

Identification of Negative cis-Acting Elements in Response to Copper in the Chloroplastic Iron Superoxide Dismutase Gene of the Moss *Barbula unguiculata*¹

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Superoxide dismutases (SODs) are ubiquitous metalloenzymes that catalyze the dismutation of superoxide radicals. Chloroplasts have two isozymes, copper/zinc SOD (Cu/ZnSOD) and iron SOD (FeSOD), encoded by nuclear genes. Because bryophytes are considered as the earliest land plants, they are one of the most interesting plant models for adaptation against oxidative stress. In a previous study, we found that the *FeSOD* gene was expressed under Cu-deficient conditions and repressed under high-Cu-supply conditions; on the other hand, the *Cu/ZnSOD* gene was induced by Cu in a moss, *Barbula unguiculata*. The expression of Cu/ZnSOD and FeSOD is coordinately regulated at the transcriptional level depending on metal bioavailability. Here, using transgenic moss plants, we determined that the GTACT motif is a negative cis-acting element of the moss *FeSOD* gene in response to Cu. Furthermore, we found that a plant-specific transcription factor, PpSBP2 (for *SQUAMOSA* promoter-binding protein), and its related proteins bound to the GTACT motif repressed the expression of the *FeSOD* gene. The moss *FeSOD* gene was negatively regulated by Cu in transgenic *Nicotiana tabacum* plants, and the *Arabidopsis thaliana FeSOD* gene promoter containing the GTACT motif was repressed by Cu. Our results suggested that molecular mechanisms of GTACT motif-dependent transcriptional suppression by Cu are conserved in land plants.

Reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (H₂O₂), are constantly produced during metabolic processes in all living species. Excessive ROS accumulation leads to cellular injury, such as damage to DNA, protein, and the lipid membrane (Mittler, 2002). Because of their potential harmful effects, excessive ROS must be promptly eliminated from the cells by a variety of antioxidant defense mechanisms, including superoxide dismutase (SOD), catalase, and various peroxidases.

SODs are ubiquitous metalloenzymes that catalyze the dismutation of superoxide radicals to H₂O₂ and molecular oxygen (2O₂⁻ + 2H⁺ → H₂O₂ + O₂; Mittler et al., 2004). Plants possess three types of SODs, classified according to their prosthetic metal: iron SOD (FeSOD), manganese SOD (MnSOD), and copper/zinc SOD (Cu/ZnSOD). FeSOD and MnSOD are likely to have arisen from a common ancestor, whereas Cu/ZnSOD appears to have evolved separately, because it exhibits only limited sequence similarity to FeSOD and MnSOD (Steinman and Hill, 1973; Stallings et al., 1983; Alscher et al., 2002; Fink and Scandalios, 2002).

FeSOD is considered to be the most primitive enzyme, because it occurs in anaerobic bacteria. Many algae contain FeSOD but lack Cu/ZnSOD; however, evolved green algae, *Charales* and *Conjugales*, do have Cu/ZnSOD (Asada et al., 1977; Kanematsu and Asada, 1989). The SOD isoforms differ in their subcellular location; FeSODs are generally located in chloroplasts, MnSODs are located in mitochondria and peroxisomes, and Cu/ZnSODs are found in the cytosol, chloroplasts, peroxisomes, and apoplasts (Alscher et al., 2002).

When life was first forming on the earth, oxygen in the atmosphere was almost nonexistent. Fe was probably the first metal used as a metal cofactor at the active site of the ancient SOD because of an abundance of Fe in a soluble Fe (II) form at that time. As the levels of O₂ in the environments increased by oxygenic photosynthesis, the mineral components in the biosphere were oxidized. The decrease in soluble Fe (II) in the biosphere caused a shift to the use of a more available metal, Mn (III), in the evolution of detoxification. When the atmosphere was completely replenished with oxygen, Fe (II) was almost completely unavailable, and insoluble Cu (I) in the ancient Earth was converted into soluble Cu (II). At this stage, Cu (II) began to be used as the metal cofactor at the active sites of SODs (Egami, 1975; Lumsden and Hall, 1975; Asada et al., 1977; Alscher et al., 2002).

Because bryophytes are considered as the earliest land plants (Waters, 2003), they are one of the most interesting plant models for adaptation against oxidative stress. Bryophytes contain three classes: liverworts, hornworts, and mosses. The liverwort *Marchantia paleacea*

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var *diptera* has only FeSOD in chloroplasts (Tanaka et al., 1995), but the moss *Barbula unguiculata*, which has been placed closer to vascular plants than to liverworts in the phylogenetic tree (Kenrick and Crane, 1997; Wellman et al., 2003), has both FeSOD and Cu/ZnSOD in chloroplasts (Yamahara et al., 1999), as do most vascular plants. Depending on metal bioavailability, various organisms coordinately regulate the alternative use of Fe- versus Cu-containing SODs with completely different apoproteins.

We previously found that chloroplastic FeSOD was replaced with Cu/ZnSOD when Cu was available in the moss *B. unguiculata* (Shiono et al., 2003). The expression of Cu/ZnSOD was repressed in a Cu-deficient medium and induced by the addition of Cu. On the other hand, FeSOD activity was increased under Cu-deficient conditions and decreased in a Cu-containing medium. This coordinated regulation of SODs by Cu has also been observed in the fern *Matteuccia struthiopteris* (Murao et al., 2004) and higher plants such as tobacco (*Nicotiana tabacum*; Kurepa et al., 1997) and *Arabidopsis* (*Arabidopsis thaliana*; Abdel-Ghany et al., 2005b). However, the molecular mechanisms of transcriptional regulation by Cu are still largely unknown.

In this study, we identified the negative cis-acting element, a GTACT motif, responsible for Cu-responsive transcriptional repression of the moss *FeSOD* gene. The

DNA fragment containing GTACTs conferred the *actin* promoter with a remarkable property of Cu responsiveness. The cis-acting element of moss is also functional in higher plants. Furthermore, overexpression of a transcription factor that binds to the GTACT motif repressed the expression of the *FeSOD* gene in transgenic moss plants. These results suggested that the molecular mechanisms for transcriptional regulation by Cu are ancient and evolutionarily conserved.

