on a PCR system 9700 (Applied Biosystems, Foster City, CA, USA) using the cDNA template. MnSOD specific primers were used as forward: 5' -...
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CACACACAAACACACTATCCATG and reverse: 5′-TCATGCAAGCATTTCTCATCTGTCATG-3′. The PCR profile was a 3 min denaturation at 95°C, then 32 cycles of 30 s at 95°C, 40 s at 52°C, and 5 min at 72°C. The PCR solution was composed of 1 x reaction buffer and 2 unit of Taq polymerase from Promega (Madison, WI, USA), 2.2 mM MgCl2, 100 nM primers, and 1 μl of cDNA made from 250 ng total RNA in 25 μl reaction solution covered with 20 μl mineral oil. The PCR products were analyzed on 1.2% agarose gel under 1 μl DNA gel extraction kit (Millipore, Bedford, MA, USA), and cloned into Topo-XL vector (Invitrogen, Carlsbad, CA, USA). Identity of the cloned PCR products was confirmed by sequencing, which was performed at the sequencing facility at Washington State University, Pullman. The ClustalW (Larkins et al., 2007) was used for DNA and protein sequence alignments.

Expression of the cloned MnSOD in SOD double mutant E.coli

Subclones without the 27 amino acid leader sequence targeted to mitochondria (Schrank et al., 1988; Wu et al., 1999) were generated with PCR using the clones described above as templates and primers (forward: 5′-ACCATGTGGCGACGTTCACG and reverse: 5′-TCATGCAAGCACTTTCTCATACTCT). The PCR product was cloned into pBAD-TOPO vector (Invitrogen, Carlsbad, CA, USA) for expressing protein in E.coli. The N-terminal leader in pBAD-Topo vector was removed by NcoI restriction digestion (Promega, Madison, WI, USA), followed by self-ligation. These synthetic MnSOD clones in the pBAD-Topo vector were used for transforming E.coli strain QC 871 (r−m+ derivative of E.coli QC 774 sodA, sodB double mutant Carlioz and Touati, 1986), which grew very slowly compared to wild type E.coli. Plasmids from the transformed QC 871 were extracted and sequenced to confirm correct configuration.

 Cultures were grown in Luria-Bertani (LB) medium (Sambrook and Russell, 2001) for 12 hr at 37°C with 200 rpm shaking to OD660 = 1 (Invitrogen pBAD Technical Manual). Expression of genes in the pBAD-Topo vector was induced by adding L-arabinose (Lee, 1980; Lee et al., 1987), therefore, various concentrations of L-arabinose with MnCl2 (Sigma-Aldrich, St. Louis, MO) with different induction time were tested to induce the MnSOD protein. The cells were collected by centrifugation at 5000 g for 10 min, and resuspended in 0.5 ml of lysis buffer (50 mM Tris-HCl, 24 mM NaCl, 2 mM EDTA, pH 8). Cell lysis solutions were added 30 μl of lysozyme (10 mg/ml) and incubated at 37°C for 30 min with slow inversion. Two μl of DNase I (10 units/ml, Stratagene, La Jolla, CA, USA) and 2.5 μl of MgCl2 at 1 M were added to the cell lysis solution, and the solution was left at 37°C for 10 min with slow inversion. The preparations were clarified by centrifugation at 14,000 g for 15 min. The supernatants were filtered through Ultrafree-15 Biomax-50 K (Millipore, Bedford, MA, USA) and the protein amount was quantified using BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). In-gel SOD activity assessments were carried out on 10% non-denaturing acrylamide gels (Sambrook and Russell, 2001) for detection of using nitroblue tetrазolium (NBT) oxidation according to Beauchamp and Fridovich (1971).

Site-specific modification in the cloned MnSOD and the expression in SOD double mutant E.coli

PCR products of 210 bp were generated by PCR reactions using the MnSOD1 and MnSOD2 sequences as templates and primers, forward: 5′-ACCATGTGGCGACGTTCACG and reverse: 5′-TCATGCAAGCACTTTCTCATACTCT. The PCR product was cloned into pBAD-TOPO vector (Invitrogen, Carlsbad, CA, USA) as templates and primers, forward: 5′-ACCATGTGGCGACGTTCACG and reverse: 5′-TCATGCAAGCACTTTCTCATACTCT. The PCR product was cloned into pBAD-TOPO vector (Invitrogen, Carlsbad, CA, USA). The N-terminal leader in pBAD-Topo vector was removed by NcoI restriction digestion (Promega, Madison, WI, USA), followed by self-ligation. These synthetic MnSOD clones in the pBAD-Topo vector were used for transforming E.coli strain QC 871 (r−m+ derivative of E.coli QC 774 sodA, sodB double mutant Carlioz and Touati, 1986), which grew very slowly compared to wild type E.coli. Plasmids from the transformed QC 871 were extracted and sequenced to confirm correct configuration.

