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RESEARCH ARTICLE

Molecular cloning and *in-silico* analysis of *Ramy3D* promoter and 5′ untranslated region from an Iranian rice (*Oryza sativa* L.) cultivar "NEMAT"

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ABSTRACT: The regulatory sequence of rice alpha amylase 3D gene (*Ramy3D*) is amongst the most successful expression systems used for recombinant protein expression in plants. In the current study a 995 bp fragment consisting of *Ramy3D* promoter and its 5′ untranslated region was amplified from the genomic DNA of an Iranian rice cultivar ″Nemat″, using polymerase chain reaction. The amplified fragment was ligated into the pTG19-T vector and the cloned fragment was sequenced. For *in silico* characterization, the rice specific consensus sequences of TATA-box and YR Rule motifs were scanned against the cloned fragment sequence using FIMO program and the cis acting elements existing in the promoter region were investigated using PlantCare database. A TATA-box motif with the rice specific pattern was identified at upstream position of the transcription start site. The identification of TATA-box in *Ramy3D* promoter is consistent with its metabolic and tissue specific regulation manner. Several cis regulatory motifs responsible for the metabolic and hormonal regulation of *Ramy3D* gene were identified including ABRE, G-Box, GC-box, GATA motif and TATCCA T/C motif. In addition, several motifs involved in response to various stimuli such as plant hormones, light and biotic and abiotic stresses were identified which include circadian motif, as-2-box, WUN-motif, TGACG-motif, Skn-1 motif, O2-site, MBS, LAMP-element, I-box, HSE, GCC Box, GATT motif, CGTCA-motif and GAG-motif.

KEYWORDS: Rice, Amylase, Ramy3D, Promoter, Regulatory cis elements, 5′ untranslated region

INTRODUCTION

α-amylase enzymes are essential for the hydrolysis of starch stored in the endosperm of plants. In this way they provide the embryo with sugar during germination of grains. In rice, there is a family of nine genes encoding the α-amylases (39). Recently, the regulatory sequences of a member of this family, the Rice alpha amylase 3D gene (*Ramy3D*) has drawn a great attention in molecular farming. In order to produce valuable recombinant proteins in a new platform, the regulatory sequences controlling the recombinant gene expression have undoubtedly a great effect on the yield production. The expression of *Ramy3D*, in both germinating grains and cell suspension culture, is strongly regulated by sugar depletion (15, 36). The transcription rate and also mRNA stability of *Ramy3D* enhances in response to sucrose starvation in the culture medium (32). Since it contains strong regulatory sequences, the Ramy3D isozyme is one of the proteins abundantly expressed and secreted into the culture medium, upon sugar depletion in the rice cell culture (38). With the aim of producing valuable recombinant proteins in rice cell suspension culture, the high expression power as well as inducibility, which are

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important factors in this issue, have turned the *Ramy3D* regulatory sequences to one of the most suitable expression systems.

Plant cell suspension cultures possess several advantages for recombinant protein production, including simplicity of the system, media safety, and easy manipulation (18). The application of an inducible expression system will effectively increase the production yield by the separation of the "growth" and the "production" phases in a manufacturing process (42). Recently, the most successful example reported on the inducible promoter used for recombinant protein expression is the rice *Ramy3D* promoter (45). The use of inducible *Ramy3D* promoter in transgenic rice cells caused a high level of recombinant protein expression .However, the cell growth rate was lower than those of the BY-2 and NT-1 of tobacco cell lines (11). The level of recombinant protein obtained using this transgenic rice cell suspension system is usually above 10 mg/L. This provides a promising start point for the developmental process (6, 49). To date several therapeutic proteins such as Human cytotoxic T lymphocyte antigen 4-immunoglobulin (hCTLA4Ig), Human growth hormone (hGH), Human lysozyme, Human granulocyte-macrophage colony stimulating factor (hGM-CSF), Human serum albumin (HSA), Human interlukin-12 (IL-12) and Human α1-antitrypsin (rAAT) have been successfully expressed using *Ramy3D* regulation elements in rice cell suspension culture (13, 14, 19, 24, 26, 33, 34). The rAAT is the protein with the highest amount of secretion (247 mg/L) recovery reported from plant cell cultures to date (24). This yield is in fact close to the production levels obtained by the mammalian cell culture (45).

