Cloning of Superoxide Dismutase (SOD) Gene of Lily 'Marcopolo' and Expression in Transgenic Potatoes

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Differential display reverse transcription PCR (DDRT-PCR) analysis was performed on lily 'Marcopolo' bulb scale for isolation of expressed genes during bulblet formation. Cu/Zn lily-superoxide dismutase (LSOD) of 872 bp gene, with ability to scavenge reactive oxygen in stress environment, was isolated. Northern blot analysis showed expression levels of LSOD maximized 12 days after bulblet formation. Ti plasmid vectors were constructed with sense and antisense expressions of LSOD gene and transformed into potato. Southern blot analysis of transgenic potatoes revealed different copies of T-DNA were incorporated into potato genome. In transgenic potatoes, lily SOD gene was overexpressed in sense lines and not in antisense lines. In native polyacrylamide gel electrophoresis analysis, additional engineered LSOD was detected in sense overexpressed transgenic line only. Transgenic potatoes were subjected to oxidative stress, such as herbicide methyl viologen (MV). Transgenic potato lines with sense orientation exhibited increased tolerance to MV, whereas in antisense lines exhibited decreased tolerance. *In vitro* tuberization of transgenic potato with sense orientation was promoted, but was inhibited in transgenic potato with antisense orientation.

Key words: *lily bulblet formation, superoxide dismutase, tuberization, transgenic potato*

Lilium species belong to the family Liliaceae as a monocot plant, with 130 native species and 500~600 varieties growing throughout the world. The bulblelt formation process of lily scale has been the subject of intensive studies at the mass propagation system. Organogenesis *de novo* in tissue cultures has provided useful systems for studying regulatory mechanism of plant development.[1)] Many investigators have studied the cultural conditions for efficient bulblet formation in various *Lilium* species scale culture *in vitro*. They found that the bulblet formation and development are influenced by cultural environmental conditions such as photoperiod, temperature, physiological stage of explants, compositions of nutrient, sucrose, and hormone in media.[2,3)] Despite considerable work on the conditions for bulblet formation, researchers have not yet identified the molecular approach during the bulblet formation. Recent findings could lead to a greater understanding of the fundamentals of plant organogenesis at the molecular level. Genes that are presumed to play critical roles in each phase of organogenesis *in vitro* are being identified largely through genetic analysis and some of them have been already isolated.[4-8)]

Some investigators are attempting to find biochemical markers for bulblet formation.[9)] However, the process or mechanism of bulblet formation is yet poorly understood, because very little information is available on the biochemical changes that take place during plant differentiation. Moreover, studies on the molecular genetics about bulblet formation have not been carried out until now. Thus, the objective of this study was to understand the process of bulblet formation in scale culture by characterizing the genes expressed during bulblet formation. Differential display reverse transcription PCR (DDRT-PCR) analysis was performed on the lily 'Marcopolo' bulb scale for the isolation of expressed genes during bulblet formation. Two genes related to proteolysis and three genes related to antioxidant mechanism in the plant were isolated through DDRT-PCR. Cu/Zn lily-superoxide dismutase (LSOD), able to scavenge reactive oxygen in stress environment, was isolated. To investigate the physiological function of identified LSOD during bulblet formation, LSOD gene was introduced into potato plants via *Agrobacterium tumefaciens* transformation. Because transformation of lily was very hard to succeed, we chose potato, another vegetative-propagated and easily transformable plant, as experimental material. The *in vitro* bulblet formation process of lily scale and *in vitro* tuber formation process of potato are very similar, and their mass propagation systems have been studied intensively. The present study shows that the LSOD-transformed plants exhibited increased resistance to oxidative stress caused by methyl viologen (MV) but changed the *in vitro* tuber formation.

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Materials and Methods

Plant materials and cultural conditions. Scales were detached from *Lilium* oriental hybrid 'Marcopolo' bulbs, washed with tap water, and surface-sterilized in 0.5% sodium hypochlorite solution. The scales were cultured on sterile medium containing 30 g/L sucrose, staba vitamin, and 100 mg/L inositol for proliferation of bulblets. *In vitro* cultured bulbs of *Lilium* oriental hybrid 'Marcopolo' were aseptically divided, and the bulb scales were used as experimental materials. The scales were grown at 24 ± 2°C, 16 h photoperiod, and 60 $\mu M \cdot m^{-2} \cdot sec^{-1}$ photosynthetic photon flux provided by a florescent lamp (Osrams).

