BioPublisher
Bioscience Methods 2015, Vol.6, No.1, 1-13
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Research Article Open Access

Eleven *StOSMs* Genes in the Potato Genome Response to Water Deficiency

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Bioscience Methods, 2015, Vol.6, No.1 doi: 10.5376/bm.2015.06.0001
Received: 08 Nov., 2014
Accepted: 13 Jan., 2015
Published: 20 Jan., 2015

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Preferred citation for this article:
Qiao et al., 2015, Eleven *StOSMs* Genes in the Potato Genome Response to Water Deficiency, Bioscience Methods, Vol.6, No.1 1-13 (doi: 10.5376/bm.2015.06.0001)

Abstract Osmotin plays an important role in plant response to low temperature and pathogen infection. To date little is known on existence and full function of *OSM* genes in genome-wide. In this study, based on an *OSM* expression sequence tag identified from a drought-treated subtractive library, *StOSMs* in the potato genome were identified and characterized in response to a water deficit gradient. BLAST and bioinformatic analysis revealed that there are at least eleven *StOSMs* in the potato genome. Among eleven *StOSMs*, eight *StOSM* mRNAs accumulated in leaf at the drought-lethal critical point (DLP) were at least 4-fold higher than in the control. The peak *StOSM*-8E mRNA accumulation was 49-fold higher than the control. Three *StOSM* mRNAs in leaf at DLP were 9.2-fold lower than in the control. This result of qRT-PCR quantification was identical with the FPKM values of all eleven *StOSMs* examined in the the Potato Genome Sequence database (PGSD). In conclusion, water deficiency does induce the regulation of the expression of the eleven *StOSMs*. Either up or downregulated by water deficiency, *StOSMs* play a positive role in enhancing potato tolerance to drought stress. Therefore, osmotins can be considered to be drought responsive molecules involved in withstanding water deficits. The result provides an evidence to reveal how potato adapts to drought stress.
Keywords OSM; Osmotin; Expression; mRNA; Drought

Introduction

Osmotin (accession No. M29279), encoding the stress response molecule osmotin, was initially identified in cultured tobacco cells (*Nicotiana tabacum* L. cv Wisconsin 38) induced by low water potential (Singh NK et al 1989). Subsequently, it was showed that abscisic acid (ABA), low temperature and NaCl can induce the accumulation of three *StOSM* mRNAs (pA13, pA35, and pA81) in *S. commersonii* grown *in vitro* (Zhu et al 1995). Later on *StOSM* was shown to exhibit antifungal activity against a range of fungal pathogens (Rivero et al. 2012, Mani et al. 2012 and Patade et al. 2013). Recently, *StOSM* overexpression in transgenic Arabidopsis and pepper results in H_2O_2 accumulation and the response of hypersensitive cell death in the leaf and reduces susceptibility to pathogen infection (Choi et al. 2013).

Remarkably, it is showed that plant osmotin, by binding to the adiponectin receptor as an agonist, activates a pathway concerned with the resistance to animal disease. Osmotin reduces ethanol neurotoxicity via the upregulation of the antiapoptotic Bcl-2 protein, and reverses synaptic dysfunction and neuronal apoptosis. The Bcl-2 protein appears to block a distal step in a common pathway for apoptosis and programmed cell death (Naseer et al. 2014 and Shah et al. 2014).

Osmotin is not only an intercellular component involved in stress tolerance but also an agonist that alternatively binds to the adiponectin receptor involved in animal apoptosis. However, although osmotin was identified in tobacco in 1989, little is known regarding the details of plant osmotin, particularly how many *OSM* members are present in a plant genome and how these members work together in response to stress.

In potato, nine *StOSM* genes were identified by screening a bacterial artificial chromosome (BAC) library. The nine *StOSM* genes are organised into two loci on chromosomes 08 and 11 (Castillo et al. 2005). Whether there are additional *StOSM* genes in the potato genome and the functional differences among the potato *StOSM* genes regarding the response to abiotic stress, such as drought, remain unknown.

With respect to the severity of the water shortage, each plant species is more or less tolerant to a certain drought level. A reliable and comparable level of plant tolerance to drought is known as the drought-lethal critical point (DLP). The availability of water in soil at a level lower than the DLP leads to plant death due to cell dehydration.

In this study, with a full-length *StOSM* cDNA isolated initially from a drought-treated subtractive library, eleven *StOSM* genes were identified using BLAST analysis of the Potato Genome Sequence database (PGSD). Leaf mRNA accumulation of *StOSMs* were assayed using RT-PCR and qRT-PCR and compared with FPKM (Fragments Per Kilobase of exon model per Million mapped reads) value of *StOSM* genes in the PGSD. The result indicated that expression of eight *StOSMs* in leafs at DLP was upregulated, three down-regulated. The result revealed that osmotins, as drought responsive molecules involved in withstanding water deficits in potato.

1. Results

1.1 Composition and structure of *StOSM* family

1.1.1 Cloning and osmotic stress response function of potato *StOSM*-3B

Through screening a subtractive cDNA library constructed from the young leaves of potato genotype ZHB at 20% ±2% water content in media (WCM) (unpublished work), an expressed sequence tag (EST) was identified. Sequence of the EST shared 99% identity with *StOSM*-3B belonging to AY737310 from *Solanum phureja* in the Genbank database (Castillo et al. 2005).

Using *StOSM*-3B ORF specific primers (O-F and O-R), a cDNA ORF with a length of 744 bp was cloned (Figure 1), which encoded osmotin with 248 amino acids. A sequence alignment showed 97%

