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Original Paper

Functional analysis of the promoter of a rice 18 kDa oleosin gene

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Abstract The 18 kDa oleosin (Ole18) is a seed-specific protein expressed specifically in embryos and the aleurone layers of rice (*Oryza sativa* L.) seeds. Sequence analysis revealed that the promoter of the gene *Ole18* contains many *cis*-acting elements, including seed-specific elements, ABA-responsive elements, and drought-responsive elements, mainly in the region between −400 and −100. To elucidate the regulatory mechanism of *Ole18* gene expression, the 1249-bp *Ole18* promoter and its internal deletion-derivatives were fused to the GUS reporter gene and introduced into rice. Histochemical analysis and fluorometric quantitative analysis in transgenic rice showed that GUS activity varied depending on the deletion-derivative construct. GUS activity decreased as the deletion region increased (from −378 to −187, to −180, to −169, and to −130), suggesting the importance of known *cis*-acting motifs such as ABA-responsive elements and drought-responsive elements, and a novel motif within these regions. In addition, either the region between −378 and −179 or that between −179 and −130 was sufficient to induce high expression in the embryos and aleurone layers. This suggests the importance of the common *cis*-acting elements in the two regions: the drought-responsive element DRE2 and the ABA-responsive element ABI4-binding motifs.

Key words: aleurone layer, *cis*-acting element, embryo, GUS, *Oryza sativa* L.

Promoters play important roles in gene expression. Identification of functional *cis*-acting elements in a promoter is a crucial step in understanding gene function. Tissue-specific promoters drive gene expression within specific tissues or organs. Many specific promoters, with different tissue specificities, have been isolated and investigated in transgenic research. Many efforts have been made to characterize *cis*-acting elements involved in seed-specific expression, such as a −300 element (Thomas and Flavell 1990), a Napin motif (Ericson et al. 1991), and an RY repeat (Ezcurra et al. 1999; Hattori et al. 1995).

Cereal seeds are composed of three major tissues: embryos, starchy endosperms, and aleurone layers. They have an important role in the accumulation of storage compounds to sustain the initial growth of seedlings. In rice (*Oryza sativa* L.), embryos and aleurone layers accumulate high levels of lipids, proteins, and phytic acid with minerals. The lipids are stored in small discrete organelles, called oil bodies.

Proteins termed oleosins cover the surface of an oil body. Oleosins are hydrophobic proteins and play a role in maintaining the structure of oil bodies (Chuang et al. 1996). Two oleosin isoforms of molecular masses 18 and 16 kDa (Ole18 and Ole16) are present in rice. Two oleosin genes showed concurrent expression patterns during seed development (Wu et al. 1998). Expression of oleosin genes is restricted to immature seeds. The promoter of the *Ole18* gene (Os03g0699000) was found to express predominantly in embryos and aleurone layers, where lipids are stored in oil bodies (Qu and Takaiwa 2004). However, detailed analysis of the *cis*-acting elements of the *Ole18* gene has not been performed. Several genes expressed in both embryos and aleurone layers, but not in endosperms, have been identified. These genes include *RINO1* (*myo*-inositol 3-phosphate synthase), a phytic acid biosynthesis-related gene (Yoshida et al. 1999), and *REG2* (7S-type globulin), encoding a seed storage protein (Miyoshi et al. 1999). The *cis*-acting elements that determine embryo-specific and aleurone layer-specific expression have not been identified.

In this report, we analyzed the *Ole18* promoter using fusion constructs of the full-length and truncated promoters with a GUS reporter gene (*β-glucuronidase*) in transgenic rice plants. We examined the transcriptional activities of the deletion-derivatives of the *Ole18* promoter in the transgenic seeds by measuring GUS activity. From qualitative and quantitative reporter analyses, several decisive promoter regions were

Abbreviations: ABRE, ABA-responsive element; DAF, days after flowering; 4-MU, 4-methyl umbelliferone; Ole18, 18 kDa oleosin.
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identified. The results from this study improve understanding of *Ole18* expression regulation and provide a guide for transgenic breeding for engineering altered seed properties.

