RESEARCH ARTICLE

*ScienceAsia* 40 (2014): **278–284**

doi: 10.2306/scienceasia1513-1874.2014.40.278

# Isolation and expression of *FMOgs-ox1* from Korean radish

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*Received 24 Feb 2014*
*Accepted 25 Aug 2014*

**ABSTRACT**: Flavin monooxygenase (FMO) is one of the most important enzymes involved in glucosinolate biosynthesis. In this study, the full length of *FMO* gene (*RsFMOgs-ox1*) encoding a putative FMO protein composed of 450 amino acids was successfully cloned using the RACE-PCR method. The amino acid sequence of RsFMOgs-ox1 has high similarities of 92% and 83% with BrFMOgs-ox1 and AtFMOgs-ox1,2,3, respectively, and the gene structure of *FMOgs-ox1* is similar to its plant homologues. Quantitative (qPCR) analysis revealed that *RsFMOgs-ox1* was highly expressed during early seedling development. In mature radish, the highest expression was observed in the leaves, while the lowest transcript was evident in the root. The expression of *RsFMOgs-ox1* was also regulated by wounding, notably 1 day after treatment. Subcellular localization in *Arabidopsis* showed that RsFMOgs-ox1 was localized in the cytoplasm and nuclei. This study allows us to understand something about *RsFMOgs-ox1* function in glucosinolate biosynthesis.

**KEYWORDS**: glucosinolate, RACE-PCR, real-time PCR, subcellular localization

## INTRODUCTION

Radish (*Raphanus sativus* L.) is an edible root vegetable crop of the Brassicaceae family widely cultivated in the world, especially in Asia. This crop has been shown to have high glucosinolate content in comparison with other Brassicaceae members[1]. This vegetable has been considered as healthy food due to its valuable nutrient contents such as vitamin C, potassium, magnesium, glucosinolate, and many other beneficial molecules (ndb.nal.usda.gov). Although more than 120 different kinds of glucosinolates have been reported in plants, the major glucosinolate in radish root is 4-methylthio-3-butenyl glucosinolate[2] as the characteristic glucosinolate with the common name of glucoraphasatin[3] (GRH).

Glucosinolate compounds have received much attention because their breakdown products display several potent bioactivities that serve as plant defence, as well as anticarcinogenesis compounds, especially in mammals. Flavin monooxygenase (FMO) is one of the important enzymes involved in glucosinolate biosynthesis. It catalyses the conversion of methylthioalkyl glucosinolate into methylsulphinyl glucosinolate through *S*-oxygenation. Isothiocyanate, a derived molecule from methylsulphinyl glucosinolate, has been proposed to be a key molecule conferring anticancer activity and plant defence[4]. In radish plant, 4-methylsulphonyl-3-butenyl isothiocyanate derived from glucoraphasatin has also been reported as a potent inducer of hepatic enzymes involved in the detoxification of chemical carcinogens[5]. Among several kinds of glucosinolates, GRH and glucoraphenin (GRE) have been shown to be able to induce phase-II xenobiotic metabolising enzymes with different induction profiles. Compare to GRH, low dosage of GRE is sufficient to trigger the cytochrome P-450 (CYP)-associated monooxygenases and the postoxidative metabolism[6].

To date, most of the FMO genes responsible to convert methylthioalkyl glucosinolate into methylsulphinyl glucosinolate have been identified and characterized in *Arabidopsis*[4,7]. There are five FMO genes (*FMOgs-ox1–5*) which have been reported to be involved in glucosinolate biosynthesis in *Arabidopsis*. FMOgs-ox2, FMOgs-ox3 and FMOgs-ox4 show broad substrate specificity and catalyse the conversion of methylthioalkyl glucosinolate to methylsulphinylalkyl glucosinolate. In contrast, FMOgs-ox5 shows substrate specificity against the long-chain 8-methylthiooctyl glucosinolate[7].

At the cellular level, aliphatic glucosinolate biosynthesis occurs in the cytoplasm, chloroplast and endoplasmic reticulum (ER), in which the initial deamination process is catalysed by BCTA4 enzyme in the cytoplasm[8]. In the next step, the side chain elongation is mediated by MAM enzymes in the chloroplast[9]. Finally, the core structure formation is

catalysed by enzymes localized in the ER-membrane such as CYPs [10–12].