Differential display. Differential display was performed using the Delta™ Differential Display kit. (Clontech, USA). Total RNA was prepared from scales extracted before (the first planting day) and 14 days after bublet formation. Total RNA was isolated using guanidinium thiocyanate followed by centrifugation in CsCl as described by Glisin[10] with some modifications. The PCR reactions were run using combinations of nine different anchor primers and eight arbitrary 13-nucleotide primers designed following the manufacturer's instructions. PCR products were separated on 5% denaturing acrylamide gels containing 8 M urea or 1% agarose (MetaPhor, BMA, USA) for a high resolution. Those cDNA bands showing differential intensities were selected for further study. The PCR product was ligated, subcloned, and sequenced. To obtain full-length coding sequences, rapid amplification of cDNA 5' ends was performed using a 5'/3' RACE kit (Roche Molecular Biochemicals, USA).

Northern blot analysis of lily SOD mRNA. The expression of *LSOD* genes was investigated by Northern blot experiments using the scales cultured for *in vitro* bulblet formation and transgenic potato plants. To recognize the expression of the *LSOD* genes, 25 μg total RNA was separated by electrophoresis in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred on to a nylon membrane. The DIG probe was made by PCR reaction with 5'-TCTAGATGCAAGCGATTC TCGCCGCC-3' and 5'-ATAGTTGCCTCCGCTACTCCTTC AGAG-3' primers directly labeled with digoxigenin (DIG)-dUTP (PCR DIG labeling Mix, BM, Germany). The membranes were hybridized and detected by same method as described in Southern blot analysis.

Construction of a LSOD sense and antisense expression vectors. A schematic representation of the LSOD sense and antisense expression constructs is given in Fig. 1. According to the cDNA sequence of LSOD cloned from *Lilium* oriental hybrid 'Marcopolo' (Genbank Accession Number, AY898945), an *sod* coding region DNA fragment starting with the start codon and continuing through the stop codon was produced by PCR amplification using *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and the cDNA clone as a template. Primers 5'-TCTAGATGCAAGCGATTCTCGCCGCC-3' and 5'-GGTA CCTCAAACAGGAGTCAACCC-3' were used for sense orientation, and primers 5'-TCTAGAGCAGTTTTAACAATC CCTC-3' and 5'-GGTACCGGGTTGATGCAAGCGATTCTC G-3' for antisense orientation. New enzyme sites of *XbaI* and *KpnI* were inserted into the ends of these primers for cloning. The resulting PCR fragments were subcloned using a Zeroblunt PCR cloning kit (Invitrogene). The *XbaI* and *KpnI* fragments were cut out from this cloning vector, and the resulting *lsod* gene fragments were ligated into the *XbaI* and *KpnI* sites of pMBP binary vector (Fig 1). We confirmed the sense-oriented pMBP-S-LSOD and antisense-oriented pMBP-AS-LSOD vector by sequencing.

Potato transformation, selection, and analysis. pMBP-S-LSOD and pMBP-AS-LSOD vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404 and introduced into *Solanum tuberosum* L. var. Desiree using the leaf disc transformation method.[11] To verify the transformed lines selected from a kanamycin treatment, PCR and Southern analysis of LSOD gene were carried out on the genomic DNA extracted from sense and antisense plants. NPT1 and NPT2 primer pairs were used to detect the transgene sequences inserted into the genomic DNA.[11] For Southern blot hybridization, the genomic DNA was isolated according to the protocol.[12] After digestion with EcoRI restriction enzyme, the DNA was subjected to electrophoresis and transferred on to a nylon membrane. The membranes were hybridized with probes labeled with DIG. Hybridization lasted for 16 h at 42°C in a hybridization buffer, a DIG Easy Hyb. The membranes were washed twice in 2 × SSC, 0.1% (w/v) SDS at room temperature for 5 min each, followed by washing twice in 0.1 × SSC, 0.1% SDS at 68°C for 15 min each. Target DNAs were detected using a DIG luminescent detection kit as described by the manufacturer's instructions (Roche Molecular Biochemicals). Transgenic lines with a high expression level of LSOD gene and high inhibition level of endogenous *sod* gene were selected by Northern blot analysis and subcultured for *in vitro* tuberization and the herbicide MV test. Microtubers from transgenic potatoes were produced according to the method described by Jeon *et al.*[13]

