Cloning and Expression of Omission of Second Division-like Genes from Carnation

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ABSTRACT. The omission of second division gene (OSD1) gene plays a fundamental role in meiosis and is associated with 2n gamete formation in Arabidopsis thaliana. The objective of this study was to unravel the mechanisms leading to 2n pollen production, and isolate and analyze the expression patterns of OSD-like (OSDL) genes in carnation (Dianthus caryophyllus). We found an absence of the second meiotic division caused the formation of 2n pollen. Three homoeologous genes were cloned and labeled as OSDLa, OSDLb, and OSDLc in a diploid carnation. The cDNAs were 1180 bp for OSDLa, 1288 bp for OSDLb, and 971 bp for OSDLc. A strong similarity was found between the amino sequences of OSDLb and OSDLc. An evident feature of OSDL proteins is the presence of D-box and MR-tail domains; however, the GzxKN-box domain, which is distinct among the other plant proteins was absent. Quantitative real time polymerase chain reaction (qRT-PCR) analysis showed that OSDL genes maintain continuous expression in buds and mature ovaries. OSDLa has the highest expression in buds of 1.1–1.2 cm long (stage 2), and OSDLb has a high level of expression in buds of 0.9–1.0 cm long (stage 1) and stage 2 buds and ovary tissues in three carnation cultivars. The expression level of OSDLc was highest in ovaries. These expression patterns strongly suggest that OSDLs in carnation involve male meiosis and ovary development. These findings can have potential applications in fundamental polyploidization research and plant breeding programs in carnation.

Received for publication 14 Mar. 2017. Accepted for publication 24 Aug. 2017.

This work was supported by grants from the National Engineering Research Center for Ornamental Horticulture (grant number 2012FU125X10), Chinese Natural Science Foundation (grant number 31460530), Yunnan Foundation Research Projects for Application (grant number 2014FA044), Yunnan Young Academic and Technical Leader Training (grant number 2015HB077), and Yunnan Science and Technology Leader Training (grant number 2016HA005).

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prevent entry into M II without impairing the prophase to M I transition. As osd1 mutants fail to enter the second division in both male and female meiosis, functional 2n gametes and tetraploidy progeny are produced (d’Erfurth et al., 2009). Male meiocytes lacking both CYCA1;2/TAM and OSD1 fail to enter the first meiotic division (d’Erfurth et al., 2010). Loss of both CYCA1;2/TAM, and OSD1 may cause a loss of CDK activity sufficient to impair entry into M I. These differences highlight the tight regulation of CDK activity required during meiosis (Brownfield and Köhler, 2011).

The A. thaliana OSD1 has the same three putative cell-cycle-related domains. These three domains are very well conserved over OSD1 homologues (d’Erfurth et al., 2009). Two of these domains are putative APC/C degradation motifs: a D-box (residues 104–110, RxxLxx) and a GxEN/KEN-box (residues 80–83, GxEN in eudicotyledon and KEN in monocotyledon OSD1 homologues) (Cromer et al., 2012). OSD1 also has a C-terminal MR-tail in common (the two last amino acid of the protein are a methionine and an arginine) (Cromer et al., 2012).

Both the interactions with APC/C and in-plant meiotic function of OSD1 are dependent on its D-box domain and its MR tail. Remarkably, the OSD1 GxEN-box is not required for OSD1 function, but its mutation allows the OSD1 protein mutated in its D-box to fulfill its function (Cromer et al., 2012).

Interestingly, OSD1 has a paralogue, uv-insensitive4 (UV14), which negatively regulates endoreplication onset in A. thaliana that play roles in the mitotic cell cycle through APC/C regulation (Heyman et al., 2011; Pesin and Orr-Weaver, 2008). However, OSD1 also has a somatic function as revealed by the mitotic phenotype of the single osd1 mutants which trigger ectopic endomitosis (Iwata et al., 2011). Iwata et al. (2011) showed that GIGI/OSD1 and UV14 encode novel plant-specific inhibitors of APC/C ubiquitin ligase.