A recent report on subcellular localization of FMO in *Arabidopsis* revealed that FMOgs-ox1 basically located in vascular tissues, endodermis flower stalk and epidermal cells in the leaf [13]. Transient expression study using tobacco leaves also indicated that FMOgs-ox1 is a cytosolic protein. In the radish however the functional study of FMO, including gene expression, subcellular localization and other molecular characterization, has not been reported. In this study, the molecular properties of *RsFMOgs-ox1* were characterized, and its potential role in the glucosinolate biosynthesis is discussed. The result of this study would be valuable for future vegetable research related with anti-carcinogenic compound.

## MATERIALS AND METHODS

### Plant materials and growth condition

Radish (*R. sativus* L.) inbred line Chungguk chongpi was used for all experiments. Seeds were sterilized with 70% ethanol and washed with distilled water to remove the seed coat, and then germinated in Murashige and Skoog medium [14]. For RNA extraction and other experiments, the radish seedling (sprouts) were harvested at indicated time, immediately frozen in liquid nitrogen, and stored at $-80$ °C until use.

### RNA isolation and cloning

Samples were collected from seed, sprout at 3, 5, 7, 9, and 11 days after germination, shoot, leaf, stem, inflorescence, flower, pod, and root. The total RNA from each sample was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to manufacturer's instruction. For cDNA synthesis, 1 µg of total RNA were mixed with oligo $(dT)_{18}$ primer and incubated at 70 °C for 5 min. Subsequently, the samples were mixed with RT-premix consisting of buffer, dNTP and reverse transcriptase (Bioneer, Daejon, Korea) in a total volume of 20 µl and incubated at 42 °C for 90 min. The reverse transcriptase was inactivated by incubating the mixture at 95 °C for 5 min.

For cloning *RsFMOgs-ox1*, the *Pfu-x* polymerase (Solgent, Daejeon, Korea) was used to amplify the target gene. For the first cloning of *RsFMOgs-ox1* (668 bp), 1.0 µl of the cDNA sample was used as a template in a 50 µl total reaction mix containing degenerate primers *RsFMO*-forward (5′-ATG GCA CCA GCT CAA AAC YCA ATC AGT TC-3′) and *RsFMO*-reverse (5′-ACG TCT CTA CTA ATA TCA CTA CCG CTC GC-3′). The PCR mixture was initially denatured at 95 °C for 5 min and then subjected to 40 cycles of the following conditions: 95 °C for 15 s, 53 °C for 15 s, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. The PCR products were analysed on a 1% (w/v) agarose gel containing ethidium bromide. The fragment was purified using gel purification system (Qiagen, Valencia, CA, USA), A-tailed, and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) using standard cloning procedures [15].

### RACE-PCR

For cloning the full-length *RsFMOgs-ox1*, 3′RACE-PCR was performed using 3′RACE System Kit according to the manufacturer's instructions (Invitrogen, USA). Briefly, the first strand cDNA was synthesized with SuperScript II reverse-transcriptase. A 2.0 µl sample of the cDNA was used for subsequent PCR amplification in a total volume of 50 µl. The gene specific primer used for PCR amplification was 5′-CGA ATG GCA CCA GCT CAA AAC CCA AT-3′ in combination with abridged universal amplification primer (Invitrogen, USA). The PCR mixture was initially denatured at 94 °C for 3 min and then subjected to 35 cycles of the following conditions: 94 °C for 15 s, 53 °C for 15 s, and 72 °C for 2 min, with a final extension at 72 °C for 5 min. Gel purification and cloning procedures were carried out as described above.

### Sequence analysis and alignment

The nucleotide sequence obtained from cloning work was used for the next sequence analysis. Amino acid sequence was obtained by translating the nucleotide sequence using the Translation program [16] (www.bioinformatics.org/sms/index.html). To determine the identity between the RsFMOgs-ox1 protein and similar proteins from other species, the amino acid sequences were analysed using the NCBI-BLAST program [17, 18] (blast.ncbi.nlm.nih.gov/Blast.cgi). The conserved domain of RsFMOgs-ox1 was determined by BLASTP and prosite [19] (www.expasy.ch/prosite).

### Quantitative real-time PCR

For quantitative real time PCR (qPCR), 1 µl cDNAs prepared from several organs were used as template in the SYBR Green PCR Master mix (Bioneer, Daejon, Korea) in a total volume of 25 µl. The primers used for qPCR were 5′-CAG GCA TGG GCA TAC AAT TC-3′ and 5′-TCT TCT GCG ACC CAG TCA AG-3′. The PCR conditions were 95 °C for 10 min, 40 cycles of 10 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. Samples were prepared in triplicate. The amplification

of target genes was analysed using the Optical System (7500 Real-time PCR software version 2.0) provided with the AB 7500 cycler (Applied Biosystems, Foster City, USA). The relative expression levels of each transcript were obtained by normalization to the radish RNA polymerase II (*RPII*) gene[20]. Calculation was based on the comparison of the distinct cycle determined by cycle threshold values (Ct) at a constant level of fluorescence. The delta-delta Ct method[21] was employed for final data analysis.

