Cloning, expression, and intracellular localization of rice SUMO genes.

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Summary

Small ubiquitin-related modifier (SUMO) is a type of ubiquitin-like proteins and regulates various protein functions through post-translational modification. The SUMO precursor proteins are processed by C-terminal cleavage reaction at the end of the di-glycine(GG) motif, and then activated and bind to substrate proteins by a series of enzymatic reaction. We cloned SUMO1-3 genes in rice, and analyzed the transient expression and intracellular localization of the DsRed fusion proteins, DsRed:SUMO1, 2, and 3 in onion epidermal cells by using confocal laser scanning microscopy. The DsRed signals from DsRed:SUMO1 and DsRed:SUMO2 were detected both in nuclei and cytoplasmic location, but not in nucleoli. In the case of DsRed:SUMO3, the DsRed signal was detected mainly in nucleus, and formed sub-nuclear domain like structure. We also tested effect of the GG motif on intracellular localization of SUMO proteins. The GG deletion mutant vectors, pDsRed:SUMO1ΔGG, pDsRed:SUMO2ΔGG, and pDsRed:SUMO3ΔGG were constructed and transiently expressed in onion epidermal cells. Result showed that the deletion mutation of GG motif suppressed the accumulation of the DsRed:SUMOΔGG proteins in the nucleus. These results indicated that the C-terminal processing of OsSUMO precursor proteins are necessary for OsSUMO localization to nucleus in onion epidermal cells.

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Key words : SUMO, rice, onion epidermal cell, GG motif, DsRed

Small ubiquitin-related modifier (SUMO) is a type of ubiquitin-like proteins and regulates various functions of plant proteins (Miura et al., 2007; Lois, 2010) through post-translational modification to various substrate proteins, for instance, identified SUMO interactome showed metabolic enzyme, stress responses signaling factor, transcripiton factor and chromatin remodeling factor, etc (Elrouby and Coupland, 2010). The SUMO precursor is processed at C-teminal GG motif, by SUMO specific protease, ULP, and following pathway involving E1 activating enzymes, E2 conjugating enzyme, and E3 ligase catalyzes binding mature SUMO to substrate protein, called SUMOylation, is similar to ubiquitination pathway (Bernier-Villamor et al, 2002; Lois and Lima, 2005;Yunus and Lima, 2006; Miura et al., 2007; Yaeno and Iba, 2008). Plant SUMO also interacts to lysine residue of ΨKXD/E motif of target protein in *E. coli,* and *in Planta* (Okada et al., 2009, Elrouby and Coupland, 2010), and mutation of the GG motif resulted lack of SUMO binding ability to the target protein (Okada et al., 2009). In Arabidopsis, ESD4 is a regulator of flowering time and is a type of SUMO specific protease, the ESD4 processes a precursor SUMO protein, and mutation of GG motif of AtSUMO prevent ESD4-mediated maturation *in vitro* (Murtas et al., 2003) . In rice, three SUMO genes and a set of SUMO related enzymes also conserved (Miura et al., 2007). According to the report, we cloned OsSUMO1-3, constructed vectors expressed fluorescent protein DsRed and OsSUMO fusion proteins, and analyzed cellular localization of the DsRed:SUMO fusion proteins in onion epidermal cells. We also analyzed cellular localization of GG mutant proteins DsRed:SUMOΔGG, and discussed.

MATERIALS AND METHODS

Plant material and RNA extraction

Oryza sativa L. cv Toride-1 was sown, and grown under 14.5L/9.5D day length, 28℃ day / 26℃ night thermo condition. Total RNA was extracted from young seedlings, and was used for synthesis of cDNA first strand by SuperScriptII (invitrogen, currently Lifetechnologies Co.).

Transient Expression of fluorescent protein in onion epidermal cells.

Onion epidermal cells were bombarded with 3 μg of DNA constructs using a helium biolistic gun (Biolistic® PDS-1000/He Particle Delivery System, BioRad). After keeping the onion epidermal cells in the dark at room temperature for 24 hrs, we observed cellular localization of DsRed fusion proteins by using confocal laser scanning microscopy (FV300-BX61, Olympus) according to Kitajima et al., 2009.