Carnation is a major floricultural crop in China and worldwide. It is a member of the Caryophyllaceae. Most carnation cultivars can produce 2n gametes, and the frequency of 2n pollen produced ranged from 0% to 4.17% (Zhou et al., 2012, 2015). Spindle abnormalities during metaphase II have been considered to be the major cytological mechanism involved in the formation of 2n pollen in carnation (Zhou et al., 2015). In this study, we describe another abnormality mechanism and found some cells were normal in the first meiotic division; however, a failure occurred in the second meiotic division, which generated dyads during microsporogenesis. The carnation OSD1 gene may be involved in the formation of 2n gametes. The role of carnation OSDL in the formation of unreduced gametes is described herein through the isolation, characterization, and analysis of expression patterns.

Materials and Methods

Plant materials. The experiments were carried out in four diploid carnation (2n = 30) cultivars: Promesa, Guernse Yellow, Nogalte, and YunhongErhao, which displayed high pollen viability. The frequency of dyad production in ‘Nogalte’ in June 2013 was 3.3%, and the frequency of 2n pollen was 1.63% (Zhou et al., 2015). The percentage of 2n pollen in ‘Promesa’, ‘Guernse Yellow’, and ‘YunhongErhao’ was 0.06%, 3.09%, and 1.72%, respectively (Zhou et al., 2015).

They were cultivated under plastic in an ambient light and temperature regimen in Sept. 2012 at Jinning district, in Kunming, Yunnan, China. During the flowering period (June 2013), the daily temperature averaged 22.84 ± 0.02 °C, and the photoperiod averaged 13 h.

Cytology. ‘Nogalte’ was used for cytological analysis of male meiosis. Observation of the final male meiotic products and chromosome spreads were carried out as previously described (Zhou et al., 2015), observed under a light microscope (E800; Nikon, Tokyo, Japan) at x1000.

Three carnation cultivars Promesa, Guernse Yellow, and YunhongErhao were sampled in June 2013. Buds were classified into eight stages (1–8) in accordance with the methods described by Zhou et al. (2015). Stage 1 and stage 2 buds usually have developed microspore mother cells (bud size 0.9–1.0 and 1.1–1.2 cm, respectively); stage 3 buds contain anthers that have generally entered meiosis (bud size 1.3–1.4 cm); stage 4 buds have developed tetrads (bud size 1.5–1.6 cm); and stages 5–8 have further progressed to the mature pollen stages (bud size 1.7–2.4 cm).

Carnation OSDL cloning. An A. thaliana OSD1 cDNA sequence was retrieved from the GenBank database (Benson et al., 1997). A homology search was conducted with BLASTX on the DNA Data Bank of Japan (DDBJ) website (Tanase et al., 2012). Accession numbers in D. caryophyllus FX309965, FX311539, FX311540, FX315550, and FX332100 resulted from this search (Tanase et al., 2012). Fragments of accession numbers FX311540 and FX311539 could be assembled into a 1262-bp segment with a polyA; FX315550 and FX332100 could be assembled into 758-bp segment with a polyA; and FX309965 was 681-bp long. These three putative homologous genes in D. caryophyllus were named OSDLa, OSDLb, and OSDLc, respectively. Total RNA from prebolting buds of ‘Promesa’ was extracted with TRIZOL Reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions.

![Fig. 1. Abnormal meiosis lacking meiosis II in carnation: (A) a wild-type tetrad; (B) a dyad; (C–H) male abnormal meiosis is indistinguishable from wild type until telophase I, but no figures characteristic of a second division were observed; (C) pachytene; (D) diakinesis; (E) metaphase I; (F) anaphase I; (G) telophase I; (H) an incipient dyad; bars = 10 μm.](image-url)
RNA was reverse transcribed to the first-strand cDNA with an oligo (dT) primer designed with an adaptor sequence according to the protocol of the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA). To isolate the 5'-ends of the carnation OSDLa, OSDLb, and OSDLc cDNA, gene-specific primers, OSDLa-5Ra-364R, OSDLb-5Ra-189R, and OSDLc-5R-468R (Supplemental Table 1) were used in combination with a universal primer mix (UPM) for 5'-rapid amplification of cDNA ends (RACE), respectively. The carnation OSDLa and OSDLb cDNA were first amplified. Initial 5'-RACE products of OSDLc gene were then used for nested polymerase chain reaction (PCR) amplifications by using a gene-specific, nested primer [OSDLc-328R (Supplemental Table 1)], in combination with a nested universal primer (NUP). To isolate the 3'-ends of the carnation OSDLa, OSDLb, and OSDLc cDNA, gene-specific primers, OSDLa-3R-103F, OSDLb-3R-147F, and OSDLc-3R-113F (Supplemental Table 1) were used, respectively. The initial 3'-RACE products of OSDLa, OSDLb, and OSDLc were then used for nested PCR amplifications by using gene-specific nested primers, OSDLa-103-length-F, OSDLb-147-length-F, and OSDLc-113-length-F (Supplemental Table 1). Both the UPM and NUP were supplied with the SMART RACE cDNA amplification kit (Clontech). All RACE reactions were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) using the following conditions: 95°C for 2 min; 35 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min.