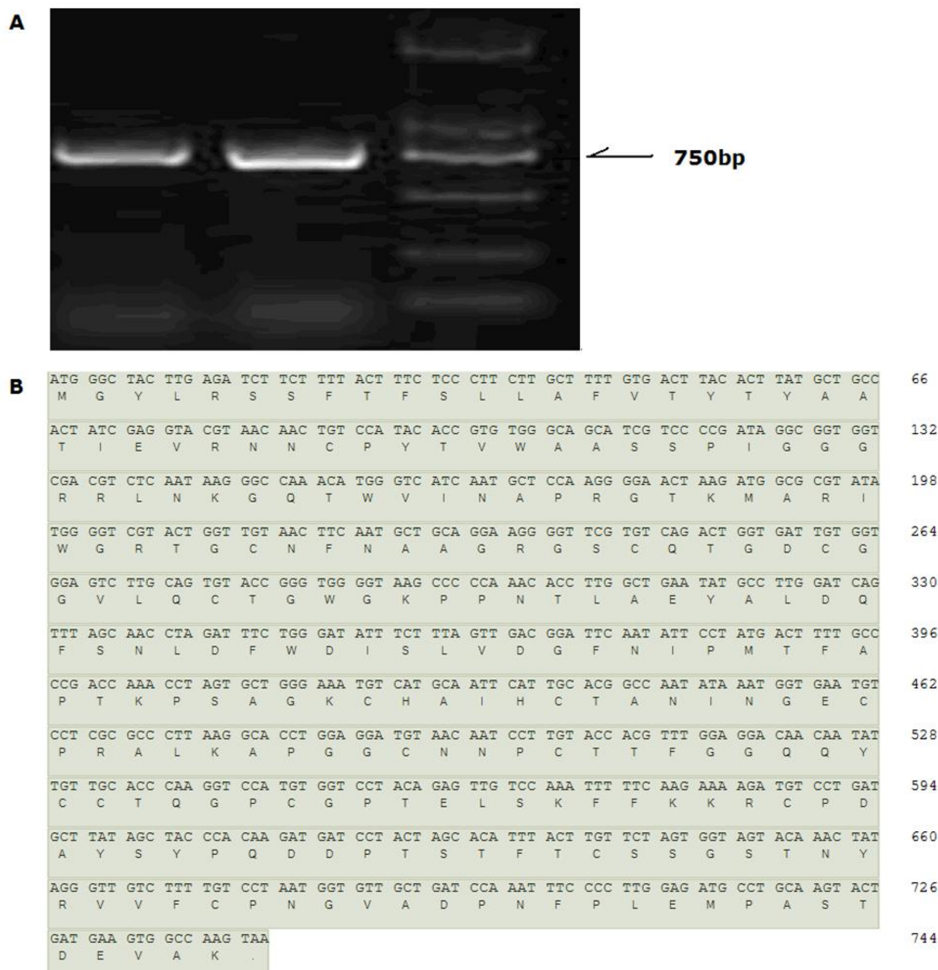

Figure 1 Cloning of the *StOSM*-3B ORF from the leaves of potato ZHB under 20% WCM. A: Product of PCR with *StOSM*-3B specific primer; B: cDNA sequence and putative amino acid sequence of *StOSM*-3B ORF isolated in this study

identity between the cDNA ORF and the *StOSM*-3B ORF. An alignment with the amino acid sequence showed that the putative amino acid sequences encoded by the isolated cDNA ORF have six residual substitutions compared with the sequence of *StOSM*-3B (Figure 2).

Figure 2 Alignment of the cDNA ORF isolated with *StOSM*-3B by putative amino acid sequence. The sequence alignment was performed using the ClustalW2 online software in EMBL-EBI. The green colour indicates the mutated amino acids (*StOSM*-3Bm: *St*OSM-3B isolated in this study; *StOSM*-3Bc: *StOSM*-3B isolated by Castillo RA et al [10])

The isolated cDNA in this study was still named after *StOSM*-3B because the cDNA sequence showed high identity with that of *StOSM*-3B. Analysis of the expression of *StOSM*-3B in *E. coli* indicated that as the *StOSM*-3B mRNA accumulates, the tolerance of recombinant *E. coli*, as described by colony livability under osmotic stress, was significantly improved, even as the osmotic stress generated by PEG6000 increased (Hu et al 2012).

Wounding, abscisic acid (ABA), low temperature, NaCl and salicylic acid (SA) increased the *StOSM*-3B (pA81) mRNA expression in *S. commersonii* cell cultures and in plants grown *in vitro*. The reason for why these conditions induce *StOSM*-3B mRNA accumulation is unknown yet (Zhu et al. 1995). In addition, the involvement of *StOSM*-3B in water stress has not yet been reported.

1.1.2 *StOSM* genes in potato

The sequence of the cloned *StOSM*-3B cDNA was retrieved in the PGSC_DM_v3.4_gene.fasta file from the Potato Genome Sequencing Consortium website (http://solanaceae.plantbiology.msu.edu) (Cory et al. 2014). A total of eleven *StOSM* genes distributed among five chromosomes in the potato genome were identified from the PGSC database. The sequence structure of the eleven potato *StOSM* cDNAs is shown in Table 1.

All potato *StOSM* genes were collected from the NCBI GenBank using BLAST analysis and aligned with these eleven *StOSM*. No one beyond eleven *StOSM* was found according the sequence alignment. Therefore, seven genes, such as *StOSM*-3B and -3F, were designated according to names used previously and registered in the database (Castillo et al. 2005). Four new *StOSM* genes were identified and named based on the length of the putative encoded peptide.

Table 1 Transcript sequence structure of the eleven *OSM* genes in potato

PGSC locus	gene	chro.	5'UTR	3'UTR	CDS	No.Intron
PGSC0003DMG400013227	OSM-8E	1	17	211	663(220 aa)	0
PGSC0003DMG400000573	OSM-297	3	0	0	894(297 aa)	2
PGSC0003DMG400026926	OSM-306	6	120	167	921(306 aa)	2
PGSC0003DMG400003040	OSM-1G	8	5	41	693(230 aa)	0
PGSC0003DMG400003041	OSM-182	8	0	0	549(182 aa)	2
PGSC0003DMG400003042	OSM-5A	8	12	211	744(247 aa)	0
PGSC0003DMG400003043	OSM-3B	8	12	201	744(247 aa)	0
PGSC0003DMG400003044	OSM-3F	8	36	138	741(246 aa)	0
PGSC0003DMG400003057	OSM-3C	8	24	183	753(250 aa)	0
PGSC0003DMG400003058	OSM-2D	8	24	157	753(250 aa)	0
PGSC0003DMG400000402	OSM-251	11	40	220	756(251 aa)	0

Overall, eleven *StOSM* genes were distributed on five of twelve potato chromosomes (Figure 3). Seven of the eleven *StOSM* genes are near 54277k–54230k on chromosome 08 (Figure 4). The other four are located on chromosomes 01, 03, 06 and 11 (Figure 3 and Table 1).