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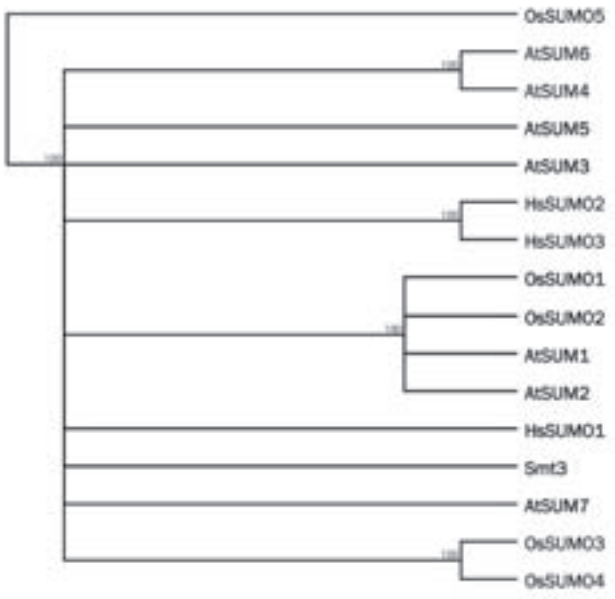

Fig. 1. Phylogenetic tree of OsSUMO family
Phylogenetic tree of previously-identified and putative OsSUMOs. Mutiple sequence alignment result of SUMOs from ClustalW (Thompson et al., 1994).

RESULTS AND DISCUSSSION

Phylogenetic analysis of OsSUMO genes.

Previously-identified and putative SUMO proteins sequences were collected and phylogenetic analysis was performed (Fig. 1). Smt3 is SUMO of bread yeast, HsSUMO1, 2, and 3 are human SUMO and AtSUM1～7 are Arabidopsis SUMO. Phylogeny indicated that the OsSUMO1, 2, AtSUM1 and 2 form a subgroup as previously reported (van den Burg, 2010) and close to human SUMO2 and 3. OsSUMO3, and 4 form a subgroup and OsSUMO5 is independent from other SUMO members and is possibly other members of a ubiquitin-like protein.

Construction of DsRed:SUMO1, DsRed:SUMO2, and DsRed:SUMO3

To analyze cellular localization of rice SUMO proteins, we constructed the vectors which express fluorescent maker DsRed, and OsSUMO fusion proteins, named pDsRed:SUMO1, pDsRed:SUMO2, and pDsRed:SUMO3.

OsSUMO2 ORF was amplified from first-strand cDNA by PCR and was used for pDsRed:SUMO2 construction (Fig. 2). Both OsSUMO2-F1 and -R1 primers have *Not*I recognition sequence at the 5' flanking region and the set of primers were used for PCR amplification of OsSUMO2 ORF fragment. The PCR amplified 0.3 kbp *Not*I-OsSUMO2-*Not*I fragment was A-tailed and cloned into pGEM-T easy vector (Promega), and resulting pGEM/SUMO2 ORF was digested with *Not*I. The purified 0.3 kbp *Not*I SUMO2 ORF fragment was ligated into *Not*I digested pDsRed (Kitajima et al., 2009). The