**Fig. 2.** Alignment of protein carnation omission of second division-like (OSDL) sequences obtained from the National Center for Biotechnology Information online database. The conserved D-box and MR tail motifs are indicated. AL = Arabidopsis lyrata ssp. lyrata, AT = A. thaliana, BN = Brassica napus, BR = Brassica rapa, BV = Beta vulgaris, CC = Citrus clementina, CM = Cucumis melo, CR = Capsella rubella, CaS = Camellia sinensis, DC = Dianthus caryophyllus, EG = Eucalyptus globulus, ES = Extrema salugineum, FV = Fragaria vesca, GM = Glycine max, GR = Gossypium raimondii, MD = Malus domestica, NS = Nicotiana sylvestris, NT = Nicotiana tabacum, PB = Prunus bretschneideri, PE = Prunus edulis, PP = Prunus persica, PT = Populus trichocarpa, SL = Solanum lycopersicum, SO = Solanum pennellii, ST = Solanum tuberosum, TH = Tarenaya hassleriana, VV = Vitis vinifera.
out according to the manufacturer’s instructions with Advantage 2 polymerase (Clontech). RACE products were subcloned into a pMD18-T vector (TaKaRa, Kyoto, Japan) and checked for the correct orientation by DNA sequence analysis. Sequencing and phylogenetic analysis were carried out as previously described (Zhou et al., 2015). Theoretical molecular weights of each ribosomal protein were calculated using the Compute pI/Mw tool (Gasteiger et al., 2003).

**Phylogenetic analysis.** After obtaining the carnation OSDLs amino acid sequences, a BLAST search for homology was performed at the National Center for Biotechnology Information [NCBI (Benson et al., 1997)]. A phylogenetic tree was constructed with MEGA 5.1 (Tamura et al., 2011) based on the converted result from BIOEDIT alignments. The construction of a phylogenetic tree was performed using maximum likelihood. The robustness of clustering was evaluated by bootstrap on 1000 replications.

**qRT-PCR analysis.** OSDL expression profiles in various organs (roots, stems, leaves, and ovaries) and developmental stages of buds were investigated using qRT-PCR in carnation ‘Promesa’, ‘Guernse Yellow’, and ‘YunhongErhao’. Total RNA was isolated and reverse transcribed to the first-strand cDNAs following the manual of the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). The GAPDH gene (GAPDH 579-F and GAPDH 788-R) was used as an internal control in all qRT-PCR reactions (Supplemental Table 1). Real time polymerase chain reaction (RT-PCR) was carried out on 50 ng of cDNA with the OSDL1a-422F and OSDL1a-656R primers for OSDLa, the OSDL1b-99-F, and OSDL1b-223-R primers for OSDLb, and the OSDL1c-133F and OSDL1c-270-R primers

![Fig. 2. (Continued)](image-url)
for OSDLc (Supplemental Table 1). Gene expression in different tissues was characterized by using the Faststart SYBR Green Master Mix kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Each result reported herein resulted from three biological replicates. For each sample, quantification was made in triplicate (technical replicates). Three plants for each cultivar with different sizes of buds were selected which were grown and arranged in the same growing area. Values are means ± SE of three replicates. Different letters indicate significant differences ($P < 0.05$). Data were subjected to analysis of variance with the least significant difference method by statistical software package SPSS 18 (IBM Corp., Armonk, NY).