### Subcellular localization

The open reading frame (ORF) of *RsFMOgs-ox1* was PCR-amplified using *Pfu*-x polymerase with primer set as follows: *RsFMO-GFPF* (5′-GAA TTC ATG GCA CCA GCT CAA AAC CCA ATC-3′) and *RsFMO-GFPR* (5′-GCG GAT CCC TAG CTA ATC AAC TTC TTA CTA GCA-3′). The enzyme sites for *Eco*RI and *Bam*HI are underlined. PCR product were digested with *Eco*RI and *Bam*HI, and ligated into pEGAD vector[22] to create the construct of pEGAD-*RsFMOgs-ox1*. The pEGAD-*RsFMOgs-ox1* construct and pEGAD vector (control) was separately introduced into *Agrobacterium tumefaciens* strain GV3101 by the heat shock method. The *Agrobacterium* cell harbouring the construct was transformed into *Arabidopsis* Col-0 to generate a transgenic plants carrying stable GFP expression using the floral dipping method[23]. Selection for transgenic lines was conducted as described previously[24]. $T_2$ seeds were grown on MS media for 1 week prior to microscopic analysis.

## RESULTS

### Cloning of *RsFMOgs-ox1* and its molecular characterization

In the beginning, the sequence information derived from wild radish (*R. raphanistrum*) end-sequence tags[7] was utilized for primer design. By using cDNA template isolated from commercial radish (*R. sativus* L.), the expected 668-bp PCR product was obtained (Fig. 1a). This partial sequence covered the start codon (ATG) similar to previously reported *RsFMOgs-ox1* from other plants. However, conventional PCR approach for isolating the C-terminal region to clone the *RsFMOgs-ox1* gene was unsuccessful. To overcome this problem, the rapid amplification of cDNA ends (RACE)-PCR method was applied to obtain the remaining sequence in the C-terminal part. After collecting the nucleotide sequence in the C-terminal part, the primer set for amplification the open reading frame (ORF) of *RsFMOgs-ox1* was designed

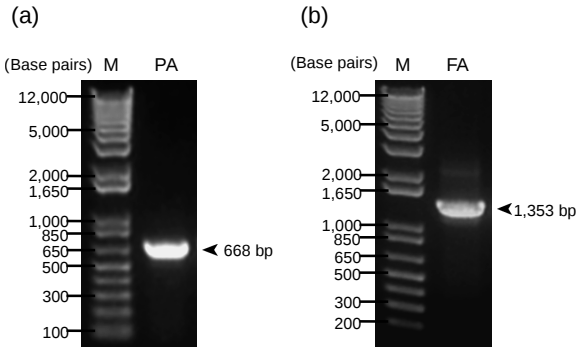


**Fig. 1** Cloning of *RsFMOgs-ox1*. PCR amplification of partial *RsFMOgs-ox1* using (a) normal PCR and (b) full-length *RsFMOgs-ox1* with 3′RACE-PCR; *M*, marker; *PA*, partial amplicon; *FA*, full amplicon.

(forward, 5′-ATG GCA CCA GCT CAA AAC CCA ATC-3′; reverse, CTA GCT AAT CAA CTT CTT ACT AGC A-3′) and used for subsequent cloning PCR. As shown in Fig. 1b, the amplicon size resulted from this reaction is 1353 bp, relatively similar size with *RsFMOgs-ox1* from other plant species reported previously[4,7].

The PCR product was then cloned into the T-vector and confirmed by sequencing. The sequence were analysed and compared with *RsFMOgs-ox* homologues from other plant species. The *RsFMOgs-ox1* composed of 1353 bp encodes a polypeptide of 450 amino acid residues with a calculated molecular mass (Mw) of 50.7 kDa and an isoelectric point (pI) of 5.79. FAD- and NADPH-binding motif (GxGxxG) is located in the N-terminal and central regions, respectively, (Fig. 2a). In addition, FMO identifying motif (FxGxxxHxxxY/F) is determined to be located at amino acid between 324 and 334 in the C-terminal region of RsFMOgs-ox1 protein.