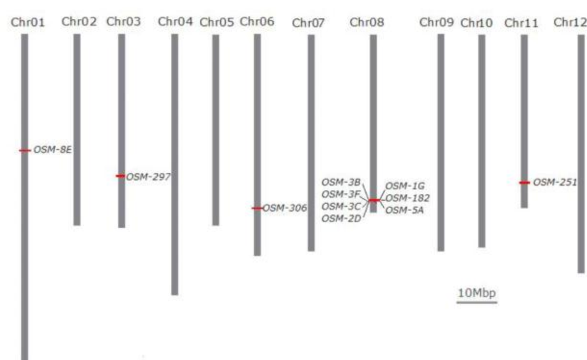

Figure 3 Genomic distribution of the *StOSM* genes throughout the potato chromosomes. The chromosome number is given at the top of each bar. The triangles next to the gene names indicate the direction of transcription

The seven *StOSM* genes on chromosome 08 identified from the PGSC database were not identical with the eight *StOSM* genes on chromosome 08 distinguished (Castillo et al. 2005). The extra *StOSM* gene on chromosome 08 identified by Castillo et al. is most likely due to two hybridised signals observed for *StOSM*-182 (PR-5X) on chromosome 08 because it contains two exons. The *StOSM* gene on chromosome 11 identified by Castillo et al. is *StOSM*-251, as shown in Figure 3.

1.1.3 Phylogeny of the putative StOSM amino acid sequences

The eleven putative amino acid sequences encoded by these *StOSM* genes were used to construct a phylogenetic tree (Figure 5). The eleven osmotins

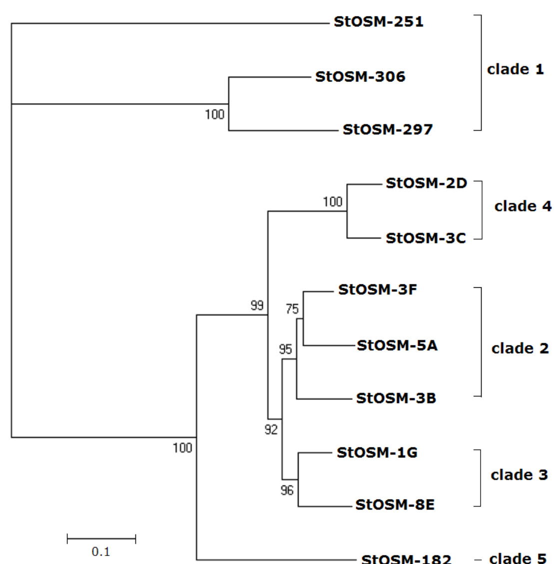

Figure 5 Phylogenetic relationship of the eleven putative potato osmotin peptides. These data were organised into a phylogenetic tree using the MEGA5.02 package and the neighbour-joining program. The numbers listed at the branching points are boot-strapping values that indicate the percentage significance for the separation of the two branches. The length of the branch line indicates the extent of the difference according to the scale on the lower left-hand side. Putative functions are listed for each gene

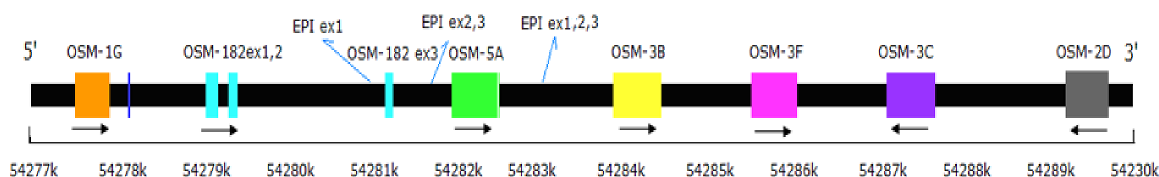

Figure 4 Distribution of the seven *StOSMs* located on chromosome 08

clustered into two main subclusters and further into four main clades. Two new osmotins, *StOSM*-297 and *StOSM*-306, were assigned to the same independent branch of the phylogenetic tree. The seven osmotins on chromosome 08 clustered into four subclades.

According to the phylogenetic relationship, one of the two main subclusters, composed of *StOSM*-251, -306 -297 (clade 1), was relatively independent of the other. After separating from *StOSM*-182 (clade 5), seven of the eight *StOSM* genes in the other subcluster evolved into three clades: clade 2, containing *StOSM*-5A, -3B and -3F; clade 3, *StOSM*-1G and -8E; and clade 4, *StOSM*-3C and -2D.

The alignment of the amino acid sequences of the eleven osmotins revealed four conserved regions: the NNCPYT, RIW and TGDCGG motifs, located in the N-terminal region, and the AYSY motif, located in the C-terminal region (Figure 6A). A Jalview of the alignment of *StOSM*-5A, -3B, -3F, -1G and -8E based on their phylogenetic relationship showed that a tandem duplication event occurred in these five genes (Figure 6B).

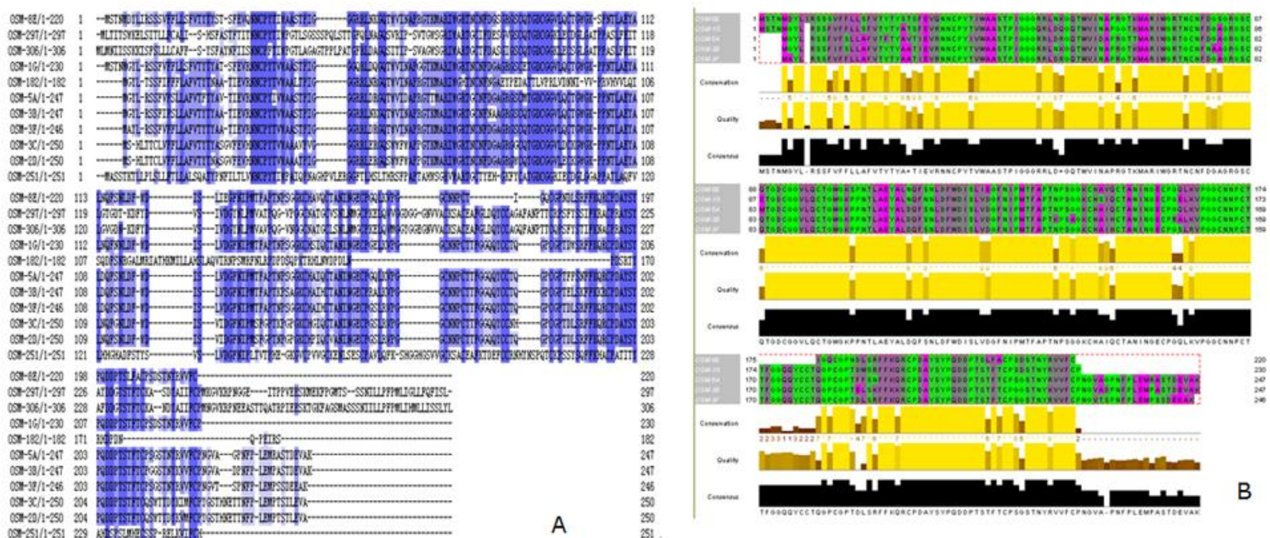

Figure 6 Amino acid sequence alignments of the putative potato osmotins

Interestingly, the putative amino acid sequences of all osmotins range from 182 aa for *StOSM*-182 to 306 aa for *StOSM*-306. *StOSM*-182 and -306 show a greater divergence towards the C-terminal region (Figure 6A). The sequences of four motifs were searched in the UniProt Knowledgebase (UniProtKB) by blastx. However, the result of zero hits from the search showed that the function of these conserved regions is unknown yet.