**Results**

**The cytological mechanism leading to 2n pollen production and 2n frequency.** To unravel the mechanisms leading to 2n pollen production, we investigated chromosome behavior during meiosis. During microsporogenesis in 'Nogalte', subsequent reductive and equatorial divisions normally result in the formation of a group of four haploid...
spores, called a tetrad (Fig. 1A). However, a low frequency of dyads (Fig. 1B) was observed, confirming that the diploid microspores are produced by a defect in meiotic chromosome segregation. Following chromosome condensation and crossover formation in pachytene and diakinesis (Fig. 1C and D), the metaphase I spindle aligns the bivalents at the equatorial plane (Fig. 1E) and subsequently, segregates the homologs toward the poles at anaphase I (Fig. 1F). Chromosomes decondensed at telophase I at the end of M I (Fig. 1G) and formed a dyad (Fig. 1H), strongly suggesting that dyad production is due to an absence of the second meiotic division.

**CLONING AND CHARACTERIZATION OF OSDL GENES IN CARNATION.** OSDL1 is involved in the entry into both M I and M II in A. thaliana. osd1 mutants produce high levels of functional 2n gametes in the absence of a second meiotic division (d’Erfurth et al., 2009). Three different OSDL cDNAs were isolated from prebolting buds of D. caryophyllus ‘Nogalte’. After the composite cDNAs were obtained, the nucleotide sequences for the open reading frames (ORFs) of all these cDNAs were confirmed by cloning the complete (full-length) cDNAs by using PCR and sequencing. The three cDNAs were designated OSDLa, OSDLb, and OSDLc, and deposited in the NCBI database under accession numbers KX622764, KX622765, and KX622766, respectively.

ORFs were analyzed using the NCBI ORF Finder (Benson et al., 2004). The structures of the three OSDL cDNAs are summarized in Supplemental Figs. 1–3, which include 5’-flanking sequences, ORFs, and 3’-flanking sequences. The cDNAs were 1180 bp long for OSDLa, 1288 bp long for OSDLb, and 971 bp long for OSDLc. The predicted proteins consisted of 223 amino acids plus a 171-bp untranslated 5’ region and a 337-bp untranslated 3’ region for OSDLa, 237 amino acids plus a 130-bp untranslated 5’ region and a 444-bp untranslated 3’ region for OSDLb, and 177 amino acids plus a 2-bp untranslated 5’ region and a 435-bp

**Fig. 3. Phylogenetic tree of plant omission of second division-like (OSDL) proteins.** Maximum likelihood phylogenetic tree of OSDL predicted proteins from 30 plant species: AL = Arabidopsis lyrata ssp. lyrata, AT = Arabidopsis thaliana, BN = Brassica napus, BR = Brassica rapa, BV = Beta vulgaris ssp. vulgaris, CC = Citrus clementina, CM = Cucumis melo, CR = Capsella rubella, CaS = Camelina sativa, CS = Citrus sinensis, DC = Dianthus caryophyllus, EG = Eucalyptus grandis, ES = Eutrema salsugineum, FV = Fragaria vesca, GM = Glycine max, GR = Gossypium raimondii, MD = Malus domestica, NS = Nicotiana sylvestris, NT = Nicotiana tomentosiformis, PB = Pyrus bretschneideri, PE = Populus euphratica, PM = Prunus mume, PP = Prunus persica, PT = Populus trichocarpa, SL = Solanum lycopersicum, SO = Spinacia oleracea, SP = Solanum pennelli, ST = Solanum tuberosum, TH = Tarenaya hassleriana, VV = Vitis vinifera.
untranslated 3’ region for OSDLc, corresponding to calculated molecular masses of 25.34, 25.95, and 19.95 kDa, respectively. Carnation OSDLs have two putative cell-cycle-related domains which are putative APC/C degradation motifs: a D-box (RxLxx, residues 104–109 for OSDLa, residues 111–116 for OSDLb, and residues 48–53 for OSDLc) and a C-terminal MR-tail (Fig. 2). However, carnation OSDLs do not have a GxEN/KEN-box motif which is otherwise very well conserved across OSDL homologues in plants (Cromer et al., 2012; Heyman et al., 2011).

The amino sequences of OSDLa have 47% and 48% similarity to OSDLb and OSDLc, respectively, and OSDLb has 55% similarity to OSDLc. A phylogenetic analysis was conducted by comparing the carnation OSDLs with 30 homologous protein sequences from different plant species (Fig. 3). The three homologues of carnation OSDLs clustered on the same basal branch. A strong similarity was found between the amino acid sequences of OSDLb and OSDLc, which clustered together as sisters.