RESULTS

FeSOD Gene Is Transcriptionally Regulated by Cu

To investigate the molecular mechanisms of transcriptional control of the *FeSOD* gene of *B. unguiculata* by Cu, we cloned the promoter region of the *FeSOD* gene (GenBank accession no. AB370198) by thermal asymmetric interlaced (TAIL)-PCR. The 5' RACE analysis showed that the transcription start site of *FeSOD* was 122 bp upstream of the translation initiation site (data not shown). To define the transcriptional regulatory region of the *FeSOD* promoter by Cu, we generated transgenic moss plants (*Physcomitrella patens* subsp. *patens*) carrying a series of 5' deletions of the *FeSOD* promoter fused to a *GUS* reporter gene (Fig. 1A). We examined the effect of Cu on the expression of

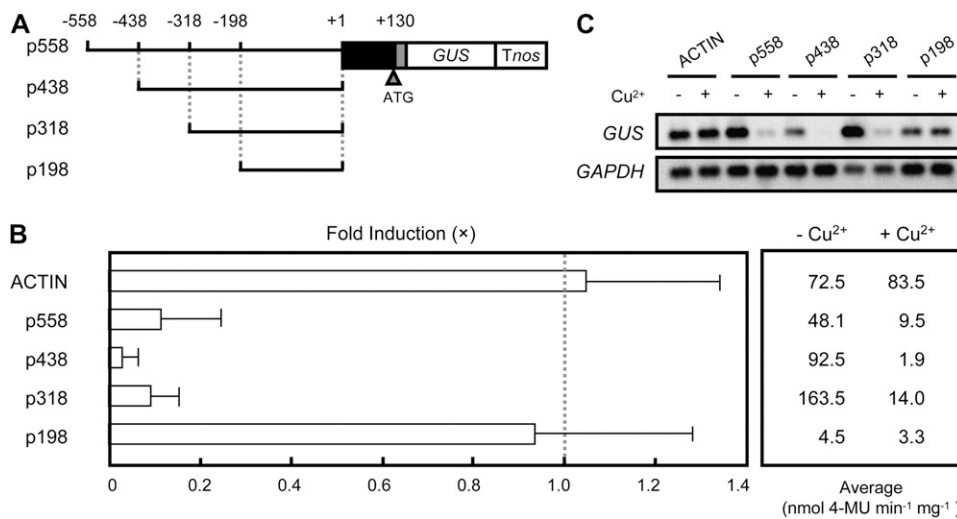


Figure 1. Identification of cis-regions for Cu responsiveness. A, Schematic diagram of constructs. A plasmid construct containing the 5' flanking region (solid line), 5' untranslated region (black box), and the coding region (gray box; three amino acids) of *FeSOD* connected to the *GUS* gene was introduced into *P. patens* subsp. *patens* by polyethylene glycol-mediated transformation. Nucleotide positions are indicated in base pairs from the transcription initiation site. *Tnos*, Nopaline synthase terminator. B, *GUS* activities of the transgenic moss plants treated with or without CuSO₄. Left, The repression of the *GUS* activities by Cu is indicated as the relative value. The bars represent the *GUS* activities of transgenic moss plants cultured in the presence of 2.0 μM CuSO₄. The *GUS* activities of the transgenic plants cultured in the absence of CuSO₄ were arbitrarily set to 1.0 (dotted line). For each construct, at least three independent transgenic plants were examined. The bars indicate ± sd. Right, The average *GUS* activities are expressed in nanomoles of 4-methylumbelliferone (4-MU) produced per milligram of extract protein per minute. The *GUS* activities of transgenic moss plants containing the promoterless-*GUS* construct were below 2 × 10⁻² nmol 4-MU min⁻¹ mg⁻¹ and were not affected by Cu (data not shown). C, RT-PCR analysis of *GUS* gene expression in transgenic moss plants cultured with (+) or without (-) CuSO₄. After amplification with specific primers, the products were detected by DNA gel-blot hybridization. *GAPDH* was used as an internal control of RT-PCR.

the *FeSOD* promoter-GUS fusion genes in transgenic moss plants. The application of Cu decreased the GUS activities in the p558 construct that contains from -558 to $+130$ nucleotides, showing that the fusion gene was under the control of Cu (Fig. 1B). Other $2.0 \mu\text{M}$ metals, including Zn^{2+} , Co^{2+} , Mn^{2+} , Ni^{2+} , and Fe^{2+} , had only a limited effect on the expression of p558 (data not shown), suggesting that down-regulation of *FeSOD* is specific to Cu. The control rice (*Oryza sativa*) *actin* promoter was not regulated by Cu treatment. Deletion of 240 bp from -558 to -319 (p318) of the *FeSOD* promoter did not affect the responsiveness to Cu. Further deletion of 120 bp from -318 to -199 (p198) resulted in a loss of repression by Cu. We confirmed these results at the mRNA levels by reverse transcription (RT)-PCR. Consistent with the GUS activities, the *GUS* mRNAs were repressed by Cu in p558, p438, and p318 but not in p198 (Fig. 1C). Therefore, there should be one or more cis-elements responsible for the repression by Cu between -318 and -199 in the *FeSOD* promoter.

Multiple cis-Elements Are Located between -318 and -199

To examine the function of the 120-bp sequence between -318 and -199 in Cu responsiveness, we carried out a gain-of-function experiment. The 120-bp DNA fragment of the *FeSOD* promoter was cloned upstream of the constitutive rice *actin* promoter (F12; Fig. 2A). The 120-bp DNA fragment endowed the reporter with a remarkable property of Cu responsiveness (Fig. 2B), suggesting that the sequences are sufficient for transcriptional repression by Cu. To further define the cis-elements for the Cu responsiveness of the *FeSOD* promoter, the function of two dissected DNA fragments of the 120-bp sequence was examined using an *actin* promoter. The Cu-dependent repression of GUS activities was not observed with the 60-bp DNA fragment between -318 and -259 (F1) or between -258 and -199 (F2), whereas Cu responsiveness was detected with the tandem repeats of the 60-bp DNA fragment between -318 and -259 ($2 \times$ F1) and that between -258 and -199 ($2 \times$ F2), respectively (Fig. 2, A and B). These results suggested that both F1 and F2 contain the Cu-responsive element and that multiple copies of the cis-element are necessary for Cu-responsive transcriptional repression.