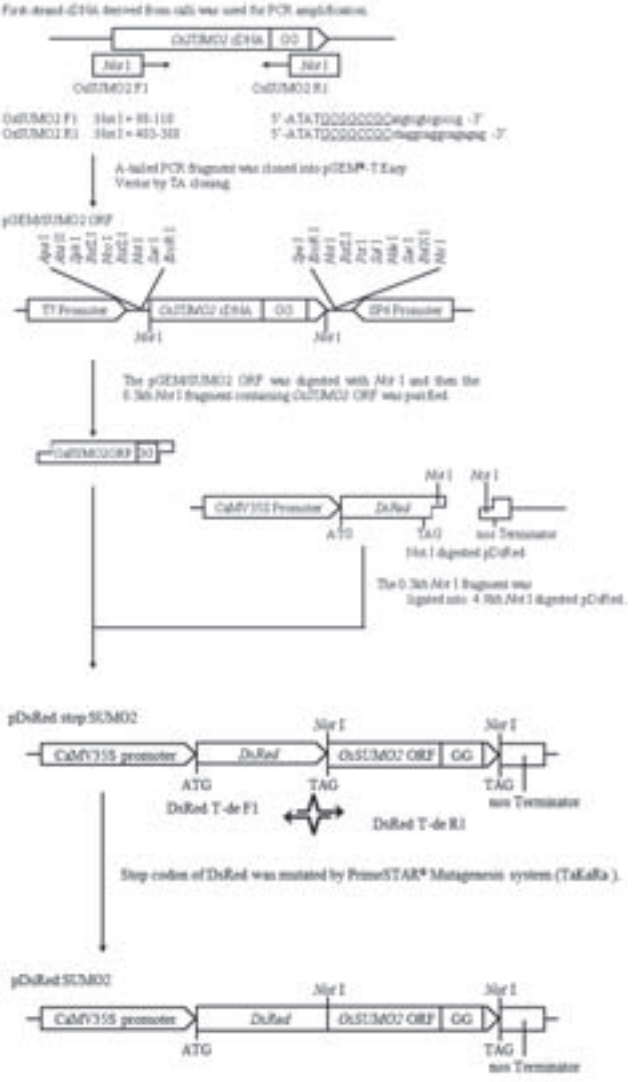

Fig. 2. Construction of pDsRed:SUMO2
OsSUMO2 cDNA was cloned and digested by *Not*I. The 0.3 kbp *Not*I fragment of the OsSUMO2 ORF was ligated into the pDsRed (Kitajima et al., 2009) *Not*I site in-flame. The stop codon at the C-terminal end of DsRed was mutated and the resulting vector pDsRed:SUMO2 was sequenced.

resulting pDsRedstop:SUMO2 was mutated by using DsRedT-deF1 and DsRedT-deR1 primers(Table 1) and PrimeSTAR Mutagenesis system (TaKaRa). Deletion of 676 bp T of *DsRed* resulted deletion of stop codon of C-terminal end of DsRed and generated inframe SUMO2 ORF downstream of DsRed. Resulted pDsRed:SUMO2 was sequenced and confirmed the structure.

Next, OsSUMO1 ORF was PCR-amplified from first-strand cDNA and was used for pDsRed:SUMO1 construction (Fig. 3). Both OsSUMO1 -F1 and -R1 primers have *Not*I recognition sequence and were used for PCR amplification of OsSUMO1 ORF fragment. The PCR amplified 0.3 kbp *Not*I-

Table 1. Information of primers for deletion mutation

primer name	position	primer sequence	cDNA sequence
DsRed T-de F1[*1]	DsRed 669-678△676 + NotI + OsSUMO2 98-101	5'- GTTCCTG/AGCGGCCGCATGT -3'	5'- GTTCCTGTAGCGGCCGCATGT -3'
DsRed T-de R1[*1]	NotI + DsRed 669-654 △676	5'- GGCCGCT/CAGGAACAGGTGG -3'	5'- GGCCGCTACAGGAACAGGTGG -3'
SUMO1 GG-de F1[*2]	OsSUMO1 378-405 △385-390 + GCG	5'- CCAGACT/TGCCTGCCTGCCTAGGCG -3'	5'- CCAGACTGGAGGCTGCCTGCCTGCCTAGGCG -3'
SUMO1 GG-de R1[*2]	OsSUMO1 398-367 △390-385	5'- GGCAGGCA/AGTCTGGTGGAGCATGGC -3'	5'- GGCAGGCAGCCTCCAGTCTGGTGGAGCATGGC -3'
SUMO2 GG-de F1[*3]	OsSUMO2 376-403 △383-388 + GCG	5'- CCAGACT/TCTCTGCCTGCCTAGGCG -3'	5'- CCAGACTGGGGGCTCTCTGCCTGCCTAGGCG -3'
SUMO2 GG-de R1[*3]	OsSUMO2 396-365 △388-383	5'- GGCAGAGA/AGTCTGGTGAAGCATGGC -3'	5'- GGCAGAGAGCCCCCAGTCTGGTGAAGCATGGC -3'
SUMO3 GG-de F1[*4]	OsSUMO3 375-396 △382-387 + GCGGCCGCC	5'- GCTGATC/GCCGCTTGAGCGGCCGCC -3'	5'- GCTGATCGGTGGCGCCGCTTGAGCGGCCGCC -3'
SUMO3 GG-de R1[*4]	OsSUMO3 395-364 △387-382	5'- CAAGCGGC/GATCAGCTCCTCGAAGAA -3'	5'- CAAGCGGCGCCACCGATCAGCTCCTCGAAGAA -3'