**Comparison of Transcript Levels of OSDLs Among Different Tissues and Different Developmental Stages of Flower Buds.** To test whether OSDLs were only expressed in the buds or also in other organs, we analyzed expression patterns. qRT-PCR analyses were performed to reveal expression levels in roots, leaves, stem, ovary, and buds. Expression analysis revealed that OSDLs are expressed in many organs, including roots, leaves, stem, ovary, and buds (Fig. 4). Interestingly, OSDLa shows the highest expression in stage 2 buds in three carnation cultivars (Fig. 4A–C). The expression level of OSDLa is significantly higher in stage 2 of buds in ‘Promesa’ (Fig. 4A) and in ‘YunhongErhao’ (Fig. 4C) than other stages of buds and tissues, and significantly higher in stage 1–3 of buds in ‘Guernse Yellow’ (Fig. 4B) than other stages of buds and tissues. OSDLb has a high level of expression in stage 1 buds and ovaries of ‘Promesa’ (Fig. 4D) and ‘YunhongErhao’ (Fig. 4E), and a high level of expression in stage 2 buds and ovaries of ‘Guernse Yellow’ (Fig. 4F). We also found that the expression level of OSDLc in ovaries is higher than in other tissues (Fig. 4G–I).

**Discussion**

We cloned and characterized OSDL genes in a diploid carnation and found three homoeologous genes that we called OSDLa, OSDLb, and OSDLc, which differ from those of A. thaliana. OSDLa and OSDLb genes...
have longer sequences than does the OSDLc gene. The evident feature of these OSDLs proteins is the presence of D-box and MR-tail domains; however, the GxEN/KEN-box domain is lacking (Heyman et al., 2011). Thus, our results suggest that the GxEN/KEN-box might not be necessary for carnation OSDL function.

Loss of OSD1 function was originally shown to cause defects in the second mitotic division during the meiotic cell cycle, thus leading to the production of diploid male and female gametes in A. thaliana (d’Erfurth et al., 2009), suggesting that OSD1 gene was expressed in buds. Bao and Hua (2014) found that meiospores cannot complete mitosis to develop into a functional female gametophyte in osd1 uv14 genotypes, and indicating the OSD1 and UVI4 together are essential for zygotene development as well. UVI4 is expressed during the mitotic cell cycle, peaking at the G1-to-S transition, whereas the expression of OSD1 peaks at the G2-to-M transition (Heyman et al., 2011). OSD1 plays an important role in determining the meristem size in roots and the cell number and size in leaves (Heyman et al., 2011). The level of OSD1 expression in leaf cells, most likely through regulating endoreduplication in carnation, suggests that OSD1 probably functions in female gametogenesis, meiotic progression, or both. However, these hypotheses regarding the function of OSDLs in carnation have not been confirmed. In future studies, we plan to focus on the function and the regulation network of OSDLs.

Conclusion

Diploid gamete formation occurred in carnation. Analysis of chromosome behavior at meiosis indicated that 2n male gamete formation is probably attributable to the absence of the second meiotic division. We cloned OSDL genes in carnation for the first time by using RACE technology and have identified three homoeologous genes, which can be distinguished from OSD1 in A. thaliana. The evident feature of these OSDLs proteins is the absence of the GxEN/KEN-box domain, suggesting that the GxEN/KEN-box is probably not necessary for gene function in carnation. Our analyses of expression levels of OSDL indicated that OSDLs in carnation may play roles in male meiosis and ovary development. These findings have potential applications in fundamental polyploidization research and carnation breeding programs.

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Supplemental Fig. 1. Open reading frame and presumed amino acid sequences of *OSDLa* in carnation.