GTACT Is Necessary for Cu-Responsive Repression

When we analyzed the DNA sequences of the *FeSOD* promoter, five copies of GTACT sequences were found between -318 and -199 (Fig. 3A). Two additional GTACT sequences are found between -198 and $+1$. Because the p198 construct that contains two copies of GTACT sequences did not respond to Cu (Fig. 1), we hypothesized that multiple copies of GTACT sequences are necessary for the transcriptional repression of the *FeSOD* promoter by Cu. If this is

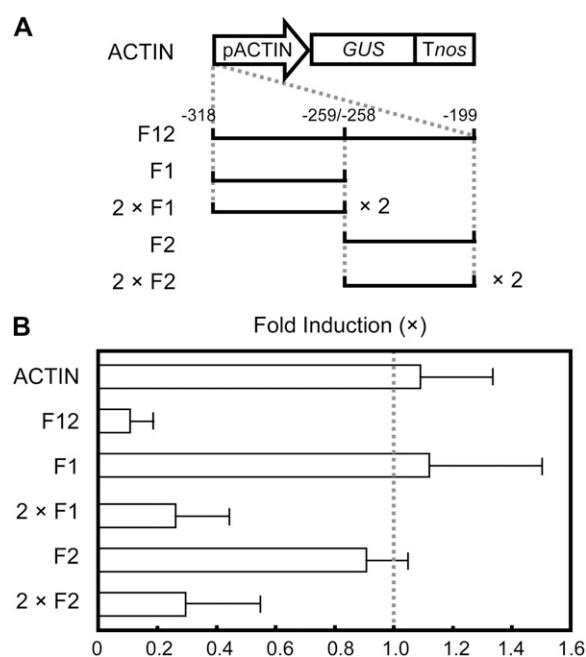


Figure 2. Gain-of-function analysis for Cu responsiveness in transgenic moss plants. A, Schematic diagram of constructs. The fragments of the *FeSOD* promoter were inserted into upstream of rice *actin* promoter (pACTIN). F12 contains 120-bp DNA (-318 to -199). F1 and F2 contain 60-bp DNAs from -318 to -259 and from -258 to -199 , respectively. $2 \times$ F1 and $2 \times$ F2 contain two tandem copies of 60-bp fragments (-318 to -259 or -258 to -199), respectively. B, GUS activities of the transgenic moss plants treated with or without CuSO_4 . The bars represent the GUS activities of transgenic moss plants cultured in the presence of $2.0 \mu\text{M}$ CuSO_4 . The GUS activities of the transgenic plants cultured in the absence of CuSO_4 were arbitrarily set to 1.0 (dotted line). The *actin* promoter-GUS (ACTIN) was used as a control. For each construct, at least three independent transgenic plants were examined. The bars indicate \pm SD.

correct, the expression of a p258 construct that contains four copies of GTACT (Fig. 3B) might be repressed by Cu. As we expected, the GUS activities decreased with an application of Cu in p258 transgenic moss plants (Fig. 3C).

To examine the functional importance of GTACT sequences on the Cu-responsive transcriptional repression of the *FeSOD* promoter, we constructed mutant versions of p258 in which mutations were introduced to four GTACT sequences (Fig. 3B). Although the mutations in B6 and B7 (mB67-p258) did not abolish the Cu responsiveness, those in B4 (mB4-p258) or B5 (mB5-p258) apparently reduced Cu-responsive transcriptional repression in the transgenic moss plants (Fig. 3C). The Cu responsiveness was completely eliminated by the mutations in B4 and B5 (mB45-p258) and by those in B4, B5, B6, and B7 (mB4567-p258). These results indicated that multiple copies of GTACT sequences are necessary for the maximum transcriptional repression of the *FeSOD* promoter in response to Cu and that B4 and B5 play important roles in the regulation.

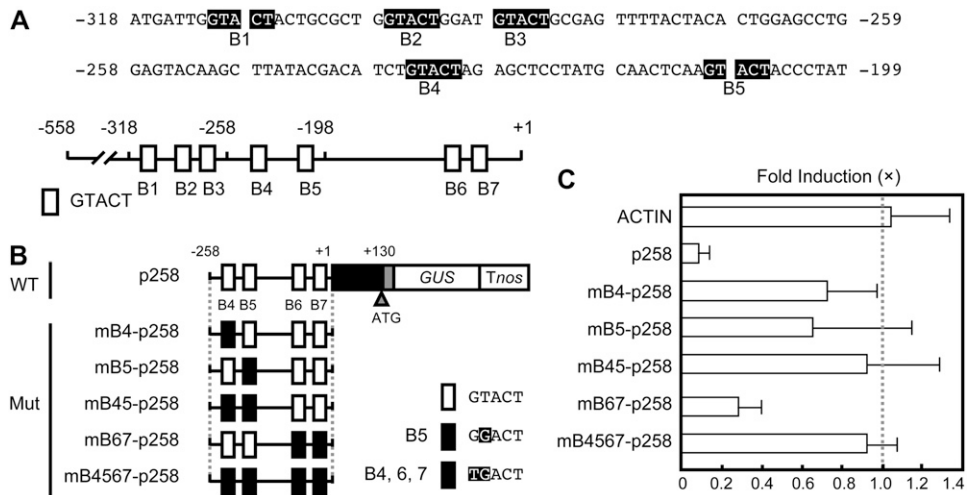


Figure 3. The GTACT motif is required for Cu responsiveness. A, Sequences of the region (–318 to –199) that are involved in Cu responsiveness. The GTACT sequences are printed in white on black. The bottom diagram represents the location of GTACT sequences. GTACTs are shown as boxes (B1–B7) and numbered from the 5' end of the *FeSOD* promoter. B, Mutagenesis of GTACT motifs. The mutated GTACT sequences are indicated by black boxes in the schematic diagram of constructs. The mutated bases in B4 to B7 are highlighted. Wild type (WT) containing –258 to +130 was used as a positive control. C, Effect of mutations in GTACT motifs on Cu responsiveness. The bars represent the GUS activities of transgenic moss plants cultured in the presence of 2.0 μM CuSO₄. The GUS activities of the transgenic plants cultured in the absence of CuSO₄ were arbitrarily set to 1.0 (dotted line). The *actin* promoter-GUS (ACTIN) was used as a control. For each construct, at least three independent transgenic plants were examined. The bars indicate ± sd.