[*1] Both DsRed T-de F1 and DsRed T-de R1 primers lack the DNA sequences corresponding DsRed stop codon, T (676), and A (676) respectively. [*2] Both SUMO1 GG-de F1 and SUMO1 GG-de R1 primers lack the DNA sequences corresponding OsSUMO1 GG-motif, GGAGGC (385-390), and GCCTCC (390-385) respectively. [*3] Both SUMO2 GG-de F1 and SUMO2 GG-de R1 primers lack the DNA sequences corresponding OsSUMO2 GG-motif, GGGGGC (383-388), and GCCCCC (388-383) respectively.
[*4] Both SUMO3 GG-de F1 and SUMO3 GG-de R1 primers lack the DNA sequences corresponding OsSUMO3 GG-motif, GGTGGC (382-387), and GCCACC (387-382) respectively. The deletion sites were indicated by slash in the primer sequences.
The GenBank accession unmbers of OsSUMO1, 2, and 3 cDNA sequences are AK058869, AK06243, and AK107923, respectively.

OsSUMO1-*Not*I fragment was A-tailed and cloned into pGEM-T easy vector, and resulting pGEM/SUMO1 ORF was digested with *Not*I. The 0.3 kbp *Not*I fragment was purified and used for ligation reaction (Fig. 3). pDsRed:SUMO2 was digested with *Not*I and the SUMO2 ORF was removed. Resulting 4.9 kbp *Not*I fragment of pDsRed:SUMO2 was ligated to the 0.3 kbp *Not*I fragment and then pDsRed:SUMO1 was generated.

OsSUMO3 ORF was PCR-amplified from first-strand cDNA and was used for pDsRed:SUMO3 construction (Fig. 4). Both OsSUMO3 -F1 and -R1 primers have *Not*I recognition sequence at the 5' flanking region and the set of primers were used for PCR amplification of OsSUMO3 ORF fragment. The PCR amplified 0.3 kbp *Not*I-OsSUMO3-*Not*I fragment was A-tailed and cloned into pGEM-T easy vector, and then resulting pGEM/SUMO3 ORF was digested with *Not*I. Then the 0.3 kbp *Not*I fragment was purified and used for ligation reaction (Fig. 4). pDsRed:SUMO2 was digested with *Not*I and then the SUMO2 ORF was removed. Resulting 4.9 kbp *Not*I fragment of pDsRed:SUMO2 was ligated to the 0.3 kbp SUMO3 ORF and then pDsRed:SUMO3 was generated.

Construction of DsRed:SUMO1ΔGG, DsRed:SUMO2ΔGG, and DsRed:SUMO3ΔGG.

To study the role of GG motif on cellular localization of SUMO proteins, we obtain a series of deletion mutant vectors pDsRed:SUMO1ΔGG (Fig. 5), pDsRed:SUMO2ΔGG (Fig. 6), and pDsRed:SUMO3ΔGG (Fig. 7).

We designed SUMO1GG-de F1 and SUMO1GG-de R1 primers(Table 1) and then the pDsRed:SUMO1 was