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Fig. 1. Amino acid sequences of wheat MnSOD variants. Three wheat MnSOD protein sequences at GenBank [AF092524 (MnSOD), U72212 (MnSOD3.1), and U73172 (MnSOD3.2)] and three MnSOD protein sequences (MnSOD1, 2, and 3) acquired in our laboratory were aligned. Arrows show the key active-site residues for SOD activity in wild-type MnSOD.
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Fig. 2. Photograph of products of RT-PCR (lane 1-6) and PCR (lane 10-12) on 1.2% agarose gel. One-step RT-PCR reaction (Superscript One-Step Kit, Invitrogen, San Diego, USA) was performed using total RNA templates extracted from clone MnSOD1 (lane 1), MnSOD2 (lane 2), MnSOD3 (lane 3), QC 871 carrying empty pBAD-Topo vector (lane 4), and original QC 871 strain (lane 5), or plasmid DNA (lane 6). PCR was also performed using total RNA as the template; clone MnSOD1 (lane 10), MnSOD2 (lane 11), and MnSOD3 (lane 12). One μg of 1 kb plus ladder (Invitrogen, Carlsbad, CA, USA) was loaded on lane 8.

Fig. 3. SOD activity staining of the 10% non-denaturing gel loaded with proteins extracted from clone MnSOD1, MnSOD2, and MnSOD3. Protein extracted from young wheat leaves (lane 1), QC 871 (lane 2), QC 871 Empty (lane 3), clone MnSOD1 (lane 4), clone MnSOD2 (lane 5), and clone MnSOD3 (lane 6) was loaded on 10% native acrylamide gel and stained for visualization of the SOD activity. The arrow on lane 5 shows the inhibition of NBT oxidation by the expressed MnSOD enzyme.

effect on the protein expression. Various L-arabinose concentrations were tested for the best working concentration for inducing highest MnSOD proteins. QC 871 carrying clone MnSOD3 expressed SOD activity only when the transformants was grown at 22°C. MnSOD activities were never detected in the clone MnSOD1 protein extracts at any grown temperatures and conditions.

Western blotting was performed to confirm no E.coli MnSOD was expressed in QC 871 after transformation with wheat MnSOD genes (not shown). All of the clones lacked E.coli MnSOD enzymatic activity (Fig. 3). SOD activity staining of the non-denaturing gels pretreated with 5 mM H₂O₂ revealed that the expressed SOD was insensitive to H₂O₂, characteristic of MnSOD protein (Rubio et al., 2001).

Amino acid swapping test of MnSOD

The sequences of wheat MnSOD1, 2, and 3 were 98~99% homologous, however, only clone MnSOD2 had SOD activity at 37°C expression conditions. The sequence comparison of the MnSOD1, 2, and 3 revealed that the only amino acid difference between these sequence MnSOD2 and sequences MnSOD1 and 3 was phenylalanine (F) at position 58 amino acid (sequence MnSOD2: F and sequences MnSOD1, 3: serine (S)) (Fig. 1). We swapped the F to S at position 58 amino acid in sequence MnSOD2 (Sequence MnSOD2S58F) (Fig. 4), and transformed QC 871 with sequence MnSOD2S58F to make clone MnSOD2S58F, which was expected not to have the SOD enzymatic activity. However, clone MnSOD2S58F still had SOD activity. Sequence N4 was made by changing F at position 58 amino acid (SF) and R at position 62 (H R) in sequence MnSOD1. The transformed QC 871 with the sequence N4 did not show any SOD enzymatic activity (data not shown).