Expression Pattern of *FeSOD*

To examine the spatial and temporal pattern of *FeSOD* expression, p558 transgenic plants were analyzed by histochemical staining of GUS activities. GUS activities were detected in protonema before the development of the gametophore (Fig. 4A) and in the rhizoids except for the gametophore (Fig. 4C) under Cu-deficient conditions. The application of Cu resulted in the loss of GUS activities in whole plants (Fig. 4, B and D), indicating that the molecular mechanisms of Cu-responsive transcriptional repression of *FeSOD* gene were operative in all tissues in which the *FeSOD* gene was expressed in *P. patens*.

Transcription Factor PpSBP2 Regulates the Expression of *FeSOD*

The unicellular green alga *Chlamydomonas reinhardtii* activates the transcription of the *Cyc6* and the *Cpx1* genes (encoding cytochrome *c*₆ and coprogen oxidase) in response to Cu deficiency to replace the Cu-dependent function of the plastocyanin function by a heme-containing cytochrome (Merchant and Bogorad, 1986; Hill et al., 1991; Hill and Merchant, 1995; Quinn et al., 1999). The GTAC sequences of the promoters are critical for Cu responsiveness in *C. reinhardtii* (Quinn et al., 2000). The COPPER RESPONSE REGULATOR1 (CRR1) is a putative transcription factor that is required for the activation of the *Cyc6* and *Cpx1* genes under Cu-deficient conditions of *C. reinhardtii*, with a *SQUAMOSA* promoter-binding protein (SBP) domain,

ankyrin repeats, and a C-terminal Cys-rich region (Kropat et al., 2005). Furthermore, the consensus DNA sequence bound by the SBP domain contains a GTAC core (Birkenbihl et al., 2005). These observations prompted us to examine whether a transcription factor with an SBP domain of moss is involved in the Cu-responsive transcriptional repression of the *FeSOD* gene.

When we started our work, the registered *P. patens* proteins with an SBP domain were PpSBP1 to 4. The amino acid sequence of these proteins exhibited high similarities in the SBP domain of *C. reinhardtii* CRR1 (Fig. 5A). In contrast, the similarities of the amino acid sequences were limited in regions outside the SBP domain for these sequences. We examined the expression of PpSBP1 to 4 by RT-PCR. Although these genes were expressed in moss plants, their expression was not affected by the application of Cu (data not shown). Because neither the PpSBP2 gene nor the *Crr1* gene has an intron within the DNA encoding the SBP domain, while other PpSBP genes carry an intron at the conserved position within the SBP domain, PpSBP2 was chosen for further analysis. We examined whether PpSBP2 binds to the cis-regulatory sequences for the Cu-responsive transcriptional repression of the *FeSOD* gene. A gel retardation assay showed that the PpSBP2 protein prepared by in vitro translation formed a specific complex with both the ³²P-labeled fragment X (–315 to –280) and Y (–239 to –200) containing GTACT sequences (Fig. 5B). To determine whether the GTACT sequences are important for binding to PpSBP2, we used mutant variants of fragments X and Y as competitors for PpSBP2 binding in gel shift

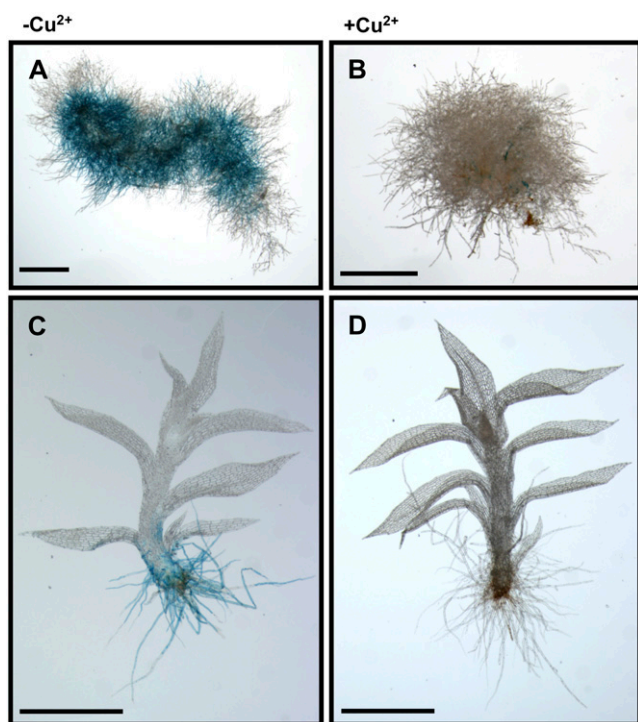


Figure 4. Histochemical analysis of p558 transgenic moss plants cultured without or with CuSO_4 . Transgenic moss plants precultured with $0.2 \mu\text{M}$ CuSO_4 for 7 d were treated without (A and C) or with (B and D) $2.0 \mu\text{M}$ CuSO_4 for 3 d. More than six independent transgenic moss plant lines were investigated. A and B, Protonema cells; C and D, gametophore. Scale bars, 1 mm.

assays. The formation of a complex of the probes with PpSBP2 was not efficiently inhibited in the presence of an excess amount of the mutated sequences. These results indicated that GTACT sequences are important for PpSBP2 binding.