Native PAGE and application of MV. The protein samples from transgenic potatoes were separated by native PAGE on a separating gel of 30% (w/v) polyacrylamide for analysis of the pattern of isoenzymes for SOD. The gels were stained for 30 min in dark using a 1 : 1 mixture of: (a) 0.06 mM riboflavin and 0.651% (w/v) TEMED, and (b) 2.5 mM nitroblue tetrazolium (NBT), both in 50 mM phosphate buffer, and developed for 20 min under moderate light condition.

Transgenic and untransformed shoots cultured *in vitro* were transferred to soil and grown in a greenhouse. Round leaf discs (9 mm in diameter) were cut with a cork borer and were treated with 0, 50, 100, 200, 500, and 1000 μM MV solutions in Tissue Culture Test plate (4 × 6). The plates were placed on the shelf in a culture room under continuous high light (150 $\mu M \cdot m^{-2} \cdot sec^{-1}$) and were observed after 24 h. To compare the resistance to oxidative stress caused by MV among transgenic lines, seven leaf discs were treated with 200 μM MV solutions. The degree of resistance to MV was calculated

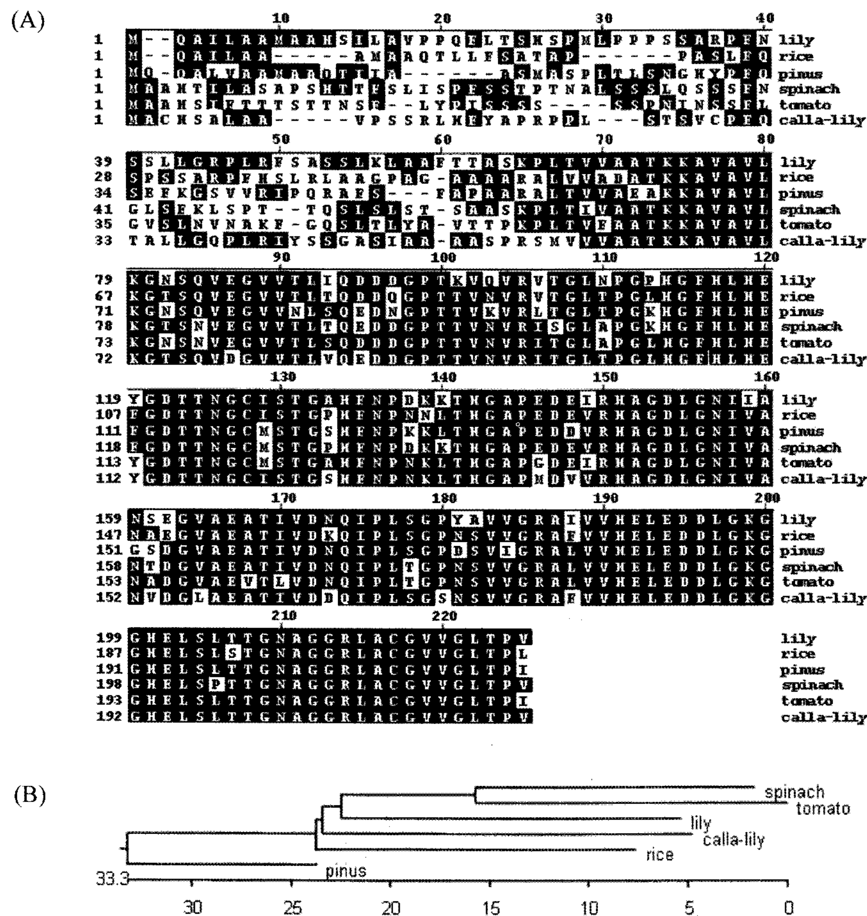

Fig. 1. Alignment of predicted amino acid sequences of superoxide dismutase of *Lilium* oriental hybrid 'Marcopolo' (*lsod*). (A) The proteins shown are superoxide dismutases from lily (AY898945), rice (P93407), tomato (AAA34195), *Zantedeschia aethiopica* (calla-lily, AAC08582), spinach (2004417A), and *Pinus pinaster* (AAL29462). The amino acid sequences conserved in more than two proteins are shaded. (B) Phylogenetic analysis of superoxide dismutase.