StOSM-182, with 182 amino acid residuals, is the shortest osmotin protein and does not contain the AYSY motif in its C-terminus (Figure 6A). In addition to the common four motifs, *StOSM*-182 and -306 share two additional motifs, FCPMKGVKRPN and ILLPFPMLI. Neither an expression sequence tag nor gDNA/cDNA clone of these two genes was registered in the database. Meanwhile, there was no hit for annotated or predicted function of these motifs when these motif sequences BLAST was done in all available protein database. This means that function of these motifs is unknown yet.

1.1.4 Introns and UTRs of *StOSMs*

The structure of all the *StOSM* genes was highly conserved. Only three *StOSM* genes, including *StOSM*-182 and -306, have two introns (Figure 7). Within the second intron of *StOSM*-182 is the first exon of a putative gene, which encodes ethylene-responsive proteinase inhibitor 1 (EPI). The second and third *EPI* exons are located in the 3’ end of the third exon of *StOSM*-182. In addition, there is one more copy of *EPI* locating within the promoter region of *StOSM*-3B (Figure 4).

The 3’ ends of the first introns of *StOSM*-182 and -306 share the same 7 nt. The 3’ and 5’ ends of the second

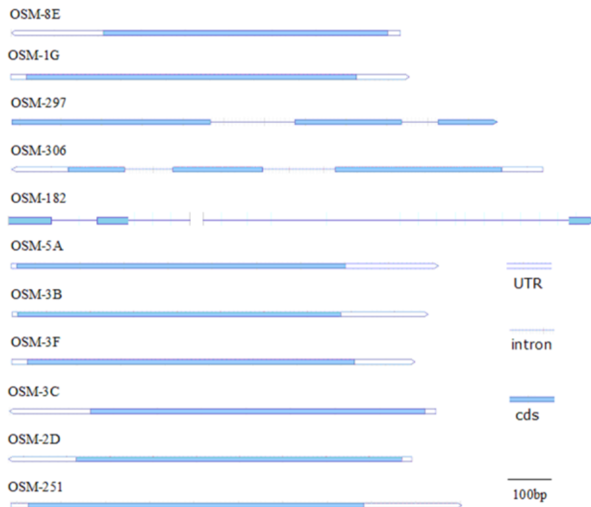

Figure 7 Diagram of the structure of the eleven *StOSM* transcripts. The diagram is drawn to scale, according to the alignment of the cDNA sequences with the corresponding genomic sequences. All genomic sequences for *StOSM* were obtained from sequencing data from the Potato Genome Sequencing Consortium website (http://solanaceae.plantbiology.msu.edu)

exon of these two genes have 5 nt and 7 nt in common, respectively. These findings imply that the two exonic splicing patterns are conserved between the two genes.

The lengths of the UTRs (untranslated region), which are important in the regulation of RNA mobility (Banerjee et al. 2006 and 2009), were compared using sequences from either the clones obtained in this study or web-based RNA data. The *StOSM*-306 5’UTR is 120 nt, and the *StOSM*-251 3’UTR is 220 nt. Both were the longest UTRs among the eleven *StOSM*s (Table 1). *StOSM*-182 and *StOSM*-297, which contain two introns, have neither a 5’UTR nor a 3’UTR.

1.2 Expression of *StOSM* family under water stress

1.2.1 *StOSM* expression analysis by mined RNA-Seq data

Among the RNA-Seq data of *S. tuberosum* genotype RH89-039-16 from the published potato genome (Xu et al. 2011 and Cory et al. 2014), the only available RNA-Seq data under water stress is from the RH leaf. Therefore, the RNA-Seq data for certain RH organs, including leaf with and without water stress, stem, roots, stolon and mature tuber, were selected and compared with respect to the expression of the eleven *StOSM* genes. The fragments per kb per million mapped reads (FPKM) in leaves were compared with the FPKM in the other four organs (Figure 8).