To investigate the function of PpSBP2 in the Cu-responsive transcriptional repression of the *FeSOD* gene, PpSBP2 was overexpressed under the control of the constitutive pE7133 promoter derived from the cauliflower mosaic virus (CaMV) promoter (Mitsuhara et al., 1996) in transgenic *P. patens* carrying the p558 construct (Fig. 5C, top). Because the mutations in the GTACT sequences eliminated both the Cu-responsive repression of the *FeSOD* promoter (Fig. 3) and PpSBP2 binding (Fig. 5), we anticipated that the overexpression of PpSBP2 would decrease the expression of the p558 construct in the moss plants. As expected, GUS activities were reduced by the overexpression of PpSBP2 in the absence of Cu (Fig. 5C, middle), suggesting that PpSBP2 and PpSBP2-related proteins function as transcriptional repressors of the *FeSOD* promoter.

The Mechanisms of Cu-Responsive Repression Are Conserved in Higher Plants

To investigate whether the mechanisms of Cu-responsive transcriptional repression are conserved

in higher plants, we generated transgenic tobacco plants carrying the moss p558 construct (Nt/p558; Fig. 6A). The application of Cu reduced the GUS activities of transgenic tobacco plants, suggesting that GTACT sequences are also functional in the transcriptional repression by Cu in higher plants. It has been reported that the expression of the chloroplastic *FeSOD* genes of Arabidopsis (Abdel-Ghany et al., 2005b) and tobacco (Kurepa et al., 1997) is repressed by Cu at the transcriptional level. Three copies of GTACT sequences were found within -200 bp of the promoter of the Arabidopsis chloroplastic *FeSOD* gene *FSD1*, whereas the chloroplastic Cu/ZnSOD gene *CSD2*, which is not regulated by Cu, has no GTACT sequences within $-1,000$ bp of the promoter region. To examine whether the Arabidopsis *FSD1* promoter is regulated by Cu, we generated transgenic Arabidopsis and tobacco plants carrying the fusion gene of the *FSD1* promoter-GUS (Fig. 6B). Cu decreased GUS activities in the transgenic Arabidopsis and tobacco plants, showing that Arabidopsis *FSD1* is regulated by Cu in the same way as the moss *FeSOD* gene. These results suggested that the molecular mechanisms of Cu-responsive transcriptional suppression are conserved between bryophytes and higher plants.

We examined whether Cu affects the pattern of expression of the *FSD1* gene or simply decreases the levels of expression in those same organs using transgenic Arabidopsis carrying the *FSD1* promoter-GUS. Although no expression of *FeSOD* was observed in aerial parts in bryophyte plants (Fig. 4), the expression of Arabidopsis *FSD1* was detected in all organs under Cu-deficient conditions (Fig. 6C). Cu decreased GUS activities in the roots and rosette leaves but not in the cotyledons of the transgenic Arabidopsis, suggesting that the Cu responsiveness was lost in cotyledons. Alternatively, the accumulation of Cu in cotyledons could be of a low level to repress the *FSD1* gene, because the absorbed nutrients from roots are preferentially transported to young leaves through the vascular system.

DISCUSSION

As sessile organisms, plants have acquired plastic developmental programs to adapt to fluctuating environments throughout their life cycles. Transition metals, including Cu, Mn, Fe, and Zn, are essential for life, because they act as effective electron acceptors and donors in the active sites of many proteins involved in oxidation and reduction reactions (Shcolnick and Keren, 2006). The acquisition of these metals is a nutritional problem for plants, because bioavailability has changed in the course of evolution owing to the increase of oxygen in the atmosphere and the movement of life from aquatic to terrestrial habitats. Plants change the expression patterns of three types of SODs (i.e. FeSOD, MnSOD, and Cu/ZnSOD) depending on the internal and external availability of the transition

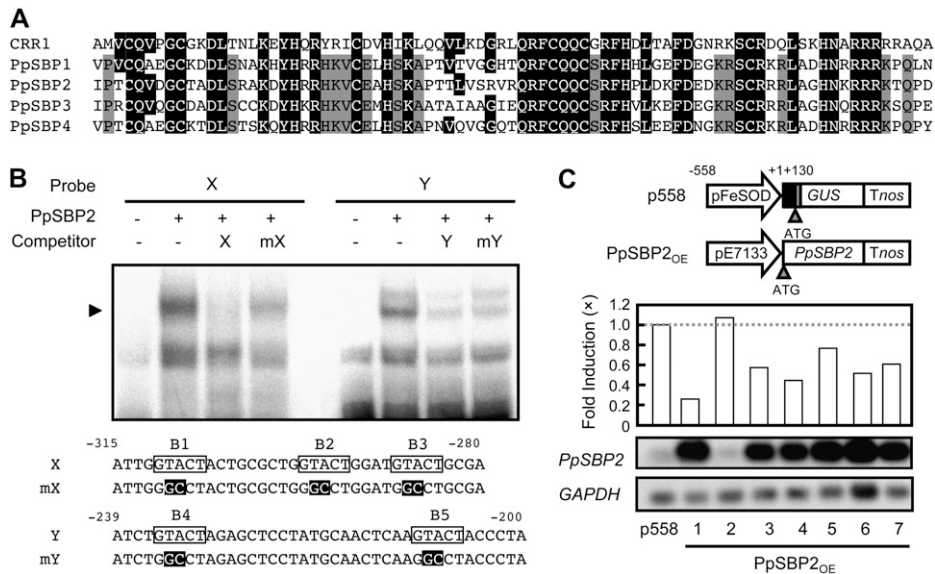


Figure 5. Binding of transcription factor PpSBP2 to the GTACT motif. **A**, Alignment of amino acid sequences of the SBP domain of CRR1 and PpSBPs. The highlighted residues indicate amino acids that are identical to those of CRR1. The gray residues indicate amino acids that are identical among PpSBPs. **B**, Gel retardation assay with in vitro-translated PpSBP2. The specific PpSBP2-DNA complexes are indicated by an arrowhead. Oligonucleotides containing the GTACT motif were used as the probes. The GTACT motif is boxed, and the mutated bases are highlighted. +, Present; -, absent. **C**, The overexpression of PpSBP2 repressed the expression of the *FeSOD* gene. Top, Schematic diagram of constructs. The p558 is the reporter construct containing -558 to +130 of the *FeSOD* gene. The effector construct PpSBP2_{OE} expresses *PpSBP2* cDNA under the control of the constitutive pE7133 promoter. Middle, Effect of overexpression PpSBP2 on GUS activities of p558. The bars represent the GUS activities of transgenic moss plants cultured without CuSO₄. The GUS activities of the mother transgenic lines containing only the reporter construct were arbitrarily set to 1.0 (dotted line). Bottom, RT-PCR analysis of *PpSBP2* expression in transgenic moss plants. After amplification with specific primers, the products were detected by DNA gel-blot hybridization. *GAPDH* was used as an internal control of RT-PCR.

metals. Therefore, elucidating the transcriptional regulation of SOD is crucial to identify the molecular mechanisms involved in plant adaptation to changes in their mineral environments.