In the current study, the *Ramy3D* promoter and its 5′ untranslated region (5′UTR) fragment were amplified from an Iranian rice cultivar ″Nemat″ genomic DNA and then cloned into the pTG19-T vector. To characterize the promoter, PlantCARE database was used and its cis acting elements were identified. Several cis-acting regulatory elements associated with metabolic and hormonal regulation of *Ramy3D* gene and its tissue specific expression were identified.

MATERIALS AND METHODS

Rice seeds were provided by Sari Agricultural Sciences and Natural Resources University. The pTG19-T vector (SinaClone) and *Escherichia coli* DH5α strain were used for the cloning purpose. The NCBI Primer-Blast tool was used for designing primers and the designed primers were synthesized by Macrogen. Amplification of the intended fragment was performed by PCR using AMPLIQON master mix (SinaClon). DNA Purification was performed using GeneAll DNA purification kit (Pishgam). DNA sequencing was performed by Bioneer.

Table 1. The list of primers used in this study.

Primer name	Sequence	Tm (◦C)
pRamy.c	AGGTGTGCGCAATCAGGAA	57.4
pRamy.r	TATCTGTGTAAGCTGAAACCGTG	56.2

Plant materials and DNA extraction

The Rice seeds were sterilized in 70% ethanol for 1 min and 2.5% (v/v) commercial bleach for 15 min. The sterilized seeds were cultured on the MS medium in a clean culture room with photoperiod condition of 14 h light (3000 lx) and 10 h darkness at 25 ◦C. After two weeks the newly sprouted leaves were collected and the genomic DNA was isolated by a CTAB method (2). The quality and quantity of the isolated DNA were analyzed by gel electrophoresis using a 1% (w/v) agarose gel.

Primer design and PCR conditions

Based on rice *Ramy3D* gene sequence (Genebank accession number M59351.1), a pair of specific primers were designed for the amplification of the promoter and 5′ UTR region with 995 bp length, using NCBI Primer-Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Table1). The designed primers features such as melting temperature, GC content, the possibility of hairpin formation, self-dimers and hetero-dimers were analyzed using online OligoAnalyzer tool (https://eu.idtdna.com/calc/analyzer). The polymerase chain reaction was carried out with a reaction mixture of 20 µl containing 10 µl AMPLIQON master mix (Cat. No. A190303), 100 ng of genomic DNA, 0.5 µM of each forward and reverse primers and nuclease free deionized water in PCR tube. The thermal cycler was programmed to the following reaction conditions: initial denaturation at 95°C for 5 min followed by 32 cycles of denaturation at 94°C for 30s, annealing at 62°C for 35s, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR product was analyzed on a 1% (w/v) agarose gel.

Cloning and sequencing of Ramy3D promoter and 5′ UTR

Following the amplification of intended fragment by PCR reaction, the PCR product was purified using a GeneAll PCR purification kit (Cat. No. 103-150). The purified PCR product was ligated into pTG19-T vector as follows: 2 µl of pTG19-T vector (25 ng/µl), 50 ng of purified PCR product, 1 µl of 10 × ligation buffer, 1µl of T4 DNA ligase (200 u/µl) and nuclease-free deionized water were mixed to a final volume of 10 µl. A 10 ng aliquot of the ligation product was used for the transformation of *E. coli* DH5α competent cells (2). Colony PCR amplification and restriction digestion by *Bam*HI were performed to ensure fragment insertion into pTG19-T vector. The prepared recombinant vector containing the desired fragment was sequenced by Bioneer.

Bioinformatic analysis and *in-silico* sequence characterization

Using local Clustal X software, the obtained nucleotide sequence was aligned with M59351.1 sequence to determine their similarity. The cis- acting elements of promoter was identified using PlantCARE database. The previously identified rice specific TATA box and YR Rule were mapped in the sequence using FIMO (http://meme-suite.org/tools/fimo) (8).