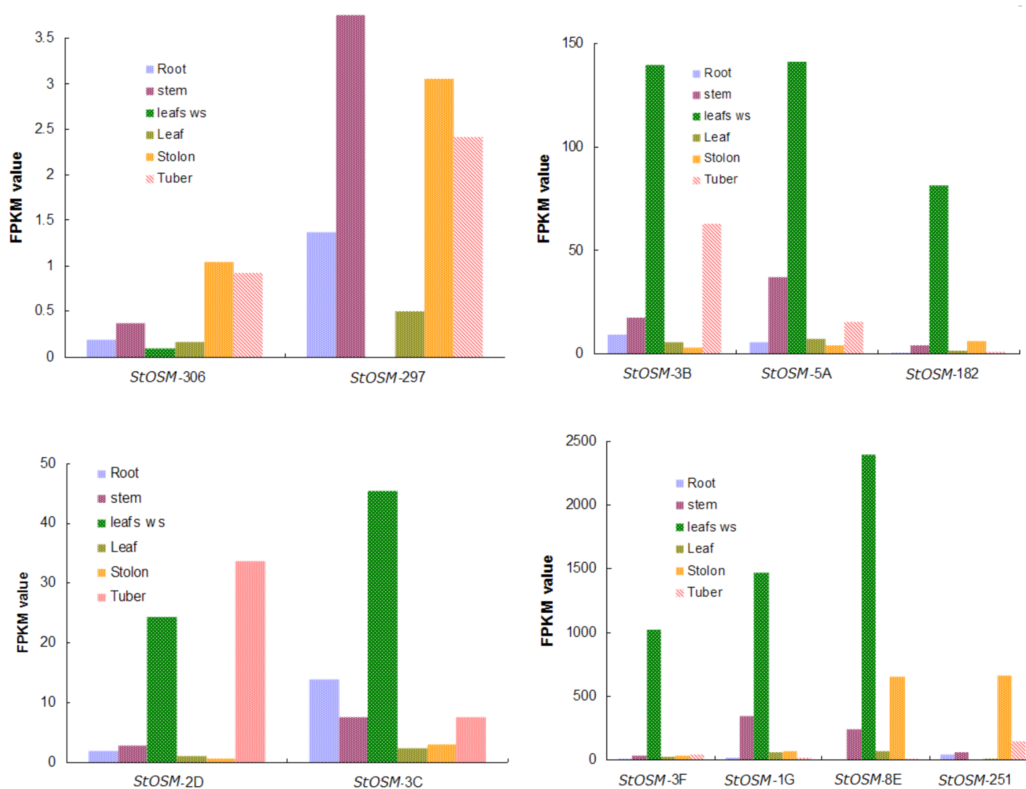

Figure 8 Expression profile for the FPKM value of eleven potato *OSM*s in selected organs. The data were mined using the RNA-seq data for the genotype RH89-039-16 haplotype from the publically available Potato Genome Database [12]. Abundance is represented in FPKM (fragments per kb per million mapped reads) values. “Leaf ws” stands for leaf under water stress

StOSM-297 mRNA occurred at a low FPKM level in the control leaf and at high FPKM levels in the other four organs. The absence of a detectable FPKM level in leaves under water stress suggests that *StOSM*-297 is only one of eleven *StOSMs* and that its expression in leaves was supressed by the induction of water stress (Figure 8). Although *StOSM*-251 is present in both the treatment and control leaves, *StOSM*-182 was expressed at a very low level in the tuber and control leaf (Figure 8), and the eleven *StOSM* transcripts were ubiquitously observed in a variety of organs.

The most striking feature of this *StOSM* mRNA expression profile, however, was that eight of the eleven *StOSM* genes in RH leaves under water stress exhibited 24-54-fold higher FPKM values than in the control leaves and the other four organs (Figure 8).

Overall, the average FPKM value of three *StOSMs*, *StOSM*-3F, -1G and -8E, in leaves under stress were 33-fold higher than in the control, exhibiting the highest FPKM values (Figure 8). In contrast, the FPKM values for *StOSM-306* and *-251* in leaves under water stress was approximately 3-fold lower than in the control.

The average FPKM value of the other 5 *StOSMs* that increased in response to water stress was 21-fold higher than in the control (Figure 8). *StOSM-8E* was the most abundant RNA of the eleven *StOSMs*, and the top three ranked *StOSM*s by expression are *StOSM*-3F, -1G and -8E (Figure 8).

Both the 21-33-fold increase and the 3-fold decrease of the FPKM values for the eleven *StOSM*s induced by drought suggest that the *StOSM*s not only are associated with low temperature, abscisic acid and NaCl, but also respond positively to water stress as drought-induced genes.

1.2.2 *StOSM* Expression Analysis by Reverse transcription PCR

When WCM decreased to 10%±2%, the leaves of the seedling became crimpy and then died. Therefore, WCM at 20%±2% was defined as the potato drought-lethal critical point (DLP) in this case. Using *StOSM* member-specific primers and total RNA from leaf samples grown under the 7 degressive WCM gradient groups, reverse transcription PCR (RT-PCR) was performed in triplicate.

RT-PCR analysis showed that eight of the eleven *StOSMs*, *StOSM*-8E, -1G, 3B, -3F, 5A, 3C, 2D and -182, were upregulated in response to the degressive gradient of water availability (Figure 9). The peak mRNA accumulation of all upregulated *StOSMs* was reached at the DLP. Among the eight upregulated *StOSM*s, *StOSM*-1G and -8E synchronously showed much stronger and higher increases than the others.

The expression attenuation of three *StOSMs, StOSM*-297, -251 and -306, was observed for the same case (Figure 9). *StOSM*-1G and -8E synchronously showed much stronger and higher increases in mRNA accumulation than the other upregulated *StOSMs* over the degressive gradient.

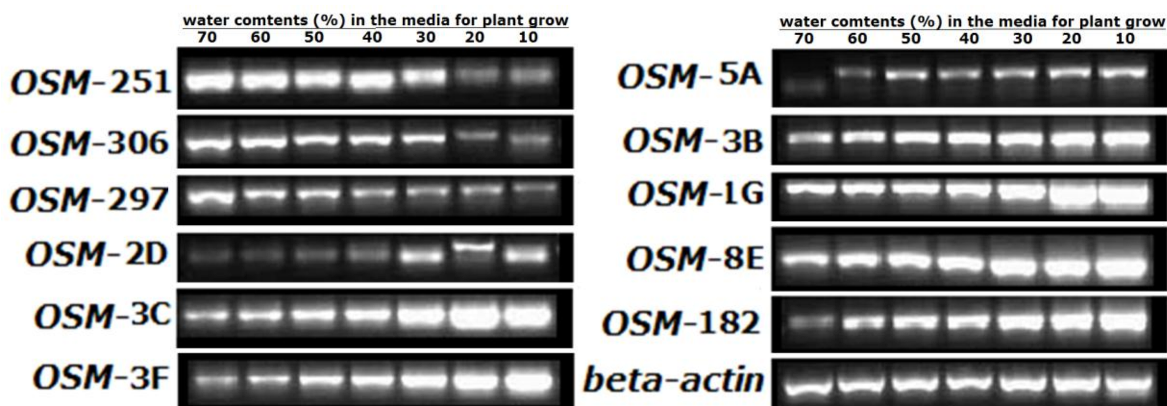

Figure 9 Dynamic quantification of leaf *OSM* mRNA accumulation over the decreasinggressive gradient. Under the degressive gradient of water stress from 70% ±5% to 10% ±2% (DLP), *OSM* mRNA accumulation was assayed by reverse transcription PCR in the leaves of the potato species, *S. tuberosum* ssp. ZHB (WCM: water content of the media in which the plants grow)

The sequences of certain *StOSM* members, such as *StOSM-3B, -3F* and -5A, share greater than 95% identity, which makes distinguishing each member precisely by RT-PCR difficult. A visible distinction of these *StOSMs* on the gel displaying the RT-PCR products confirmed the specificity and reliability of the eleven primer pairs to distinguish each gene by RT-PCR. Therefore, these primers were used to quantify the expression of the eleven *StOSM* mRNAs in leaves.