In this article, we have identified the GTACT motif as a cis-acting element that is involved in the Cu-responsive transcriptional repression of the moss *FeSOD* gene. Seven copies of the GTACT motif (B1 to B7) were located within 400 nucleotides upstream of the transcription start site of the *FeSOD* promoter. We found that both B4 and B5, but not B6 and B7, are necessary for repression of the *FeSOD* gene (Fig. 3), suggesting that two copies of the GTACT sequences could repress the transcription in a Cu-dependent manner and that the GTACT sequences of B4 to B7 are not functionally equivalent. The latter could be due to the effect of surrounding sequences of each GTACT motif and/or the position of the GTACT motif on the promoter DNA sequences. Furthermore, a tandem repeat of the 60-bp DNA fragment between -258 and -199 of *FeSOD* that contains B4 and B5 conferred Cu responsiveness to the *actin* promoter, whereas the solo 60-bp DNA fragment did not (Fig. 2). This implied that the copy number of the GTACT motif that is required for Cu-responsive repression varied with the primary structure of the promoter. Taken together, our

results suggested that the GTACT motif plays a central role in the Cu-responsive transcriptional repression of the *FeSOD* gene and that its function is affected by the surrounding DNA context.

We showed that the overexpression of the transcription factor PpSBP2 resulted in the repression of the *FeSOD* gene in transgenic moss plants (Fig. 5); however, the effect was moderate, i.e. overexpression of PpSBP2 is not sufficient for full repression of the *FeSOD* gene. This suggests the involvement of the posttranslational modification of the transcription factor. One possible mechanism for the functional regulation of PpSBPs is the interaction with accessory proteins, including other transcriptional repressors, activators, general transcription factors, coactivators, chaperones, or chromatin-remodeling factors that mobilize nucleosomes. In this context, we observed that PpSBP1 bound to GTACT sequences and repressed the expression of the *FeSOD* gene as well as PpSBP2 (our unpublished data). Heterodimer formation of PpSBP2 with PpSBP1 or with other PpSBPs could enhance the repression activity of PpSBP2. Alternatively, covalent modification of PpSBPs, including acetylation, methylation, and phosphorylation, could be involved in the transcriptional regulation of the *FeSOD* gene in response to Cu.