Materials and methods

Quantitative real-time RT-PCR analysis

Total RNAs were prepared from roots and leaves of 7-day-old seedlings, flowers, and pistils prior to flowering, immature embryos 10 days after flowering (DAF), and dehusked seeds at 5, 7, 10, 14, 21, 28 DAF, using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). Contaminated genomic DNA was eliminated by treatment with DNase I. The RNA was purified using the RNeasy Micro Kit (Qiagen). Gene-specific primer pairs for *Ole18* were 5′-GTC GAT CGA CGT CGA GAA TG-3′ and 5′-GAC GGC CAG CGT GAG GAA GC-3′. Detailed methods have been described previously (Suzuki et al. 2007). The RT-PCR reactions were performed in triplicate, three separate RNA preparations, using independent tissue samples. To quantify *Ole18* mRNA, in vitro-transcribed *Ole18* RNA was used as a standard.

Sequence analysis

Putative *cis*-regulatory elements within the 1249 bp of the *Ole18* promoter region were predicted according to the PLACE (http://www.dna.affrc.go.jp/PLACE/; Higo et al. 1999) and JASPAR (http://jaspar.genereg.net) databases.

Construction of promoter deletion segments and generation of transgenic plants

The 1249-bp promoter region of the *Ole18* gene cloned into the pT7Blue vector (pT7Blue-Ole18pro) was provided by Dr. Fumio Takaiwa. Internal deletion series of the *Ole18* promoter in pT7Blue-Ole18pro were generated by inverse PCR, using the KOD-Plus-Mutagenesis kit (Toyobo, Osaka, Japan). The primers used are listed in Table S1. All constructs were confirmed by sequencing. The promoter internal deletion series were inserted into the pGWB3 vector (Nakagawa et al. 2007), which contained the GUS reporter gene and nopaline terminator, using the Gateway System (Invitrogen, Carlsbad, CA, USA). Transgenic plants were generated by transferring T-DNA regions of pGWB3 into rice (cv. Nipponbare) via the Agrobacterium-mediated method, as described previously (Kuwano et al. 2006).

GUS analyses

To examine *Ole18* promoter activities in seeds, mature seeds from transgenic rice plants containing various promoter fragments were subjected to histochemical analyses, as described in Qu and Takaiwa (2004). The seeds were cut into median longitudinal sections using a razor blade and then immersed in GUS staining solution. Three to four independent lines were used in the GUS staining analysis and demonstrated similar results.

A fluorometric assay for GUS activity was performed, according to the method of Jefferson et al. (1987). Seeds were homogenized in GUS extraction buffer, and the resulting supernatants were used to assess GUS activity. The protein concentration in the extracts was measured by the Bradford method (1976). GUS activity was represented as nM 4-methyl umbelliferone (4-MU) formed/90 min/mg total protein.

Results and discussion

Expression patterns of Ole18

Gene expression profiles of *Ole18* were examined by quantitative real-time RT-PCR (Figure 1). High levels of *Ole18* transcripts were observed in immature embryos, in contrast to the very low levels observed in roots, leaves, flowers, and pistils (Figure 1A). This result agreed well with the exclusive presence of the 18 kDa oleosin protein in rice embryos and aleurone layers (Wu et al. 1998) and with the potential of the *Ole18* promoter to drive specific expression in embryos and aleurone layers (Qu and Takaiwa 2004). During seed development, *Ole18* mRNA was detected at 5 DAF and reached the maximum level at 21 DAF (Figure 1B). After 21 DAF, the level of *Ole18* transcripts in the seeds decreased slightly.