1.2.3 Real-time quantitative reverse transcription-PCR (qRT-PCR) for *StOSM* expression analysis

Real-time qRT-PCR was performed for all *StOSM* members to determine the leaf RNA accumulation patterns in response to decreasing water availability. The mRNA accumulation of eleven *StOSMs* in the leaf induced by 10% WCM (also DLP) as the treatment and by 70% WCM as the control was quantitatively assayed (Figure 10). Similar to the qualitative assay results, eight of eleven *StOSMs* under the 10% DLP condition displayed significant increases in RNA accumulation compared with the control condition.

The changes in the eleven *StOSMs* induced by drought varied from gene to gene (Figure 4). Leaf *StOSM* mRNA accumulation for the genes upregulated by DLP was 4-49-fold higher than in the control. The peak *StOSM*-8E mRNA accumulation was greater than 49-fold higher than in the control. *StOSM*-297 mRNA accumulation decreased 9-fold in response to drought compared with the control.

StOSM-251 showed exceptional leaf mRNA accumulation. No significant difference was observed in the relative levels of transcripts between the treatment and control leaves (Figure 10). This finding was not in agreement with the FPKM value and qualitative assay results.

This comparison clearly revealed that the result of the qualitative assay is in agreement with the mRNA quantification and FPKM value results from the RNA-Seq analysis (Table 2). This observation indicates that drought does induce the regulation of the expression of the eleven *StOSMs*. With eight members of *StOSM* being upregulated and three being downregulated, *StOSM*s play a positive role in enhancing plant tolerance to stress.

Table 2 Comparison of the qualitative and quantitative *OSM* mRNA accumulation under water deficiency

TF	*OSM*										
	182	297	251	8E	1G	5A	3B	3F	3C	2D	306
FPKM	+	−	−	+	+	+	+	+	+	+	−
RT-PCR	+	−	−	+	+	+	+	+	+	+	−
qRT-PCR	+	−	−	+	+	+	+	+	+	+	−

Note: − stands for down-regulated; + for up regulated

Therefore, osmotins can be considered to be drought responsive molecules involved in withstanding water deficits. Recent reports provide direct evidences to support osmotin as a drought responsive molecule. Expression of a osmotin-like SnOLP from *Solanum nigrum*, 93.0% identity on aminoacid sequence with OSM-3F (PGSC0003DMG400003044) in *S. tuberosum* confers drought tolerance in transgenic soybean (Weber et al., 2014). Aslo, overexpression of OSM-3F gene confers tolerance to salt and drought stresses in transgenic tomato (*Solanum lycopersicum L.*) (Goel et al., 2010).

Drought stress is an important environment factor limiting crop production in some areas. Up to date, a litter is known on how to enhance crop tolerance to drought stress. This study not only provides a method on how to screen isoforms of a gene from a genome database, but also disclose response of osmotin in drought tolerance. This is a practical option to dig a genome for member identification of a gene family.

2 Discussion

From a metabolic point of view, the plant response to water stress involves the generation of intercellular components concerned with the regulation of water potential. Osmotin is a component that accumulates substantially when induced by water stress. Previous studies revealed that the enhancement of osmotin expression can increase plant tolerance to fungi and abiotic stress. However, little is known regarding the mechanism of osmotin in the response to stress.