by determining the contents of the remaining chlorophylls a and b from the damaged leaf discs. After MV treatment, the concentrations of chlorophylls a and b were determined spectrophotometrically in DMSO extracts of leaf discs using the method of Porra *et al.*[14]

Results and discussion

Cloning of SOD gene from lily scale. Total RNA samples isolated from scales before and after bulblet formation were subjected to RT-PCR using different primer pairs, and the amplified cDNAs were separated on polyacrylamide gels. The cDNA bands showing increased intensity during bulblet formation were reamplified using the same primer pairs. After cloning and sequencing, BLAST analysis was performed, and the five partial cDNA fragments were revealed as cystein proteinase, Cu/Zn superoxide dismutase, Ubiquitin-conjugating enzyme, Photosystem II, and Fe-S complex protein.[15]

Reactive oxygen species (ROS) such as superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) cause oxidative damage to cells, and are also involved in various biotic and abiotic stresses. Plants have both nonenzymatic and enzymatic ROS detoxification systems. Several enzymes can efficiently detoxify ROS, while SODs disproportionately detoxify superoxide radicals. Moreover, hydrogen peroxide is destroyed by catalases (CATs) as well as by different kinds of peroxidases (POD).[16] Cu/Zn SOD gene was selected for further study of characterizations, because this gene was expected to be involved with the bulblet formation in aspect of stress or storage protein degradation during adventitious bud initiation in lily scale.

Complete cDNA sequences were derived by rapid amplification of cDNA ends (RACE) PCR. LSOD cDNA fragments were cloned by a series of PCRs and 5'RACE of total RNA isolated from the scales at the bulblet formation stage. Complete DNA sequencing of LSOD revealed an open reading frame capable of encoding a polypeptide of 223 amino acids, with a molecular mass of 23 kD and a predicted pI of 6.67. The sequence homology was compared with those of other SOD clones by the NCBI BLAST[15] network service. Figure 1 shows that the deduced amino acid sequences of cloned cDNA for the LSOD gene. Cu/Zn SOD amino acid of lily showed high homologies with rice (P93407), tomato (AAA34195), and spinach (2004417A) (70.6, 70, and 68.5%

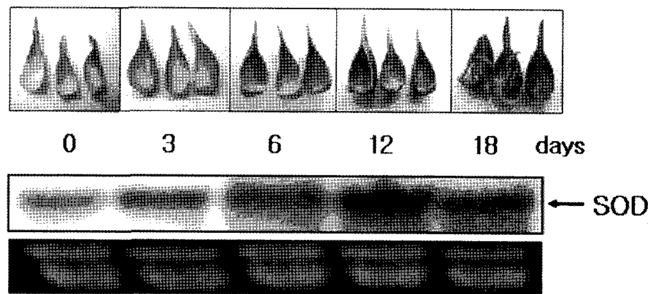

Fig. 2. Northern hybridization analysis of LSOD from lily at different stages of *in vitro* bulblet formation and photographs of bulblets at 0, 3, 6, 12, and 18 days after induction.

identities, respectively). The dendrogram of the alignment of SOD genes using a multiple sequence alignment program (Clustal) revealed the SOD gene of lily is more related to rice, also a monocotyledonous plant, than tomato and other plants.