RESULTS and DISCUSSION

Cloning of Ramy3D promoter

The *Ramy3D* promoter and 5′ UTR were amplified using PCR from rice genomic DNA. As shown in Fig 1a, a 1000 bp fragment was obtained from PCR which was consistent with our expected length (995 bp). The amplified fragment was ligated into the pTG19-T vector to obtain pTG19-RamyPro recombinant vector. Following digestion of the pTG19-RamyPro vector with *BamH* I (its recognition sites flank the cloning region), the desired fragment with predicted length of ~1000 bp was released from vector that confirmed the cloning process (Fig 1b).

Promoter sequence analysis

The sequence of the cloned fragment was scanned against PlantCARE (20) database and as a result a total number of 50 cis elements belonging to 19 different motif types were identified in the promoter region (Table 2). The locations of the identified motifs are represented in Fig 3.

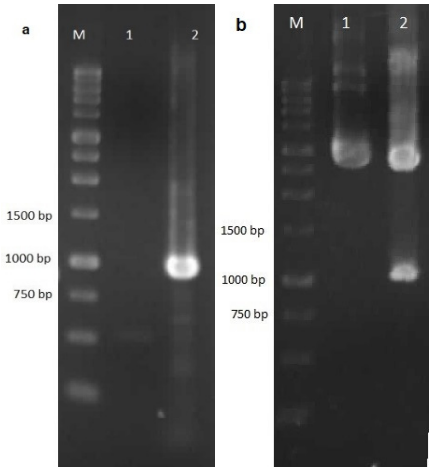

Fig 1. a) Ramy3D PCR product on agarose gel. M: 1kb ladder, lane 1: control negative; lane 2: PCR product with the size of 995 bp. No PCR product is observed in control negative lane. **b)** The pTG19-RamyPro digested by *Bam*HI on agarose gel. M: 1 kb ladder; lane 1: undigested plasmid; lane 2: the digestion product. The two bands in lane 2 show the excision of the cloned fragment from multiple cloning site of the recombinant vector.

Gene promoters could be simply defined as DNA sequences located upstream of gene coding regions which contain multiple cis-acting elements, and are specific sites for binding the proteins involved in the initiation and control of transcription (12). The core promoter comprises cis- elements required for binding and assembly of basal transcription machinery and directs basal transcription. TATA-box is the most well characterized cis element of core promoter, defined by the 'TATAWAWAR' consensus sequence. The upstream 'T' is often located around −30 in relation to A +1 (or G +1) position of the initiator (*Inr*) sequence for the transcription start site or TSS (4, 37). Clivan and Svec, (2009) reported that rice TATA-box as 'CTATAWAWA' is located within a C-rich region around −49 to −20 (3). Based on this result we investigated the *Ramy3D* promoter for the presence of TATA box using FIMO and 'CTATAWAWA' as rice specific TATA-box consensus sequence. As a result, 'CTATATATG' motif, located in −49 to −40 was specified as Ramy3D TATA-box (Fig 3). Previous studies on yeast, Human and Arabidopsis have shown only 13%, 10% and 29% of their promoters contain the TATA-box (25, 48). Similarly, about 19% of promoters in rice contain the TATA-box (3). With regard to metabolically regulation of *Ramy3D* promoter by sugar in the embryo, the presence of TATA-box in rice

Table 2. Putative cis-acting regulating elements in *Ramy3D* promoter.