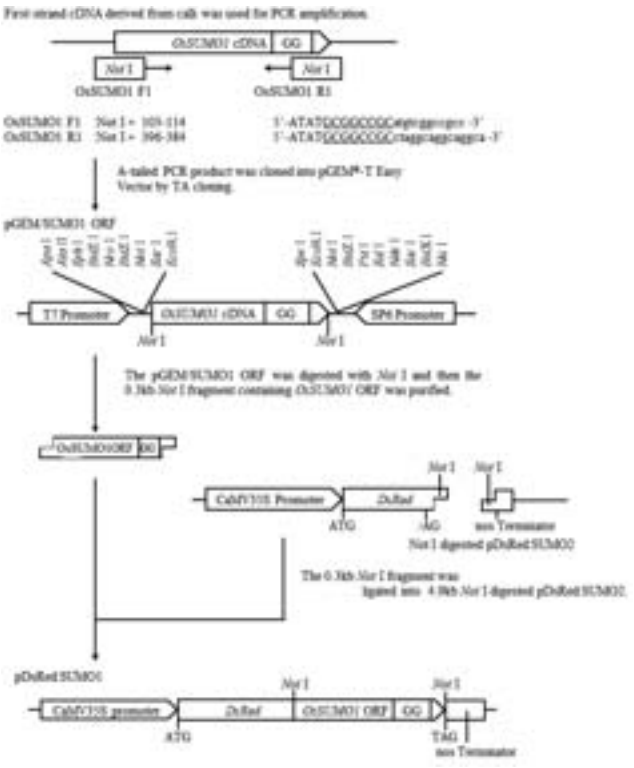

Fig. 3. Construction of pDsRed:SUMO1
OsSUMO1 cDNA was cloned and digested with *Not*I. The 0.3kbp fragment of OsSUMO1 ORF was ligated into the 4.9 kbp fragment of *Not*I digested pDsRed:SUMO2 without SUMO2 fragment. Resulting vector pDsRed:SUMO1 was sequenced and confirmed the structure.

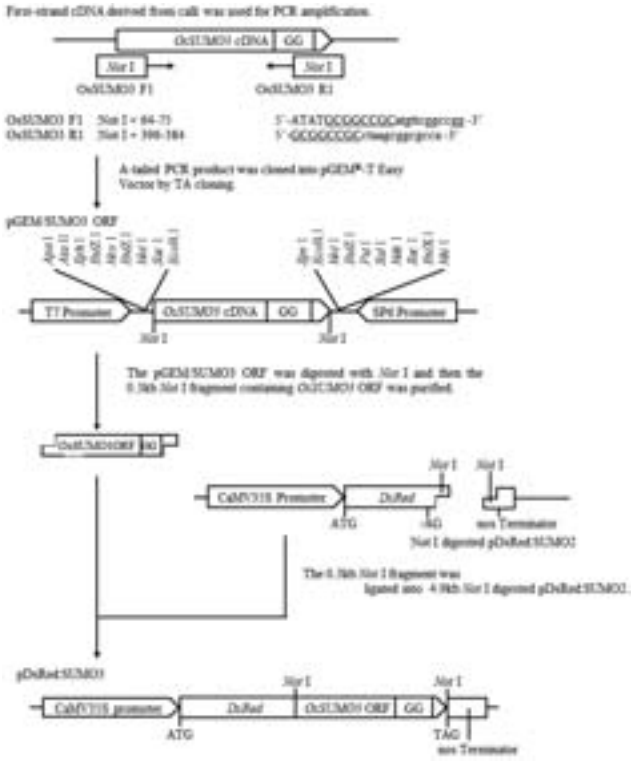

Fig. 4. Construction of pDsRed:SUMO3
OsSUMO3 cDNA was cloned and digested with *Not*I. The 0.3kbp fragment OsSUMO3 ORF was ligated into the 4.9 kbp fragment of *Not*I digested pDsRed:SUMO2 without SUMO2 fragment. Resulting vector pDsRed:SUMO3 was sequenced and confirmed the structure.

mutagenized and deleted the GG motif of the SUMO1 C-terminal by using the set of primers and PrimeSTAR Mutagenesis system (TaKaRa). Resulting pDsRed:SUMO1 Δ GG was sequenced and confirmed the vector structure (Fig. 5). In the same way, the pDsRed:SUMO2 was mutagenized and deleted the GG motif of the SUMO2 C-terminal by using SUMO2GG-de F1 and SUMO2GG-de R1 primers(Table 1) and then pDsRed:SUMO2 Δ GG was created (Fig. 6). Next, the pDsRed:SUMO3 was mutagenized and deleted the GG motif of the SUMO3 C-terminal by using SUMO3GG-de F1 and SUMO3GG-de R1 primers (Table 1), and then pDsRed:SUMO3 Δ GG was created (Fig. 7). These pDsRed:SUMO1 Δ GG (Fig. 5), pDsRed:SUMO2 Δ GG (Fig. 6), and pDsRed:SUMO3 Δ GG (Fig. 7) were used for analysis of cellular localization in onion epidermal cells.