Phylogenetic tree was generated by comparing RsFMOgs-ox1 protein with its homologues from other plant species. The result showed that RsFMOgs-ox1 has high identity (92%) and located in the same cluster with FMOgs-ox1 from *Brassica rapa* (BrFMOgs-ox1) (Fig. 2b). Hence our RsFMO clone is designed as RsFMOgs-ox1.

### Expression pattern of *RsFMOgs-ox1*

In previous reports, the *FMOgs-ox* member in *Arabidopsis* expressed in several organs such as leaves, flowers, and seeds[4,25,26]. In this study, the quantitative real-time PCR (qPCR) was performed to determine the expression pattern of *RsFMOgs-ox1*. Total RNA from seed, seedling (sprout) at several stages, and

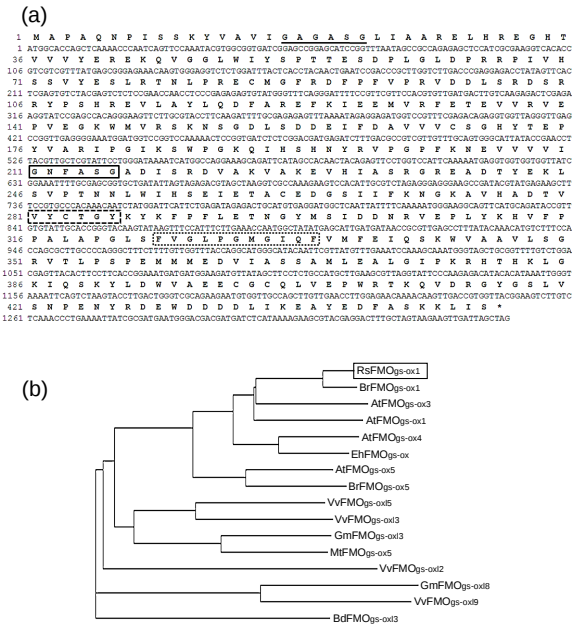


**Fig. 2** Sequence of *RsFMOgs-ox1* and phylogenetic tree analysis. (a) Nucleotide and deduced amino acid sequences of *RsFMOgs-ox1*. The FAD binding site is underlined and the NADPH binding motif is boxed (bold line). The conserved motifs for FMO family are boxed with dashed- and dotted-lines, respectively. (b) Phylogenetic comparison of RsFMOgs-ox1 to other plant FMOgs-ox homologues. BrFMOgs-ox1 (accession no. ACR10274.1), AtFMOgs-ox3 (NP 176444.1), AtFMOgs-ox1 (NP 176761.1), AtFMOgs-ox4 (NP 564797.1), EhFMOgs (ABJ98059.1), AtFMOgs-ox5 (NP 172678.3), BrFMOgs-ox5 (ACR10275.1), VvFMOgs-oxl5 (XP 003631450.1), VvFMOgs-oxl3 (XP 003631451.1), GmFMOgs-oxl3 (XP 003538706.1), MtFMOgs-ox5 (XP 003611271.1), VvFMOgs-oxl2 (XP 002265001.1), GmFMOgs-oxl8 (XP 003524682.1), VvFMOgs-oxl9 (XP 002281491.2), BdFMOgs-oxl3 (XP 003562230). Phylogenetic tree shows a graphical representation of evolutionary relationships and were constructed using the EBI-CLUSTALW algorithm. Rs, *Raphanus sativus*; Br, *Brassica rapa*; At, *Arabidopsis thaliana*; Eh, *Eutrema halophilum*; Mt, *Medicago truncatula*; Vv, *Vitis vinifera*; Gm, *Glycine max*; Bd, *Brachypodium distachyon*.

other organs were prepared and used as templates in the qPCR reaction. Weak transcriptional level was observed in seeds. During seedling development, the highest expression of *RsFMOgs-ox1* was observed 5 days after germination (Fig. 3a). The expression of *RsFMOgs-ox1* was also examined in vegetative organs (stem, leaves, roots) and generative organs such as inflorescence, flower, and pod. Among them, leaves