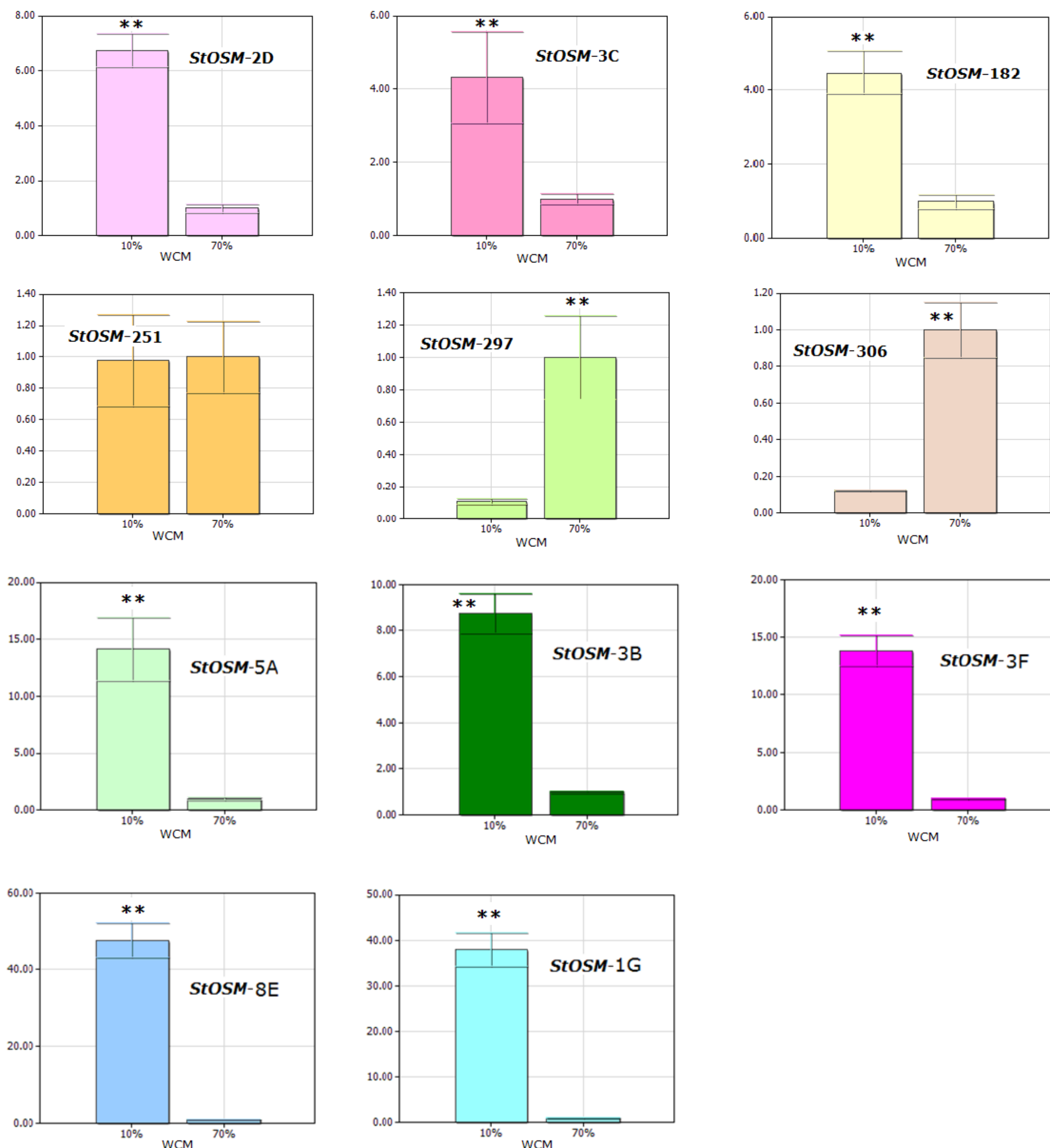

Figure 10 Comparison of the relative levels of *StOSM* transcripts in leaves. The potato species *S. tuberosum* ssp. *ZHB*, was grown under 10% ±2% WCM as the treatment and under 70% ± 5% as the control. The relative levels of the *StOSM* transcripts are given on the *y*-axes and were quantified using total RNA extracted from new leaves (leaf) from plants grown under 10% WCM (or DLP) as the water stress condition or under 70% WCM as the control condition. Leaf samples under water stress were harvested when the water content reached the expected level based on the water content in the media, which was regularly monitored every 12 hours. Real-time qRT-PCR with gene-specific primers was used to calculate the relative amounts of RNA for each *StOSM* gene. The expression of each *StOSM* gene was calculated as the $2^{-\Delta Ct}$ value and normalised to the endogenous reference gene, beta-actin. The standard errors of the means of three biological replicates are shown, with asterisks indicating significant differences (***P*<0.01) by Student's *t*-test

A recent study in animals showed that plant osmotin can activate a pathway involved in disease resistance by binding to the adiponectin receptors. Through the upregulation of the antiapoptotic Bcl-2 protein, plant osmotin reduces ethanol neurotoxicity and reverses synaptic dysfunction and neuronal apoptosis (Naseer et al. 2014 and Shah et al. 2014).

The functions of plant osmotin in animal cells may provide a clue to understand how osmotin in plant

cells responds to water stress at the molecular level. More importantly, plant genomes annotated have revealed that a gene has diverse functions. Therefore, to reveal a specific role of a gene or its product and its interaction with other genes/proteins in a stress response pathway, a prerequisite is to clarify and specify the function(s) of the gene and any differences from other genes with the same or similar functions.

Based on the consideration above and the genome sequence database, starting with an EST from a *StOSM* gene isolated from the stress subtractive library for water stress, the drought responses of eleven *StOSM* genes were revealed. Eight *StOSM*s were upregulated and three were downregulated in response to water deficits. Using the RegSite Plant Database and the online software TSSP, the binding sites for *cis*-elements in the *StOSM* promoter were analysed, revealing that the pattern of binding sites in the *StOSM* promoters varies among the eleven *StOSM* genes. The resulting expression of the different *StOSM* genes varies during the response to water stress.

The drought tolerance capacity of a plant, similar to its tolerance to other stresses, has been formed during the long process of evolution. No water conditions except extreme drought induce the expression of potential drought responsive genes and the accumulation of drought responsive molecules. Based on the potato drought-lethal critical point (DLP) defined in this study according to the phenotype under water deficit, *StOSM* mRNA accumulation reaches its peak at DLP along a degressive gradient toward the DLP. This observation has not been previously reported.

The qualitative and quantitative expression of the *StOSM* genes in this study is in agreement with the data analysis of the potato genome sequence (Xu et al. 2011). Osmotins, as response molecules, are clearly involved in drought tolerance in the potato. However, the extent to which the osmotins are associated with drought tolerance in the potato, such as the specific stages in the adult plant and during the restoration of water after DLP, has yet to be determined. The answers to these questions are indispensable to establishing the dynamic network involved in drought response.

In addition, osmotins, as drought responsive players, surely interact or crosstalk with other molecules in the drought response network. In this study, the qualitative and quantitative results of StOSM in response to drought provide a background in which the interaction or crosstalk of StOSM with other response molecules can be revealed to establish the network.

Although the first *StOSM* (accession No. M29279) was isolated almost 25 years ago, eleven-*StOSM* in *S. tuberosum* identified in this study is the first one to disclose OSM members in a plant genome. This is necessary not only for understanding *StOSM* function in the drought response network, but also for engineering improvements in plant tolerance to drought.

3. Materials and Methods

3.1 Plant materials

S. tuberosum genotype ZHB is a tetriploid cultivar. ZHB seedling can survival at the level of relative soil water content as low as 10%. RH (RH89-039-16) genotype is a diploid clone derived from a cross between a *S. tuberosum* ‘dihaploid’ (SUH2293) and a diploid clone (BC1034). RH was selected as a diploid model for potato genome sequencing. In this study ZHB minitubers were sown in a pot containing peat as the medium (Pindstruo Mosebrug, DK) and grown under the following conditions: water content of 70% ± 5% in the medium, light intensity of 100 μmol m^{-2} s^{-1}, temperature of 20-25 ℃ and relative humidity of 60-80%, as it was done by Anne J.K (Anne et al., 1996, Liu et al. 2012)).

On the 35th day after minituber germination (when the stored starch and other nutrition in the minituber is completely exhausted as observed in our case), the seedlings were kept under the same conditions, with the exception that no water was provided. The water content in the medium (WCM) was assayed every 12 hours until seedling death. Leaf sampling with three replicates was performed along with a water deficit gradient above the drought-lethal critical point (DLP) ranging 70, 60, 50, 40, 30, 20 and 10 %. Ten-gram leaf samples were collected, frozen in liquid nitrogen and stored at –80 ℃ for subsequent experiments.

3.2 Cloning of a *StOSM* with its EST sequence.

Total RNA was isolated from the leaf sample at 70% ±5% and 20% ±2% WCM using *TRIZOL* Reagent (15596-026, *Invitrogen* Corp., Carlsbad, CA, USA).

Reverse transcription of mRNA was performed from the total RNA using a Prime Script™ RT reagent Kit (TaKaRa Biotechnology, Dalian, China). The construction and screening of a subtractive library was performed as described previously (Ma et al.2011).

Based on BLAST analysis with the ESTs, a *StOSM* specific primer, O-F/O-R, was designed and synthesised. Using the primers O-F (5'-ATGGG CTACTTGAGATCTTC3') and O-R (5'-TTACTTGG CCACTTCATCAG-3'), the general protocol of molecular cloning was followed to clone the full-length *StOSM* cDNA.