Motif	# of motifs	Description
ABRE	5	Abscisic acid responsiveness
TATA box	1	Common cis-acting element in core promoter
CAAT-box	12	Common cis-acting element in promoter and enhancer
CGTCA-motif	2	Methyl jasmonate responsiveness
GCC box	1	ERF binding site in Pathogen responsive genes
HSE	1	Heat stress responsiveness
MBS	2	MYB binding site involved in drought inducibility
GC-box	1	Enhancer-like element involved in anoxic specific inducibility
G-box	2	Light responsive element
GAG-motif	1	Light responsive element
GATA-motif	2	Light responsive element
GATT-motif	1	Light responsive element
I-box	4	Light responsive element
LAMP-element	3	Light responsive element
as-2-box	1	Light responsive element
O2-site	1	Endosperm expression
Skn-1 motif	7	Endosperm expression
WUN-motif	1	Wound responsive element
Circadian	2	Circadian control

Ramy3D promoter core is consistent with the previous reports on yeast and human in which the TATA-box is generally related to tissue specific expression and mostly modulated by stress stimuli (48). Previously, the transcription start site (TSS) of Ramy3D was mapped (23). A dimer motif called YR Rule (C/T A/G) was identified at the transcription start site (-1/+1) of both Arabidopsis and rice promoters (47). We also identified the YR Rule motif with 'CA' sequence in TSS of Ramy3D (Fig 3). The CAAT box is another well conserved core promoter and plays an important role during transcription (1). Totally, 12 copies of CAAT-box were identified in the studied promoter.

There are some regulatory sequences such as enhancers, silencers, insulators, and cis-elements at the proximal and distal regions of the promoter that are involved in the regulation of gene expression at the transcriptional level (12). The expression of α-amylase genes in both rice cell suspension and germinating embryos is inhibited by sugars and this mechanism involves transcriptional regulation (23). Scanning the promoter region through PlantCARE search tool revealed the presence of several cis acting elements involved in the metabolic and hormonal regulation of *Ramy3D* gene. A total number of five Abscisic acid Responsive Element (ABRE) motifs were identified in the proximal region of promoter while one of them was also identified as the G-Box cis element due to the presence of the same core motif, 'ACGT'. The ABA responsive element ABRE (ACGTGG/T) controls dehydration and salt stress responses in Arabidopsis and Rice (12, 46). Multiple ABREs or an ABRE with other types of 'non-ACGT' coupling elements such as CE1, CE3 and DRE (drought response element), comprising the minimal ABA-responsive complex or ABRC (for review see (4)), are required for ABA-responsive gene expression. Recently, a genome-wide study on the co-expressed rice genes carried out by de los Reyes et al. (2015) based on over-representation of the CGMCACGTB' consensus sequence 'within -1,000 upstream regions estimated that more than 10% (>400) of total genes are regulated by the ABA signaling pathway. As mentioned above, in rice α-amylase (Ramy3D) gene promoter the ACGT core sequence is also known as a consensus sequence of the G-Box (cctACGTggc). This is an important cis-acting sequence for the metabolic modulation of this gene by glucose starvation (16, 23, 41). Therefore, the ACGT core sequence might be a consensus sequence for ABA and glucose responses. The G-motif sequence has been discovered to reside in the promoters of many genes that are turned on in response to diverse stimulatory pathways (i.e. light, anaerobiosis and phytohormones such as ABA) (40).

Previously, comprehensive efforts have been made to identify promoter regions and cis acting elements involved in the regulation of *Ramy3D* promoter by sugar. In addition to G-Box, three other cis elements including