Measurement of the enzymatic activity of the expressed MnSOD

The SOD activities of the protein extract from MnSOD2 or MnSOD2S58F at 37°C expression conditions composed of LB liquid medium, 0.004% final concentration of L-arabinose, and 1 μM MnCl₂ were measured by the oxidation hindrance of NBT at different temperatures (Fig. 5). Due to unsuccessful induction of other sequence variants, only SOD enzymatic activities of MnSOD2 or MnSOD2S58F were determined. The clone MnSOD2 had SOD activity ranging from 0.65 to 0.75 units and clone MnSOD2S58F had SOD activities ranging from 0.85 to 1.05 units when they were incubated from 0 to 30°C. The highest activities for SOD were observed when the protein extracts from clones MnSOD2 and MnSOD2S58F were incubated at 30°C (0.75 and 1.05 units, respectively). The biggest difference in the SOD enzymatic activities between protein extract from clone MnSOD2 and that from clone MnSOD2S58F was observed at 10°C reaction condition, in which condition
Fig. 4. Alignment of the amino acid sequences for the wheat MnSOD clones using ClustalW program. The MnSOD amino acid sequences were compared between MnSOD2 and MnSOD2S58F. The sequence of MnSOD2S58F was artificially made by changing one amino acids of MnSOD2. Arrows show the key active-site residues for SOD activity in wild-type MnSOD.

Fig. 5. Measurement of SOD activities from the protein extracts of clone MnSOD2 and clone MnSOD2S58F at different temperatures. 120 µg of protein extracts were incubated with SOD detection media for 10 min under 140 µmol quanta \( \cdot \) \( m^{-2} \cdot s^{-1} \) for 10 min. One unit of SOD activity was defined as the amount of SOD which catalyzes a 50% inhibition in the rate of reduction of NBT under the specific conditions. The protein extract of original QC 871 was used for the blank control for detecting the SOD activity.

protein extract from clone MnSOD2S58F had 48% more activity than that from MnSOD2 (Fig. 5).

Discussion

All QC 871 transformants carrying wheat MnSOD clone MnSOD1, 2, or 3 expressed the wheat mRNA, however, only MnSOD2 expressed SOD activity when expression was induced at 37°C. MnSOD3 expressed SOD activity only when induced at 22°C and MnSOD1 did not express activity under the conditions we tested. Heterologous expression of plant proteins in E.coli frequently results in the protein being expressed in inactive inclusion bodies (Fisher et al., 1993; Rudolph and Lilie, 1996). We do not know whether MnSOD1 and MnSOD3 formed inclusion bodies under the conditions we tested or whether position 58 amino acid, the only different amino acid between MnSOD2 and MnSOD1 and 3, prevented expression. When this amino acid in MnSOD2 sequence was swapped from F to S, the newly made variant (MnSOD2S58F) expressed SOD activity. In fact, the protein extract from clone MnSOD2S58F had greater enzymatic activities than clone MnSOD2 at all temperatures tested (Fig. 5). In human MnSOD, formation of a tetrameric interface by two identical four-helix bundles stabilizes the active sites (Borgstah et al., 1996). There is Ile58Thr human MnSOD, naturally occurring polymorphic MnSOD variant which causes two packing defects in each of the two four-helix bundles of the tetrameric interface. In solution, Ile58Thr MnSOD is primarily dimeric, and is significantly less thermostable than the normal enzyme. The sequence variations embedded in MnSOD1 and 3 might be a reason for the no sign of SOD enzymatic activity in solution. Furthermore, as shown in human
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Ile58Thr MnSOD, which had decreases of 15°C in the main melting temperature and 20°C in the heat-inactivation temperature, MnSOD2S58F might have more stability than MnSOD2 by the single amino acid change. The stability of our newly characterized MnSOD protein products emphasizes again that MnSOD is an ideal scavenging enzyme protecting cells from ROS in various temperature conditions.

These data showed that even a single amino acid modification in MnSOD significantly changed SOD enzymatic activities. Although these experiments were done with heterologous expression of wheat MnSOD in E.coli, the results suggest that single amino acid variants in wheat plants also may have significantly different levels of expression. Since it now is known that wheat MnSOD genes comprise a multigene family, it is possible that highly efficient forms of MnSOD may be identified and utilized through a molecular breeding approach. Furthermore, the active wheat MnSOD sequence can be used for transforming monocot crops to make them more tolerant to environmental stresses relevant to producing oxidative stresses. Many environmental stresses, e.g., heat, chilling, freezing, salt, metal, ultraviolet light, etc., produce high amounts of superoxide anion directly and indirectly. As shown in transgenic corn or alfalfa, MnSOD can be the most appropriate SOD for increasing tolerance for environmental stresses, therefore, selection for the highest enzymatic activity for MnSOD will be one of the most important step for plant transformation.

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References