To examine the expression of the genes corresponding to clones LSOD, northern analysis was carried out on total RNA isolated from lily at various stages of bulblet formation. Figure 2 shows the results of northern analysis of lily at various bulblet formation stages by probing with the LSOD clone. Weak hybridizing transcript of LSOD was detected at day 0, its intensity increased at day 3, and strong signals were detected at days 6 and 12. Thereafter, the hybridization intensity remained detectable throughout the bulblet formation and growth. At this various bulblet formation stages, browning was observed on the surfaces of the wounds around tissues, which was attributed to the oxidation of phenolic compounds. Strong Cu/Zn SOD was activated during wound healing about 6 days after culture. Further experiments were carried out to clarify the physiological function of the identified LSOD using transgenic potato plants that overexpress or downregulate each gene.

Introduction of the lily-SOD gene into potato. To investigate the physiological function of LSOD during bulblet formation, LSOD gene was introduced into potato plants. A schematic representation of the pMBP-S-LSOD and antisense-oriented pMBP-AS-LSOD vector constructs is given in Fig. 3. The binary vector used in these experiments was pMBP, which includes the CaMV and *nos* sequences within its T-DNA. Cu/Zn SOD sequences of lily were inserted between these sequences in antisense and sense orientations relative to the CaMV promoter. A *NPTII* gene is also located within its

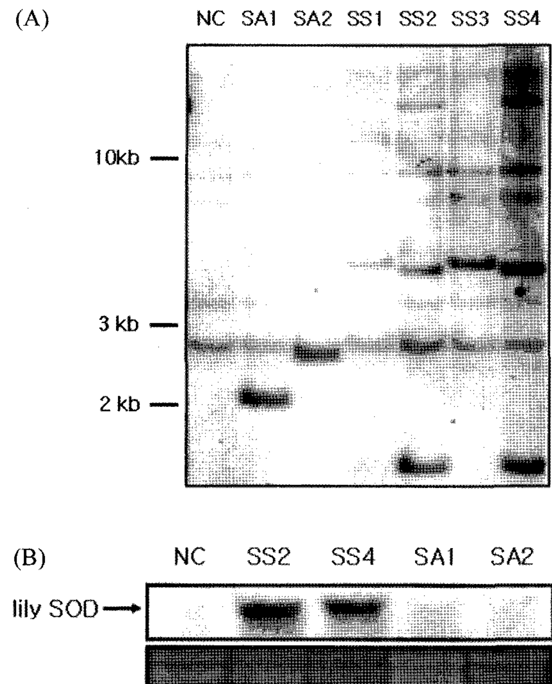

Fig. 4. Genomic Southern blot analysis of LSOD from transgenic potatoes and expression profile of LSOD gene from different transgenic potato lines by Northern blot experiments. (A) Different copy numbers were incorporated into transgenic potatoes. (B) Northern analysis of lsod from transgenic potato tubers 20 days after tuberization. Transgenic lines: sense expression of lsod (SS1, SS2, SS3, and SS4), antisense expression of lsod (SA1, SA2); NC, negative control (cv. Desiree).

T-DNA. The regenerated potato shoots were again selected by treatment with kanamycin. To verify the transformed lines selected from the kanamycin treatment, PCR analysis was carried out on genomic DNA extracted from the transgenic plants. NPT1 and NPT2 primer pairs were used to detect the transgene sequences inserted into the genomic DNA.[11] Two highly inhibited and four highly overexpressed transgenic plants were selected by Northern blot analysis from the PCR positive lines for the constructs (Fig. 4B). Southern blot analysis showed that different copies of T-DNA were incorporated into the potato genome (Fig. 4A). To test the expression of the LSOD transgene, leaf total protein extracts of sense- and antisense-oriented transgenic potato plants were electrophoresed in native polyacrylamide gels. Subsequent activity staining revealed five distinguishable SOD isozymes.

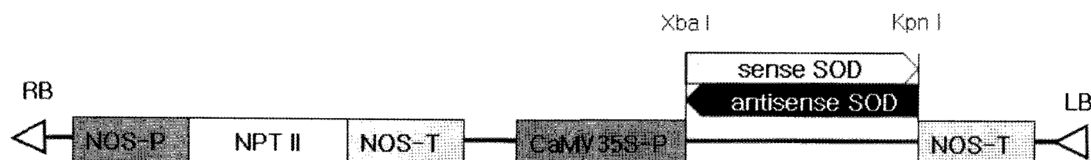

Fig. 3. Schematic diagrams of sense- and antisense- oriented plant expression vectors, pMBP-S-LSOD and pMBP-AS-LSOD, for potato transformation. CaMV35S-P, CaMV 35S promoter; NOS-P, nopaline synthase promoter; NPTII, neomycin phosphotransferase; NOS-T, nopaline synthase terminator; LB and RB, T-DNA left and right border sequences, respectively.