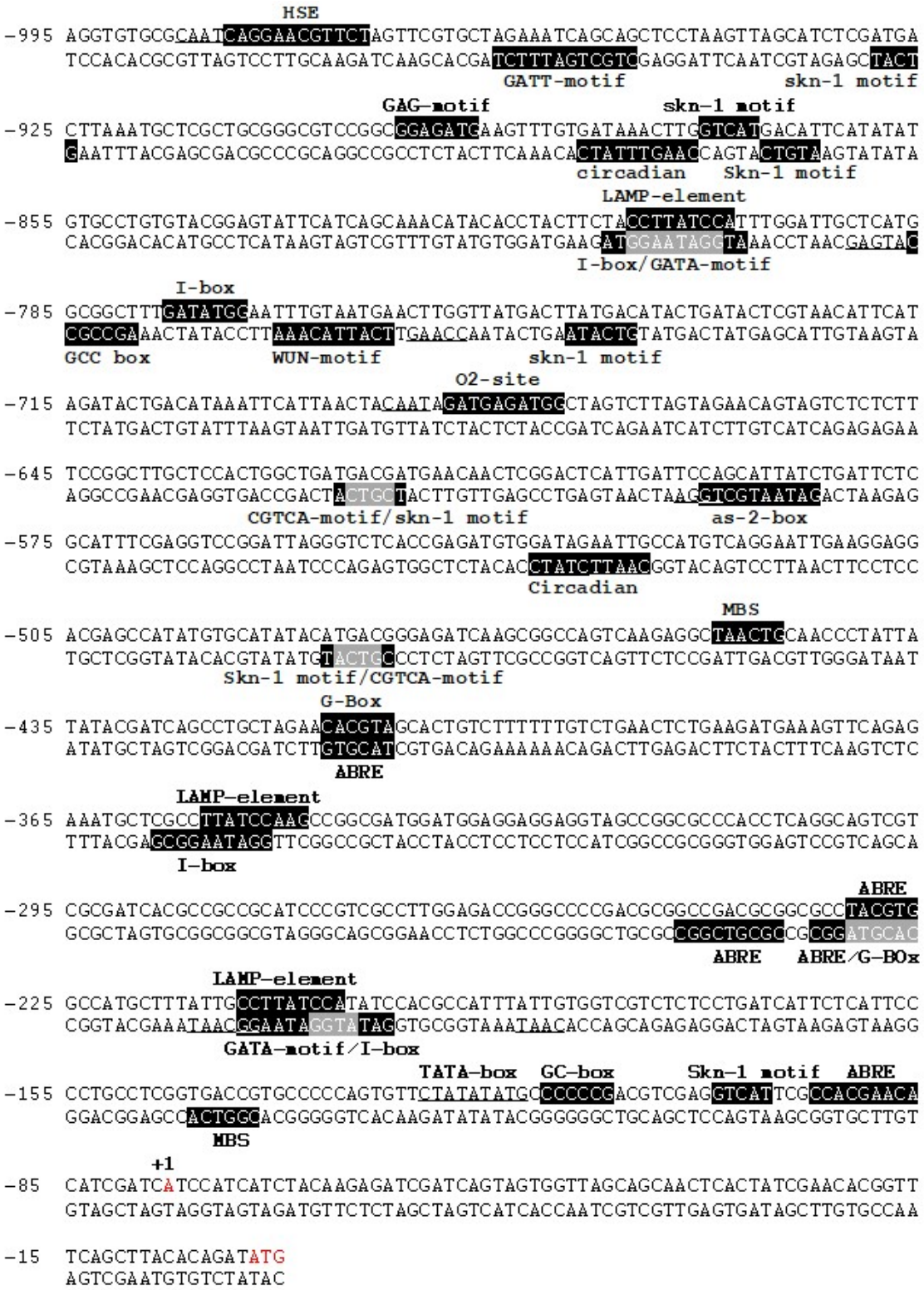

Fig 3. Putative cis-acting regulatory elements predicted using PlantCare database in the promoter of *Ramy3D*. The TATA- and CAAT-box sequences are underlined. The gray highlights indicate the shared regions between adjacent motifs. The +1 indicate transcription start site. The start codon (ATG) is in red.

GC-box, GATA and TATCCA T/C motif (GATA motif as its antisense sequence) were identified as essential elements regulating sugar response and act synergistically to cause a high level of induced expression upon glucose starvation (16, 23, 41). A GC-box motif downstream to TATA-box and two copies of the GATA motif were identified in the promoter. This has been reported that the G-Box and GATA elements occur several times in every potential upstream regulatory region (27, 31). In plants, GATAbox is necessary for light and nitrate-dependent control of transcription (21, 28).