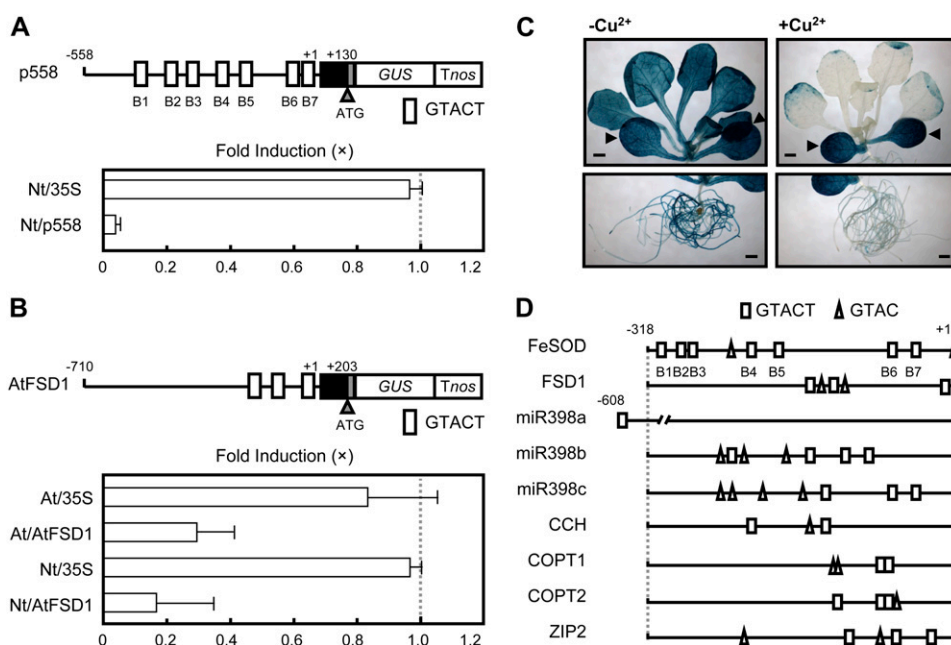


Figure 6. The mechanisms for Cu responsiveness are conserved in higher plants. **A**, The moss *FeSOD* promoter was repressed by Cu in the transgenic tobacco plants. Top, Schematic diagram of the p58 construct. The white boxes represent GTACT sequences. Bottom, GUS activities of the transgenic tobacco plants. The bars represent the GUS activities of transgenic plants cultured in the presence of $1.0 \mu\text{M}$ CuSO_4 . The GUS activities of the transgenic plants cultured in the absence of CuSO_4 were arbitrarily set to 1.0 (dotted line). The transgenic tobacco plants containing the CaMV 35S promoter fused to the *GUS* gene were used as a negative control (Nt/35S). The bars indicate \pm s.d. **B**, The Arabidopsis *FeSOD* gene *FSD1* is regulated by Cu. Top, Schematic diagram of the construct. The site of transcription initiation is indicated (+1). Black box, 5' Untranslated region; gray box, coding region (three amino acids). Bottom, GUS activities of transgenic Arabidopsis (At/35S and At/AtFSD1) and tobacco plants (Nt/35S and Nt/AtFSD1). The 35S-GUS construct was used as a negative control. The bars represent the GUS activities of transgenic plants cultured in the presence of $1.0 \mu\text{M}$ CuSO_4 . The GUS activities of the transgenic plants cultured in the absence of CuSO_4 were arbitrarily set to 1.0 (dotted line). The bars indicate \pm s.d. **C**, Histochemical analysis of transgenic Arabidopsis plants carrying the AtFSD1-GUS construct cultured without or with CuSO_4 . Transgenic Arabidopsis seedlings precultured with $0.1 \mu\text{M}$ CuSO_4 for 7 d were treated without (left; $-\text{Cu}^{2+}$) or with $1.0 \mu\text{M}$ CuSO_4 (right; $+\text{Cu}^{2+}$) for 7 d. The arrowheads indicate the cotyledons. More than three independent transgenic lines were investigated. Scale bars, 1 mm. **D**, Schematic diagrams of the promoter region of Arabidopsis genes regulated by Cu. *FeSOD*, moss *FeSOD* (AB370198); *FSD1*, *FeSOD* (At4g25100); miR398a, microRNA 398a (At2g03445); miR398b, microRNA 398b (At5g14545); miR398c, microRNA 398c (At5g14565); *CCH*, Cu chaperone (At3g56240); *COPT1*, Cu transporter (At5g59030); *COPT2*, Cu transporter (At3g46900); *ZIP2*, ZRT/IRT-like protein (At5g59520). The white boxes and triangles represent GTACT sequences and GTAC sequences, respectively.

The molecular mechanisms for Cu-responsive transcriptional repression seemed to be conserved in higher plants. The Arabidopsis chloroplastic *FeSOD* gene *FSD1* containing three copies of GTACT sequences was regulated by Cu at the transcriptional level (Fig. 6). On the other hand, the mRNA levels of the chloroplastic Cu/ZnSOD gene *CSD2* of Arabidopsis have been shown to be up-regulated by Cu (Abdel-Ghany et al., 2005b). Although Cu did not activate the transcription of the *CSD2* gene, it promoted the expression of the *CSD2* gene at the posttranscriptional level (Sunkar et al., 2006; Yamasaki et al., 2007). MicroRNAs are a class of regulatory small RNAs that posttranscriptionally regulate gene expression by directing mRNA cleavage or translational inhibition (Bonnet et al., 2004; Jones-Rhoades and Bartel, 2004). MicroRNA miR398 targeted *CSD2* mRNA and negatively regulated the expression of the *CSD2* gene (Sunkar and Zhu, 2004; Sunkar et al., 2006; Yamasaki

et al., 2007). The transcription of miR398 was repressed by Cu (Sunkar et al., 2006), and multiple copies of GTACT sequences were found in *MIR398* promoter sequences (Fig. 6D). This suggests that Cu promotes the expression of the Cu/ZnSOD gene *CSD2* posttranscriptionally by repressing the transcription of miR398 through GTACT sequence motifs. Thus, GTACT sequences play key roles in the replacement of *FeSOD* by Cu/ZnSOD in response to Cu by directly repressing the transcription of the *FeSOD* gene and indirectly inducing the transcription of the *Cu/ZnSOD* gene in Arabidopsis. Moreover, it has been reported that high Cu availability reduced the expression of several Cu-related genes of Arabidopsis, including *COPT1*, *COPT2* encoding Cu transporters, *ZIP2* encoding divalent metal transporters, and *CCH* encoding Cu chaperones (Himmelblau et al., 1998; Sancenón et al., 2003; Wintz et al., 2003). It is worth noting that the GTACT motifs are commonly found in the promoter

of these genes (Fig. 6D). The expression of the Arabidopsis gene, CCS, encoding the Cu chaperone for SOD, is up-regulated by Cu (Abdel-Ghany et al., 2005a); however, its molecular mechanisms are unknown at present.

Cu is one of the toxic, but essential, metals for all aerobic organisms, and its metabolism is tightly controlled (Balamurugan and Schaffner, 2006; Puig et al., 2007). For example, in *Saccharomyces cerevisiae*, a transcription factor Ace1p regulates Cu homeostasis by activating Cu-sequestering proteins under Cu-excess conditions (Thiele, 1988; Gralla et al., 1991). Cu directly binds to Ace1p and promotes Ace1 binding to DNA and transcriptional activation (Fürst et al., 1988; Dameron et al., 1991). In addition, a transcription factor Mac1p is required for the up-regulation of the expression of Cu transporters under Cu-deficient conditions (Labbé et al., 1997; Keller et al., 2005). The target sequences of Ace1p and Mac1p are TNNNGCTG and TTTGCTC (Huibregtse et al., 1989; Evans et al., 1990; Gralla et al., 1991; Labbé et al., 1997), respectively, clearly different from that of the Cu-responsive motif containing the GTAC core sequence of *C. reinhardtii*, moss, and higher plants. Furthermore, occurrence of SBP-related transcription factors that bind to the GTAC motif is limited to the plant kingdom. These results suggest the existence of plant-specific mechanisms of Cu-responsive signaling and transcriptional regulation.

In *C. reinhardtii*, the Cu-responsive element, together with its transcription factor, CRR1, positively regulates the transcription of the *Cyc6* and *Cpx1* genes (Kropat et al., 2005). However, our results suggested that the Cu-responsive element gene and the transcription factor PpSBPs negatively regulate the transcription of the *FeSOD* gene in response to Cu in moss. This discrepancy may be attributable to the functional difference of transcription factors, namely, a transcriptional activator for CRR1 and a repressor for PpSBP2. In this context, the similarities in the amino acid sequence of *C. reinhardtii* CRR1 and moss PpSBPs were very limited in regions outside the DNA-binding SBP domain (Fig. 5). Because the expression of both the moss *FeSOD* promoter and the Arabidopsis *FSD1* promoter is repressed by Cu in transgenic Arabidopsis (Fig. 6), the mechanisms for Cu-dependent negative transcriptional regulation may be conserved between moss and higher plants. Collectively, the molecular mechanisms of Cu responsiveness seem to be more sophisticated during the evolution of plants from green algae to vascular plants, keeping the GTAC core cis-regulatory sequence for Cu responsiveness through adaptation to terrestrial habitats and differentiation from higher plants. Further investigation regarding how the function of PpSBPs is controlled by Cu will help to reveal the molecular mechanisms behind the fine transcriptional regulation by Cu and provide insights into the evolution of SODs, which constitute the first line of defense against oxidative stress and signal the emergence of land plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Physcomitrella patens Bruch & Schimp subsp. *patens* Tan (Ashton and Cove, 1977) was cultured at 25°C under continuous light in a BCDATG agar medium (Nishiyama et al., 2000). For vegetative propagation, the plants were ground with a pestle and mortar in sterile water and soaked in the BCDATG agar medium overlaid with a layer of cellophane.