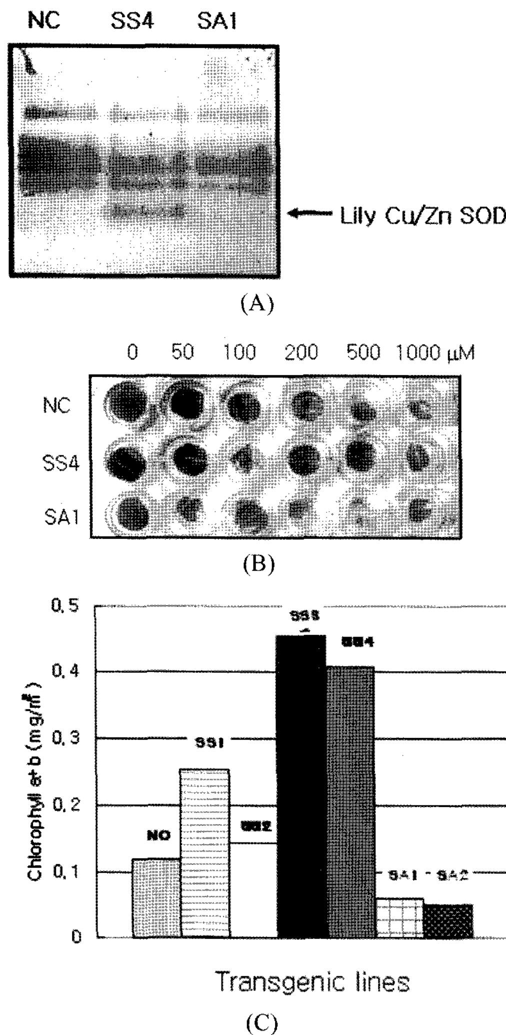

Fig. 5. Native PAGE assay for SOD isoenzyme pattern analysis (A) and visual symptoms of resistance to oxidative stress by MV treatments of non-transgenic (NC), pMBP-S-LSOD sense orientation (SS4), and pMBP-AS-LSOD antisense orientation (SA1) transgenic potato plant (B). The degree of resistance to MV was calculated by determining the contents of the remaining chlorophylls a and b from the damaged leaf discs (C).