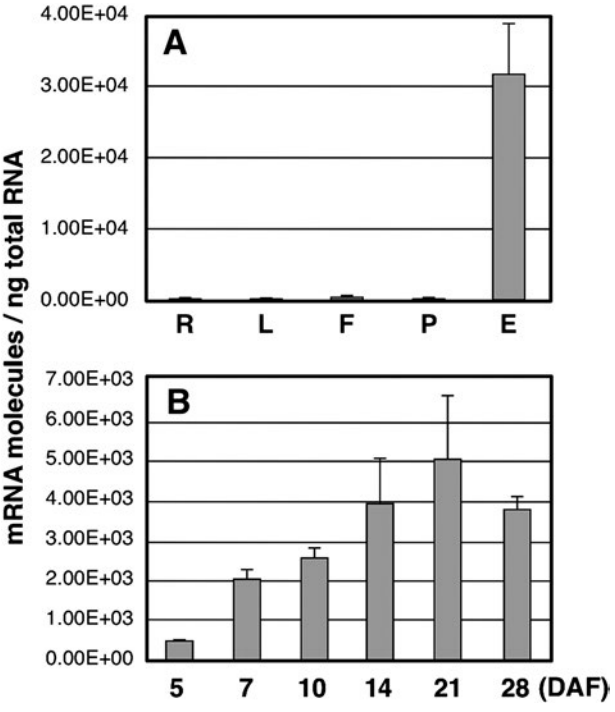

Figure 1. Quantitative real-time RT-PCR analysis of *Ole18* in non-transgenic plants. (A) mRNA levels of *Ole18* in roots (R) and leaves (L) at 7 days after germination, flowers (F) and pistils (P) at 1 day before flowering, and embryos (E) at 10 DAF. (B) mRNA levels of *Ole18* in dehusked whole seeds at 5, 7, 10, 14, 21, and 28 DAF. The mean values of mRNA molecules of total RNA were plotted for the different samples. Each value is the mean ± SD of three biological replicates.

Table 1. Putative *cis*-acting elements presented in the *Ole18* promoter regions found in PLACE or JASPAR database.

Motif sequence	Motif name	Position[a]	Species	Reference
PLACE				
Seed-specific element				
TACACAT	Napin motif	−969	Rape	Ericson et al. (1991)
TGHAAARK	−300 element	−993	Wheat	Thomas and Flavell (1990)
CATGCA	RY repeat	−231, −229, −158	Rape	Ezcurra et al. (1999)
CATGCATG	RY repeat	−229	Rice	Hattori et al. (1995)
ABA-responsive element				
RTACGTGGCR	ABRE	−136	Rice	Mundy et al. (1990)
TACGTGTC	ABRE	−112	Rice	Hattori et al. (1995)
CAAACACC	Prox-box	−316	Rape	Ezcurra et al. (1999)
ACACNNG	DPBF	−168	Carrot	Kim et al. (1997)
Drought-responsive element				
RYCGAC	DRE	−375, −174	Barley	Xue (2002)
ACCGAC	DRE2	−174	Rice	Dubouzet et al. (2003)
JASPAR				
ABA-responsive element				
CAACGCACCG	ABI4-binding motif	−260	Maize	Niu et al. (2002)
AGACGCGCCG	ABI4-binding motif	−197	Maize	ibid.
CACCCCCGCC	ABI4-binding motif	−142	Maize	ibid.
CACCCCCTCC	ABI4-binding motif	−127	Maize	ibid.
CACCGCTCTC	ABI4-binding motif	−103	Maize	ibid.

[a] Number of bases from start codon

Putative cis-acting elements in the Ole18 promoter

To elucidate the potential *cis*-acting elements responsible for seed-specific expression, 1249 bp of the *Ole18* promoter region were analyzed using the PLACE and JASPAR databases. Several motifs responsible for seed-specific expression of storage proteins are listed in Table 1. The Napin motif (Ericson et al. 1991) and −300 element (Thomas and Flavell 1990) were found at positions −969 and −993, respectively. RY repeat elements (CATGCA[TG]; Ezcurra et al. 1999; Hattori et al. 1995) were found at positions −231, −229, and −158.

Many genes expressed in developing seeds contained ABA- and drought-responsive elements in their promoter regions. ABA plays a critical role in seed development, including induction of seed dormancy, accumulation of storage products, and acquisition of desiccation tolerance. Using the PLACE database, typical ABA-responsive elements (ABRE; Hattori et al. 1995; Mundy et al. 1990) were found at positions −136 and −112 of the *Ole18* promoter. In addition to ABRE, Prox-box (Ezcurra et al. 1999) and DPBF (Kim et al. 1997) elements were found at positions −316 and −168, respectively. Using the JASPAR database, many ABI4-binding motifs (Niu et al. 2002) were found at positions −260, −197, −142, −127, and −103. The transcription factor ABI4 is responsible for ABA signaling during seed development and germination (Wind et al. 2013). During seed maturation, dehydration for long-term storage causes severe drought stress. Therefore, many drought-responsive protective proteins are induced in developing seeds (Delahaie et al., 2013). Using the PLACE database, the drought-responsive elements DRE and DRE2 (Dubouzet et al. 2003; Xue 2002) were found in the *Ole18* promoter at positions −375 and −174, respectively. The majority of the *cis*-acting elements responsible for seed-specific expression were found to be located between −400 and −100 (Table 1). These *cis*-acting elements located within 400 bp of the promoter region are summarized in Figure 2. Next, we conducted a deletion analysis that focused on the region between −400 and −100.