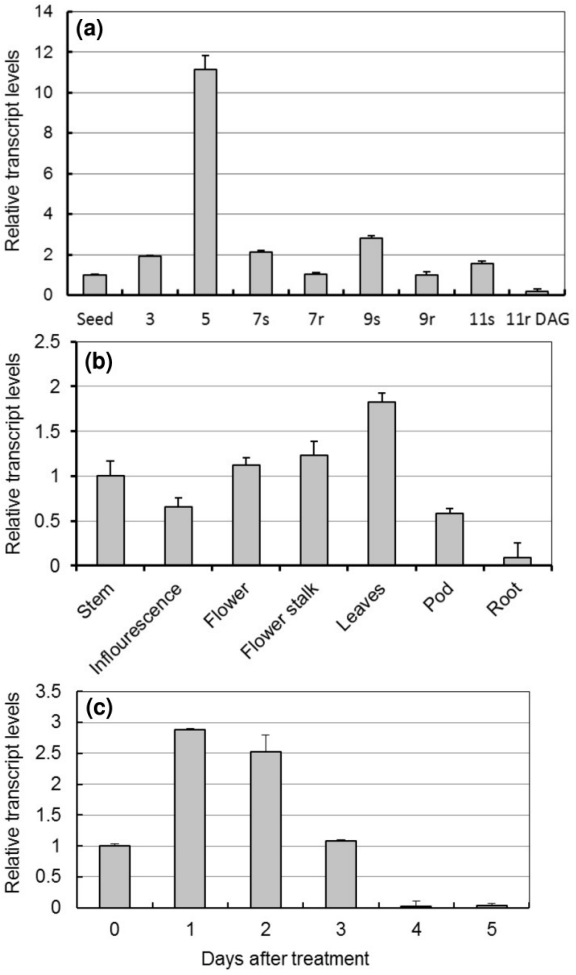


**Fig. 3** Expression pattern of *RsFMOgs-ox1*. The expression of *RsFMOgs-ox1* gene in (a) seedling, (b) radish organs, and (c) in response to wounding treatment; *s*, shoot; *r*, root; *DAG*, days after germination.

and roots showed the highest and lowest expression of *RsFMOgs-ox1*, respectively, (Fig. 3b). In addition to organ specific expression, the transcriptional level of *RsFMOgs-ox1* was also explored under a wounding treatment. The leaves were wounded with scissors and harvested at indicated time (Fig. 3c). The results showed that *RsFMOgs-ox1* transcript was high at 1 and 2 days after treatment (Fig. 3c).

## Subcellular localization of RsFMOgs-ox1

Localization within the cell can explain the protein's function. *Arabidopsis* FMOgs-ox1 has been reported to be localized in cytoplasm[13]. To investigate the subcellular localization of RsFMOgs-ox1, the ORF of *RsFMOgs-ox1* was fused to the C terminus of GFP and expressed under the control of the 35S promoter.

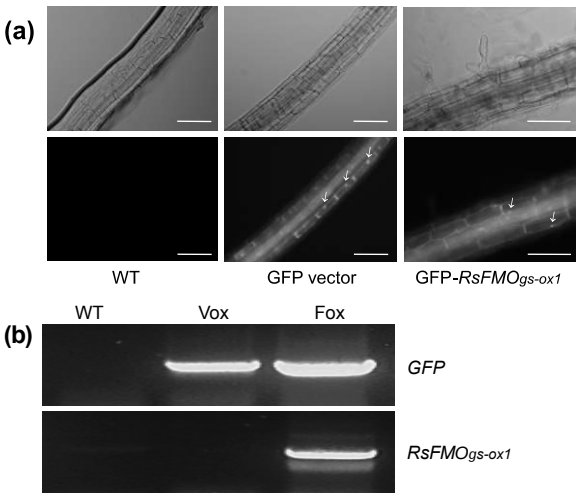


**Fig. 4** Subcellular localization of RsFMOgs-ox1. (a) The vector constructs were stably expressed in *Arabidopsis* transgenic lines. One week *Arabidopsis* roots were observed under bright light (upper panel) and GFP channel (lower panel). The arrow indicates nuclei. (b) PCR result confirmed the successful transformation. WT, non-transformant; Vox, *Arabidopsis* transformed with vector control; Fox, *Arabidopsis* transformed with *GFP-RsFMOgs-ox1*.

The resulting gene fusion (35S:GFP-*RsFMOgs-ox1*) and empty vector, pEGAD[22], was separately transformed into *Arabidopsis thaliana*. As shown in Fig. 4a, RsFMOgs-ox1 appeared to be localized in the nucleus and cytoplasm. To confirm a successful transformation in *Arabidopsis*, the genomic DNA isolated from seedling was used as templates in the PCR mixture with specific primer set for *GFP* and *RsFMOgs-ox1*. The results showed that the constructs were successfully introduced in the system (Fig. 4b).