Analysis of expression and cellular localization of OsSUMO proteins.

DsRed:SUMO1, DsRed:SUMO2, and DsRed:SUMO3 were transiently expressed in onion epidermal cells and the fluorescent DsRed signals were observed by confocal laser scanning microscopy (Fig. 8).

The DsRed:SUMO1 and the DsRed:SUMO2 signals are

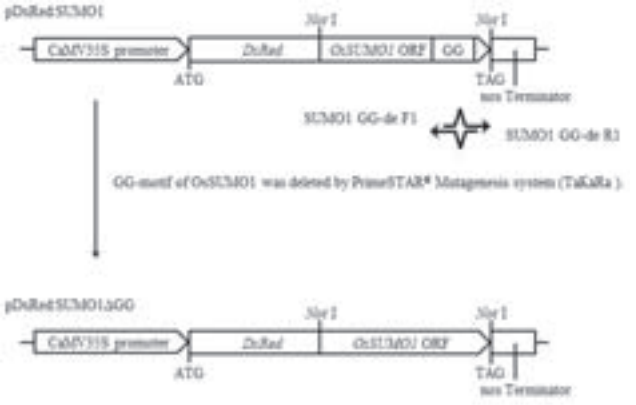

Fig. 5. Construction of pDsRed:SUMO1 Δ GG
GG motif of pDsRed:SUMO1 was deleted and resulting vector pDsRed:SUMO1 Δ GG was sequenced and confirmed the structure.

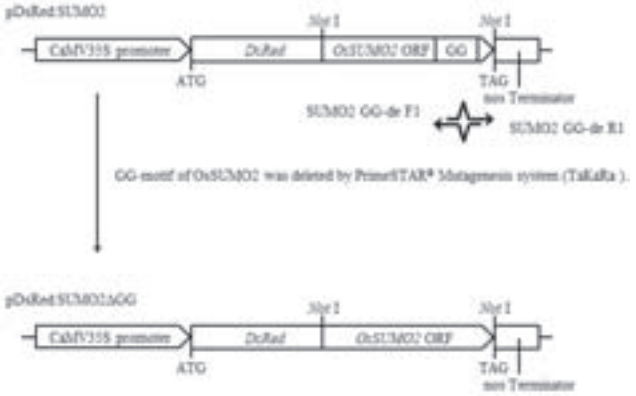

Fig. 6. Construction of pDsRed:SUMO2 Δ GG
GG motif of pDsRed:SUMO2 was deleted and resulting vector pDsRed:SUMO2 Δ GG was sequenced and confirmed the structure.

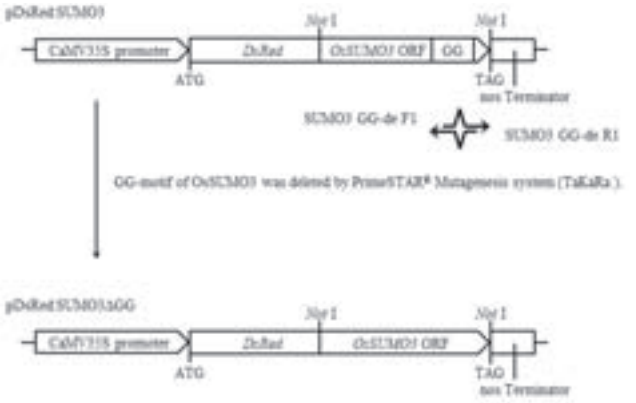

Fig. 7. Construction of pDsRed:SUMO3 Δ GG
GG motif of pDsRed:SUMO3 was deleted and resulting vector pDsRed:SUMO3 Δ GG was sequenced and confirmed the structure.

detected in both cytoplasm and nucleus but not in nucleolus (Fig. 8 A, B). Control DsRed signal also detected in both cytoplasm and nucleus but not in nucleolus (Fig. 8 D). Control DsRed showed clear gap at the borderzone around nucleus, whereas DsRed:SUMO1/2 did not showed any gap around nucleus(Fig. 8 A, B). The result suggested that the DsRed:SUMO1/2 also located in nuclear envelop or nuclear pore complex as expected from previous study (Zhang et al., 2002).