As well as the mentioned motifs, several other functional cis-acting regulatory elements were identified in *Ramy3D* promoter including circadian motif, as-2-box, WUN-motif, TGACG-motif, Skn-1 motif, O2-site, MBS, LAMP-element, I-box, HSE, GCC Box, GATT motif, CGTCA-motif, GAG motif and 4 unnamed motifs. The CGTCA motif deals with responses to methyl jasmonate (29). GAG motif is known as light responsive cis-elements (LREs) (17). GCC-box is present in promoters of many genes responsive to pathogens and has been shown to function as an ethylene-responsive element (35). The Heat Stress Element (HSE) is a cis-acting regulatory element involved in heat stress responsiveness (30). I-box and LAMP element are GATA-related motifs recognized as cis-acting elements regulated by light (7, 9). The MYB Binding Site (MBS) is related to drought response (22). The O2-site, also known as the endosperm motif, occurs in the promoters of many cereal storage protein genes highly expressed in endosperm tissue (5, 10, 43). The Skn-1 motif is found in a number of seed-specific promoters which also causes endosperm specific gene expression (44). WUN is a wound responsive element.

CONCLUSION

In this study the upstream regulatory region of *Ramy3D* gene from an Iranian rice cultivar was cloned and analyzed through a bioinformatic survey for the identification of the core promoter and other important cis regulatory motifs. Our study represented the *Ramy3D* as a TATA-box containing promoter. The presence of multiple ABRE motifs in the studied promoter showed that the *Ramy3D* gene could be under hormonal regulation by abscisic acid in addition to its metabolic regulation through sugar level. Moreover, the *in silico* analysis revealed the presence of several cis-acting regulatory elements involved in response to different stimuli and tissue specific expression.

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همسانه سازی مولکولی و آنالیز *in silico* پیش بر و ناحیه ترجمه نشده ′5 ژن *Ramy3D* از رقم برنج (*Oryza sativa* L.) ایرانی "نعمت"

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چکیده

توالی تنظیم کننده ژن آلفا آمیلاز 3D گیاه برنج (*Ramy3D*) از موفق‌ترین سیستم‌های بیانی مورد استفاده برای بیان پروتئین نوترکیب در گیاهان است. در این مطالعه با استفاده از واکنش زنجیره‌ای پلیمراز، قطعه‌ای متشکل از پیش‌بر و ناحیه ترجمه نشده ′5 ژن *Ramy3D* با طول ۹۹۵ جفت باز از DNA ژنومی یک رقم برنج ایرانی با نام نعمت تکثیر شد. قطعه تکثیر شده در حامل pTG19-T درج و بدنبال همسانه سازی، ناحیه یاد شده توالی یابی شد. به‌منظور آنالیز کامپیوتری، با استفاده از نرم افزار FIMO الگوی حفاظت شده موتیف‌های TATA-box و YR Rule گیاه برنج در توالی قطعه همسانه سازی شده مورد جستجو قرار گرفت و همچنین عناصر تنظیمی سیس موجود در ناحیه پیش بر با استفاده از پایگاه داده PlantCare مشخص گردید. یک موتیف TATA-box دارای الگوی اختصاصی برنج در موقعیت بالادست جایگاه شروع رونویسی شناسایی شد. ردیابی TATA-box در پیش بر *Ramy3D* در این مطالعه با الگوی تنظیم متابولیکی و اختصاصی بافت این ژن مطابقت دارد. چندین موتیف درگیر در تنظیم متابولیکی و هورمونی ژن *Ramy3D* شامل ABRE، G-Box، GC-box، موتیف GATA و موتیف TATCCA T/C در ناحیه پیش بر شناسایی شد. همچنین چندیدن موتیف مرتبط با پاسخ به محرک‌های مختلفی همچون هورمون‌های گیاهی، نور و تنش‌های زیستی و غیر زیستی شامل موتیف circadian، as-2-box، موتیف WUN، موتیف TGACG، موتیف Skn-1، O2-site، MBS، عنصر LAMP، I-box، HSE، GCC Box، موتیف GATT، موتیف CGTCA و موتیف GAG شناسایی شد.

کلمات کلیدی: آمیلاز، پیش بر، Ramy3D، عناصر تنظیمی سیس، ناحیه ترجمه نشده' ۵