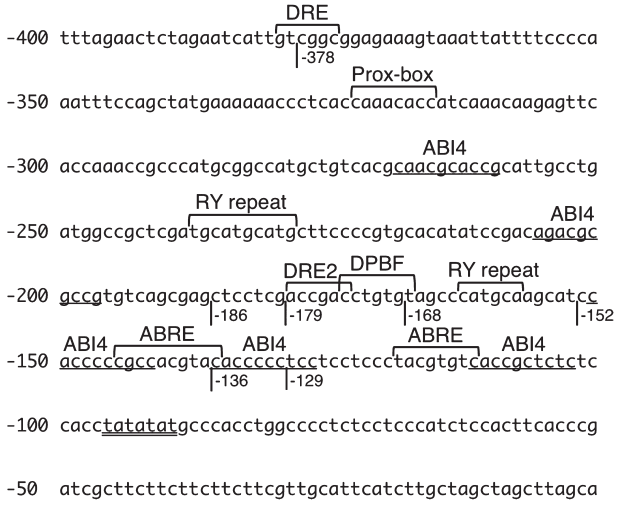

Figure 2. Distribution patterns of putative *cis*-acting elements within the −400 promoter region of the seed-specific *Ole18* gene. The seed-specific, ABA-responsive, and drought-responsive elements are indicated. The ABI4-binding motif is indicated as underline. The putative TATA-box is indicated as double underline.

Deletion analysis of the Ole18 promoter

To determine the functional significance of these elements in embryo-specific and aleurone-specific expression, we introduced several internal deletions within this region (Figure 3) and fused the deleted sequences to a GUS reporter gene. Tissue specificity and promoter activity in mature seeds were estimated using GUS assays.

GUS expression in the mature transgenic rice seeds was examined (Figure 4). The transgenic seeds containing the deletion construct in which the sequence from −378 to −187 (−378/−187) was deleted showed strong GUS activity, which was comparable to that with the 1249-bp full-length promoter (Figure 4). In this deletion region, one DRE, one Prox-box, one RY-repeat, and two ABI4-binding motifs were identified (Figures 2, 3). GUS staining was restricted to the embryo and aleurone layers and was not observed in the starchy endosperm. GUS activity was slightly reduced but remained strong in seeds containing the −378/−180 deletion construct. GUS activity was markedly reduced in seeds containing the −378/−169 deletion construct. The ABA-responsive DPBF motif ACA CNNG and drought-responsive DRE2 motif ACC GAC were found in the region between −179 and −169 (Figures 2, 3). Therefore, DPBF and/or DRE2 motifs might be responsible for the high level of *Ole18* expression in the embryos and aleurone layers. When the promoter was deleted from −378 to −153 (−378/−153) or from

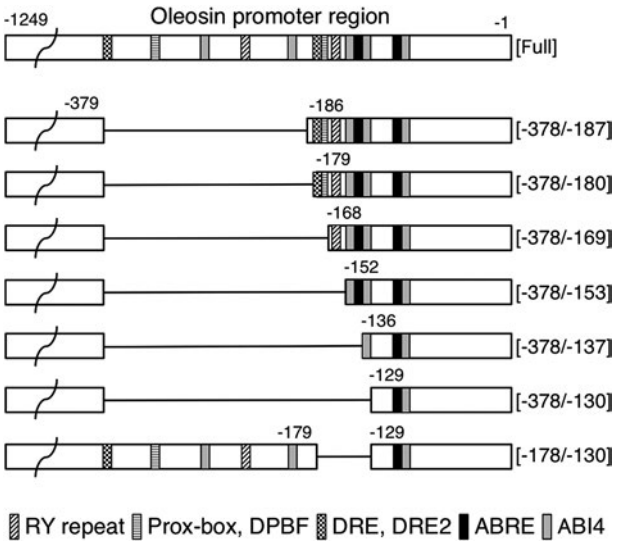

Figure 3. Schematic representation of deletion constructs used for GUS expression analysis and location of putative *cis*-acting elements in the *Ole18* promoter.