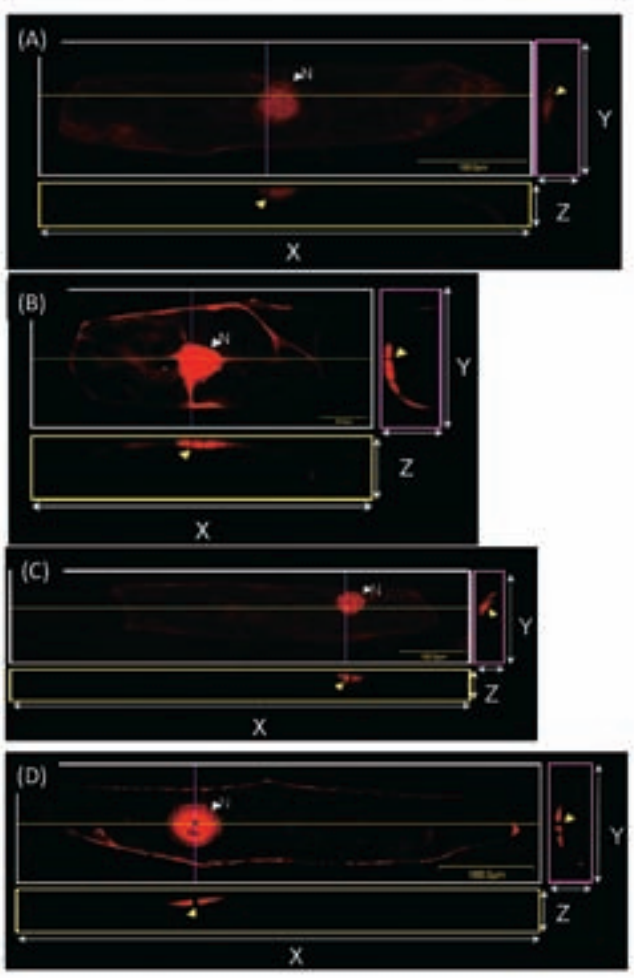

Fig. 8. Transient Expressions of DsRed:SUMOs in Onion Cells. pDsRed:SUMOss were transiently expressed in onion epidermal cells, and the DsRed signals were detected by confocal scanning laser microscopy. *White open culmns* showed 2D image, X-Y matrix showed horizontal scanning images of DsRed signals. *Yellow open columns* and *magenta open columns* showed vertical stacked images of DsRed signals. *White arrow heads* showed nucleus, and *yellow arrow heads* showed nucleolus. (A) Distribution of DsRed signals of DsRed:SUMO1 in onion epidermal cell. (B) Distribution of DsRed signals of DsRed:SUMO2 in onion epidermal cell. (C) Distribution of DsRed signals of DsRed:SUMO3 in onion epidermal cell. (D) Distribution of DsRed signals by expression of 35S::DsRed.

In contrast to the DsRed:SUMO1/2, the DsRed:SUMO3 signal is almost detected in nucleus, and form nuclear subdomains (Fig. 8 C). Such nuclear substructure was previously observed, when the SUMO was co-transformed with SCE1 (Lois et al., 2003). Therefore, the result indicated that DsRed fused OsSUMO3 was processed, activated and bind to certain substrate proteins by SUMO activating system internal onion epidermal cells. Moreover, the DsRed:SUMO3 does not locate on nuclear envelope or nuclear pore complex. The result suggested that the activated DsRed:SUMO3 does not locate on nuclear envelope or nuclear pore complex.

Analysis of effect of GG motif on cellular localization of SUMO proteins.