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nt        aa
1  acatggggacaccattaaaactctttttttcttcccttttaattcgacaccattttat
   tatattactcttttatattaactacctctctctatacctccccatatttctgtaaat
121  caactcagaaattgtctctgtgtaaaacccggttagaattttgttagacagaatagacctga

181  tccaggggtatagttctgcagacccgtagacataatcgacgccactataaaatgcacacgcg
   srdrlsrpdvddisslllpnanaqr
241  cgaatttactctgtagatgaaaccaggggtttcgaccttaggggactatcgtgcataat
   rvnlnvvdpeglrlrlrgllsln
301  ggtcctacagtaaaacctagtggtagctacgcagctgcagcagctgacagctcaatat
   asiskpsgsasrlrrrssqy
361  agagccgcttagagacaaaaacccgtactcttggtctctgcctacccaagaatattcg
   ratlrrqqrlyralrllpdpqener
421  acacccgtggtttaacgaatgtacaaggttagacggaggtcgctctttctttggtgcacccg
   tpvglrnvqgrrssvlpwhp
481  cgaacctctctaggatatatttctgcattgtagggcaatagagagggagagagccaa
   rtdplrditaiaraierare
541  cttcaagagacagacgagaaaaaactgtctaatcaatcgtgctacgtcaggtttctca
   lqeqrrretssvpeessaasssq
601  cctgagaacggaacccacactaccgacctcaaaacccccgattccgttaaaaatttagac
   penetniptptptipvkild
661  agccagtagttaaaagagcagatttcgaggagacctcagaatccatcgaacccccgagaagc gastric
   sdvkdedeiaagdsestpkrkl
721  cttaacactcaatcagatcagacacaggttgggtagggagcaaaaaaagtggagaa
   lnsidtvrqvvvveeqekvek
781  acctccagccgctaaaaaggtgtaagagacgacagagagttccgctacattgtgcagctga
   tpaaakkaerdrkvrtlmsnr
841  tgtatatacatattatattggtttctgtgttgatttggtgtcgccctctttttcatatagat
   *

901  taggccccgtgttagagacacggagatcctgcccttttaattctgctggtcgtagctggttttaggc
961  gtactgtgacgcgacctttagcttttttggtctgtagtaaactctcctcgtcctcctt
1021  gcattctgtgcgccatctgttattctttttctcctcggttaaataggtttagagggcactc
1081  agggccatccattttgactcaacgggtttggataaacccggtttggatcgctcttcatatctcttaa
1141  agtatcattatatctccagatttggtaacaalaaalaa
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Supplemental Fig. 2. Open reading frame and presumed amino acid sequences of OSDLb in carnation.
Supplemental Fig. 3. Open reading frame and presumed amino acid sequences of OSDLc in carnation.
Supplemental Table 1. Primers used in this study [forward primer (FP), reverse primer (RP)].

| Name               | Primer sequence 5’ to 3’                          |
|--------------------|---------------------------------------------------|
| OSDL1a-5Ra-364R    | RP CCTAGCCAAGTACCGTTGTTGCTCTCA                    |
| OSDL1a-82-length-F | FP ACTTTCCTCCTCCTAAATTCC                         |
| OSDL1a-103-length-F| FP CCATTTATCTTGTGTTATTCACCTCA                    |
| OSDL1b-5Ra-189R    | RP GCTTCTTCTCGCGGTCTACGA                         |
| OSDL1b-138-length-F| FP ATGCCTGTACCAGTGTCAAG                          |
| OSDL1b-147-length-F| FP ATGCCTGTACCAGTGTCAAGAGATAGGT                  |
| OSDL1c-5R-468R     | RP CTTGTCGTCTTGTAGTATGG                         |
| OSDL1c-328R        | RP GTGGCTTCTTCTCATCACCA                         |
| OSDL1c-69-length-F | FP ACGCGGTGACGTGCTCAGACAGT                      |
| OSDL1c-113-length-F| FP GAACAGGGGTCAACGTTTCTGCTTTGCTTGTA            |
| GAPDH 579-F        | FP CAGTGGGACAGCAGGAAGC                          |
| GAPDH 788-R        | RP TGGCATCGTTGACGTTCT                           |
| OSDL1a-422F        | FP CCGAGTGTCTACCTCCTT                           |
| OSDL1a-656R        | RP CTCTGCACATCTCGTTT                           |
| OSDL1b-99-F        | FP CTCTCAGCAACAACACTCA                          |
| OSDL1b-223-R       | RP CACACTTTCCTCACCCTT                          |
| OSDL1c-133F        | FP CTGAACCTCATTGAAAAAGGTAAGGC                   |
| OSDL1c-270-R       | RP GTAACAGCACGAGGACTATCGCAT                     |