## DISCUSSION

The availability of gene bank or whole genomic sequences in certain species is an essential factor for convenience and successful cloning work. Up to date, the complete genome sequence is available for the model plant *Arabidopsis*[27]. The gene bank for several crops and vegetables such as rice and tomato have also been released[28,29]. However, radish (*R. sativus* L.) whole genomic sequence is still partially available although has been released to the public[30]. In this study, the cloning work for *RsFMOgs-ox1* using the RACE-PCR[31] is reported. The RACE-PCR method was selected since gene screening using cDNA library in combination with DNA probe labelling is quite time consuming and laborious. Several genes from plants with limited genome sequence information, such as peach, seepweeds, and azuki bean, were isolated with above approach[32–34]. Using RACE-PCR, the coding sequence of *RsFMOgs-ox1* was successfully isolated from cDNA isolated from radish sprouts.

The sequence of *RsFMOgs-ox1* consists of 1353 bp, encodes 450 amino acid residues for flavin-containing monooxygenase (FMO). Similar size for FMOgs-ox proteins have been reported from other species such as *Arabidopsis*, human, yeast, and rice[4,13,35–37]. In silico analysis of conserved sequences for FMO protein attributes such as FMO identifying motif (FxGxxxHxxxY/F), FAD- and NADPH-binding motif (GxGxxG) are evident to exists in *RsFMOgs-ox1*. Amino acids sequence comparison revealed that RsFMOgs-ox1 has high identity with FMO from *Brassica rapa* (BrFMOgs-ox1) that probably plays a role in glucosinolate biosynthesis. However, except sequence information in NCBI, there is no follow-up report for the functional study of *BrFMOgs-ox1*. Hence it is worth to notice that the *RsFMOgs-ox1* reported here probably also involved in glucosinolate biosynthesis. However, further work is required to confirm this feasibility.

During seedling development, *RsFMOgs-ox1* is highly expressed at 5 days after germination. The fact that radish seedling (sprouts) contains high glucosinolates content indicates that *RsFMOgs-ox1* play a potential role in the biosynthesis of these molecules. However, additional functional characterization is necessary to elucidate the mode of action of *RsFMOgs-ox1* in glucosinolates biosynthesis pathway. In radish, although the glucosinolates content is different among cultivars, the general pattern of glucosinolates distribution is almost similar. In the seed, the major glucosinolate is GRE, while GRH is abundant in other organs. During germination, GRE content was slowly decreased and the GRH being increased[38]. The conversion of GRH into GRE is mediated by FMOgs-ox enzyme. Interestingly, although the *FMOgs-ox* is mainly involved in GRH-GRE conversion, the *Arabidopsis* knock out mutant did not show any significant difference for glucosinolates content in the seeds and leaves, indicating a compensation mechanism for *FMOgs-ox* genes member in *Arabidopsis*. In radish, at the moment, it is still not clear whether *FMOgs-ox* also exists in multi copy genes. More extensive genome wide study is required for the identification of *FMO* genes member in radish.

Glucosinolate-derived molecules also play a role in several other biological processes such as defence against insect attack and function as disease suppression caused by fungal pathogen[39,40]. Experi-

mental data from wounding treatment revealed that *RsFMOgs-ox1* expression is up-regulated at 1 day after wounding. This result suggests that *RsFMOgs-ox1* is involved in early signalling pathway for wounding stress.

Protein localization studies should provide information for the proper residence of certain protein to execute their function. Previously, Li et al[13] reported that *Arabidopsis* FMOgs-ox1 is localized in the cytoplasm. Bioinformatics analysis using pSORT[41] revealed that RsFMOgs-ox1 was predicted to be localized in cytoplasm. However, our data which is observed from stable expression in *Arabidopsis* revealed that RsFMOgs-ox1 is both cytosolic and nucleic protein. These data indicates that cytoplasm and nucleus are the appropriate intracellular location for RsFMOgs-ox1 to perform its function.

In summary, the result obtained from this study will be helpful for further pursuing functional studies of RsFMOgs-ox1 and its modification. Generating transgenic lines with ectopic or knock down expression of *RsFMOgs-ox1* in radish and its biochemical compounds analysis, such glucosinolates content, should be very interesting challenge.

*Acknowledgements*: This study was supported by iPET (Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries), Ministry of Agriculture, Food and Rural Affairs and by the GRRC Program of Gyeonggi Province (GRRC-CAU-2014-B01, Development of Baemoochae Kimchi and Postharvest Technology).

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