To analyze the effect of GG motif on cellular localization of SUMO proteins, pDsRed:SUMOΔGG vectors were transiently expressed in onion epidermal cells, and then the expression signals were observed (Fig. 9). Faint DsRed:SUMOΔGGs signals were detected in both cytoplasm and nucleus. In case of DsRed:SUMO3ΔGG, subnuclear structures were disappeared, and smear signal was detected in nucleus but not in nucleolus. Because of GG motif is also necessary to

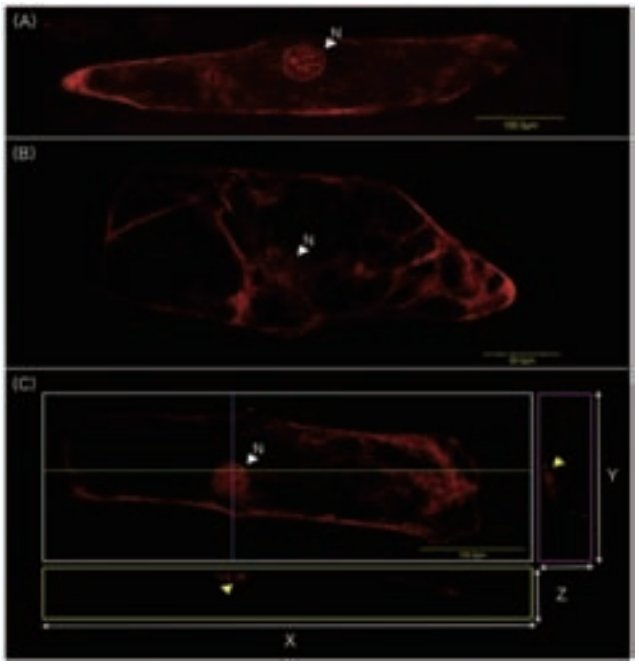

Fig. 9. Transient Expressions of DsRed:SUMOΔGG s in Onion Cells.
pDsRed:SUMOΔGGs are transiently expressed in onion epidermal cells, and the DsRed signals are detected by confocal scanning laser microscopy. Each panel showed stacked 2D image of DsRed signals. (A) Distribution of DsRed signals of DsRed:SUMO1ΔGG in onion epidermal cell. (B) Distribution of DsRed signals of DsRed:SUMO2ΔGG in onion epidermal cell. (C) Distribution of DsRed signals of DsRed:SUMO3ΔGG in onion epidermal cell.

processing of SUMO precursor in plant (Murtas et al., 2003), unprocessed DsRed:SUMO1ΔGG and DsRed:SUMO2ΔGG might be unstable and inhibited to intranuclear accumulation. Similarly, DsRed:SUMOΔGG might be unstable, and failure to form the subnuclear structures. These results indicated that the GG motif of SUMO1, 2, and 3 is necessary for processing, activation and accumulation of OsSUMO1, 2, and 3 proteins in the nucleus.

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イネ SUMO 遺伝子の単離および発現と細胞局在性の解析

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要　約

Small-ubiquitin related modifier (SUMO) はユビキチン様タンパク質の一種であり、翻訳後修飾により多様なタンパク質の機能を調節する。SUMO 前駆体タンパク質はＣ末側に存在するジグリシン（GG）モチーフの末端でプロセシングされ、一連の酵素反応により活性化され基質タンパク質に結合する。私たちはイネ SUMO1-3遺伝子を単離し、DsRed 融合タンパク質である DsRed:SUMO1，２、および３をタマネギ表皮細胞において一過的に発現させ、その細胞局在性を共焦点レーザー顕微鏡により解析した。DsRed:SUMO1および DsRed:SUMO2のシグナルは核と細胞質にも分布したが、核小体には観察されなかった。DsRed:SUMO3の場合は、主に核に局在し、そのシグナルは細胞核ドメイン様構造を形成した。私たちはまた、GG モチーフ欠失変異細胞内局在性に関わる影響をテストした。GG モチーフ欠失変異を持つベクター、pDsRed:SUMO1ΔGG、pDsRed:SUMO2Δ GG、および pDsRed:SUMO3ΔGG を構築し、タマネギ表皮細胞において発現させた。GG モチーフの欠失変異は DsRed:SUMO Δ GG タンパク質の核への集積を抑制した。これらの結果から、OsSUMO 前駆体タンパク質のＣ末端のプロセシングがタマネギ表皮細胞における OsSUMO タンパク質の核への局在性に必要であることが示唆された。

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キーワード：SUMO、イネ、タマネギ表皮細胞、GG モチーフ、DsRed

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