For the GUS assay and histochemical analysis, the transgenic plants were precultured in a BCDATG agar medium containing 0.2 μM CuSO_4 for 7 d at 25°C and then transferred to a BCDATG liquid medium with (2.0 μM CuSO_4) or without CuSO_4 for 3 d at 25°C under continuous light ($<50 \mu\text{mol m}^{-2} \text{s}^{-1}$).

The Arabidopsis (*Arabidopsis thaliana*) lines used were in the Columbia-0 background. Seeds were sterilized and sown in a Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.8% agar (Wako) and 3% Suc. The plates were incubated in the dark for 3 d at 4°C and subsequently transferred to a long-day condition (18 h of light/6 h of dark) at 22°C ($<100 \mu\text{mol m}^{-2} \text{s}^{-1}$). For the Cu treatment, 7-d-old seedlings were transferred to plates with (1.0 μM CuSO_4) or without CuSO_4 for 7 d at 22°C. The tobacco (*Nicotiana tabacum*) lines used were in the 'Petit Havana SRI' background. Seeds were sterilized and sown in the same medium composition as Arabidopsis. The plates were cultured at 28°C under continuous light ($<100 \mu\text{mol m}^{-2} \text{s}^{-1}$). For the Cu treatment, 7-d-old seedlings were transferred to the plates with (1.0 μM CuSO_4) or without CuSO_4 for 7 d at 28°C.

Plasmid Construction

The *FeSOD* gene promoter was isolated by TAIL-PCR using a random primer, 5'-NGTCGA(G/C)(A/T) GANA(A/T) GAA-3', and gene-specific primers, 5'-GAGACTTCTCCACCATCAACTTG-3', 5'-CGAGCTCCGTCCTCCTGATCTGC-3', and 5'-TCGTCAAAGGCAACGGACGGCAGCC-3'. Genomic DNA was used as the template for TAIL-PCR. The amplified product was cloned into a pGEM-T Easy vector (Promega). A series of 5' deletion constructs was generated by PCR using this plasmid as a template with primers, generating a *Sall* site at the 5' and the 3' ends. The primer pairs were as follows: for p558, 5'-GGGGGTCGACAGAAGAAGTGAAGAC-3' and 5'-GGGGGTCGACGGCAGCCATCCTGGT-3' (primer-R); for p438, 5'-GGGGTTCGACACGGAGAACTGATATCAG-3' and primer-R; for p318, 5'-GGGGGTCGACATGATTGGTACTAC-3' and primer-R; for p258, 5'-GGGGTTCGACGAGTACAAGCTTATACGAC-3' and primer-R; and for p198, 5'-GGGGGTCGACCATCAAAAACACTCAC-3' and primer-R. The amplified DNA fragment was digested with *Sall* and cloned into *Sall* of pGUSmutNPTII, which contained the coding sequence of *GUS*, the nopaline synthase polyadenylation signal, and the NPTII cassette (NPTII; Nishiyama et al., 2000). To make the gain-of-function experimental constructs, the PCR-amplified fragment was cloned into *KpnI* of pCRII-ACTIN, which contained the rice (*Oryza sativa*) *actin* promoter derived from the pTN90 plasmid, the coding sequence of *GUS*, the nopaline synthase polyadenylation signal, and the NPTII cassette. The primer pairs were as follows: for F12, 5'-GGGGGTACCATGATTGGTACTACTGCGC-3' (primer-F12-F) and 5'-GGGGGTACCATAGGGTACTACTTGAAGTTG-3' (primer-F12-R); for F1, primer-F12-F and 5'-GGGGGTACCATAGGGTACTACTGCGC-3'; and for F2, 5'-GGGGGTACCGAGTACAAGCTTATACGAC-3' and primer-F12-R. The mutant version constructs were generated by PCR using primers including specific mutations. The *PpSBP2* was amplified by a PCR reaction from *P. patens* cDNA library with primers 5'-GGGGGCCCATGTCTGCCGTGACC-3' and 5'-GGGGGCCCTACAGATGCAGGGACAC-3'. To make the *PpSBP2*_{OE} construct, the PCR-amplified fragment was cloned into *ApaI* of the pCMAK1 plasmid, which contained pE7133 elements derived from the CaMV 35S promoter and some enhancers (Mitsuhashi et al., 1996). The pGUSmutNPTII-derived constructs (p558, p438, p318, and p198), pCRII-ACTIN-derived constructs (F12, F1, and F2), and *PpSBP2*_{OE} construct were linearized with *SpeI*-*XhoI*, *ApaI*, and *NotI*, respectively; the subsequent polyethylene glycol-mediated transformation followed the method of Hiwatashi et al. (2001). To make the p558 construct for transgenic Arabidopsis and tobacco plants, the *Sall*-digested promoter DNA fragment from the p558 construct was cloned into the pBI101 vector to generate *GUS* fusion genes. The promoter of *FSD1* was amplified by a PCR reaction from Arabidopsis genomic DNA with primers 5'-GGAAGCTTACTGTATAAGATACAAGGTG-3' and 5'-GGGGATCCAGCAGCCATCTTTGTAATG-3'. The amplified DNA fragment was digested with *HindIII*-*BamHI* and cloned into the pBI101 vector. The integrity of all constructs was confirmed by DNA sequencing. These constructs were

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