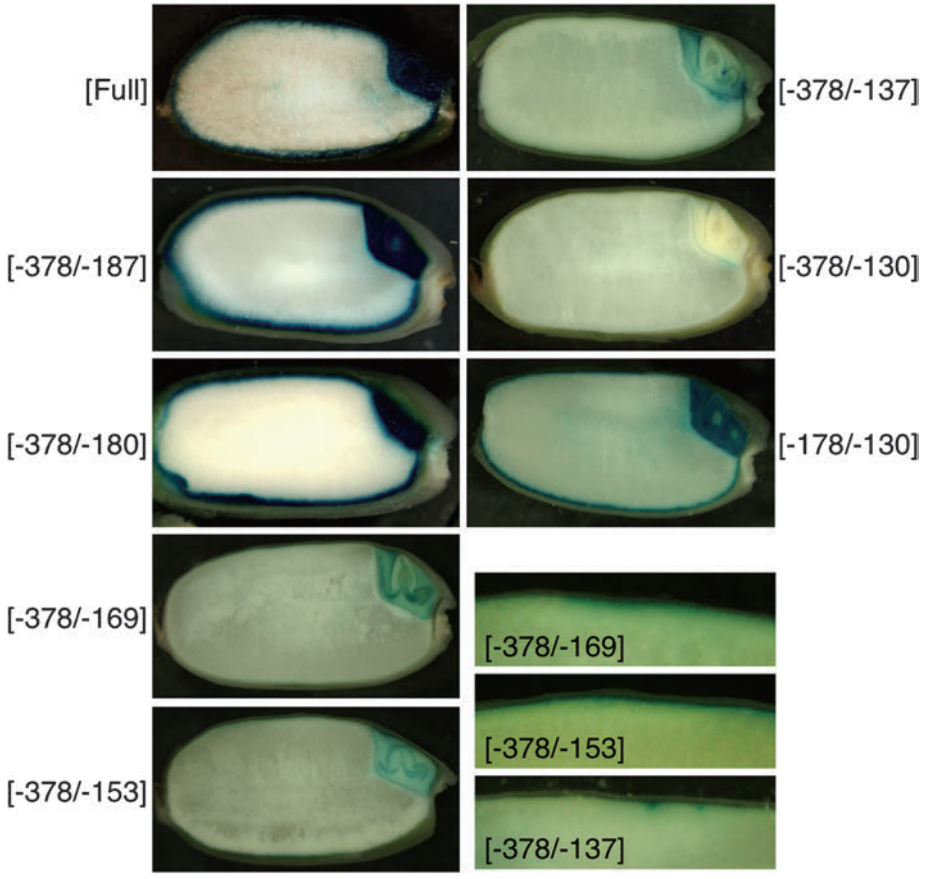

Figure 4. Histochemical localization of GUS expressions in rice transgenic seeds containing various *Ole18* promoter fragments. In the lower right, close-up views of aleurone layers are shown.

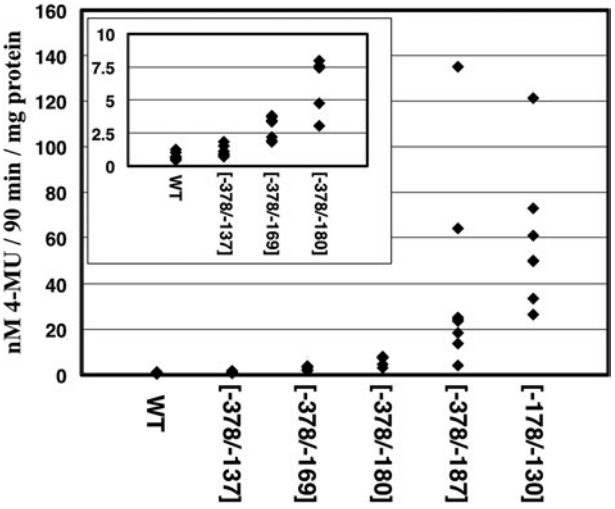

Figure 5. Fluorometric GUS assay of the mature seeds of transgenic plants containing various *Ole18* promoter fragments (n=5 to 8). The inset gives the enlarged graph of WT, [−377/−137], [−377/−169], and [−377/180].