3.3 Phylogenetic and gene structure analysis

The sequence of the cloned *StOSM* cDNA was compared against the NCBI files and the PGSC_DM_v3.4_gene.fasta files from the Potato Genome Sequencing Consortium (PGSC) website (http://potatogenomics.plantbiology.msu.edu) (Xu et al. 2011 and Cory et al. 2014). Using the downloaded sequences for the StOSM and promoter from the PGSC database, two phylogenetic trees for the StOSM peptides and promoters were constructed using the neighbour-joining method in the MEGA 5.02 software package developed by (Saitou and Nei1987 and Tamura et al. 2007). Briefly, copy sequences in FASTA format to the Alignment Explorer in the MEGA 5.02. Align sequences by Clustal W. Import and save alignment file in MEGA format. Construct Neighbor Joining tree with 1000 Bootstrap Replications. The Genomic distribution of the *StOSM* genes throughout the potato chromosomes was drawn in proportion to their positions in the PTGS (Release: Potato Genome Brower v4.03). The selected *StOSM* expression on FPKM values was compared with data downloaded from the RH genotypes in the Potato Genome Sequencing Consortium website (http://potatogenome.net), Annotation report v3.4. RNA-Seq Gene Expression Data. DM_RH_RNA-Seq_FPKM_ expression_ matrix.

3.4 Reverse transcription PCR for *StOSM* expression analysis

A total of 300–350 ng of RNA was extracted from leaves at WCM concentrations of 70%, 60%, 50%, 40%, 30%, 20% and 10%. Following the instructions for Premix Ex TaqW Version 2.0 (TaKaRa), reverse transcription PCR was performed using primers (Table 3) designed specifically for each of the eleven *StOSMs*. The PCR product amplified from the treatment templates using beta-actin-specific primers was used as an internal control.

Table 3 *StOSM* member-specific primers for reverse transcription PCR

StOSM-	Primer	Primer sequence	Product (bp)
1G	F	5'TGCCCCGACCAATCCTAG 3'	183
	R	5'GAAAAATCTCGACCAGTCAGTAGG 3'	
2D	F	5'TGAATCAATTTAGCAACTTAGA 3'	131
	R	5'CATTTATATTGGCAACACATT 3'	
3B	F	5'TTCTCCCTTCTTGCTTTTGTGACT 3'	150
	R	5'CCCTTGGAGCATTGATGACC 3'	
3C	F	5'ATACGCTTTGAACCAGTTTG 3'	129
	R	5'TTGGCTGTGCATTGAATT 3'	
3F	F	5'TTCTTCCTCCTTGCTTTTGTGAC 3'	149
	R	5'CCTCGGCGCATTGATAACC 3'	
5A	F	5'TTCTCCCTTCTTGCTTTTGTGAC 3'	144
	R	5'GCGCATCAATAACCCACGT 3'	
8E	F	5'CGCCCCGACCAATCCTA 3'	154
	R	5'TGAAAAATCTGGACAAGTCGTTAG 3'	
182	F	5'GGTGCCCTAATGCGTATA 3'	183
	R	5'CGGGGTCGTGCCTATA 3'	
251	F	5'ACAAGCCACCTACCCTAAT 3'	134
	R	5'CGGTGGGTAAGTGAGTGA 3'	
297	F	5'CGTCCATCATTCTATTCAAG 3'	147
	R	5'CGTTGGGCCTCTTCAC 3'	
251	F	5'AACTTGTCAACCATCTTTTTAC 3'	159
	R	5'TTCTTCATTAGGCCTCTTTAC 3'	

Reverse transcription reactions with template-free were used as a negative control to exclude DNA contamination. Using a standardised procedure, all PCRs were optimised with respect to the denaturing temperature and extension time to yield a product of the expected size.

3.5 Real-time quantitative reverse transcription-PCR (qRT-PCR) for *StOSM* expression analysis

Total RNA was treated with RNase-free DNase (Qiagen). The quantity and quality of the RNA samples were estimated using a Nano spectrophotometer (ND-1000; Thermo Scientific). RNA samples with a 260:280 ratio ranging from 1.9 to 2.1 and a 260:230 ratio ranging from 2.0 to 2.5 were used for the qRT-PCR analyses.

Real-time quantitative reverse transcription-PCR analysis was performed using the PrimeScript™ RT Master Mix and SYBR® Premix Ex Taq™ II (RR820A and RR036A, TaKaRa Biotechnology, Dalian, China) following the manufacturer's protocol. Briefly, 50-ng aliquots of the total RNA template were subjected to each qRT-PCR in a final volume of 20 μL containing 4 μL of the PrimeScript RT Master Mix and 0.4 μL of qScript Reverse Transcriptase along with target-specific primers (200 nm).

All reactions were performed in triplicate using 1 μl of template cDNA in a final volume of 20 μl in a Bio-Rad Real Time PCR System (Bio-Rad, CA, USA). The Illumina Eco qPCR machine with fast quantitative PCR cycling parameters was used for complementary DNA synthesis with the following conditions: 37 ℃ for 15 min followed by 85 ℃ for 5 s; initial denaturation − 95°C for 30 s for 1 cycle; 2 step PCR − 95°C for 5 s and 60 ℃ for 30 s for 40 cycles.

Beta-actin (accession number DQ252512) was used as an internal control. The relative gene quantification method was used to calculate the expression levels of different target genes (Livak and Schmittgen 2001). Primer specificity was determined by melting curve analysis and qualification analysis by RT-PCR. A standard curve was generated based on serial dilutions of cDNA to calculate the gene-specific PCR.

The relative expression levels of each *StOSM* gene were analysed using the IQ5 software and are presented as the fold-difference from the baseline expression levels. Using Origin Pro 8.0 software (Origin Lab Corp., Northampton, MA, USA)，paired t-tests were performed to assess the significance of expression level differences between the treatments and the controls.

Author's contributions

YXL designed the experiment and primers used, performed data analysis, wrote and revised the manuscript and supervised performance of the project. Performed the experiments: Qiao LB, Dai HX, Fan HH, Zhang XP and Wu F. Analysed the data: Yao XL, Qiao LB, and Fan HH. Wrote the paper: YAO XL and Qiao LB.

Acknowledgments

This work was jointly supported by National Natural Science Foundation of China (NSFC 31360275, 31160236, To Yao XL).

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