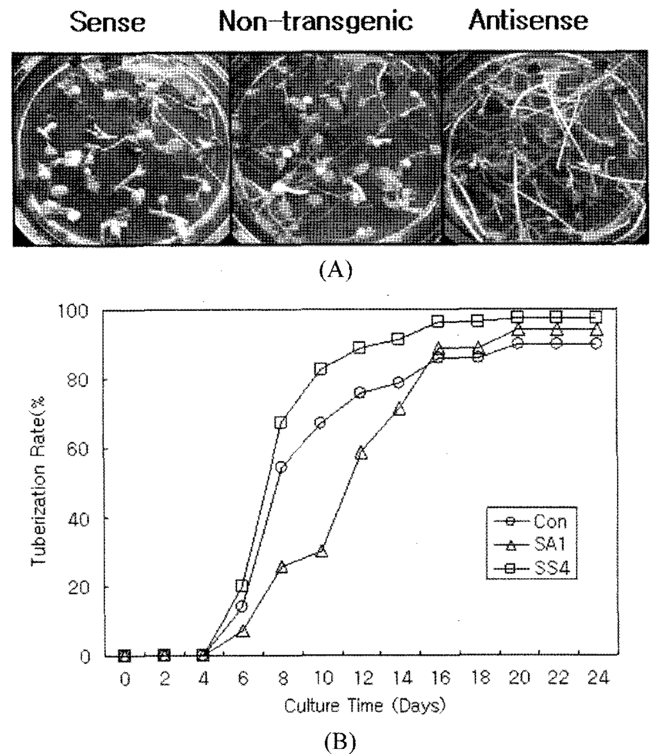

Fig. 6. In vitro tuberization from transgenic potatoes. (A) Photographs of *in vitro* tuberization of nodal explants from transgenic potatoes for 14 days after tuberization. (B) Tuberization rates were detected by two-nodes stem segments system from transgenic potatoes. Ten two-nodes stem segments were cultured in a Petri dish for microtuberization with 10 replicates of each transgenic potato lines. Con, non-transgenic; transgenic lines: sense expression of LSOD (SS4), antisense expression of LSOD (SA1).

In LSOD overexpressed transgenic line, we also clearly detected the additionally engineered lily Cu/Zn SOD band, but not in antisense- and non-transgenic lines. The transgenic lines expressing the S-LSOD and AS-LSOD genes at the highest level were subcultured for microtuberization, and further grown in a greenhouse for treatments known to cause oxidative stress, such as herbicide MV. During tissue culture of transgenic potatoes, some differences in microtuberization and plant morphologic characteristics were observed between control and transgenic plants.

Resistance to oxidative stress and *in vitro* tuberization of transgenic potatoes. Active oxygen species such as superoxide, H_2O_2, and hydroxyl radicals are by-products of normal cell metabolism. These active oxygen species result in the peroxidation of membrane lipids, breakage of DNA strands, and inactivation of enzymes. The conditions leading to damages caused by active oxygen species are referred to as oxidative stress. Both chloroplast and mitochondria can produce active oxygen species either under normal growth conditions or during exposure to various stresses.[17] SODs are a group of metalloenzymes that protect cells from superoxide radicals by catalyzing the dismutation of the superoxide radical into the molecular O_2 and H_2O_2. For monocotyledonous plants, only cDNAs for chloroplast Cu/Zn SOD and mitochondrial MnSOD have been isolated from rice and corn.[18, 19] Several studies have reported that the upregulation of SOD levels may enhance the oxidative stress tolerance.[20] We also attempted to detect the increased tolerance to oxidative stress by treatment of MV from transgenic potatoes expressing the S-LSOD and AS-LSOD genes at the highest level. The transgenic potatoes with overexpressing LSOD gene exhibited increased tolerance to stress by the treatment of up to 500 μM MV (Fig. 5A). However, antisense lines showed higher decrease in the tolerance to oxidative stress than the non-transgenic potatoes. These results are consistent with the results of previous studies, where constitutive SOD overexpression has led to increased tolerance of various plant species to oxidative stress.[20-22]

The selected transgenic lines expressing the S-LSOD and AS-LSOD genes showed different morphologic characteristic and growth patterns during tissue culture (data not shown). To examine the effects of introduced SOD genes of lily on *in vitro* tuberization of transgenic potatoes, microtuberization experiment was carried out on transgenic potatoes with sense and antisense expressions of LSOD gene. Tuberization was promoted in SOD-overexpressed transgenic lines, whereas delayed in SOD-inhibited antisense transgenic lines (Fig. 5B). In SOD-overexpressing sense transgenic lines, direct *in vitro* tuber formation from axillary bud of nodal segments was detected. Contrary to the results of sense transgenic lines, the growth of *in vitro* stolon-like axillary bud was induced firstly in antisense transgenic lines, followed by the delayed formation of *in vitro* tuber (Fig. 5A). These results indicate that lily Cu/Zn SOD transgenes provide a developmental shift to tuberization rather than growth. Interestingly, the overexpression of Cu/Zn SOD induced *in vitro* tuberization, whereas antisense inhibition of endogenous SOD activity inhibited the *in vitro* tuberization. It is therefore evident that one of the functions of SOD genes is crossly related to the tuberization process of potatoes. Northern analysis result of lily at various bulblet formation stages, revealed expression level of SOD was highest for 12 days after induction, the initiation time of small bulblets (Fig. 2), suggesting that one of the functions of SOD gene in lily may involve the induction of bulb formation. Because potatoes and lily are vegetative-propagated plants, they may possess a common controlling mechanism for tuber or bulb formation.

Acknowledgments

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