−378 to −137 (−378/−137), the GUS expression pattern and activity were unchanged in comparison with the −378/−169 deletion construct (Figure 4). This was confirmed in close-up views of the aleurone layer. On the other hand, GUS activity was barely observed in both the embryo and aleurone layer when the promoter was deleted from −378 to −130 (−378/−130; Figure 4). In the sequence from −136 to −130, an ABA-responsive ABI4-binding motif was found (Figures 2, 3). There was only a single ABI4-binding motif in the −378/−130 deletion construct. It is plausible that at least two ABI4-binding motifs are needed to maintain minimum expression in developing seeds. To confirm this, point mutation experiments are required.

To confirm the differences in promoter activity among the *Ole18* promoter deletion constructs, a fluorometric GUS assay was performed on protein extracts from whole mature seeds (Figure 5). Expanding the deletion area from −187 (−378/−187) to −180 (−378/−180), −169 (−378/−169), or −137 (−378/−137) caused a gradual reduction in GUS activity. This is consistent with results from the histochemical analysis of GUS expression (Figure 4) and indicates the presence of positive regulatory elements in these regions. Like *Ole18*, the seed expression of *Ole16*, *RINO1*, and *REG2* was restricted to embryos and aleurone layers (Qu and Takaiwa 2004; Yoshida et al. 1999). According to sequence data from the RAP-DB (http://rapdb.dna.affrc.go.jp), the promoters of *Ole16*, *RINO1*, and *REG2* also contain the sequence CTCCTC, which is located between −186 and −181 in the *Ole18* promoter; however, the sequence CTCCTC was not predicted in the PLACE or JASPAR database. The sequence CTCCTC is located from −122 to −117, from −281 to −276, and from −51 to −46 in the *Ole16*, *RINO1*, and *REG2* promoter, respectively. These facts suggest that CTCCTC is a novel *cis*-acting element that enhances gene expression in seeds. This hypothesis should be tested via point mutation experiments in this region.

To determine the significance of the *cis*-regulatory region between −179 and −130, a construct deleted from −178 to −130 (−178/−130) was prepared (Figure 3). Unexpectedly, the −178/−130 construct induced strong GUS expression in both the embryos and aleurone layers (Figures 4, 5). This result suggests that either the region between −378 and −179 or that between −179 and −130 is sufficient to drive high expression in the embryos and aleurone layers. This indicates that two discrete regions of the *Ole18* promoter, from −378 to −179 and from −179 to −130, ensure strong expression in seeds. Both regions have several common *cis*-acting elements: a DRE, an RY-repeat, and ABI4-binding motifs.

Ole18 and *Ole16* expression was regulated in the same manner (Wu et al. 1998). The promoter of *Ole16* also contains many ABI4-binding motifs (JASPAR) and two DRE2 motifs (RAP-DB). In conclusion, a drought-responsive element and two or more ABI4-binding motifs play an important role in enhancing the expression level of *Ole18* in rice seeds. Although the strength of the promoter activities depended on the internal deletion construct, the tissue specificity of these promoters never changed. Expression of the GUS reporter gene in embryos and aleurone layers was reduced simultaneously and to the same extent. It is possible that the tissue specificity of gene expression is determined by a common unknown factor, such as a transcription factor, present in both embryos and aleurone layers.

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Table S1. List of primers for construction of deletion constructs.

Primer type	Construct name	Primer sequence
Reverse primer	[-378 /]	ACAATGATTCTAGAGTTCTA
Reverse primer	[-178 /]	TCGAGGAGCTCGCTGACACG
Forward primer	[/ -187]	CTCCTCGACCGACCTGTGTA
Forward primer	[/ -180]	ACCGACCTGTGTAGCCCATG
Forward primer	[/ -169]	TAGCCCATGCAAGCATCCAC
Forward primer	[/ -153]	CCACCCCCGCCACGTACACC
Forward primer	[/ -137]	CACCCCCTCCTCCTCCCTAC
Forward primer	[/ -130]	TCCTCCTCCCTACGTGTCAC